FACULTÉ DES ÉTUDES SUPÉRIEURES
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FACULTY OF GRADUATE AND
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GRADE / DEGREE

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FACULTÉ, ÉCOLE, DÉPARTEMENT / FACULTY, SCHOOL, DEPARTMENT

On the role of chromatin in the regulation of adenovirus vector transgene expression

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On the role of chromatin in the regulation of adenovirus vector transgene expression

by

P. Joel Ross

Thesis

Submitted to the Faculty of Graduate and Postdoctoral Studies
In partial fulfillment of the requirements of the degree of

Doctorate of Philosophy

Program of Microbiology and Immunology

Department of Biochemistry, Microbiology, and Immunology
Faculty of Medicine
University of Ottawa

Library and Archives
Canada

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395 Wellington Street
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Direction du
Patrimoine de l'édition

395, rue Wellington
Ottawa ON K1A 0N4
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Your file Votre référence
ISBN: 978-0-494-59537-4
Our file Notre référence
ISBN: 978-0-494-59537-4

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Abstract

Adenovirus vectors are undergoing evaluation in clinical trials for the treatment of a variety of human diseases. However, little is clearly understood regarding interactions between non-replicating adenovirus (Ad) vectors and the host nucleus. Here I have examined the role of chromatin in the regulation of helper-dependent Ad (hdAd) vector-encoded transgenes. Eviction of the Ad DNA packaging-protein VII, histone deposition, and transgene expression initiated within 2 hours of infection; hdAd assembled into physiologically-spaced nucleosomes within 6 hours. Histone deposition was transcription-independent, dependent on the histone chaperone HIRA, and essential for efficient viral gene expression. Having established that hdAd vectors assemble into chromatin, I examined the role of epigenetic factors in mediating the repressive effects of prokaryotic DNA attached to eukaryotic transgenes. To this end, I used helper-dependent adenovirus (hdAd) vectors with identical expression cassettes, but containing 22 kb of prokaryotic or eukaryotic stuffer DNA (hdAd-prok and hdAd-euk, respectively). I hypothesized that abundant CpG motifs in the hdAd-prok backbone are methylated, resulting in the formation of chromatin that is refractory to transcription. Within the host cell, the hdAd-prok transgene promoter was not associated with epigenetic markers of heterochromatin, although it did exhibit reduced association with acetylated histones H3 and H4. Inhibition of histone deacetylases or insertion of an insulator element between the transgene and stuffer DNA abrogated the inhibitory effects of prokaryotic DNA. However, I did not detect methylation of hdAd-prok DNA. Therefore, I examined the role of promyelocytic leukemia (PML) bodies and their constituents, Sp100 and

Daxx - which have all been implicated in repression of DNA virus transcription - in repression of hdAd-prok. Intact PML bodies and the PML protein were not necessary for repression of hdAd-prok. Rather, Sp100 isoforms B and HMG, which bind to unmethylated CpGs, and Daxx, which interacts with histone deacetylases, repressed expression of hdAd-prok. Therefore, "foreign" prokaryotic DNA is recognized by Sp100B/HMG and Daxx, which repress associated genes via induction of histone deacetylation. I found that chromatin plays an important role in the regulation of Ad vector-encoded transgenes, suggesting that both genetic and epigenetic factors must be considered in the design of Ad vectors for gene therapy.

Acknowledgments

First, and foremost, I have to thank my family: Mom, Dad, and Addy. You are the foundation upon which I have built my life. Dad, you stand as the man that I strive to be. Mom, you have always understood how hard life can be, and how only you can make it little bit better. Addy, you have always been there to keep me sane and grounded. No matter what hardships I encounter, I always have all of your encouragements and your belief in me, to keep me on the right track.

To my friends: Adam, we share the one of the closest bonds that any friends can. We've supported each other through the darkest days of our lives, and shared in some the best we'll ever see. You always made the end of an unhappy day the beginning of a better one. Lisa, you are pure joy. Every minute with you is a pleasure. I can't wait to be a part of the amazing life that you and Adam will share. Louis and Karie, your friendship (especially in the last few months) has been immeasurably important to me. Without you, I could have never moved-on to the next step. Kristin Heinz and Shawn, I am happy to have known that all of our experiences together have made us better people. I am also happy to have shared so many incredible moments with you.

To Catherine Sadler: you helped to make me who I am today. You gave me the strength to follow my dreams. You also showed me how much joy one person can give another, and opened me up enough to let the world in.

To my labmates: Catherine Barrett, Marisa, and Kristin, you each validated my belief that mentoring is one the most important things that a scientist can do. Each day spent working with you was a pleasure. To Adam Smith, our discussions, from the ridiculous to the profound, made each day in the lab fun and exciting. I can't imagine having a better foil for sharing ideas and planning experiments. To all of my other labmates over the years: Kathy, Rob, Lauren, Bobby, Koralee, Karen, Mike, and Milagros thank you for technical help and support. I would also like to thank Mike for many stimulating discussions.

To Robin, my supervisor: we have each grown in the wake of many challenging experiences. You gave me just the right amount of independence to find my place, but the guiding hand that I needed to develop into an independent scientist. Thank you for having the patience to allow me to see this project through to an end that I can be proud of.

Most importantly, I would like to thank Kristin. You brought me to new peaks of happiness; and we always helped each other through the troughs that life brings. You encouraged my belief in myself and made me understand the importance of community and professionalism. I spent some the happiest times that I can remember with you. I will never forget the incredible impact that you have had on me. Thank you for giving me the strength to keep fighting.

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List of abbreviations

AAV - adeno-associated virus
Ad - adenovirus
ACF - ATP-dependent chromatin assembly and remodeling factor
Amino terminus - N-terminus
Antibody - Ab
APS - ammonium persulfate
Asf1 - anti-silencing function 1 homologue
ATP - Adenosine triphosphate
AZA - 5-azacytidine
β-gal - beta galactosidase
BME - β-mercaptoethanol
bp - base pairs
CAF-1 - chromatin assembly factor 1
Carboxy-terminus - C-terminus
CBP - cyclic AMP response element binding protein (CREB) binding protein
Chd1 - chromodomain helicase DNA binding domain 1
ChIP - Chromatin Immunoprecipitation
CMV - cytomegalovirus
CpG - cytosine-guanine dinucleotides
Cre - cyclization-recombination
CREB - cyclic AMP response element binding protein
CTCF - CCCTC binding factor
Daxx - fas death domain associated protein
DID - Daxx interaction domain
DID2/3 - Daxx interaction domains 2 and 3
DMEM - Dulbecco's Modified Eagle Medium
DNMT - DNA methyltransferase
DRB - 5,6-dichlorobenzimidazole 1-ß-D-ribofuranoside
E1 - early region 1 gene
E1A - early region 1A gene
E1B - early region 1B gene
E2 - early region 2 gene
E3 - early region 3 gene
E4 - early region 4 gene
EDTA - ethylenediaminetetraacetic acid
fgAd - first-generation Adenovirus
Gam1 - *Gallus* anti morte
GCN5 - general control of amino acid synthesis 5
GR - glucocorticoid receptor
HAT - histone acetyltransferase
HCl - hydrogen chloride
HD1 - histone deacetylase 1 (original name)
HDAC - Histone deacetylase
hdAd - helper-dependent Adenovirus

HIRA - histone cell cycle regulation defective homolog A
HP1 - heterochromatin protein 1
hpi - hours post-infection
HPRT - hypoxanthine phosphoribosyltransferase
HS - DNase I hypersensitive site
HS4 - DNase I hypersensitive site 4 of the chicken β-globin locus
HSV - herpes simplex virus
IND - Innate nuclear defense
ITR - inverted terminal repeat
IU - infection units
K - lysine
kb - kilobase pairs
kDa - kilo-Dalton
LB - Luria broth
LiCl - lithium chloride
*lox*P - locus of X over P1
MCMV - murine cytomegalovirus
MDB1 - methyl-CpG-binding domain 1
MeCP2 - methyl-CpG binding protein 2
MEM - minimal essential medium
MMTV - murine mammary tumour virus
MOI - multiplicity of infection
Myt1 - myelin transcription factor 1
$Na_3C_6H_5O_7$ - sodium citrate
NaCl - sodium chloride
NaOH - sodium hydroxide
NAP1 - nucleosome assembly protein 1
Oct1 - octamer-binding transcription factor 1
orf - open reading frame
PBS - phosphate-buffered saline
PCNA - proliferating cell nuclear antigen
PEV - position effect variegation
PML - promyelocytic leukemia
pp32 - phosphoprotein of 32 kDa
RLU - relative light units
RNApII - RNA polymerase II
SAND domain - Sp100, autoimmune regulator 1, NucP41/P75, deformed epidermal autoregulatory factor 1 domain
SDS - sodium dodecyl sulphate
SDS-PAGE - sodium dodecyl sulphate polyacrylamide gel electrophoresis
SETB1 - Su(var)3-9, enhancer of zeste, trithorax domain bifurcated 1
SET (protein domain) - Su(var)3-9, enhancer of zeste, trithorax
SET (oncogene) - patient SE translocation-associated
Sp100 - speckle protein of 100 kDa
Spt16 - suppressor of Ty 16 homologue
SUMO-1 – small ubiquitin-like modifier 1

Suv39H1 - Suppressor of variegation 3-9 homologue 1
TBP - TATA box binding protein
TSA - Trichostatin A
USF1 - upstream transcription factor 1
X-gal - 5-bromo-4-chloro-3-indolyl-ß-D-galactopyranoside

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Chapter 1 - Introduction

1.1 - Literature review

1.1.1 - Gene therapy

Modern medicine has achieved levels of sophistication that could not have been dreamed-of as few as 30 years ago. The molecular revolution in biology has led to a dramatic improvement in our understanding of the mechanisms of disease (270). Deaths resulting from infection have become increasingly rare in developed countries (119). In some cases, horrific diseases that once plagued humanity, such as smallpox, have been eradicated. However, genetic diseases such as cystic fibrosis (227) and Duchenne muscular dystrophy (44) remain nearly as devastating as ever. Although we now have an intricate understanding of the etiology and mechanism of progression of these diseases, we have been unable to use this information to develop cures.

For nearly 20 years there has been hope for the development of a new form of therapy that could be used to cure genetic disease, called gene therapy (149, 301). Conceptually, the most-effective way to cure disorders that arise from functional loss of a single gene is to replace it. This is the rational basis of gene therapy. In a broader sense, gene therapy is any therapeutic regimen that uses genetic information to treat disease. In addition to gene-replacement paradigms, a wide variety of diseases can theoretically be treated by using gene products to alter cell physiology: some examples include using pro-apoptotic genes to kill cancer cells (171) or anti-apoptotic genes to promote cell survival in the treatment of neurodegenerative disorders (121).

However, gene therapy is easier said than done: DNA is not easy to deliver to human cells. To have a therapeutic effect DNA must be delivered into the nucleus of a cell and express the encoded gene (a process called "transduction") (301). Means of DNA delivery are generally divided into two classes: nonviral vectors and viral vectors. Nonviral delivery of DNA can be accomplished using direct injection of bare DNA or DNA complexed with cationic lipids or nanoparticles. Although safe, cost-effective, and scalable, current nonviral technologies suffer from poor target-cell transduction, and are therefore ineffective (98).

Viral vectors

As a result of millions of years of co-evolution with their hosts, viruses have become tremendously-efficient in the delivery of their own genetic information to host cells. Therefore, a great deal of effort has been expended in identifying ways to disarm viruses and use them as vectors for the delivery of therapeutic transgenes (301). A number of viruses have been used as platforms for the generation of gene-delivery vectors. The most prevalent of these vector systems are retrovirus, lentivirus, adenovirus (Ad), herpes simplex virus (HSV), and adeno-associated virus. These systems all have relative advantages and disadvantages (1) (reviewed in (301)). Of the viral vector systems for gene delivery, Ad exhibits the greatest potential for widespread usage in gene therapy applications (reviewed in (12) and (237)). Ad has a well-characterized life cycle and genome, is not associated with any serious human disease, its genome is easily manipulated using molecular tools, and Ad

Table 1. Viral vectors used in gene therapy applications. Reprinted by permission from Macmillan Publishers Ltd: Molecular Therapy (99), copyright 2005.

Vector	**Viral genome**	**Cloning capacity**	**Tropism**	**Inflam-mation**	**Vector genome Forms**	**Main limitations**	**Main advantages**
Enveloped							
Retrovirus	RNA	8 kb	Dividing cells only	Low	Integrated	Integration might induce oncogenesis in some applications	Transduces dividing cells
Lentivirus	RNA	8 kb	Broad	Low	Integrated	Integration might induce oncogenesis in some applications	Stable gene expression in replicating cells; transduces non-diving cells
HSV-1	dsDNA	40 kb[a] 150 kb[b]	Neurons/ broad	High	Episomal	Toxic and inflammatory; transient transgene expression in target tissues except neurons	Stable gene expression in most tissues; large cloning packaging capacity; broad cell tropism and strong tropism for neurons
Unenveloped							
AAV	ssDNA	<5 kb	Broad	Low	Episomal (>90%), Integrated (<10%)	Small cloning capacity	Broad cell tropism; non-inflammatory and nonpathogenic
Adenovirus	dsDNA	7.9 kb[a] 30 kb[c]	Broad	High	Episomal	Capsid mediates an inflammatory response; preexisting anti-Ad antibodies in most humans	Highly efficient transduction of most tissues; large cloning capacity; high titer and long-term expression[c]

HSV-1, herpes simplex type 1 recombinant vector; AAV, adeno-associated viral vector; ssDNA, single-stranded DNA; dsDNA, double-stranded DNA.

[a] Replication defective

[b] Amplicon

[c] Helper-dependent high capacity adenoviral vectors

vectors are easily grown to high titers. Furthermore, Ad efficiently infects a wide range of cell types in a cell cycle-independent manner.

Ad vectors are currently under evaluation in preclinical and clinical trials for the treatment of numerous human diseases (70). According to the Gene Therapy Trials Worldwide database maintained by the Journal of Gene Medicine (http://www.wiley.co.uk/genetherapy/clinical/) there were 1472 gene therapy clinical trials underway at the time of writing (December 28, 2008). Ad was under evaluation in 367 of these trials, making it the most widely-used vector system for gene delivery in humans.

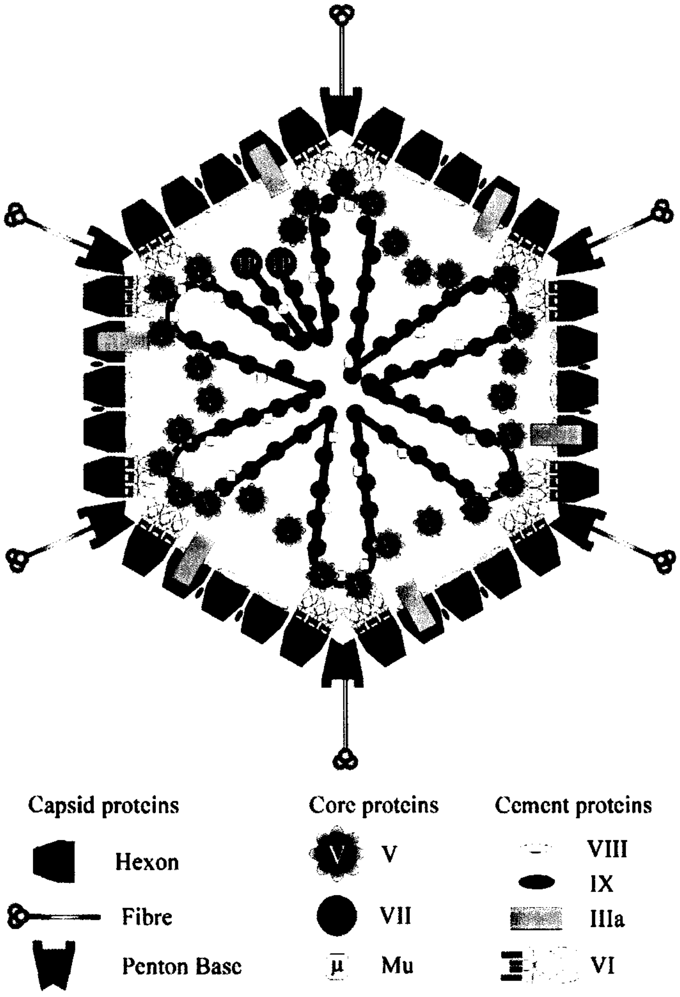
1.1.2 – Adenovirus

Biology, genome, and replication

Ad infects a wide range of hosts throughout the animal kingdom ((259); unless otherwise specified, references to Ad biology are attributed to this comprehensive review). There are currently 52 known serotypes of Ad that infect humans, which are divided into 7 subgenera, or classes (A-G) (142). Of these subgenera, Group C (which consists of Ad serotypes 1, 2, 5, and 6) is home to the most commonly-used serotypes for the generation of Ad vectors: the highly similar serotypes 2 and 5 (12). Group C Ad are endemic in most human populations and are associated with few human diseases, which makes them relatively safe for gene delivery vehicles.

Ad5 is a medium-sized DNA virus with a 36 kb linear double-stranded genome that is packaged within a non-enveloped, protein capsid (Fig. 1). The Ad genome is flanked by short inverted terminal repeats (ITRs), which serve as the

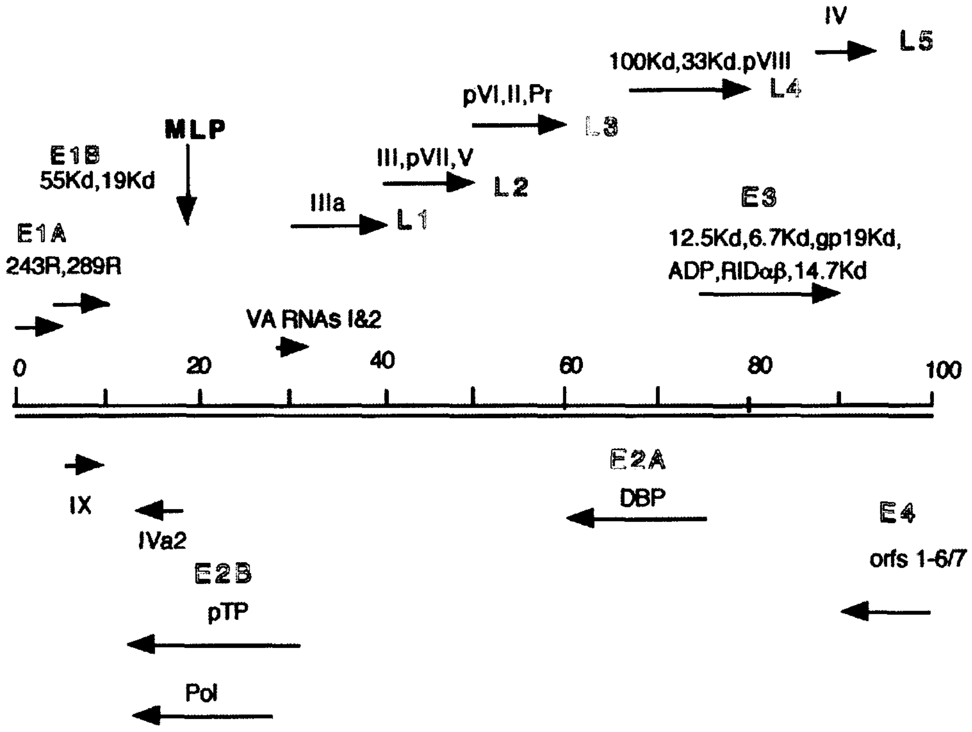
Figure 1. The adenovirus capsid. Displayed is a schematic representation of a cross-section of an Ad capsid. Reproduced with permission from the Society for General Microbiology (79), copyright 2000.


Capsid proteins
Hexon
Fibre
Penton Base
Core proteins
V
VII
μ
Mu
Cement proteins
VIII
IX
IIIa
VI

origins of DNA replication. Conventional terminology refers to the ITR adjacent to the early region 1 (E1) genes as the "left" ITR and the ITR adjacent to the E4 genes as the "right"; schematic representations of the linear Ad genome are oriented to reflect this terminology (Fig. 2). Between the left ITR and the E1 region is the packaging sequence, which is necessary for encapsidation of viral DNA. The remainder of the genome encodes genes and transcriptional regulatory sequences.

Ad-encoded genes are generally divided into three classes, early, delayed, and late (Fig. 2); early and late designations refer to whether genes that are expressed before or after genome replication, respectively; delayed genes (i.e., pIX, IVa2, and VA RNAs I and II) are expressed after early genes, but prior to DNA synthesis. Early gene products are responsible for creating a cellular environment that is conducive to Ad replication and for replication of viral DNA: E1A promotes expression of Ad genes and forces the host cell into S phase; E1B gene products inhibit apoptotic death of the host cell; the E2 gene products replicate the viral genome; the E3 gene products interfere with innate and adaptive immune responses to Ad infection; finally, the E4 products are involved in several aspects of the Ad lifecycle including selective nuclear-export of viral mRNA, inhibition of apoptosis, and inhibition of the host cell's double-strand break machinery, which prevents genome concatemerization during replication (280). The delayed genes have several different functions: pIX is a component of the Ad virion (192, 246), Iva2 is important for the activation of the major late promoter (213), which drives expression of Ad late genes, and VI RNA I and II abrogate both RNA interference and activation of the anti-viral state via inhibition of protein kinase

Figure 2. The adenovirus genome. The early and late transcripts are labelled in green and blue, respectively, with direction of the arrows indicating the direction of the transcription units. For description of the function of gene products, see text. The ~36 kb Ad genome is depicted in terms of units on a scale of 100. Reproduced with permission from the Society for General Microbiology (79), copyright 2000.


IV
L5
100Kd,33Kd.pVIII
L4
pVI,II,Pr
L3
MLP
E1B
55Kd,19Kd
III,pVII,V
L2
IIIa
L1
E3
12.5Kd,6.7Kd,gp19Kd,
ADP,RIDαβ,14.7Kd
E1A
243R,289R
VA RNAs I&2
0
20
40
60
80
100
E2A
DBP
IX
IVa2
E4
orfs 1-6/7
E2B
pTP
Pol

R signalling (14, 55). The late genes encode the 11 structural proteins required for Ad virion assembly (238).

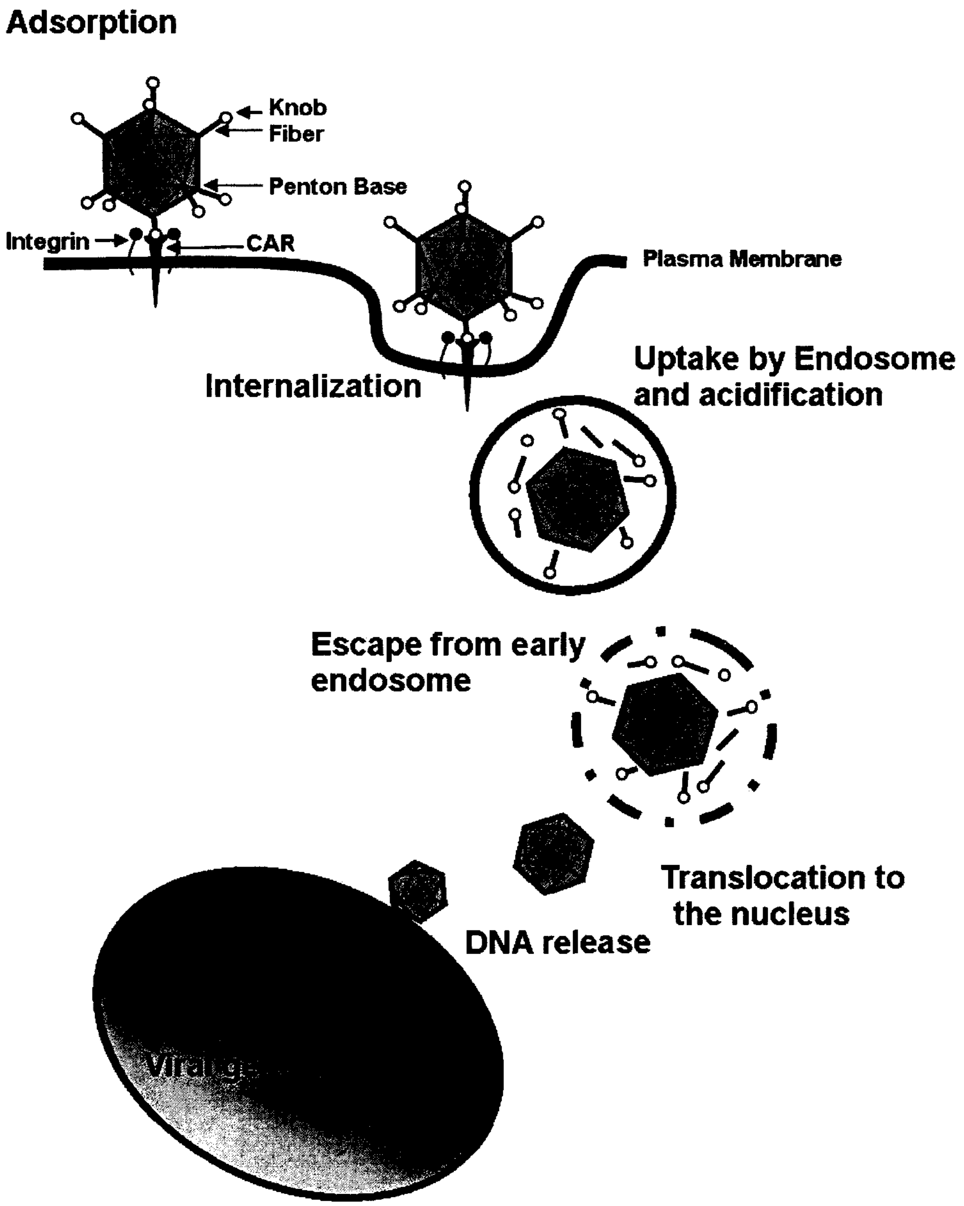
Entry, trafficking, and nuclear translocation

The Ad virion is icosahedral in shape and at each five-fold axis of symmetry a trimeric complex called the fiber protrudes (238) (Fig. 1). Fiber mediates host cell binding by interacting with the primary receptor for host cell internalization, the coxsackievirus and Ad receptor (CAR) (23) (Fig. 3). The high-affinity interaction between fiber and CAR promotes a secondary interaction between the Ad penton proteins at the base of the protruding fiber molecule and cellular integrins, which induces clathrin-mediated internalization of the Ad (304, 307, 308) and subsequent trafficking to the early endosome (165). Once in the endosome the Ad capsid undergoes pH-dependent partial disassembly: fiber and penton fall away from the capsid and protein VI is released, resulting in endosome disruption and escape of Ad into the cytosol (102, 309). Once in the cytosol the partially-disassembled capsid traffics to the nucleus via dynein-mediated translocation along cellular microtubules (164, 189). This process of internalization and nuclear translocation takes less than 1 hour (165, 192).

Adenovirus vectors

The simplest and most commonly-used Ad vectors, so-called first-generation Ad (fgAd) vectors, resulted from deletion of E1A and E1B genes. This disarms the virus by making it replication-incompetent. However, cells such as 293 cells, which

Figure 3. Attachment and entry of host cells by adenovirus. Depicted is a schematic representation of the mechanism of host cell transduction of Ad. For details, see the text. Figure provided courtesy of Kristin Willemsen.


Adsorption
Knob
Fiber
Penton Base
Integrin
CAR
Plasma Membrane
Internalization
Uptake by Endosome and acidification
Escape from early endosome
Translocation to the nucleus
DNA release
Viral ge

encode the left-most 11% of the Ad genome and stably express E1A and E1B (101), complement the E1 deletion and are permissive to Ad vector replication. The Ad virion has an upper packaging limit 37.8 kb (105% of the 36 kb wild-type genome) (26); deletion of E1 therefore confers a cloning capacity of 4.8 kb. Further deletion of the E3 region, which is unnecessary for replication in culture, increases the cloning capacity to 8 kb (214).

Although fgAd are replication-defective in the absence of the E1 region, they are still able to express immunogenic late gene products and to replicate in proliferating cells (71, 205). Even at low levels, replication of Ad vectors can be toxic (206); furthermore, immune responses to genes encoded by the vector backbone result in elimination of transduced cells (71, 320). These limitations of fgAd vectors led to the development of so-called “second-generation” Ad vectors. In addition to deletion of E1, these vectors were deleted-of either E2A (the Ad DNA polymerase) and/or E2B (the Ad pre-terminal protein) (9, 10, 71) and/or E4 (16). These deletions increased vector cloning-capacity to 8-13 kb. However, results suggesting improved safety or efficacy of second-generation Ad vectors have been inconsistent (11, 79).

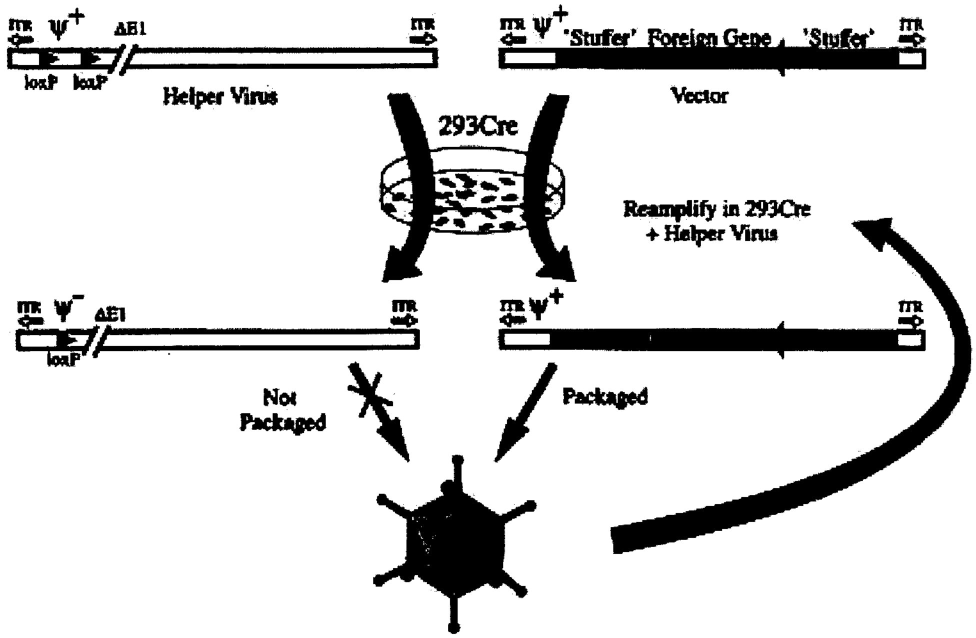
Further improvements of Ad vectors have culminated in the development of vectors devoid of Ad genes, termed helper-dependent Ad (hdAd) vectors (also referred-to as third-generation or gutted Ad vectors (108, 216)). hdAd consist simply of DNA of a size that is within the biophysical packaging capacity of the Ad capsid (i.e., 27-38 kb) (26, 217) bearing the minimal *cis*-acting elements required for vector replication and packaging: the ITRs and packaging sequence (216). The Ad

DNA encoded by these vectors amount to little-over 500 bp; therefore, hdAd have a cloning-capacity of 37 kb. These vectors must be propagated in the presence of a helper virus (i.e., a modified first-generation Ad vector), which produces the *trans*-acting factors required for hdAd genome replication and packaging (216) (Fig. 4).

The packaging sequence of the helper virus is flanked by *lox*P (abbreviated from “locus of X over P1) elements, which are recognition sequences for the recombinase Cre (cyclization-recombination) (216). In 293 cells stably-expressing Cre, the helper virus packaging sequence is excised, resulting in selective packaging of the hdAd vector DNA (Fig. 4). Further purification is accomplished by designing the hdAd genome to have a size that is small enough (i.e., <30 kb) to allow separation from any contaminating helper virus by the use of cesium chloride buoyant density centrifugation. Current technologies allow for the production of high-titer hdAd stocks (i.e., ~1-5 x 10^{12} infectious units (IU)/ml) with helper virus contamination as low as 0.01% (210, 212).

As a result of deletion of viral genes, hdAd vectors exhibit reduced toxicity *in vivo*, reduced immune-mediated elimination of transduced cells, and improved stability of transgene expression (12, 214). In fact, hdAd vectors can persist and express a therapeutic transgene for the lifetime of mice (151) and for at least two years in non-human primates (211). However, much is still uncertain regarding interactions between the non-replicating (216), episomal (110) hdAd genome and the host cell nucleus that allow for such durable transgene expression.

Figure 4. The Cre/*lox*P system for production of helper-dependent adenovirus vectors. Depicted is a schematic representation of propagation of hdAd vectors using a helper virus that encodes a packaging sequence (ψ) that is flanked by *lox*P sites. In the presence of the Cre recombinase, the hdAd vector is selectively packaged. Serial passaging is used to amplify the hdAd vector until desired titers are reached, as described in the text. Reproduced with permission from the lead author (Dr. Robin Parks) (208), copyright 2006.


ITR
Ψ+
ΔE1
ITR
loxP
loxP
Helper Virus
ITR
Ψ+
'Stufer' Foreign Gene
'Stufer'
ITR
Vector
293Cre
Reamplify in 293Cre
+ Helper Virus
ITR
Ψ-
ΔE1
ITR
loxP
ITR
Ψ+
ITR
Not
Packaged
Packaged

Transcriptional targeting

Although many of the drawbacks associated with the use of Ad vectors have been overcome with the development of hdAd, one major problem exists. As mentioned above, Ad has a broad tropism *in vivo*. This is a problem when delivery to a specific cell or tissue type is desired. Firstly, this squelches the therapeutic potential that could be achieved with a single infusion, as transduction of non-target tissues decreases the amount of vector that is available for entry into the target cell-type (25). Secondly, widespread transduction of antigen-presenting cells promotes immune-mediated elimination of transduced cells (145). Thirdly, many transgenes - particularly genes used in cancer gene therapy applications - may be toxic to non-target cells (171). Therefore, great effort has been expended in developing novel ways to target Ad to specific cell types.

Two primary approaches are used to retarget Ad vectors: transductional (97) and transcriptional targeting (239). The aim of transductional targeting is to modify the Ad capsid in such a way that it enters specific cell types. Transcriptional targeting uses genetic regulatory elements to limit transgene expression to the desired cells. Although transductional targeting is theoretically an ideal approach, it has yielded few clinically-relevant successes to date (97). Therefore, transcriptional targeting continues to grow in popularity.

A large number of promoters have been used with varying degrees of success to target Ad gene expression to specific cell types. Ad vectors encoding transgenes driven by a 1.35 kb muscle creatine kinase (MCK) promoter (which confers muscle-specific gene expression in transgenic mice (140)) exhibited strong, muscle-specific expression, despite the presence of viral DNA in numerous non-muscle tissues (162). A truncated, recombinant, 600 bp MCK promoter (called CK6) drove transgene expression that was 600-fold greater in muscle than in liver (112). Expression was also undetectable in human dendritic (antigen-presenting) cells. It should be noted, however, that CK6-driven expression in muscle tissue was only ~16-fold higher than background levels of expression.

Interestingly, orientation of insertion of the transgene expression cassette within the first-generation Ad vector backbone influenced the tissue-specificity of the CK6 promoter: when transcription was driven towards the left ITR, expression was muscle-specific, whereas transcription in the opposite direction resulted in leaky expression in non-myogenic cells (112). Similar results with regards to transcription orientation have also been obtained by others (235). The left ITR partially-encodes the E1A enhancer (111). Therefore, interaction between tissue-specific promoters and transcriptional regulatory elements present within the backbone of both first generation and hdAd vectors may represent an important consideration in the design of Ad vectors with transcription targeted to specific cell types.

Numerous promoters have also been used to restrict Ad vector transgene expression to tumour cells. The human telomerase reverse transcriptase (hTERT) gene is exclusively expressed in immortal cells (152). Few cells in adult humans express this gene, whereas >85% of tumour cells exhibit detectable expression (136). The hTERT promoter has been used to limit expression of Ad-encoded transgenes (105) and replication of conditionally-replicative Ad vectors to immortal cells (92). Other promoters have been used to limit gene expression to specific tumour types, such as prostate (235, 262) and breast cancer cells (261, 263). The major drawbacks that have been reported from studies of transcriptional targeting have been the weak expression of cellular promoters (64, 275, 316) and “leaky” gene expression in non-target cells (124, 125, 262, 263, 323).

My primary concern with regards to transcriptional targeting is that whether Ad DNA assembles into chromatin is unknown (discussed below). This is cause for concern because eukaryotic transcriptional regulatory elements have evolved to function within a chromatin environment. The majority of chromatin assembly is coupled with DNA synthesis. However, hdAd do not replicate; and studies of plasmid DNA suggest that replication-independent chromatin assembly results in the formation of disorganized chromatin (271). Replication-coupled chromatin assembly is also necessary for repression of basal transcription (8). Therefore, hdAd vector promoters may be constitutively-accessible in all cell types. This is particularly alarming in light of the fact that Ad vectors expressing the potent apoptosis-inducing factor p53 (reviewed in (88)) are commercially-available in China (220) and are under evaluation in dozens of clinical trials, in which hundreds of participants have been enrolled (http://www.wiley.co.uk/genetherapy/clinical/).

Studies of plasmid DNA may or may not be predictive of the state of hdAd DNA after nuclear translocation. The only way to determine if Ad assembles into chromatin, and the role of chromatin the regulation of hdAd-encoded genes, is by experimental analysis. Such experiments are essential for a complete understanding of interactions between hdAd vector DNA and the host cell, and for developing safe and effective vectors for gene therapy applications.

The fate of Ad core proteins in the host nucleus

Once the Ad has arrived at the nucleus, the outer-layer of the capsid is fully-disassembled and the viral genome is released (102). Ad DNA is packaged in the form of a nucleoprotein structure called the Ad core (36, 180) (Fig. 1). The Ad core is a sub-virion structure consisting of Ad DNA and the three highly-basic, Ad-encoded proteins V, VII, and mu (36), of which core VII is the major DNA-binding protein (45). After complete capsid disassembly, the viral genome and core VII translocate into the nucleus via a process that is dependent upon the nuclear pore complex protein Nup214, histone H1, and H1 nuclear import factors (294).

The fate of core VII after nuclear translocation is not clearly understood. Early *in vitro* studies showed that isolated Ad cores are not accessible to the basal transcription machinery until they have been "decondensed" by eviction of Ad core proteins (183). The prototypical VII chaperone is template activating factor 1 (TAF-1), which was first identified as a cellular factor that mediates eviction of Ad core proteins (183), but is now better known as the SET (patient SE translocation oncogene (302)). Other proteins that exhibit Ad core decondensation activity include the histone chaperone nucleosome assembly protein-1 (148), B23 (208), and phoshoprotein of 32 kDa (pp32) (318). These proteins are all highly acidic and likely mediate eviction of core proteins from DNA via charge neutralization. Interestingly, Ad core proteins are similar to the protamines that package sperm DNA (42, 282) and many of the proteins implicated in Ad core decondensation have also been implicated in removal of protamines from incoming sperm DNA in the oocyte (182, 221, 222, 236) (reviewed in (187)).

Conflicting data suggest that VII either stably associates with Ad DNA throughout the early phase of infection (45, 318), or is evicted within a few hours (277). Analysis of ^{3}H-arginine-labelled Ad revealed that core VII is the only Ad protein that is detectable in the host nucleus 2 hpi (45). Further analysis of proteins cross-linked to ^{32}P-labelled Ad DNA suggested that core VII associated with wild-type Ad DNA throughout the early phase of infection (45, 46). However, this interaction did not change from 1-51 hpi, despite detectable levels of late protein expression. Although newly translated pre-VII (a VII precursor, which is cleaved by the Ad protease during virion maturation (13)) was detected in these cells, only mature, cleaved VII was associated with parental, ^{32}P-labelled Ad DNA. Therefore, the association between core VII and Ad DNA was likely an artefact attributable to defective Ad particles that were unable to enter the nucleus (102).

Immunofluorescence-based experiments also suggested that core VII remains associated with viral DNA after host cell transduction and that transcription is necessary to evict core VII from hdAd vector DNA (48). However, the hypothesis that transcription was necessary for core VII eviction was not tested directly. Rather, the authors used an hdAd vector that did not encode an exogenous promoter and assumed that it was not transcribed. However, the likelihood that active transcription was driven away from the right ITR was very high, considering that the hdAd backbone that was used encodes the E4 promoter (244). This fact calls into question the validity of the authors' conclusions.

In contrast to the results described above, work by David Spector suggests that core VII dissociates from Ad DNA within a few hours of nuclear translocation.

When chromatin was obtained from Ad infected cells, sheared by sonication, and analyzed by Southern blot, shear-sensitive and shear-resistant Ad DNA was observed. The proportion of DNA that was shear-sensitive increased from ~35% to ~90% between 1.5 and 4.5 hpi, which is indicative of a change in the Ad nucleoprotein structure in the host nucleus (278). Furthermore, chromatin immunoprecipitation analyses showed that the majority of VII still present within the nucleus was not associated with Ad DNA (277). Therefore, whether VII dissociates from Ad DNA after host cell transduction has not been clearly determined.

Ad and chromatin

A number of early studies suggested that soon after nuclear translocation, wild type Ad DNA assembled into chromatin similar to that of the host cell (chromatin is discussed in detail in **1.1.3**). This conclusion was based upon experiments using micrococcal nuclease (MNase), a secreted endonuclease encoded by *Staphylococcus aureus* (113). MNase has been a commonly-used reagent for probing chromatin structure for over 30 years, since Felsenfeld and colleagues discovered that DNA assembled into chromatin is resistant to MNase digestion (54), as MNase preferentially digests the bare linker DNA between nucleosomes (257). When chromatin is digested with MNase, deproteinated and analyzed by agarose gel electrophoresis, an oligomeric ladder is produced of DNA bands with a repeat-size of ca. 180-200 bp (19, 257). These bands represent DNA that was assembled into oligonucleosomes and the linker DNA between those nucleosomes.

Ad core DNA from capsids exhibits no significant protection from MNase (300). Therefore, DNA released from the Ad capsid into the nucleus should exhibit distinct patterns of MNase-resistance compared to cellular chromatin. A number of studies have suggested that wild type Ad exhibits protection from MNase at some points during its life cycle; however, contradictory data abound. Two studies in which Ad DNA from MNase-digested nuclei was analyzed by Southern blot suggested that Ad DNA was protected from MNase within 3-6 h post-infection (hpi); this nucleoprotein structure was also observed during the late phase of infection (255, 290). Results from another study suggested that assembly of Ad DNA into chromatin is coupled with DNA replication (67). Also, during the late phase of infection, several groups have observed Ad DNA assembling into a non-repeating nucleoprotein complex that is distinguishable from cellular chromatin (37, 63, 67).

Two groups that used chemical cross-linking experiments to examine Ad nucleoprotein complexes within the host cell each found no evidence for association with histones or assembly into chromatin (46, 313). These results led one group to repeat Southern analyses of MNase-digested chromatin from Ad infected cells (313). They initially observed DNA resembling cellular chromatin. However, when they increased the stringency of their washes, the "nucleosomal" signal disappeared, although the ability of the probe to hybridize to control viral DNA was unaffected. This led them to conclude that cross-hybridization between Ad probe DNA and cellular DNA was responsible for the appearance of Ad DNA assembled into chromatin. In conclusion, whether Ad or Ad-derived vectors assembly into chromatin is unknown. This is surprising in light of the fundamental importance of chromatin in regulating gene expression within the cell (167, 271).

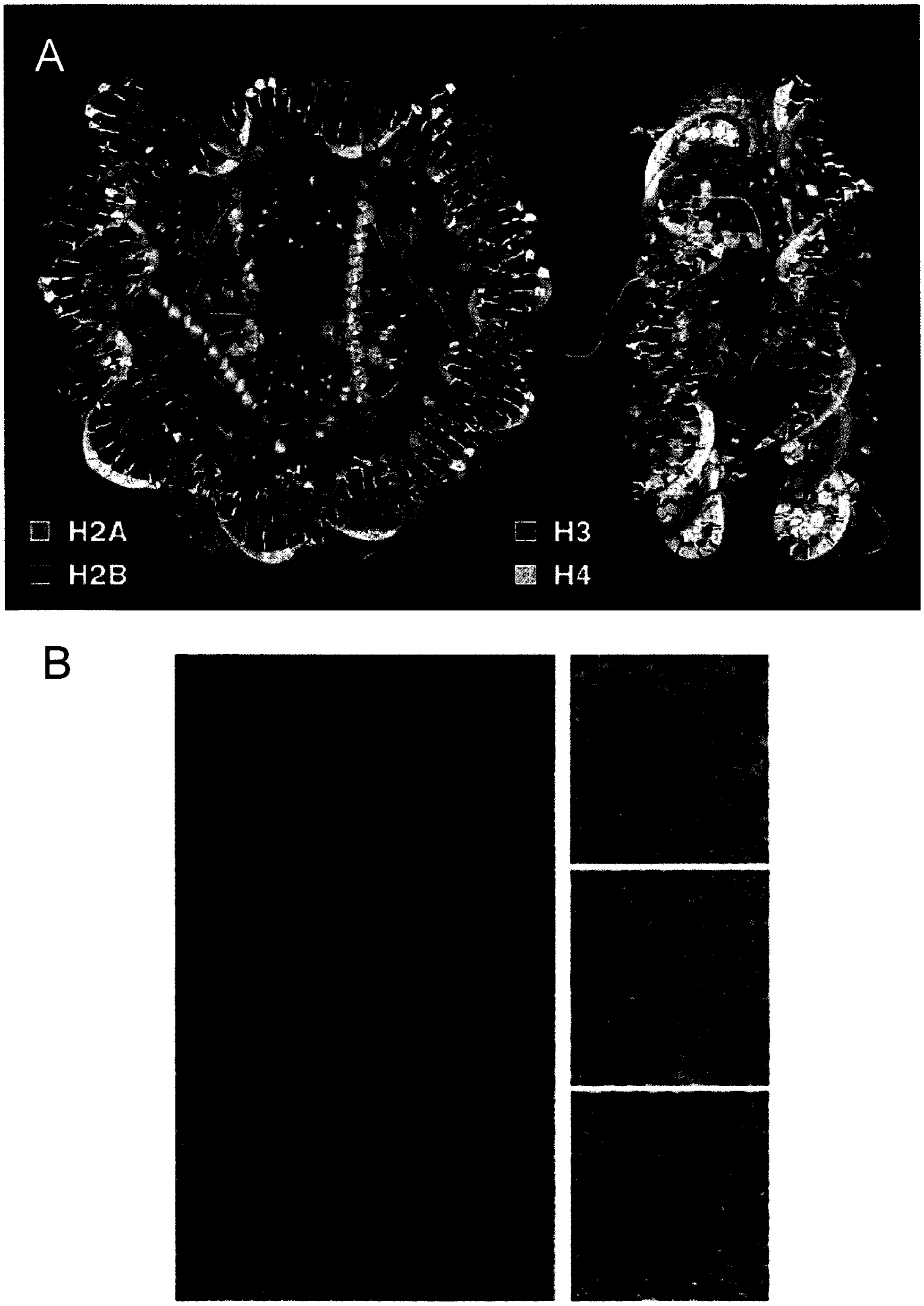
1.1.3 - Chromatin

Chromatin structure

If the DNA of each chromosome in a single human cell were isolated and joined end-to-end, it would be at least 1 meter in length; in most of the billions of cells in the human body, this DNA is packaged into a nucleus with a diameter of about 10 micrometers (311). This remarkable degree of compaction is accomplished by packaging of DNA into a nucleoprotein complex, called chromatin (for a comprehensive review, see (311)).

The fundamental unit of chromatin is the nucleosome, which consists of ca. 146 base pairs of DNA wrapped 1.5 turns around an octamer of proteins called histones (two copies each of H2A, H2B, H3, and H4) (7) (Fig. 5A). Nucleosomes are regularly-spaced on DNA and are separated by ca. 40 bp in most human cells (57). Due to their appearance in electron micrographs, nucleosomes are referred to as having a "beads-on-a-string" structure (209, 293) (Fig. 5B). DNA between nucleosomes (i.e., the string) is called linker DNA and is bound by linker histones, most notably histone H1 (311). The linear of arrangement of nucleosomes in DNA represents the primary level of chromatin structure. Secondary and higher levels of chromatin structure represent two- and three-dimensional folding of primary

Figure 5. The nucleosome. (A) Ribbon trace of the nucleosome at 2.8 angstrom resolution. The DNA backbone is depicted in brown and turquoise; histones are depicted in the indicated colours. Left is a "top-down" depiction of the nucleosome, whereas right is a "side-view". Ribbons that protrude from the nucleosome core represent the histone tails, whose post-translational modifications are discussed in detail in the section detailing the histone code. Reprinted by permission from Macmillan Publishers Ltd: Nature (167), copyright 1997. (B) Electron micrographs of chromatin. Left shows the "beads on a string" primary structure of nucleosomes, which is described in the text (arrows indicate "beads"). Right shows individual nucleosomes that have been released from bulk chromatin by digestion with MNase, as described in the text. Reprinted by permission from Macmillan Publishers Ltd: Nature Reviews Molecular Cell Biology (201), copyright 2003.


A
☐ H2A
☐ H2B
☐ H3
▨ H4
B

structures (reviewed in (314, 315)). Higher-order chromatin structures culminate in the architectural landscape of the chromosome, which often take the form of large loops of hundreds of kb of DNA (311, 314).

Cytological analyses of chromosomes revealed that some regions were more densely-packed than others throughout the cell cycle (114) (reviewed in (218)). These dense regions were called heterochromatin. We know now that heterochromatin contains primarily transcriptionally-inactive DNA, whereas its cytological opposite, euchromatin, contains transcriptionally-active genes (123). Heterochromatin comes in two forms: constitutive and facultative (reviewed in (61)). Constitutive heterochromatin packages primarily pericentromeric and telomeric DNA and repetitive elements, and exhibits little variability between cell types within a species. Facultative heterochromatin consists of once-euchromatic genes that have been actively repressed by assembly into heterochromatin; the identity of these genes differs in a cell-type specific manner (103). The molecular hallmarks of heterochromatin and euchromatin will be discussed below.

Chromatin assembly

The minimal requirements for assembly of physiologically-spaced chromatin (i.e., one nucleosome every 180-200 bp, depending upon organism and tissue type (57)) are DNA, histones, a histone chaperone, and an ATP-dependent chromatin remodeling protein (223). In the absence of a chaperone, basic histones non-specifically interact with acidic DNA, resulting in the formation of an insoluble, non-nucleosomal aggregate (69). Chaperones mediate the ordered deposition of histones on DNA, resulting in random nucleosome placement and unordered spacing. However, upon addition of an ATP-dependent chromatin remodeling protein to the assembly reaction, the result is an ordered array of physiologically-spaced nucleosomes (130).

The first histone chaperone identified was nucleoplasmin (69). Nucleoplasmin is the most abundant protein in *Xenopus* oocytes and participates in the sequestration and deposition of the massive histone store that is necessary for swift assembly of incoming sperm DNA into chromatin (221, 222). Orthologues of nucleoplasmin have been identified in flies (132) and mammals (248) (called B23/nucleophosmin in humans), and their roles in chromatin assembly appear to be evolutionarily conserved. Other human proteins that have histone chaperone activity are NAP-1 (129), histone cell cycle regulation defective homolog A (HIRA) (228), anti-silencing function 1 homologue (ASF1) (62), suppressor of Ty 16 homologue (Spt16) (22), and chromatin assembly factor 1 (CAF-1) (272).

ATP-dependent chromatin remodeling proteins are molecular motors that provide catalytic activity to protein complexes that modify chromatin structure. The first chromatin remodeling complex to be implicated in chromatin assembly was the 220 kDa ATP-dependent chromatin assembly and remodeling factor (ACF) complex, which is composed of the ATPase domain-containing protein imitation switch homologue (ISWI) and another protein termed Acf1 (131). In addition to ACF, ISWI provides catalytic activity to several other chromatin remodeling complexes (reviewed in (177)). Chromodomain helicase DNA binding domain 1 (Chd1) is the only other chromatin remodeling protein that exhibits chromatin assembly and spacing activity although it does so as a monomer and, unlike ISWI-containing complexes, it is unable to incorporate histone H1 into chromatin (178).

The vast majority of chromatin assembly occurs during DNA synthesis and is coupled to passage of the replication fork (311). The proliferating cell nuclear antigen (PCNA) protein is essential for replication-coupled chromatin assembly: PCNA forms a multimeric complex that encircles replicating DNA and coordinates assembly of chromatin on both the mother and daughter strands (299) by recruiting the H3/H4 chaperone CAF1 (264) and the ISWI homologue SNF2H (224) to the replication fork. Although the majority of chromatin is assembled during DNA replication, cells can also assemble chromatin independently of replication.

The mechanism of replication-independent chromatin assembly is best understood in two biological processes: reassembly of chromatin in the wake of elongating RNA polymerase II (RNApII) (22, 139, 194, 250, 251) and *de novo* assembly of sperm DNA (which is packaged in protamines) into chromatin after entry into the oocyte (32, 155, 174, 221, 236). Both of these processes are distinguished from replication-coupled chromatin assembly by their exclusive incorporation of the H3 variant H3.3 and requirement of the H3.3 chaperone HIRA (4, 32, 174, 285). During DNA replication CAF-1 cooperates with Asf1 to deposit the canonical histone H3.1 (245, 285). HIRA also interacts with Asf1; however, biochemical studies have shown that CAF-1 and HIRA exclusively bind H3.1 and H3.3, respectively, *in vivo* (285). During RNApII elongation, Spt16 (as a member of facilitates chromatin transcription (FACT) complex) (181) and Asf1 (249) mediate eviction and deposition of H2A/H2B and H3/H4, respectively. However, whereas the preexisting chromatin downstream of RNApII contains H3.1, newly assembled chromatin contains H3.3 (4, 194, 251).

Yeast mutants lacking either FACT (181) or Asf1 (249) activity exhibit transcriptional elongation defects. The absence of these proteins also results in aberrant transcription from cryptic promoters within transcribed genes, due to their inability to properly assemble chromatin that has been disrupted by passage of RNApII. Elongating RNApII interacts not only with histone chaperones, but also with Chd1 (157, 185, 268), a protein that is necessary for *de novo* chromatin assembly in *Drosophila* (155). Therefore, transcriptional elongation could promote *de novo* assembly of Ad DNA into chromatin.

Mechanisms of replication-coupled and -independent chromatin assembly are distinct; so too are the templates for transcription that are produced by these processes. Comparison of the murine mammary tumor virus (MMTV) long terminal repeat promoter encoded by either transiently-transfected or stably-replicating templates has revealed that the mechanism of chromatin assembly plays an important role in the regulation of gene expression (reviewed in (271)). MMTV integrates into the host genome as a part of its lifecycle (301). Therefore, the MMTV promoter has evolved to function within the context of chromatin that was assembled during host DNA replication (311). In the context of stably-replicating chromatin, the MMTV promoter is assembled into four positioned nucleosomes, which exclude transcription factors and repress basal transcription (34, 86). Ligand-bound glucocorticoid receptor (GR) enters the nucleus, binds specific sequences in the MMTV promoter, and recruits proteins that remodel local chromatin (84, 141, 295). This chromatin remodeling event promotes accessibility of MMTV promoter DNA and allows the transcription factors nuclear factor I (NFI) and octamer-binding transcription factor 1 (Oct1) to bind their recognition sequences within the promoter (15, 58). These factors then recruit the basal transcription machinery, resulting in activation of gene expression (15). In contrast, when DNA encoding the MMTV promoter is transiently-transfected into cells, the newly-assembled chromatin is relatively poorly-compacted and nucleosomes are not properly organized (43, 138). This disorganized chromatin structure fails to occlude NFI and Oct1, resulting in constitutive, GR-independent transcription (15). Whether hdAd vector DNA and plasmid DNA undergo chromatin assembly via similar or distinct mechanisms is unknown. However, elucidation of structure and function of hdAd chromatin is necessary for the development of safe and effective hdAd vectors for gene therapy.

Chromatin Dynamics

Chromatin is an inherently repressive structure that blocks access to DNA by cellular factors involved in nuclear processes such as transcription, DNA replication, and recombination (311). Therefore, chromatin structure must be dynamic (reviewed in (242)). Chromatin fluidity is accomplished via three mechanisms: (1) histone exchange, (2) ATP-dependent chromatin remodeling and (3) post-translational histone modifications.

Histone exchange involves removal of canonical histones deposited during S phase and replacement with specialized histone variants (4, 107, 116). H2A has three variants: H2AX, H2AZ, and macroH2A (17). H2AX is phosphorylated in response to double stranded DNA breaks and coordinates recruitment of repair enzymes (219). H2AZ and macroH2A play poorly-understood roles in transcriptional activation and repression, respectively (17). Mammals have five H3 variants: H3.1, H3.1t, H3.2, H3.3, and Cenp-A (107, 116). H3.1 is the canonical H3. H3.2 differs from H3.1 at only one amino acid (C96S) and is also deposited during S phase (5). H3.1t is a testis-specific H3 variant (107). Human H3.3 differs from H3.1 and H3.2 at five and four amino acid positions, respectively. *Drosophila* encode only one canonical H3 (which is simply called H3), and mutation of three amino acids - 87, 89, and 90 - from H3 to H3.3 identities confers replication-independent assembly into chromatin (5). Cenp-A replaces canonical H3 in centromeric nucleosomes, where it plays an important role in centromeric identity and chromosome segregation (107).

ATP-dependent chromatin remodelling proteins are molecular motors that assemble into multi-subunit protein complexes that utilize energy from ATP to assemble, disassemble, or mobilize nucleosomes (reviewed in (242)). Each nucleosome has a core unit consisting of a heterotetramer of H3/H4 proteins and two peripheral H2A/H2B dimers (311), and ATP dependent chromatin remodelers regulate H2A/H2B dimer exchange (39). They also participiate in exchanging canonical and variant histones (195).

Post-translational histone modifications are thought to represent a “histone code” that is interpreted by protein-protein interaction domains, which mediate recruitment of proteins to specifically-modified histones (137). Therefore, these modifications are key signals that regulate accessibility to DNA within the chromatin landscape (137, 281). The best-studied histone modification is acetylation of lysine (K) residues in the N-terminal tails of H3 and H4, which is associated with transcriptionally active euchromatin. Acetylation is a reversible process, and deacetylase activity is associated with proteins that repress transcription (137).

Two papers published in 1996 unified the studies of transcription and chromatin by providing mechanistic insight into the role of histone acetylation in the regulation of gene expression, and also initiated a revolution in the study of epigenetics. These papers, by David Allis and colleagues (38) and Stuart Schreiber and colleagues (291), described the earliest cloning and characterization of genes encoding histone-modifying enzymes: *Tetrahymena* histone acetyltransferase (HAT) A and human histone deacetylase (HDAC) (originally called HD1), respectively. The most significant aspect of these studies was that both molecules exhibited homology with proteins that previously had been identified as putative transcriptional regulators in genetic studies in budding yeast. Specifically, HAT1 and HD1 were remarkably-similar to the transcriptional activator Gcn5p (96) and transcriptional repressor Rpd3p (96), respectively. These studies provided the first direct evidence for chromatin in mediating the effects of transcriptional regulatory proteins and provided the foundation for the development of the histone code hypothesis.

The histone code

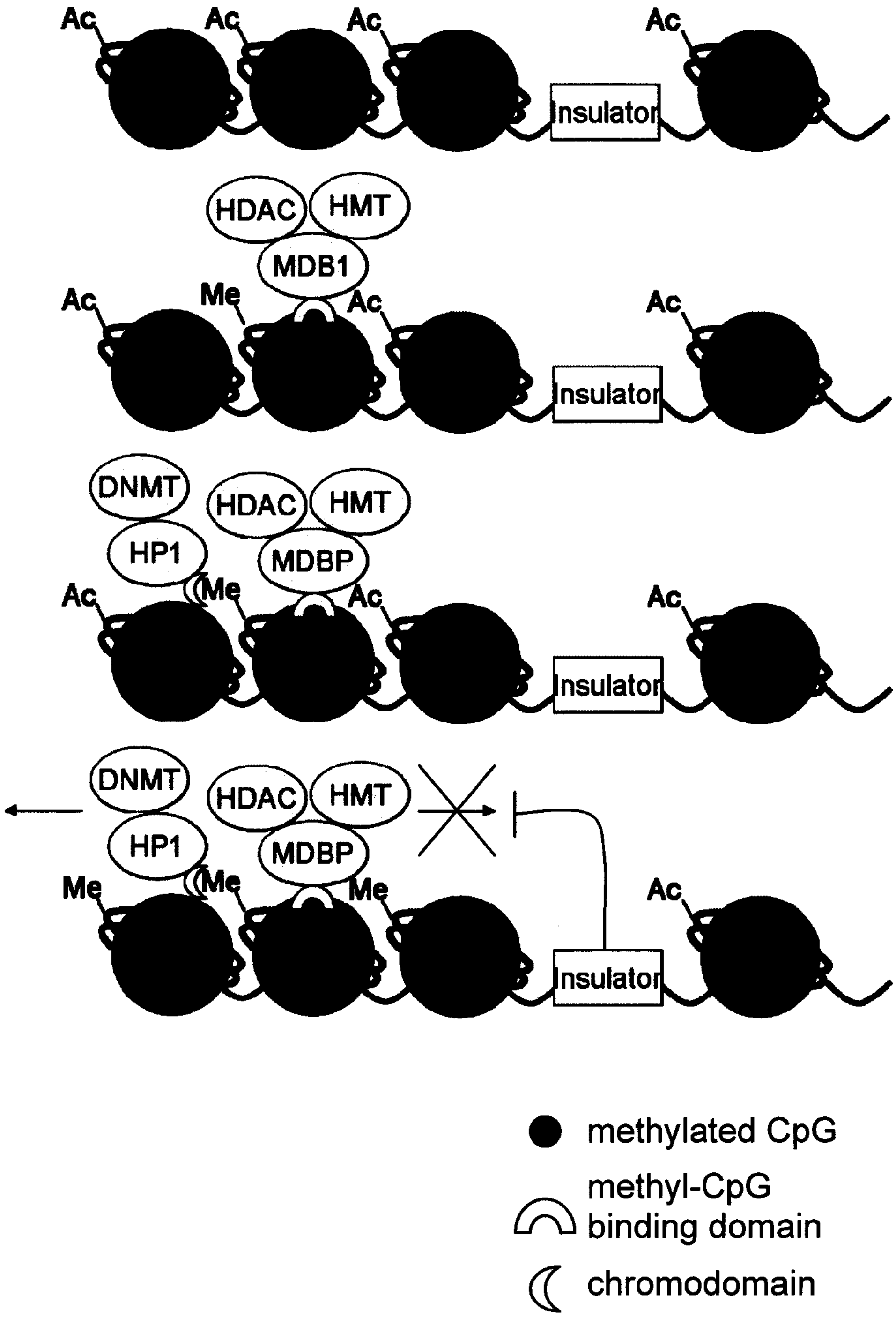
The histone code was originally conceived by analysis of post-translational histone modifications that were exclusively present at transcriptionally-active (euchromatic) or transcriptionally-silent (heterochromatic) genes (137). For example, acetylation of H3 K9, diacetylation of H4 K5/K9 or K12/K16, and methylation of H3 K4 are associated with euchromatin, whereas methylation of H3 K9 or H3 K27 is associated with heterochromatin. The transcriptional effects of these modifications are mediated by proteins with motifs that interact with histone tails with highly-specific affinity. The best-understood of such motifs are the bromodomain and chromodomain, which interact with acetylated and methylated lysine residues, respectively (137). Bromodomains are typically-found in proteins that are associated with transcriptional activation. For example, the largest subunit of the TATA box binding protein (TBP) complex, TBP-associated factor 1, has two adjacent bromodomains whose structure and spacing confer specificity for diacetylated H4 (134). Chromodomains, on the other hand, are found in proteins that participate in both activation and repression of gene expression. For example, heterochromatin protein 1 (HP1) and polycomb group proteins are recruited to methylated H3 K9 and H3 K27, respectively, to mediate transcriptional repression (83); whereas the Chd1 chromodomain binds methylated H3 K4 and recruits HATs to promote gene expression (225).

In addition to affecting the local chromatin environment, histone modifications can have far-reaching effects due to spreading by self-propagation. The best-studied example of the spreading of histone modifications is heterochromatin spreading, which was first identified as position effect variegation (PEV) in *Drosophila*. PEV was first observed in flies that had an X-ray-induced X-chromosome inversion (197) (reviewed in (123)). The phenotype of these flies was a mosaic, mottled red and white eye color that resulted from random silencing of the *white* gene, which is necessary for red eye color. This random inactivation of a euchromatic gene was the result of the inversion, which placed the *white* gene adjacent to pericentromeric heterochromatin. In some cells the heterochromatin spread from the nearby pericentromeric locus into the *white* gene, resulting in its heritable inactivation. Several proteins that are necessary for heterochromatin formation in mammalian cells, such as the histone methyltransferase Suppressor of variegation 3-9 homologue 1 (Suv39H1) and HP1, were originally identified as dominant suppressors of variegation in genetic studies of *Drosophila* (303).

In mammalian cells DNA methylation plays an important role in heterochromatin formation (reviewed in (27)). DNA methyltransferase enzymes catalyze the addition of a methyl group to carbon 5 of cytosine residues present in cytosine-guanine (CpG) motifs. In mammalian genomes, CpG motifs are rarely found in abundance outside of promoter regions (so-called CpG islands) and DNA of transcriptionally-inactive promoters are often hypermethylated (28). DNA assembled into constitutive heterochromatin is also hypermethylated.

Although the mechanistic basis of heterochromatin spread is not perfectly understood, a feed-forward mechanism has been suggested (303) (Fig. 6). One known catalyst of heterochromatin formation is CpG methylation (146, 147). *De novo* methylation of CpG elements results in the recruitment of proteins that have methyl-CpG binding domains, such as methyl-CpG binding protein 2 (MeCP2) and methyl-CpG-binding domain 1 (MDB1) (115, 202). These proteins interact with chromatin modifying enzymes, such as HDACs (144) and histone methyltransferases (91). Deacetylation of H3 K9 allows methylation by MeCP2-

Figure 6. Heterochromatin formation and spreading. Schematic representation of formation of heterochromatin after methylation of nucleosomal-DNA. Spreading of heterochromatin is only stopped by an insulator (barrier) element. For details, see text. Adapted by permission from Macmillan Publishers Ltd: Nature (79), copyright 2001.


Ac
Ac
Ac
Ac
Insulator
HDAC
HMT
MDB1
Ac
Me
Ac
Ac
Insulator
DNMT
HDAC
HMT
HP1
MDBP
Ac
Me
Ac
Ac
Insulator
DNMT
HDAC
HMT
HP1
MDBP
Me
Me
Me
Ac
Insulator
● methylated CpG
methyl-CpG binding domain
chromodomain

associated proteins (94) and by MDB1-associated SETDB1 (which stands for SET (Su(var)3-9, enhancer of zeste, trithorax) domain bifurcated 1) (247) to catalyze H3 K9 methylation, which provides a docking site for recruitment of HP1 (133). HP1 is a key mediator of the feed forward mechanism of heterochromatin spreading, as it interacts not only with itself (273), but also with the *de novo* DNA methyltransferase (DNMT) proteins DNMT3a and DNMT3b and with the H3 K9-specific histone methyltransferase Suv39H1 (93).

Heterochromatin requires a nucleation point (e.g., methylated DNA); however, once heterochromatin begins to form, continual recruitment of factors that promote heterochromatin at nearby loci results in the spread of DNA and histone methylation and HP1 recruitment (91, 123, 144, 146, 247). Why then is the entire human genome not assembled into heterochromatin? The answer is "insulator elements", which block the spread of both transcriptionally-active and -repressive chromatin between genomic loci (81, 176) (Fig. 6). Insulators are defined by having one or both of the following activities when placed between a genetic regulatory element and a promoter: (1) enhancer-blocking and (2) barrier activity. Enhancer-blocking is self-explanatory; barrier activity refers to the ability of an insulator to block heterochromatin spreading. The best-studied insulator element was derived from the DNase hypersensitive (HS) 4 site of the chicken β-globin locus. This element encodes binding sites for two transcription factors, upstream transcription factor 1 (USF1) (306) and CCCTC binding protein (CTCF) (21), which are primarily responsible for the HS4 element's barrier and enhancer blocking activities, respectively. USF1 recruits enzymes that catalyze methylation of H3 K4 and acetylation of numerous lysine residues, thus preventing the spread of heterochromatin (306). CTCF homo-oligomerizes to promote the formation of chromatin loops. This activity contributes to enhancer blocking, although the mechanism is poorly understood (reviewed in (95)). Two tandem copies of the minimal elements of the HS4 insulator have been assembled into 500 bp element called the HS4 double core insulator (229). Other genomic elements with barrier activity include transfer RNA genes in *Saccharomyces pombe* and small interspersed nuclear elements in mice (176).

1.1.4 - hdAd stuffer DNA

In addition to examining whether hdAd vectors assemble into chromatin, a second goal of my research was to test the role of chromatin in the regulation of gene expression from hdAd vectors. Specifically, I chose to examine the role of chromatin in repression of gene expression from hdAd vectors encoding 22 kb of prokaryotic DNA.

As discussed above, hdAd vectors typically require "stuffer" DNA to meet the minimal size required for packaging within the Ad capsid (i.e., 28 kb). As a simple proof of principle that the Cre/*lox*P system (described above) could be used to propagate hdAd, a vector called pRP1001 was generated. This vector was devoid of Ad structural genes and encoded 8.4 kb of stuffer DNA derived from bacteriophage lambda, the E1A and E4 genes, and the β-galactosidase (β-gal) reporter gene (*lacZ*). This vector was successfully propagated and purified using the Cre/*lox*P system and exhibited expression levels that were nearly identical to first generation vector encoding the same expression cassette (R. Parks, personal communication). As a next step, Parks et al. generated a vector entirely devoid of Ad genes (originally called hdAd-RP1030, herein-referred to as hdAd-prok) (215), which was stuffed with 22 kb of DNA derived from bacteriophage lambda. However, when compared with hdAd-RP1001 hdAd-prok expression was much lower, both *in vivo* and *in vitro*. The authors tested whether the origin of the stuffer DNA was in any way responsible for the precipitous drop in gene expression by replacing the lambda DNA with human genomic DNA derived from the hypoxanthine phosphoribosyltransferase (HPRT) gene (originally called hdAd-RP1050, herein-referred to as hdAd-euk). This simple change in non-coding stuffer DNA resulted in a dramatic improvement in gene expression.

The repressive effects of prokaryotic DNA on transgene expression have become well-known in the gene transfer community, although the mechanistic basis of this phenomenon is poorly understood. In hdAd, HSV, and plasmid vectors, covalent linkage of eukaryotic transgenes to prokaryotic DNA results in poor expression that is swiftly silenced (49, 51, 215, 232, 283). Removal of prokaryotic antibiotic resistance genes and replication origins from HSV amplicons increased transgene expression by 20-fold *in vitro* and extended the duration of expression *in vivo* (283). Silencing of transgene expression in HSV amplicons and plasmid DNA was at the level of transcription, and distinct histone modifications indicative of transcriptionally inactive chromatin were associated with the presence of the prokaryotic DNA (232, 283). These data indicate that prokaryotic DNA represses transgene expression by stimulating the formation of repressive chromatin on the transgene. Studies using plasmid DNA devoid of bacterial sequences also clearly showed that prokaryotic DNA must be covalently-linked to the transgene in order to repress transgene expression (49-51, 232, 233). Prokaryotic DNA therefore represses the expression of eukaryotic transgenes in *cis*, likely at the level of chromatin-based transcriptional repression; however, the mechanism of this phenomenon is poorly understood.

How do sequence or base-composition biases in prokaryotic DNA repress covalently linked transgenes? Unlike vertebrate DNA, in which CpG dinucleotides are under-represented, prokaryotic DNA contains the statistically expected number of CpG motifs (28). In eukaryotic cells, CpG motifs are often found in abundance exclusively in promoter regions, and CpGs are typically methylated in transcriptionally inactive promoters (27). CpG methylation can actively induce the silencing of gene expression by recruiting factors that deacetylate and methylate K residues in histone tails, leading to the formation of chromatin structures that are refractory to transcription, as described above (27, 29, 35, 137, 154). I, among others, hypothesized that the repressive effects of prokaryotic DNA on eukaryotic expression cassettes are due to the presence of abundant CpG motifs (49, 51, 215, 232, 233, 283); methylation of these CpGs may lead to the formation of nucleation sites for transcriptionally repressive chromatin, which would spread from prokaryotic DNA to transgenes and, thus, silence gene expression.

1.1.5 - Innate nuclear defense

Although DNA methylation is one possible explanation for the repressive effects of prokaryotic DNA, a second possible mechanism is active repression of a linked transgene by nuclear defense factors. Metazoans have evolved innate mechanisms that recognize and defend against intracellular pathogens (reviewed in (188)). The best studied arm of the innate defense system is innate immune signalling, which involves recognition of pathogen-associated molecular patterns and induced secretion of factors that communicate the potential danger to the immune system and nearby cells. Another form of innate defense that has recently come to the forefront of virology research is here termed "innate nuclear defense" (IND). IND involves recognition of foreign DNA by mechanisms that are still poorly-understood, and subsequent inhibition of expression of encoded-genes.

PML bodies

Innate immunity and IND are likely linked by promyelocytic leukemia (PML) bodies (also referred-to as PML nuclear bodies, PML oncogenic domains, and nuclear dot 10 elsewhere) (reviewed in (72, 74, 230)). PML bodies are large (ca. 1 micron in diameter) subnuclear domains that require PML for their assembly and have been implicated in nearly every aspect of cellular physiology, including cell division, senescence, apoptosis, transcription, protein degradation, chromatin structure and dynamics, and response to cellular stress (24). In addition to PML, other proteins that constitutively localize to PML bodies are speckle protein of 100 kDa (Sp100), the fas death domain-associated protein (Daxx), and small ubiquitin-like modifier 1 (SUMO-1) (a protein that is covalently-linked to PML, which is necessary for PML body assembly (53, 198, 279)). All of these proteins have been implicated in IND, particularly in response to DNA virus infection (41, 198, 204, 241).

PML bodies are thought to be important in innate defense against pathogens for five key reasons (72, 74, 230). (1) PML body size and number increase in response to interferon stimulation. (2) PML and Sp100 are transcriptionally-upregulated by interferon. (3) Large DNA viruses associate with PML bodies after nuclear translocation. (4) These viruses encode gene products that reorganize PML bodies, which are necessary for productive infection. (5) Viral genomes do not traffic to PML bodies; rather, PML bodies assemble *de novo* near viral genomes. Therefore, there has been great interest in determining the role of PML bodies in the regulation of viral replication.

Early immunofluorescence-based studies suggested that many DNA viruses, such as Ad (68), HSV (184), and cytomegalovirus (CMV) (150), among others, swiftly accumulated at PML bodies after nuclear translocation (please note that I will refer to herpes simplex virus 1 as HSV; any reference to "herpes viruses" is a reference to the phylogenetic lineage of viruses that includes HSV, CMV, and Epstein-Barr virus, among others). These viruses encode phylogenetically-distinct gene products that are responsible for reorganizing or disrupting PML bodies (reviewed in (72)). Ad E4 open reading frame (orf) 3 reorganizes PML bodies into filamentous structures (166). Avian Ad-encoded *Gallus* anti morte 1 (Gam1) disrupts PML bodies by blocking SUMOylation of PML (56). The HSV-1-encoded infected cell protein 0 and cytomegalovirus-encoded immediate-early 1 target PML for proteasome-dependent (47, 73) and -independent degradation, respectively

(317). Mutation of any of these genes results in a dramatic reduction in viral replication.

Sp100 and Daxx

Other PML body constituents have been implicated in regulation of viral gene expression. Human cells encode four Sp100 isoforms (204): A, B, C, and high mobility group (HMG; the name refers to the fact that this isoform has an HMG-like putative DNA binding domain (106)). Sp100A is exclusively-associated with PML bodies and is a known activator of herpes virus gene expression (30, 170), whereas the other three isoforms are diffusely localized throughout the nucleus and repress HSV transcription and replication (204), and expression of transfected reporter genes (310). Interestingly all three putative repressor isoforms – but not Sp100A – have a DNA binding domain that specifically binds unmethylated CpG motifs (127). Therefore, in the absence of DNA methylation, Sp100 repressor isoforms may repress expression of hdAd-prok.

Another PML body constituent that has been implicated in antiviral defense is the co-repressor protein Daxx. CMV capsids contain a protein called phosphoprotein of 71 kDa (pp71) that translocates to the nucleus upon host cell infection (117); this process is necessary for efficient expression of CMV immediate early genes (40). The primary function of pp71 is to degrade Daxx (40, 41, 120, 126, 128, 226, 240, 241). A mutant virus expressing pp71 that is unable to bind Daxx is nearly incapable of replicating *in vitro* at low multiplicity of infection (MOI) (41). Biochemical fractionation studies have shown that Daxx interacts with HDACs

(122) and Daxx-mediated histone deacetylation represses expression of murine CMV gene expression (289). Importantly, although PML is necessary for formation of PML bodies, both Sp100 and Daxx are recruited to newly-delivered viral genomes in the absence of PML (76). Therefore, Sp100 and Daxx may mediate IND against DNA viruses.

What hallmarks might separate "foreign" DNA from host DNA? Like large DNA viruses, small DNA viruses also associate with PML bodies (65, 288). However, PML promotes establishment of papillomavirus infection (65) and PML bodies are likely to be the major site of papillomavirus capsid assembly (284). Polyomaviruses, such as john cunningham virus (266), BK virus, and SV40 (266) also assemble into virions near PML bodies. Small DNA viruses have CpG and GC content that is similar to that of host DNA, whereas large DNA viruses typically have high GC content and encode the statistically-expected number of CpG motifs (256). Interestingly, hdAd-prok stuffer DNA has GC and CpG content that are similar to those of large DNA viruses (215, 256). Therefore, like large DNA viruses, hdAd-prok may be recognized as foreign by IND factors, resulting in repression of gene expression.

1.2 – Objectives

At the outset of my research, my goal was "simple": to determine the mechanism by which transgenes covalently-linked to prokaryotic DNA are repressed in mammalian cells. Upon analysis of my initial results I developed three objectives for my thesis research:

1) Determine the kinetics and mechanism of hdAd vector chromatin assembly.

2) Determine the role of chromatin in mediating the repressive effects of prokaryotic DNA on eukaryotic transgenes.

3) Examine the role of innate nuclear defense proteins in mediating the repressive effects of prokaryotic stuffer DNA.

1.3 - Hypothesis

The underlying hypothesis for all of the research described here is that chromatin will have an important role in regulating hdAd-encoded transgenes. However, each of my three objectives began with working hypotheses:

1) Based-upon studies of transfected, non-replicating plasmid DNA summarized above, hdAd vectors likely assemble into chromatin slowly and inefficiently.

2) Since prokaryotic DNA only represses gene expression when covalently-linked to eukaryotic expression cassettes, I hypothesize that prokaryotic DNA assembles into chromatin that is refractory to transcription, which spreads from the hdAd-prok stuffer DNA to the expression cassette.

3) Nucleation of transcriptionally-repressive chromatin may be due to methylation of abundant CpG motifs within the hdAd-prok stuffer DNA.

4) Prokaryotic DNA may be recognized as foreign by host cell proteins involved in innate nuclear defense, such as PML, Sp100, and Daxx, resulting in repression of hdAd-prok transgene expression

Chapter 2 – Materials and Methods

2.1 - Reagents

For a list of reagents, see Appendix 1. All biochemical supplies were obtained from Fisher, unless indicated.

2.2 - Cell Culture, Ad vectors, and transfections

Cell culture

Propagation of 293 (101) (a kind gift of Dr. Frank Graham, McMaster, University, Hamilton, ON), 293-N3S (100) (Microbix), 116 cells (293-based, Cre-expressing cells) (210) (a kind gift of Dr. P. Ng, Baylor College of Medicine, Houston, TX), and A549 human lung adenocarcinoma cells (American Tissue Type Collection (ATCC)) was as previously described (212, 234). HeLa cells were obtained from the ATCC and maintained in Dulbecco's modified Eagle's medium (DMEM; Sigma-Aldrich), supplemented with 10% fetal calf serum, 2mM GlutaMAX, and 1x antibiotic/antimycotic (all from Invitrogen).

To generate cells stably-expressing H3.3-YFP, HeLa cells were transfected with pJR123 (pCDNA3-H3.3-YFP). Twenty-four hours later, the cells were placed under selection with 800 μg/ml G418. After ~10 days a H3.3-YFP colony was identified and isolated using cloning rings (Corning). Specifically, medium was removed, the cells were rinsed twice with phosphate buffered saline (PBS), sterile cloning rings were dipped in sterile vacuum grease (just enough to create a seal with the dish). Several drops of 1x trypsin were added to the cloning ring. When the cells detached, they were suspended in selection medium and removed to a well of a 12-well plate. The cells were expanded and maintained in selection medium.

Drug treatments

Trichostatin A (Upstate), 5-azacytidine (Sigma), and 5,6-dichlorobenzimidazole 1-β-D-ribofuranoside (Sigma) were added to the indicated concentrations in growth medium for pre-treatments (for duration of pre-treamtent, see figure legends). Throughout the duration of all drug treatment experiments, all buffers or medium that came into contact with the cells were supplemented with drug until the cells were harvested for reporter gene expression.

Stress induction

Heat stress was induced by moving cells to a 5% CO_2 tissue culture incubator with the temperature set to 43°C. Cells in 35 mm dishes were placed in direct contact with the shelves of the incubator and then left for 2 h. Ultraviolet (UV) radiation stress was induced by placing cells (in 35 mm dishes, with lids-on, and 2 ml growth medium covering the monolayer) in a UV cross-linker set to deliver 100 joules/m^2 of UV radiation for the indicated timepoints. After either stress, cells were moved back to 37°C, 5% CO_2 for a 30 min recovery period.

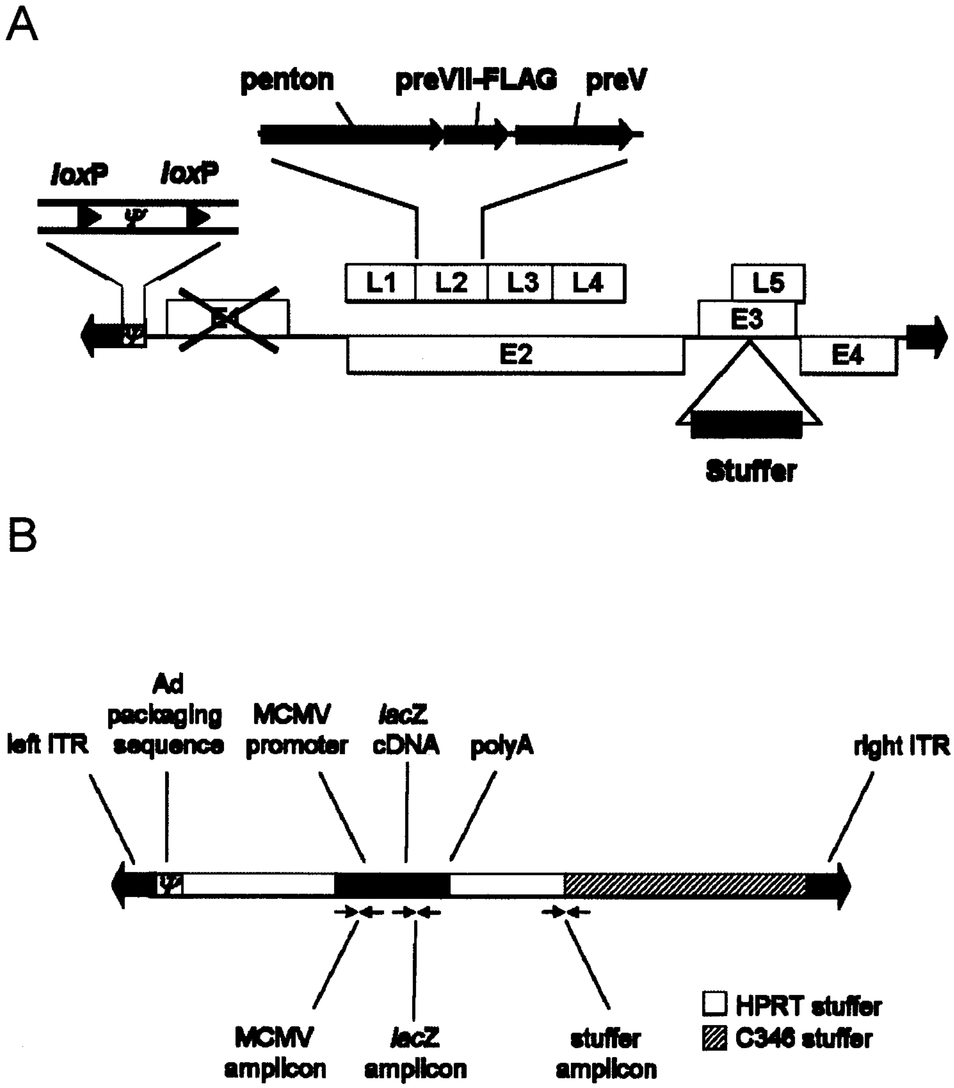
Adenovirus vectors

hdAd-*lacZ* (originally-described as hdAd-delta28E4-*lacZ*) (210), hdAd-PGK-mSEAP (originally-described as hdAd-RP2203) (199), hdAd-euk (originally-described as hdAd-RP1050) (215), hdAd-prok (originally-described as hdAd-RP1030) (215), AdNG163 (210), and AdRP2050 (33) were described previously. hdAd-*lacZ* and AdNG163 were kind gifts of Dr. Philip Ng. AdJR46 encoded the core VII-FLAG open reading frame in the endogenous core VII locus of AdRP2050 (Fig. 7) and will be described in detail below. AdNG163, AdJR46, and AdRP2050 were propagated and titered in 293 cells as described (234). All vectors were purified by cesium chloride buoyant density centrifugation as described (210, 234).

Ad vector titration and infections

Infectious hdAd particles were scored as infectious units (IU). Vectors were titered on 293 cells using previously established protocols (82, 216). Eighteen to twenty-four hours after infection with a range of vector dilutions (typically 10^{-4} to 10^{-9}), medium was removed and cells were fixed with β-gal fix for 5 min at room temperature. Cells were rinsed once with PBS and then twice for 5 min with PBS. Upon removal of the third PBS rinse, β-gal staining solution was added to the cells. Plates were incubated overnight in the dark (i.e., wrapped in foil or in a cupboard or drawer). Next day, blue cells were counted. The vector dilution that resulted in 25-50 blue cells/field of view (fov) was typically chosen for counting blue cells. The average number of blue cells from 10-20 fov was multiplied by the dilution factor, the number of fov in a 35 mm dish (300), and finally by 10 (so that the number of IU/100 μl could be converted to IU/ml). Detection of individual cells infected by hdAd-prok was difficult due to poor transgene expression. To enhance detection of β-gal

Figure 7. Model system for analysis of hdAd vector chromatin assembly. To track the fate of Ad core VII, a helper virus was generated that encoded VII-FLAG in the endogenous core VII locus (A). This helper virus is an E1-deleted fgAd with a packaging sequence (ψ) that is flanked by *lox*P sites and an E3 gene that has been disrupted by insertion of stuffer DNA from the human low-density lipoprotein receptor-related protein gene. Adapted from (292), with permission. (B) The hdAd vector encodes the Ad ITRs and packaging sequence and *lac*Z reporter gene driven by the murine cytomegalovirus (MCMV) immediate early promoter/enhancer. Three primer sets were used for ChIP analysis of hdAd vector DNA. These primers amplified DNA in the mCMV promoter, the *lac*Z gene, or in the non-transcribed stuffer DNA. The stuffer DNAs were derived from the human hypoxanthine-guanine phosphorybosyltransferase (HPRT) gene located at Xq26 and the C346 cosmid, which encodes human genomic DNA from Xq28 (285).


A
penton
preVII-FLAG
preV
loxP
loxP
Ψ
L1
L2
L3
L4
L5
E1
E2
E3
E4
Stuffer
B
left ITR
Ad packaging sequence
MCMV promoter
lacZ cDNA
polyA
right ITR
MCMV amplicon
lacZ amplicon
stuffer amplicon
☐ HPRT stuffer
▨ C346 stuffer

positive cells infected with hdAd-prok, titering was performed in the presence of 250 ng/m trichostatin A (TSA) (Upstate). Particle counts were performed as described (234). Contamination of hdAd vector stocks with helper virus was determined by plaque-assays as described, and scored as plaque-forming units (PFU) (234). Importantly, all hdAd vectors had similar ratios of IU to PFU (approximately 0.01 to 0.05% helper virus contamination).

Ad infections were performed in small volumes (i.e., 100 μl for 35 mm dishes and 1 ml for 15 cm dishes). Vectors were diluted in DMEM to achieve the desired MOI (i.e., the number of IU added to the monolayer for each cell). Prior to addition of dilute vector to cellular monolayers, all growth medium was removed. After addition of the vector, cells were incubated 1 h in a 37°C, 5% CO_2 incubator. Dishes were rocked every 15 min throughout the period of infection. After infection, inoculum was removed and replaced with growth medium or maintenance medium. Where indicated, infections were synchronized by regulating the timing of internalization (102). Specifically, medium was removed and monolayers were rinsed three times with ice-cold DMEM. Upon removal of the third rinse, hdAd (diluted in ice-cold DMEM) were added to cells and allowed to attach to host cells for 2 h at 4°C; the plates were rocked every 15-30 min. After 2 h, the DMEM was removed, unattached hdAd was removed by rinsing three times with ice-cold DMEM, and internalization of attached hdAd was stimulated by addition of pre-warmed (37°C) growth medium.

hdAd vector propagation

hdAd were propagated in 116 cells as described (210, 216). hdAd-*lacZ* and hdAd-PGK-mSEAP were propagated using AdJR46 and AdNG163, respectively; hdAd-prok and -euk were propagated using AdRP2050.

Plasmids encoding hdAd vectors were either left undigested (hdAd-prok and -euk) or digested with PacI (hdAd-PGK-mSEAP) or PmeI (hdAd-*lacZ*) (both New England Biolabs (NEB)) overnight to release the hdAd vector genome from the bacterial origin of replication and antibiotic resistance gene. hdAd plasmid DNA was then transfected into 116 cells in 35 mm plates using Superfect (Qiagen), as recommended by the manufacturer. Three hours after the transfection, DNA/lipid complexes were removed and the cells were rinsed with PBS. After addition of growth medium, cells were allowed to recover for one hour in the incubator, and were then infected with the indicated helper virus (MOI = 2.5-5 PFU/cell). Seventy-two hpi, the medium and cells were scraped into a 5 ml cryo-vial (this is passage 0 (P0)); sucrose was added to 4%, and the cells were either subjected to one round of freeze/thaw to release hdAd or were stored at -80°C. After thawing, the inoculum was vortexed and 0.1 ml was added to 116 cells along with the indicated helper virus (MOI = 2.5-5 PFU/cell). Forty-eight hpi, the monolayer was detached from the plate by tituration, and cells and medium were removed to a 5 ml cryo-vial (this is P1); sucrose was added to 4%, and the cells were either subjected to one round of freeze/thaw to release hdAd or were stored at -80°C. At each passage, 293 cells were infected to determine the titer of the inoculum. hdAd vectors were serially-propagated in this manner, until the titer of the inoculum was sufficient to infect a 15 cm dish of 116 cells with a MOI of 5-10 IU/cell (i.e., ~$2x10^8$ IU/ml). Two to three days after co-infection of 116 cells in 15 cm dishes, the cells and medium were harvested by tituration, sucrose was added to 4% and the inoculum was stored at -80°C. This inoculum was then used for large-scale production of hdAd vector, as described (210, 212, 234).

Transfections

pCMV-H3.3-YFP (135) was purchased from Addgene. Plasmids encoding FLAG-Sp100A, FLAG-Sp100B, and FLAG-Sp100HMG were kindly provided by Dr. Hans Will (Heinrich-Pette Institute for Experimental Virology, Hamburg, Germany). Plasmids encoding HA-pp71 and HA-pp71delDID2/3 (which is deleted of daxx interaction domains (DID) 2 and 3) (241) were kindly provided by Dr. Robert Kalejta (University of Wisconsin, Madison, USA). Plasmids expressing wild type and mutant Gam1 (56) were kindly-provided by Susanna Chiocca (European Institute of Oncology, Milan, Italy). The plasmid encoding the dsRed-PML fusion (287) protein was kindly provided by Dr. David Picketts (Ottawa Health Research Institute). Pooled siRNA targetting Sp100 and Daxx were obtained from Dharmacon. Transient transfections were performed using Lipofectamine 2000 (Invitrogen), according to the manufacturer's instructions. The amount of DNA and lipofectamine were doubled for transfection with plasmids encoding Sp100 isoforms.

Pooled siRNA targeting human HIRA, Sp100, and Daxx and control non-targetting pooled siRNA were obtained from Dharmacon. Cells were transfected with 200 nM siRNA using Lipofectamine 2000 as recommended by the manufacturer. In co-transfection experiments, cells were transfected with 200 nM siRNA and 5 μg/ml pCMV-H3.3-YFP, which were complexed with 20 μl/ml Lipofectamine 2000; 42 h after transfection, cells were infected with hdAd-*lacZ*; 6 hpi, cells were processed for ChIP.

2.3 - Cloning

Construction of helper virus encoding VII-FLAG

As a first step, I removed an 8.89 kb *Nde*I fragment from pRP2014 (a plasmid encoding an E1/E3-deleted Ad vector, equivalent to pAdEasy1), generating pRP2014delNde. Removal of the NdeI fragment resulted in AscI having a single recognition sequence, which facilitated subsequent cloning steps. A 1.4 kb AscI /KpnI (both from NEB) fragment encoding the Ad core VII cDNA was subcloned from pRP2014delNde into AscI/KpnI-digested pNEB193 (NEB), generating pJR15.

Polymerase chain reaction (PCR) was used to add a C-terminal FLAG tag to the core VII open reading frame (ORF). Specifically, primers were designed (Sigma) that would amplify pJR15 in opposite directions from the 3' end of the core VII cDNA (Table 2); one primer (REV) encoded the DNA complementary to a FLAG tag in frame with the 3'end of core VII cDNA, and the other primer (FWD) was complementary to DNA immediately downstream of the core VII cDNA. Each primer also encoded a 5' XmaI recognition sequence. The PCR product was gel purified and then digested with XmaI (NEB). The digested DNA was then self-ligated and transformed into DH5α cells. The resultant plasmid, pJR16, was identical to pJR15, with 2 exceptions: (1) the plasmid encoded core VII with a C-terminal FLAG tag, and (2) immediately downstream of the core VII cDNA, there was a unique XmaI site.

Table 2. Oligonucleotides used in this study.

Application[1]	Name	Sequence	Temp[2]	Reference
ChIP	β-actin FWD	CAAAACTCTCCCTCCTCCTCTTC	60	(286)
	β -actin REV	GAGCCATAAAAGGCAACTTTCGGAA	60	(286)
	IFN- β FWD	CCTCGAGTCCCAAGTCTTGTTTTACAATTTGC	60	-
	IFN- β REV	CAAGCTTTTGACAACACGAACAGTGTCGC	60	-
	Myt1 FWD	CAGGAAGACACCTCTCACAC	65	(153)
	Myt1 REV	ACAGTGTCCAGGGGCTTTGC	65	(153)
	MCMV FWD	GTTCTTCGAGCCAATACACGTCAATG	56	(289)
	MCMV REV	GTACCGACGCTGGTCGCGCC	56	-
	*lac*Z FWD	TGAAGCAGAAGCCTGCGATGTCGG	60	-
	*lac*Z REV	CACAGCGGATGGTTCGGATAAAGCG	60	-
	hdAd-delta stuffer FWD	GTAAAGCCGAACCCGGGAAACTG	56.5	-
	hdAd-delta stuffer REV	CGTGTGGGAGAAGGGCAGGATC	56.5	-
bisulfite conversion	MCMV bis con FWD	AATCCATAACAAAATCCTCTAACAACTTAAATT	65	-
	MCMV bis con REV	GTATATAAGGTTAATAGGGGTGAGTTATTGGG	65	-
	prok stuf mid FWD	GCGAAGCTTATCCCTTCTAATACTATCATCAACATTAC	50, 65	-
	prok stuf mid REV	GCGCTCGAGTTTATGATGTTTTGTTGGATATGTATTT	50,65	-
Cloning	VII-FLAG FWD	GCGCCCGGGTAGATTGCAAGAAAAAACTACTTAGAC TCG	45	-

VII-FLAG REV	GCGCCCGGGTTACTTATCGTCGTCATCCTTGTAATCGTTGCGCGGGGGGCGG	45	-
H3 FLAG FWD	GCGGCGGCCGCATGGCTCGTACGAAGCAAACAGCTCG	45	-
H3 FLAG REV	CGCTCTAGATTACTTATCGTCGTCATCCTTGTAATCTGCCCTTTTCCCCACGGATGC	45	-
H3 YFP fusion FWD	GCGTCTAGAATGGCTCGTACGAAGCAAACAGCT	50	-
H3 YFP fusion REV	GCGCGGCCGCTTATCGTCCATCCTTGTAATCTGCC	50	-

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[1] Abbreviations: ChIP (chromatin immunopreciptation); bis con (bisulfite conversion).
[2] Temp: annealing temperature (in °C) for PCR with the oligonucleotide pair. The two temperatures displayed for prok stuf mid were used for the first 10 and last 30 rounds of amplification, respectively.

The AscI/KpnI fragment of pRP2014delNde was replaced with that of pJR16, generating pJR32. The 8.9 kb NdeI fragment of pRP2014 was cloned into the unique NdeI site of pJR32, generating pJR34. pJR34 was a E1/E3-deleted Ad vector encoding VII-FLAG in the endogenous core VII locus. To generate a helper virus encoding VII-FLAG, a 9.62 kb AscI fragment of pRP2050 (33) (which encodes an E1-deleted virus with a floxed packaging signal, and in which the wild-type E3 sequence contains an insertional-interruption encoding a segment of intron 2 from the human low-density lipoprotein receptor-related protein) was replaced with a 9.65 kb AscI fragment from pJR34. This generated pJR46, which encoded an E1/E3-deleted fgAd encoding VII-FLAG in the endogenous VII locus, and with a floxed packaging signal. AdJR46 was used as a helper virus to generate hdAd vectors packaged in VII-FLAG (Fig. 7).

Construction of plasmids expressing H3.1-YFP and H3.3-YFP

As I first step, I generated pJR67, which was a platform for generating bicistronic plasmids consisting of a ubiquitin C (UbC) promoter (kindly provided by Dr. Doug Gray, Ottawa Health Research Institute, Ottawa, Canada), followed by a multiple cloning site, an encephalomyocarditis virus (EMCV) internal ribosome entry site (IRES), and a hygromycin resistance gene. pJR67 was generated by subcloning a HincII/XbaI fragment containing the UbC promoter into SstI (ends were then repaired with T4 DNA polymerase (NEB)) and XbaI-digested pHygroCB (kindly provided by Dr. Frank Graham, McMaster University, Hamilton, ON).

Initially, I generated plasmids encoding H3.1 and H3.3 bearing C-terminal FLAG epitope tags. To do so, I employed PCR to amplify H3.1 from human genomic DNA and H3.3 from the plasmid CMV-H3.3-YFP (Addgene). The primers (Table 2) were designed to incoporate a 5' *Xba*I site and 3' NdeI site into the amplicons, as well as adding a C-terminal FLAG tag to H3.1 or H3.3. These amplicons were sub-cloned into pJR67, generating pJR69 and pJR70, respectively. However, I was never able to detect expression of H3.1- or H3.3-FLAG. Therefore, I generated expression cassettes encoding H3.1- and H3.3-YFP fusion proteins.

H3.1 and H3.3 were amplified from human genomic DNA and plasmid DNA, respectively using primers (Table 2) that were designed to incorporate a 5 XbaI site and 3 EagI. The PCR products were digested with these enzymes, and the fragments (438 bp each) were subcloned into pCMV-H3.3-YFP (replacing the original XbaI/EagI fragment), in frame with the YFP tag. These plasmids, expressing H3.1-YFP and H3.3-YFP, were called pJR129 and pJR130, respectively. A plasmid expressing H3.3-YFP and a selectable marker encoding resistance to G418 was generated by subcloning the 1.9 kb MluI to EcoRI fragment encoding H3.3-YFP from pCMV-H3.3-YFP into MluI/EcoRI-digested pCDNA3 (Invitrogen). This vector was called pJR123 (pCDNA3-H3.3-YFP).

Insertion of insulator elements between transgenes and stuffer DNA

hdAd-prok-ins

The double core insulator was kindly provided by Dr. Gary Felsenfeld (National Institute of Diabetes and Digestive and Kidney Diseases, Bethesda, MD). The double core insulator had previously been subcloned into pGem7(f+) (Promega) by another member of the Parks laboratory (Robert A. Meulenbroek), generating pRM2. A EcoRI/BamHI fragment from pRM2 containing the double core insulator was subcloned into EcoRI and BamHI-digested pSP72 (Promega), generating pJR24.

As a first step, a 2.7 kb EagI fragment in the plasmid encoding hdAd-prok (i.e., pRP1030) was excised, generating pJR25. The *Eag*I fragment contained a *Bgl*II site; excision of this fragment made a BglII site downstream of the poly-adenlyation signal in the expression cassette unique. A 0.88 kb BglII/Eco0109I fragment of pJR24 containing the insulator element was subcloned from into pJR25, immediately downstream of the expression cassette, generating pJR26. Next, the 2.7 kb *Eag*I fragment from pRP1030 was subcloned back into pJR26, generating pJR28. As a result of the cloning strategy used, pJR28 contained 0.3 kb of pSP72 DNA (i.e., DNA between the multiple cloning site and the Eco0109I site) and was missing 2.4 kb of pRP1030 DNA (i.e., the BglII to Eco0109I fragment). A 5.1 kb BglII/BamHI fragment of pRP1030, which contained the BglII/Eco0109I fragment was subcloned into pGem7(f+), generating pJR37. From this plasmid, a 2.4 kb HindIII/Eco0109I fragment was subcloned into pJR28, resulting in pJR30, which encoded hdAd-prok with the 0.5 kb double core insulator element between the transgene expression cassette and prokaryotic stuffer DNA.

hdAd-euk-ins

hdAd-prok and hdAd-euk have identical expression cassettes, and each have a

*Pac*I site in their transgene promoters. This PacI site was unique in the plasmid encoding hdAd-euk (pRP1050). Furthermore, pRP1050 had a unique NotI site downstream of the transgene expression cassette. pJR26 (described above) was digested with HindIII and the ends were then repaired with T4 DNA polymerase. This DNA was then digested with Pac I. The PacI/HindIII (blunt) fragment was subcloned into pRP1050, which (1) was digested with NotI, (2) had its ends repaired with T4 DNA polymerase, and (3) digested with PacI. The resultant plasmid, pJR31, encoded hdAd-euk with the 0.5 kb double core insulator element between the transgene expression cassette and eukaryotic stuffer DNA.

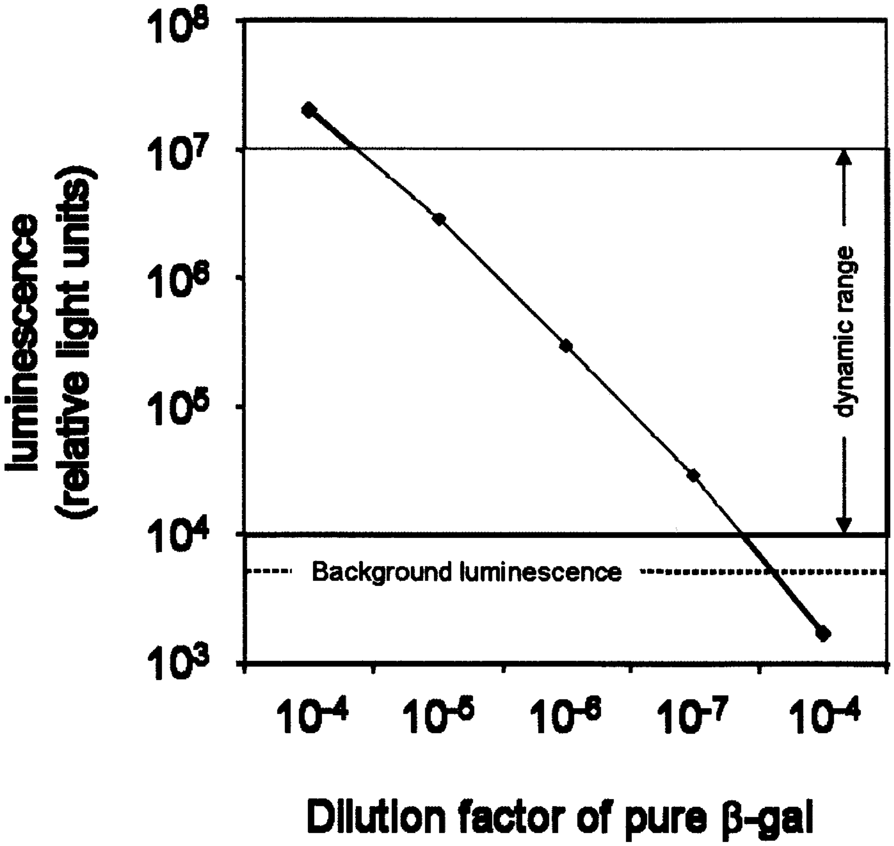
2.4 - Reporter Assays

Cell lysate from 35 mm plates (i.e., ~10^6 cells) was harvested in 0.5 ml 1 x reporter lysis buffer (Promega) at the indicated timepoints for analysis of reporter gene expression using a chemiluminescent β-galactosidase Assay Kit (Roche). After 5-15 min incubation of reporter lysis buffer, cell lysate was scraped into 1.5 ml eppendorf tubes. Cell lysate was snap-frozen and either stored at -80°C or thawed. Thawed lysate was cleared by centrifugation (20,000 x *g* for 3 min at room temperature) and then analyzed for reporter gene activity using chemiluminescent β-gal or SEAP assay kits (Roche). For β-gal assays, raw data (in relative light units) were normalized by comparison with purified β-gal (Roche), which was assayed in parallel (see below).

As a first step, we determined the dynamic range of detection of β-gal activity under our reaction conditions. Purified β-gal was serially-diluted (10^{-4} to 10^{-8}) in lysate from mock-infected cells. Note that dilutions of less that 10^{-4} results in RLU values that are too high to be read (which are reported as "overload") and that dilution of greater than 10^{-8} results in background levels of β-gal (data not shown). Dilute β-gal (5 μl) was added to 5 ml polystyrene tubes. The substrate buffer was diluted (1:20) in reaction buffer of the β-gal assay kit to create the assay buffer, of which 100 μl was added to each tube; tubes were then vortexed briefly. Addition of assay buffer to tubes was staggered by 10 s. After 15-25 min incubation, β-gal activity (expressed as relative light units (RLU) was determined using an EG&G Berthold Lumat LB 9507 Luminometer set to measure light output for 10 s. Analysis of purified β-gal revealed that the linear range of detection was between ~10,000 and ~10,000,000 RLU (Fig. 8). Whenever samples exhibited RLU values that were higher than 10,000,000, the samples were diluted and re-analyzed. β-gal data presented in Chapter 3 is expressed in RLU. Data presented in Chapters 4 and 5 were normalized by background subtraction and comparison with purified β-gal (10^{-5}, 10^{-6}, or 10^{-7}), which was assayed in parallel with experimental samples in each experiment:

1. "Background" β-gal activity was inferred from the activity present in lysate from mock-infected cells. This value (typically ~5000 RLU) was subtracted from the mean RLU samples of lysate from cells infected in duplicate.

Figure 8. Standard curve from analysis of purified β-gal. Pure β-gal was diluted (10^{-4} to 10^{-8}) in lysate from untreated HeLa cells and subjected to a chemluminescent β-gal assay, as described in the materials and methods. The RLU values produced from this analysis are plotted on a logarithmic (log)-log scale. The dotted line indicates the approximate value typically obtained upon analysis of mock-infected cells. The dynamic range of the detection is highlighted in yellow.


luminescence
(relative light units)
10^8
10^7
10^6
10^5
10^4
10^3
dynamic range
Background luminescence
10^{-4}
10^{-5}
10^{-6}
10^{-7}
10^{-4}
Dilution factor of pure β-gal

2. If lysate was diluted, the RLU values were then multiplied by the dilution factor.
3. The amount of pure β-gal present in the 10^{-5}, 10^{-6}, or 10^{-7} dilutions was 250, 25, and 2.5 pg, respectively. The mean RLU/pg value from these three samples gives the normalization value.
4. Corrected RLU values obtained in steps 1 and 2 are divided by the normalization value obtained in step 3, which yields values describing the number of pg β-gal present in the 5 μl of the lysate that was assayed.
5. Multiplication of the values obtained in step 4 by 100 gives the amount of β-gal that was present in the entire monolayer of infected cells (which were harvested in a volume of 500 μl); therefore, data from these experiments is described as pg β-gal/10^6 cells.

Unless otherwise indicated, displayed data represents mean from the indicated number of experiments ± standard error of the mean.

For experiments designed to determine whether prokaryotic DNA had to be linked to the transgene to repress gene expression, HeLa cells were mock infected or infected with hdAd-prok or -euk (MOI = 1). These cells were either pre- or co-infected with hdAd-PGK-mSEAP (MOI = 10). Pre-infections were performed 24 h prior to infection with hdAd-prok or -euk. Cell lysate was harvested 24 h after infection with hdAd-PGK-mSEAP. Chemiluminescent analyses of SEAP expression were performed as recommended by the manufacturer (Roche) with two exceptions: whole cell extract was used for the reporter assay and the heat inactivation step was excluded (although the Inactivation Buffer was used).

2.5 - Immunoblot

Whole cell extracts were obtained in 2x protein sample buffer and immunoblot was performed using standard techniques (243). In some cases, lysate that was harvested 1x reporter lysis buffer for reporter assays was brought to 1x protein sample buffer by addition of 5x buffer. These samples were applied-to 7.5-15% polyacrylamide gels (depending upon the size of the antigen) and resolved for 1.5-2 h at 100-125 V. Protein was transferred to polyvinylidene fluoride membrane (BioRad) using a semi-dry transfer apparatus (BioRad). Transfers were performed using constant amperage (0.8 x the size of the membrane in cm2 for 45-90 min, dependending on the size of the antigen). Membranes were probed with the indicated antibodies (Appendix 1). When indicated, antibodies were removed from the membrane by incubating 30-60 min at 37 or 65°C with Restore Western Blot Stripping Buffer (Pierce).

2.6 - Micrococcal nuclease accessibility assays

A549 cells (2.5 x 10^7) in 15 cm dishes were infected with hdAd-PGK-mSEAP (MOI = 500 IU/cell). At the indicated timepoints after infection, nuclei were isolated as described (201). Monolayers were rinsed twice with PBS, and cells were scraped into 15 ml polypropylene tubes (Corning). Cells were pelleted (750 x *g*, 5 min, 4°C) and suspended in homogenization buffer. Cells were transferred to a dounce homogenizer and nuclei were released by 5 strokes with a Dounce A pestle. Nuclei were sedimented through a 10% sucrose wash buffer cushion (1400 x *g*, 20 min, 4°C) and suspended in 1 ml of 1 mM $CaCl_2$ MNase wash buffer. Aliquots of 0.2 ml

(~5 x 10^6) were treated with 0, 1.5, 5, 10, or 25 units MNase (Roche) for 5 min at 22°C. Digests were terminated by addition of 0.2 ml stop solution, followed by overnight incubation at 42°C. DNA was purified by phenol/chloroform extraction and isopropanol-precipitation. Purified DNA (10 μg) from the 1.5, 5, and 10 unit treatments were separated on 20 cm 1.25% agarose gels, transferred to a nylon membrane, and subjected to Southern blot (276) with a digoxygenin-labeled 2.15 kbp BglII to HincII fragment from hdAd-PGK-mSEAP containing the PGK promoter and mSEAP open reading frame. DNA labelling and probe detection were performed using the DIG High Prime DNA Labeling and Detection Starter Kit II (Roche), exactly as described by manufacturer's instructions.

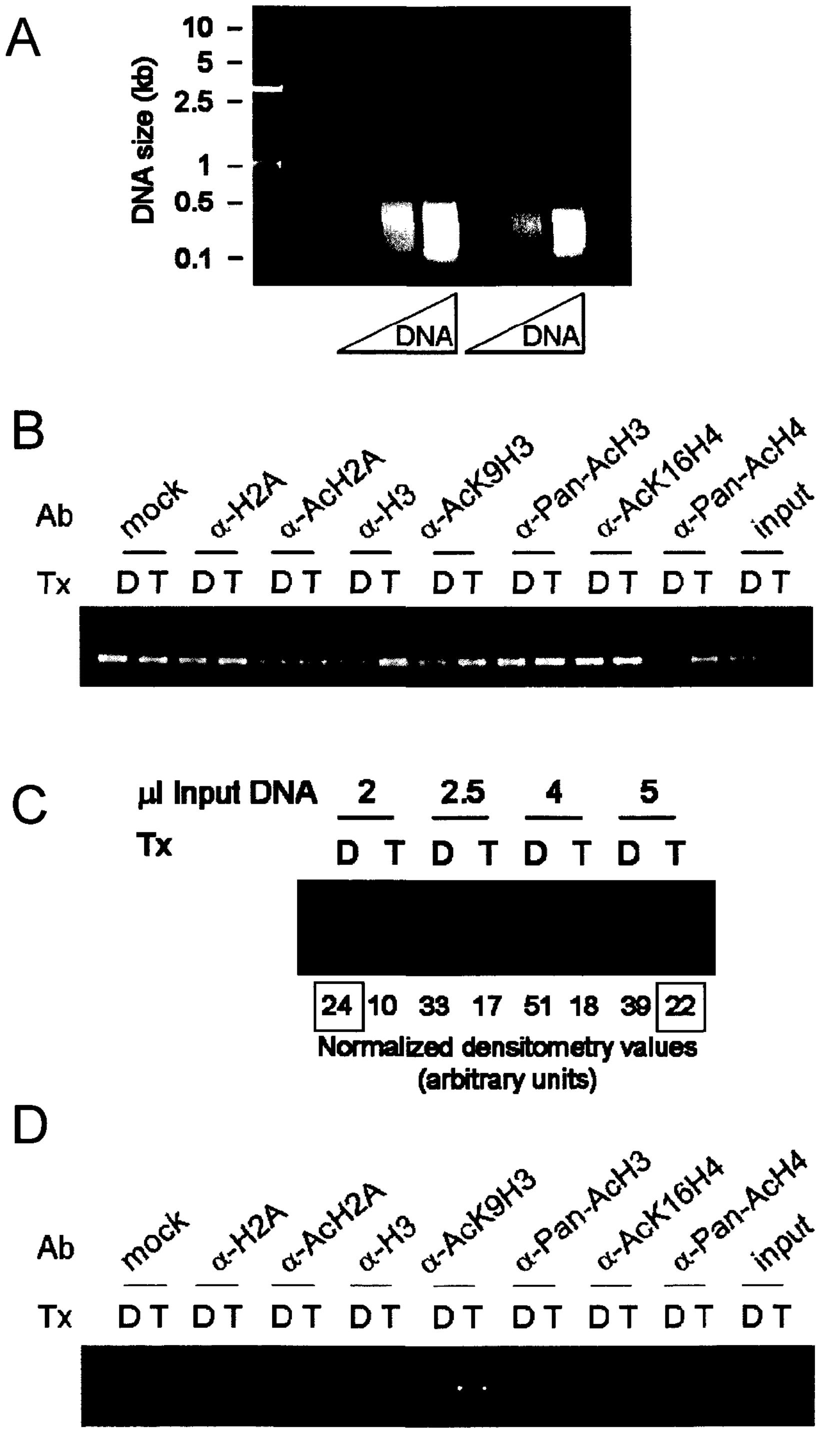
2.7 - Chromatin Immunoprecipitation

Chromatin immunoprecipitation (ChIP) assays were performed using a modified version of the Upstate Biotechnology protocol, and all buffers (unless indicated) were made according to Upstate's specifications. Lysis buffers and ChIP dilution buffer were supplemented with 1 mM phenylmethylsulphonyl fluoride (PMSF) (OmniPur), 10 μg/ml leupeptin (Roche), and 10 μg/ml aprotinin (Roche). Confluent HeLa or A549 cells in 15 cm dishes (ca. 2.5x 10^7 cells) were infected with the indicated virus and indicated MOI. Twenty-four hours post-infection (hpi), formaldehyde (Sigma) was added to culture medium to a concentration of 1% and the plates were incubated 10 min at room temperature. After a subsequent 5 min incubation in 0.125M glycine, monolayers were rinsed twice with 2 ml ice-cold PBS, and then scraped into 0.75 ml ice-cold PBS supplemented with 1 mM PMSF in 1.5

ml microcentrifuge tubes; the plates were then rinsed with 0.5 ml 1 mM PMSF PBS, which was then added to the previous 0.75 ml. Cells were pelleted by centrifugation (750 x *g*, 5 min, 4°C) and suspended in 0.5 mL cell lysis buffer (5 mM PIPES, pH 8, 85 mM KCl, 0.5% NP-40 (BDH Laboratory Supplies)), and incubated 45 min at 4 °C with constant rotation. The nuclei were pelleted by centrifugation (2000 x *g*, 5 min, 4 °C) and lysed in 0.25 mL nuclear lysis buffer (50 mM Tris-HCl, pH 8, 1% SDS, 10 mM EDTA) for 10 min.

Chromatin was sheared to an average size of ~500 bp (range of ~100-800 bp) (Fig. 9A) by sonication: 5-10 pulses of 10 s with a VibraCell VCX600 ultrasonic processor (Sonics and Materials) equipped with a stepped microtip, at a setting of 30-35% of maximal amplitude. After shearing, 2.5 μl aliquots of chromatin were analyzed for shearing efficiency. The aliquot was diluted to 500 μl in 200 mM NaCl TE and crosslinks were reversed by overnight incubation of 65°C. Next day, the samples were deproteinated by addition of 1 μl 20 mg/ml proteinase K (Sigma) and incubation for 2 h at 37-42°C. DNA was purified by phenol/chloroform extraction and isopropanol-precipitation. DNA was suspended in 25 μl H_2O, to which 5 μl RNase loading buffer was added. RNA was digested by incubation at 65 °C for 2-5 min and 37 °C for 5-15 min. Increasing amounts of DNA (i.e., 4, 8, and 16 μl) was resolved on a 1% agarose gel. Shearing efficiencies displayed in Figure 9A were deemed optimal. If suboptimal shearing was noted, the chromatin was subjected to further rounds of sonication.

Figure 9. ChIP shearing and input DNA normalization. (A) Chromatin was sonicated as described in the materials and methods. Displayed is an example of ideal ChIP chromatin shearing efficiency. (B) A typical initial semi-quantitative (sq) PCR from a single ChIP experiment is displayed. In this experiment (described in the legend of Figure 16) cells were treated (Tx) with DMSO (D (abbreviation)) vehicle control or TSA (T) and then infected with hdAd-*lac*Z. DNA (2 μl) was amplified for 30 cycles with *lac*Z primers. DNA was resolved on a 2% agarose gel and DNA visualized by EtBR staining. Note the following: (1) mock ChIP DNA is visible, suggesting that the cycle number and/or template volume must be reduced; (2) DNA in both the mock ChIP lane and unmodified H2A lanes exhibit greater signal for DMSO-treated cells, suggesting that input DNA concentration was likely higher for all ChIPs from the DMSO treated cells; and (3) despite what was just stated, no difference is apparent in the signal of input DNA from these samples; this, combined with the high intensity of the signals, suggests that the input DNA may have been out of the linear range of amplification. (C) The indicated volumes of input DNA from the two treatment groups in panel B were amplified for 25 cycles with *lac*Z primers. Note the following: upon using the lower cycle number, input DNA from the DMSO treated cells clearly contained more *lac*Z DNA than the TSA-treated cells. Comparison of densitometry signals from the DMSO band with 2 ml and the TSA band with 5 ml suggests that the DMSO-treated samples contained ~2.5 times more hdAd DNA. (Panel D) Once this dilution factor was determined, all DMSO ChIP DNA was diluted for a subsequent sqPCR with 25 cycles of amplification. This normalization approach was used when the amount of input DNA from two samples to be compared by sqPCR was found to differ.


A
DNA size (kb)
10
5
2.5
1
0.5
0.1
DNA
DNA
B
Ab
mock
α-H2A
α-AcH2A
α-H3
α-AcK9H3
α-Pan-AcH3
α-AcK16H4
α-Pan-AcH4
input
Tx
D T D T D T D T D T D T D T D T D T
C
µl Input DNA
2
2.5
4
5
Tx
D T D T D T D T
24 10 33 17 51 18 39 22
Normalized densitometry values (arbitrary units)
D
Ab
mock
α-H2A
α-AcH2A
α-H3
α-AcK9H3
α-Pan-AcH3
α-AcK16H4
α-Pan-AcH4
input
Tx
D T D T D T D T D T D T D T D T D T

Sheared chromatin was diluted 10-fold with ChIP dilution buffer supplemented with 22.2 μg/mL sheared herring sperm DNA (Roche) and 1.11 mg/mL bovine serum albumin (BSA; Sigma). Dilute chromatin was precleared with 50 μl of a 50 % slurry of protein G beads (Upstate) (which had been preincubated 1 h in the presence of 200 μg/mL sheared herring sperm DNA and 1 mg/mL BSA) for 2 h at 4 °C with constant rotation. Precleared chromatin was then incubated overnight with the indicated antibody and 50 μl of a 50 % slurry of protein G beads at 4 °C with constant rotation. For antibodies, see Appendix 1. After immunopreciptation, the beads were rinsed sequentially with low salt immune complex wash buffer (ICWB), high salt ICWB, lithium chloride ICWB, and twice with TE. The beads were then incubated twice for 15 min with 0.25 mL immune complex elution buffer. Eluates were combined, NaCl was added to 200 mM, and the samples were incubated 4-6 h at 65°C to reverse the crosslinks. After crosslink reversal, 10 μl 0.5 mM EDTA, 20 μl 1 M Tris-HCl pH 6.5, and 1 μl 20 mg/ml proteinase K were added to samples, which were then incubated 2 h at 42°C. DNA was purified by phenol/chloroform extraction and precipitated with isopropanol and 20 μg glycogen (Roche). ChIP DNA was suspended in 50 μl H_20; input DNA was suspended in H_20 (0.02% of input/μl).

ChIP DNA was analyzed by semi-quantitative PCR (sqPCR) using *Taq* polymerase (Invitrogen) (Fig. 9 B-D). Semi-quantitative analyses typically involved 25-30 cycles of amplification; annealing temperatures for all primer sets are displayed in Table 2. PCR products were subjected to electrophoresis on 1.8 or 2 % agarose gels and stained with ethidium bromide. In some cases, input DNA or control ChIPs would reveal that samples from different treatment groups exhibited differential amounts of hdAd vector DNA (Fig. 9B). This was suggestive of differential transduction. In such cases, several volumes of input DNA were subjected to PCR, and the amplification efficiencies were compared (Fig. 9C). These analyses would reveal the approximate fold-difference in the amount of vector DNA between the samples (e.g., ~2.5-fold, as in Figure 9C). The amount of hdAd DNA in subsequent analyses would be normalized accordingly, by modifying the amount of DNA used (although total volume of "template" was always controlled) (Fig. 9D).

2.8 - Analysis of DNA methylation

Endonuclease accessibility assays

To examine the status of CpG methylation prior to host-cell transduction, DNA was isolated from $1x10^{11}$ hdAd-prok particles by SDS-proteinase K extraction as described (234), and digested with MspI or HpaII (NEB). Plasmid DNA encoding hdAd-prok (0.5 μg) was also digested with HpaII or MspI. Digested DNA was separated on a 1.0 % agarose gel and visualized by staining with ethidium bromide.

Methylation of vector DNA after cell transduction was analyzed by Southern blotting. A549 cells were infected (MOI = 250 IU/cell). At the indicated timepoints, the monolayers were rinsed twice with PBS and the cells were scraped into 1.5 ml Eppendorf tubes in 0.5 ml PBS. Cells were pelleted (5 min, 750 x *g*) and the cell pellets were lysed for 2-18 h at 37-42°C in genomic DNA extraction buffer. DNA was then phenol/chloroform-extracted, isopropanol-precipitated, and suspended in

TE. Purified genomic DNA (10 μg) was digested with MspI, HpaII, SphI, and/or Mcr B/C (all NEB). DNA was separated on a 1% agarose gel, transferred to a nylon membrane, and probed with DIG-labeled hdAd-prok plasmid DNA. DNA labeling and probe detection were performed using the DIG High Prime DNA Labeling and Detection Starter Kit II (Roche), according to the manufacturer's instructions.

Bisulfite Sequencing

HeLa cells were infected with Ad-prok or Ad-euk (MOI = 1 BFU/cell). Genomic DNA was obtained 24 hpi. Genomic DNA was bisulfite converted using previously established conditions (89, 118) with some modifications. Genomic DNA (1 μg) in 50 μl H_2O was denatured by addition of NaOH to 0.2 M and boiling for 2-3 min. Unmodified cytosines were chemically converted to thymine by addition of 30 μl 10 mM hydroquinone (Sigma) and 520 μl 3 M sodium bisulfite (Sigma) and overnight incubation in a 50°C water bath. Next day, the DNA was purified from the bisulfite using the Illustra GXF PCR DNA and gel band purification kit (Amersham) (NOTE: the Illustra kit that I initially-used was ~4 years old. When the kit was replaced with a new, equivalent kit from GE Healthcare, bisulfite conversion became very inefficient and I had great difficulty amplifying DNA for sequencing; therefore, repetition of these results will require another protocol for purifying DNA from the bisuflite solution). The DNA was eluted in 50 μl and 5.5 μl 3 N NaOH was added to complete the conversion. The DNA was incubated 5 min at 22°C and then ethanol-precipitated with 20 μg glycogen. Converted DNA was suspended in 20 μl; 5 μl was used for PCR using 40 amplification cycles. Annealing temperatures for the primer sets used are displayed in Table 2. The ends of the murine cytomegalovirus (MCMV PCR) products were repaired with T4 DNA polymerase (NEB), gel purified, and cloned into the SmaI (NEB) site of pBluescript KS+ (Stratagene); the hdAd-prok stuffer PCR product was digested with Hind III (NEB) and XhoI (Invitrogen), gel-purified, and cloned into pBluescript KS+ using compatible ends. Bisulfite-converted DNA was sequenced by the StemCore Laboratories DNA sequencing facility (Ottawa, ON) using the T3 and T7 sequencing primers.

Chapter 3 - Rapid assembly of adenovirus DNA into chromatin promotes efficient gene expression *in vivo*

3.1 - Introduction

The fate and structure of Ad vector DNA after host cell transduction is not clearly understood. My goals were to determine whether: (1) core VII remains associated with hdAd vector DNA after nuclear translocation, (2) hdAd vectors associate with cellular histones and assemble into chromatin, and (3) whether chromatin has any role in the regulation of hdAd vector-encoded transgenes.

To determine the fate of VII after infection, I generated a helper virus that encoded FLAG-tagged VII (VII-FLAG) in the endogenous core VII locus (Fig. 7A). This helper virus was then used to propagate a hdAd vector that encoded the reporter gene *lac*Z (hdAd-*lacZ*) (Fig. 7B). Histone deposition could be analysed by ChIP; for PCR analysis of ChIP DNA, I designed primers that amplified three sites unique to the hdAd genome: the MCMV promoter, the *lac*Z open reading frame, and the junction between two fragments of stuffer DNA (Fig. 7B). Therefore, hdAd-*lac*Z could be used to track the fate of core VII after nuclear translocation, and to examine histone deposition and chromatin assembly. Using the model, I show that hdAd DNA assembles into chromatin, and that histone deposition promotes gene expression *in vivo.*

3.2 - Results

Ad core proteins are evicted after nuclear translocation

Ad DNA within the capsid is condensed by association with the virus encoded protein VII. To determine the fate of VII after infection, we infected cells with hdAd-*lacZ* packaged in VII-FLAG (Fig. 7) and examined the quantity of VII-FLAG within the cell at various times post-infection. Between 1 h and 2 h of infection, VII-FLAG levels dropped to approximately 20% of initial levels; between 2 and 3 hpi, VII-FLAG became nearly undetectable (Fig. 10A). Furthermore, the kinetics of VII-FLAG eviction mirrored the kinetics of nuclear translocation (164, 192) and the initial detection of hdAd vector gene expression (Fig. 10B).

Data from recent immunofluorescence-based experiments suggested that transcription is necessary to evict core VII from hdAd vector DNA (48); however, this hypothesis was not tested directly. To block transcription, we treated cells with a pharmacological inhibitor of RNA polymerase II elongation (5,6-dichlorobenzimidazole 1-β-D-ribofuranoside (DRB)). Treatment of cells with 500 μM DRB was sufficient to ensure that hdAd-encoded transgene expression was undetectable for 6 hpi (Fig. 11). We found that treatment with DRB had no effect on efficiency of VII eviction (Fig. 10A), suggesting that transcription is unnecessary for removal of VII from vector DNA.

hdAd chromatin exclusively incorporates H3.3

The swift dissociation of VII-FLAG led us to examine whether VII is replaced by cellular histones. Preliminary ChIP analyses suggested that hdAd DNA was associated with histone H3, H2A, and the linker histone H1 (Fig. 12). However, immunoblot analyses revealed that our anti-H1 and anti-H3 antibodies cross-reacted

Figure 10. Ad core protein VII is evicted from hdAd DNA after host cell transduction. (A) A549 cells were pre-treated 1h with 500 μM DRB or DMSO vehicle control and then infected with hdAd-*lac*Z (MOI = 5000 infectious units (IU)/cell). At the indicated timepoints, whole-cell lysate was harvested for immunoblot with anti-FLAG; the membrane was then stripped and probed with anti-α-tubulin. (B) A549 cells were infected with hdAd-*lac*Z (MOI = 100 IU/cell); at the indicated times after infection, cell lysate was harvested for analysis of β-gal activity (expressed in relative light units (RLU)). Representative data from two similar experiments are displayed; error bars represent range of values obtained from two replicate samples.

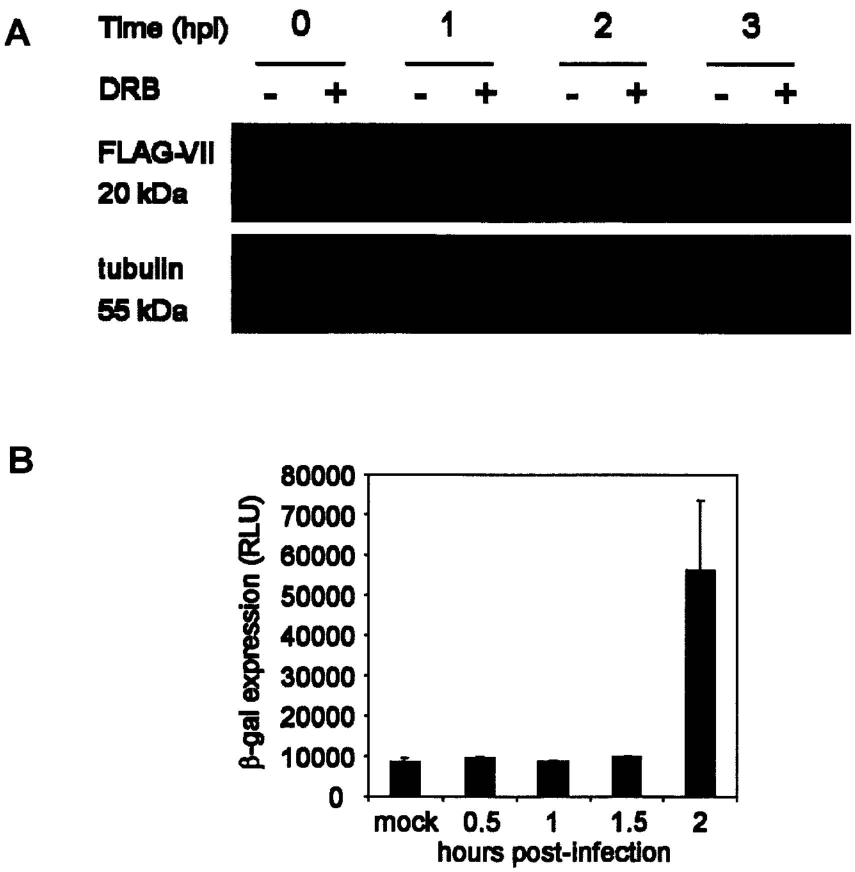

A
Time (hpi)
0
1
2
3
DRB
- +
- +
- +
- +
FLAG-VII
20 kDa
tubulin
55 kDa
B
80000
70000
60000
50000
40000
30000
20000
10000
0
β-gal expression (RLU)
mock
0.5
1
1.5
2
hours post-infection

Figure 11. hdAd vector gene expression is undetectable for 6 h in the presence of 500 mM 5,6-Dichlorobenzimidazole 1-β-D-ribofuranoside (DRB). A549 cells were pre-treated 1 h with the indicated concentration of DRB. Cells were then infected with hdAd-lacZ (MOI = 25 IU/cell). Six hpi, cell lysate was harvested for a chemiluminescent β-gal assay. β-gal activity is displayed in RLU.

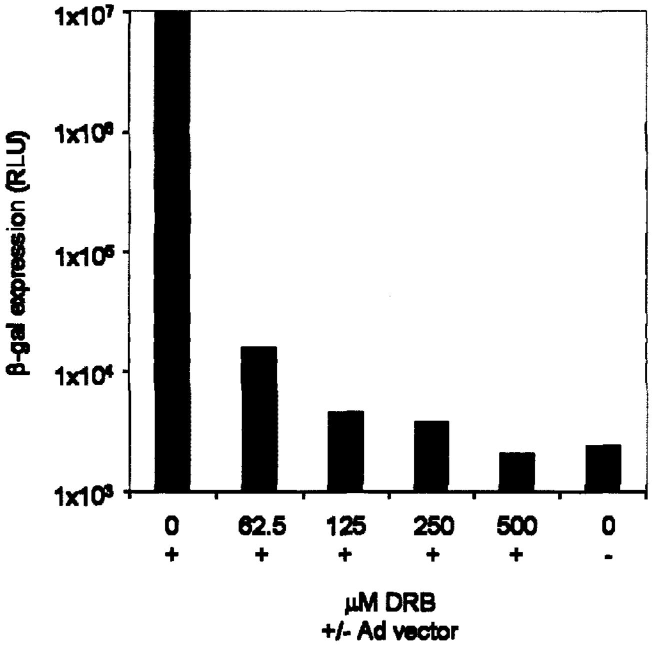
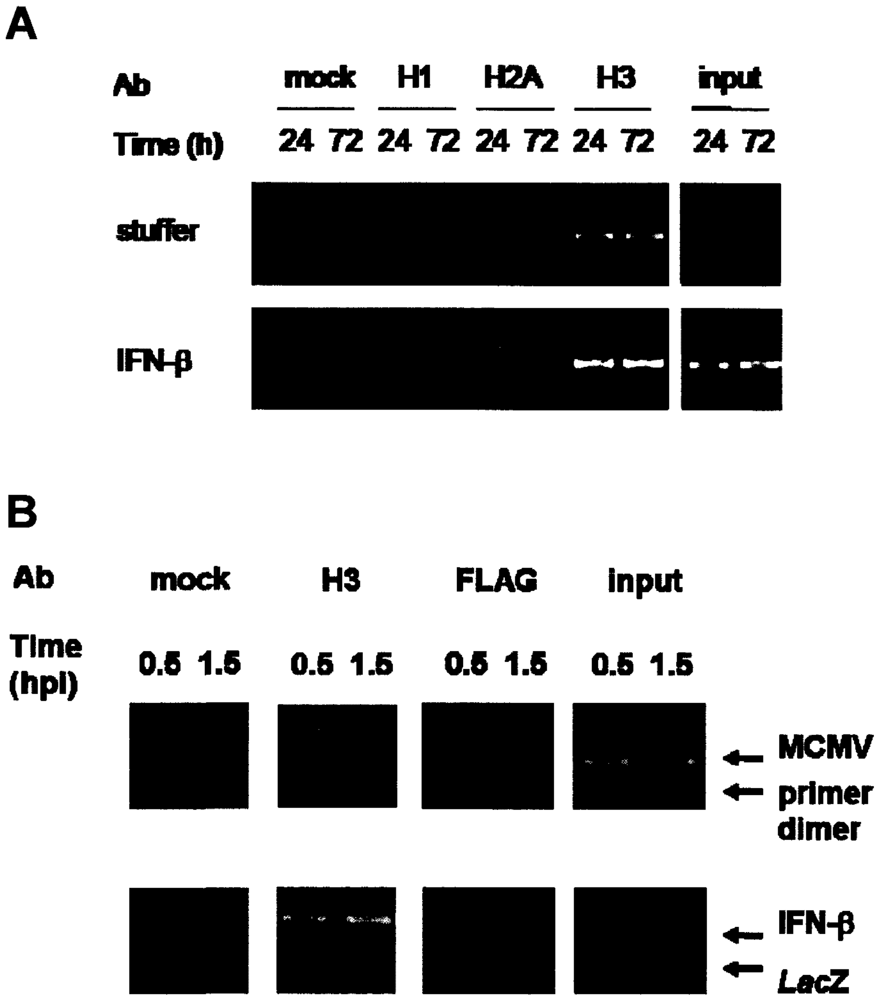

1x10^7
1x10^6
1x10^5
1x10^4
1x10^3
β-gal expression (RLU)
0
+
62.5
+
125
+
250
+
500
+
0
-
μM DRB
+/- Ad vector

Figure 12. ChIP analysis of histone association with hdAd vector chromatin. (A) A549 cells were infected with hdAd-*lac*Z (MOI = 5 IU/cell) or (B) HeLa cells were infected with hdAd-*lac*Z (MOI = 10 IU/cell). At the indicated timepoints cells were processed for ChIP with the indicated antibodies. ChIP DNA was analyzed by semi-quantitative PCR. PCR products were resolved on agarose gels and visualized by EtBR staining.


A
Ab
mock
H1
H2A
H3
input
Time (h)
24 72 24 72 24 72 24 72 24 72
stuffer
IFN-β
B
Ab
mock
H3
FLAG
input
Time (hpi)
0.5 1.5 0.5 1.5 0.5 1.5 0.5 1.5
MCMV
primer dimer
IFN-β
LacZ

with Ad core proteins (Fig. 13). Therefore, we decided to use ectopically-expressed, epitope-tagged histone H3 for kinetic analyses of histone deposition. To this end, we used plasmids encoding H3.1 and H3.3 fused in frame with a C-terminal YFP tag. The YFP fusion protein was selected because of its availability and because addition of an auto-fluorescent tag has no effect on H3 deposition (5, 186). Due to significant similarity with GFP (i.e., 234/239 identical amino acids), an anti-GFP polyclonal Ab was used for ChIP analyses of H3 deposition.

Before performing kinetic experiments, two important control experiments were performed. We wished to determine (1) whether anti-GFP would non-specifically enrich hdAd DNA sequences in ChIP and (2) which of the two major H3 variants - H3.1 or H3.3 - were deposited on hdAd vector DNA. To this end, we transfected cells with YFP-tagged H3.1 or H3.3, or a GFP control and examined association with vector DNA via ChIP. Anti-GFP did not enrich hdAd vector or host cell DNA in the presence of GFP alone (Fig. 14A). Similar amounts of H3.1-YFP and H3.3-YFP were expressed in these cells (Fig. 14B). Each H3 variant was associated with cellular DNA, whereas hdAd vectors exclusively incorporated H3.3 (Fig. 14A). Since H3.3 is only deposited in by a replication-independent mechanism (5), these data confirm that association of hdAd vector DNA with cellular histones was not due to aberrant vector replication or non-specific association of vector DNA with host chromatin.

Having determined that hdAd exclusively incorporate H3.3, we next examined the kinetics of histone deposition. We detected H3.3 deposition as early as 2 hpi; deposition was increased over the following 2 h (14C). Therefore, eviction

Figure 13. Two antibodies raised against unmodified histones cross-react with Ad core proteins. (A) Increasing amounts of purified Ad5 (AdJR34) particles (i.e., 2 x 10^9, 4 x 10^9, and 1x 10^{10}) and increasing amounts of acid extracts from uninfected HeLa cells (i.e., extracts from ca. 1.33 x 10^4, 3.33 x 10^4, 1.33 x 10^5 cells) were examined by immunoblot with a-unmodified H3, stripped, and reprobed with a-Ad core V. (B) The same increasing amounts of purified hdAd-*lacZ* particles as used in A and whole cell extracts from ca. 2 x 10^4 HeLa cells were examined by immunoblot with anti-unmodified H1. Bands representing the indicated proteins are indicated with labeled arrows.

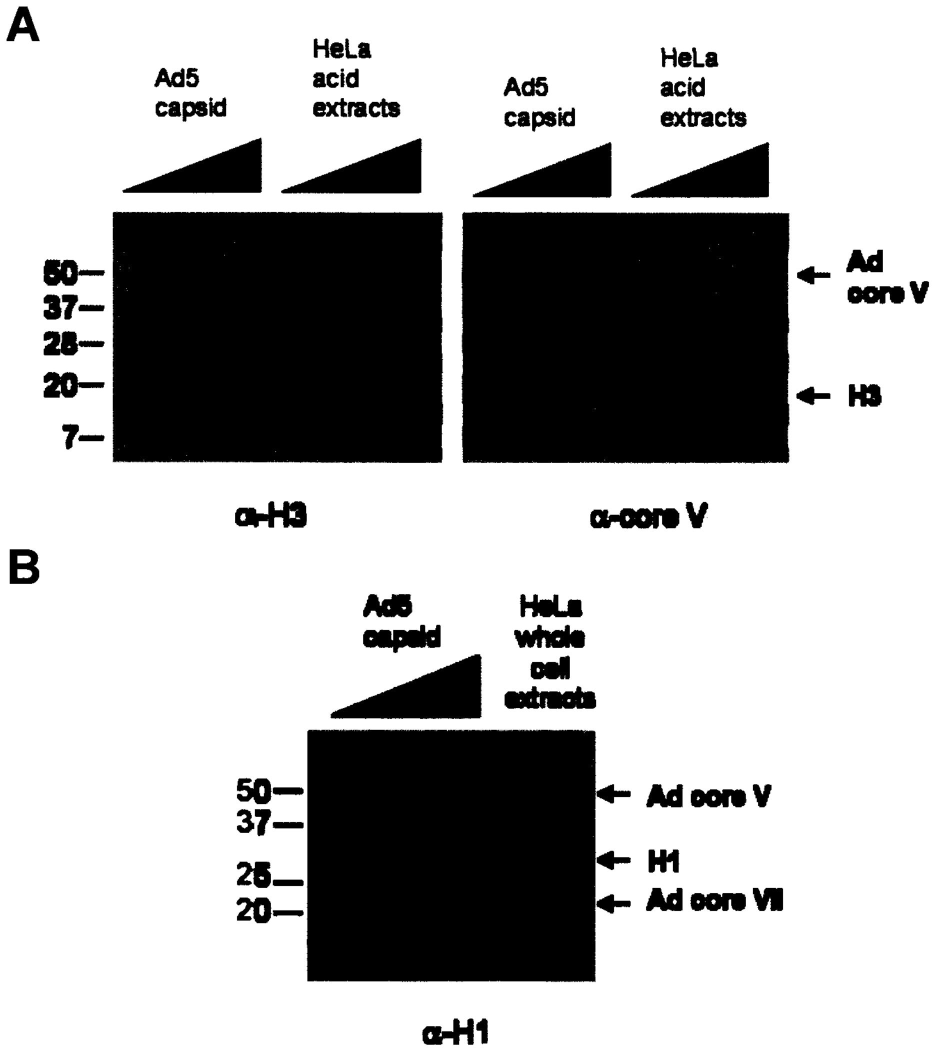

A
Ad5 capsid
HeLa acid extracts
Ad5 capsid
HeLa acid extracts
50—
37—
25—
20—
7—
Ad core V
H3
α-H3
α-core V
B
Ad5 capsid
HeLa whole cell extracts
50—
37—
25—
20—
Ad core V
H1
Ad core VII
α-H1

Figure 14. hdAd vector chromatin exclusively incorporates the histone variant H3.3, (A) HeLa cells were transfected with plasmids encoding GFP, H3.1-YFP, or H3.3-YFP; note that addition of an auto-fluorescent protein tag does not affect deposition patterns of H3 variants (5). Twenty-four hours post-transfection (hpt), the cells were infected with hdAd-*lac*Z (MOI = 10 IU/cell). The next day, cells were processed for ChIP with the indicated antibodies. (B) Flow-through from the mock ChIP (1%) was analyzed by immunoblot with anti-GFP (A). The blot was then stripped and reprobed with anti-α-tubulin. (C) A549 cells stably expressing H3.3-YFP were synchronously infected with hdAd-*lac*Z-VII-FLAG (MOI = 10 IU/cell). At the indicated timepoints after infection, cells were harvested for ChIP with the indicated antibodies. ChIP DNA was analyzed by semi-quantitative PCR with the indicated primers sets. PCR products were resolved on agarose gels and visualized by EtBr staining.

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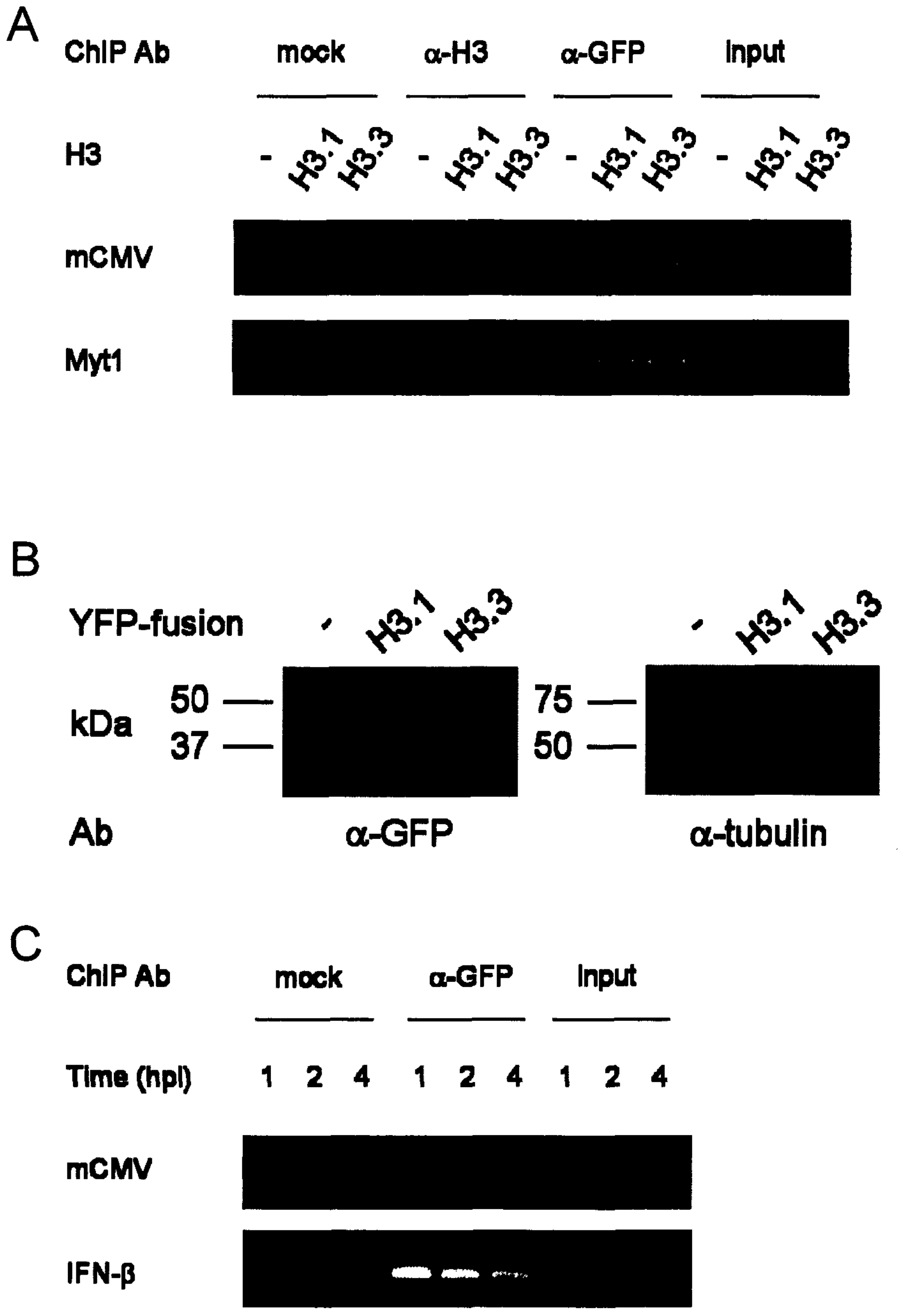

A
ChIP Ab
mock
α-H3
α-GFP
Input
H3
- H3.1 H3.3 - H3.1 H3.3 - H3.1 H3.3 - H3.1 H3.3
mCMV
Myt1
B
YFP-fusion
- H3.1 H3.3
- H3.1 H3.3
kDa
50
37
75
50
Ab
α-GFP
α-tubulin
C
ChIP Ab
mock
α-GFP
Input
Time (hpi)
1 2 4 1 2 4 1 2 4
mCMV
IFN-β

of VII (Fig. 10A), intiation of hdAd gene expression (Fig. 10B), and deposition of cellular histones all occur contemporaneously.

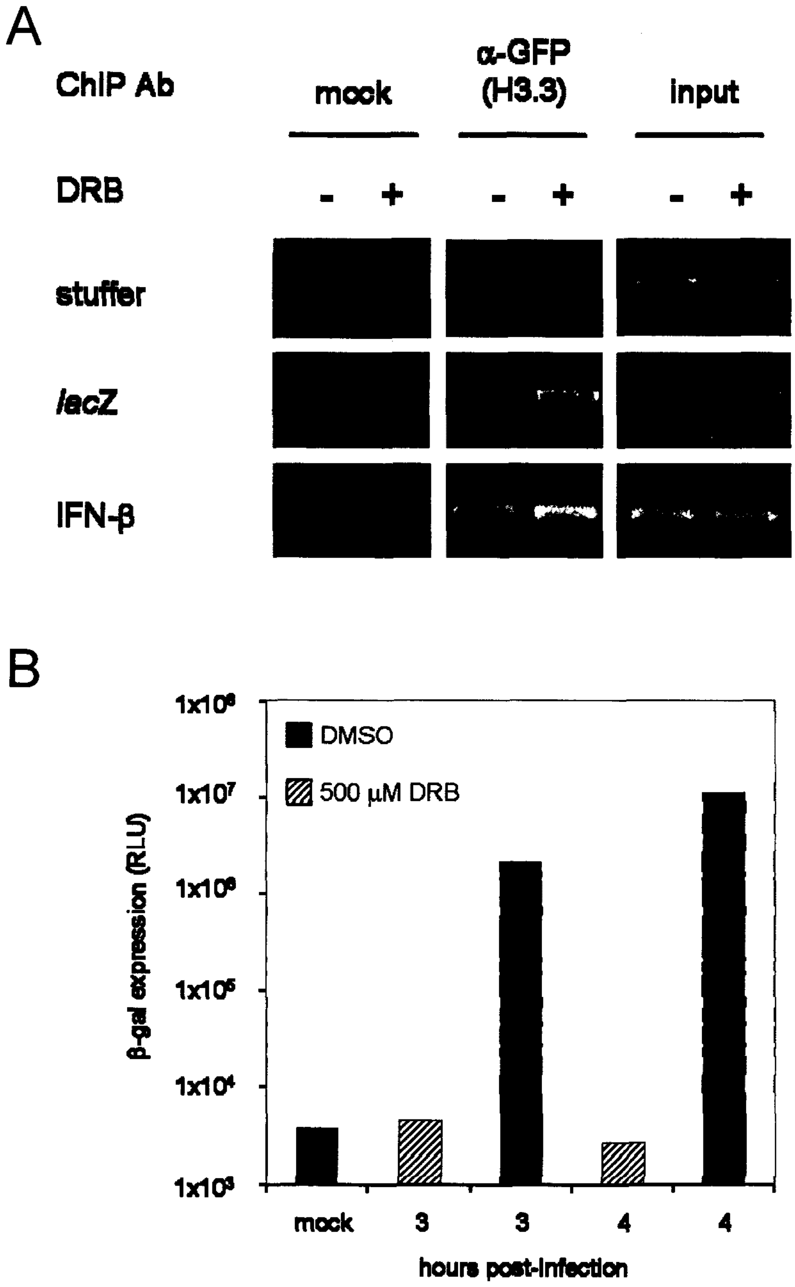
H3.3 deposition is transcription-independent

As discussed in the introduction, H3.3-containing chromatin is assembled in the wake of RNApII. Also, RNApII associates with Chd1, which is necessary for *de novo* chromatin assembly in *Drosophila*. Furthermore, a great deal of experimental evidences suggests that transcriptional elongation may be necessary for H3.3 deposition. To address the role of transcriptional elongation in the deposition of H3.3 in our system, cells were again treated with 500 μM DRB and H3.3 deposition was analyzed at 3 hpi by ChIP. DRB treatment had no effect on the association of cellular DNA or hdAd non-coding DNA with H3.3-YFP (Fig. 15A). Indeed, DRB appeared to increase association of the *lac*Z open reading frame with H3.3-YFP (Fig. 15A), suggesting that transcription may actually impair H3.3 deposition. As expected, cells treated with DRB and infected in parallel exhibited no transgene expression at 3 or 4 hpi, confirming inhibition of transcription (Fig. 15B). Therefore, although deposition of H3.3-YFP on hdAd DNA coincided with gene expression we have shown that deposition of H3.3 is transcription-independent.

Epigenetic regulation of hdAd vector transgene expression

Chromatin plays a crucial role in regulating gene expression (167). Since hdAd vector DNA associated with cellular histones (Fig. 14), we wished to determine whether this would influence expression of hdAd-encoded genes. To this end, we

Figure 15. Transcriptional elongation is not necessary for deposition of H3.3. (A) A549 cells stably expressing H3.3-YFP were treated with DMSO vehicle or DRB for 1h, and then infected with hdAd-*lac*Z-VII-FLAG (MOI = 10 IU/cell) and, at the indicated timepoints, processed for ChIP analysis with the indicated antibodies. ChIP DNA was analyzed by semi-quantitative PCR with the indicated primers sets. PCR products were resolved on agarose gels and visualized by EtBr staining. (B) To confirm that our experimental protocol would inhibit transgene expression at a higher MOI, for both the desired timepoint of analysis (3 hpi) and 1 h later, A549 cells were infected with hdAd-*lac*Z-VII-FLAG (MOI = 100 IU/cell); at the indicated timepoints after infection cell lysate was harvested and assayed for b-gal activity, which is displayed as RLU.


A
ChIP Ab
mock
α-GFP (H3.3)
input
DRB
- + - + - +
stuffer
lacZ
IFN-β
B
1x10^8
1x10^7
1x10^6
1x10^5
1x10^4
1x10^3
β-gal expression (RLU)
■ DMSO
▨ 500 µM DRB
mock
3
3
4
4
hours post-infection

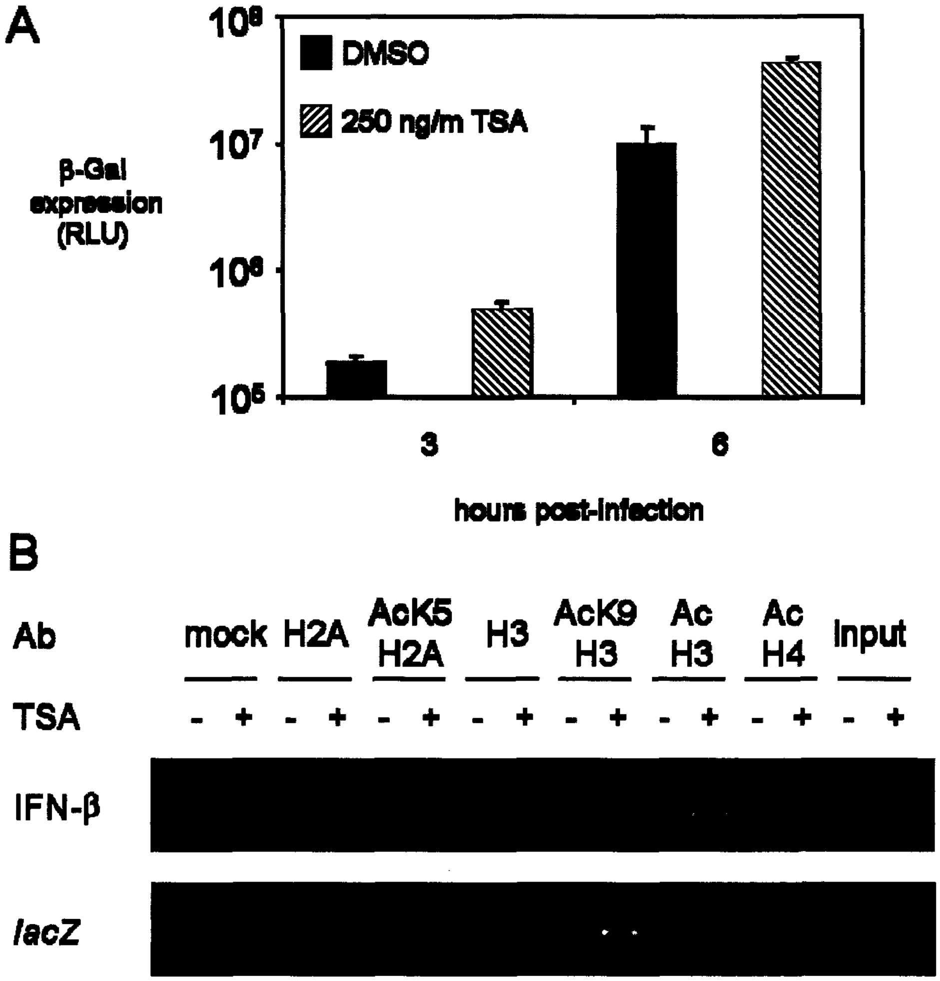
tested whether Trichostatin A (TSA), an inhibitor of class I and II histone deacetylases (291), would affect hdAd transgene expression. TSA promotes acetylation of histone tails, which results in increased expression of genes assembled into chromatin (104). Treatment with TSA resulted in 2- and 3-fold increases in hdAd transgene expression at 3 and 6 hpi, respectively (Fig. 16A). These data suggest that association with cellular histones has a physiologically-relevant impact on transgene expression.

To confirm that TSA acted directly on hdAd chromatin, and was not due to non-specific effects, we examined association of hdAd DNA with acetylated histones. To probe the acetylation state of hdAd-associated histones, we performed ChIP with several antibodies to acetylated residues with well-characterized roles in transcription (160). Treatment with TSA did not alter association of cellular or hdAd DNA with unmodified histones H2A and H3 (Fig. 16B). As expected, TSA increased association of cellular DNA with acetylated H2A, H3, and H4 (Fig. 16B). Although TSA treatment increased association of hdAd DNA with acetylated histone H3, no change was observed in association with K5-acetylated H2A, anfd association with acetylated H4 was decreased (Fig. 16B). Therefore, the observed increase in hdAd gene expression in the presence of TSA was due to a direct effect: increased acetylation of hdAd-associated histone H3.

HIRA is necessary for deposition of H3.3 on hdAd vector DNA

Deposition of H3.3 on incoming *Drosophila* sperm DNA requires the histone chaperone HIRA (32, 174). Biochemical analyses of human cells have shown that

Figure 16. hdAd vector transgene expression is induced by trichostatin A. (A) HeLa cells treated with 250 ng/ml TSA or DMSO control were infected with hdAd-*lac*Z-HPRT (MOI = 25 IU/cell); 3 and 6 hpi cell lysate was harvested for analysis of β-gal activity, which is displayed in RLU (N = 2). (B) HeLa cells treated with 500 ng/ml TSA were infected with hdAd-*lac*Z-VII-FLAG (MOI = 25 IU/cell); 6 hpi, cells were processed for ChIP with the indicated antibodies. ChIP DNA was analyzed by sqPCR with the indicated primers sets. PCR products were resolved on agarose gels and visualized by EtBr staining.

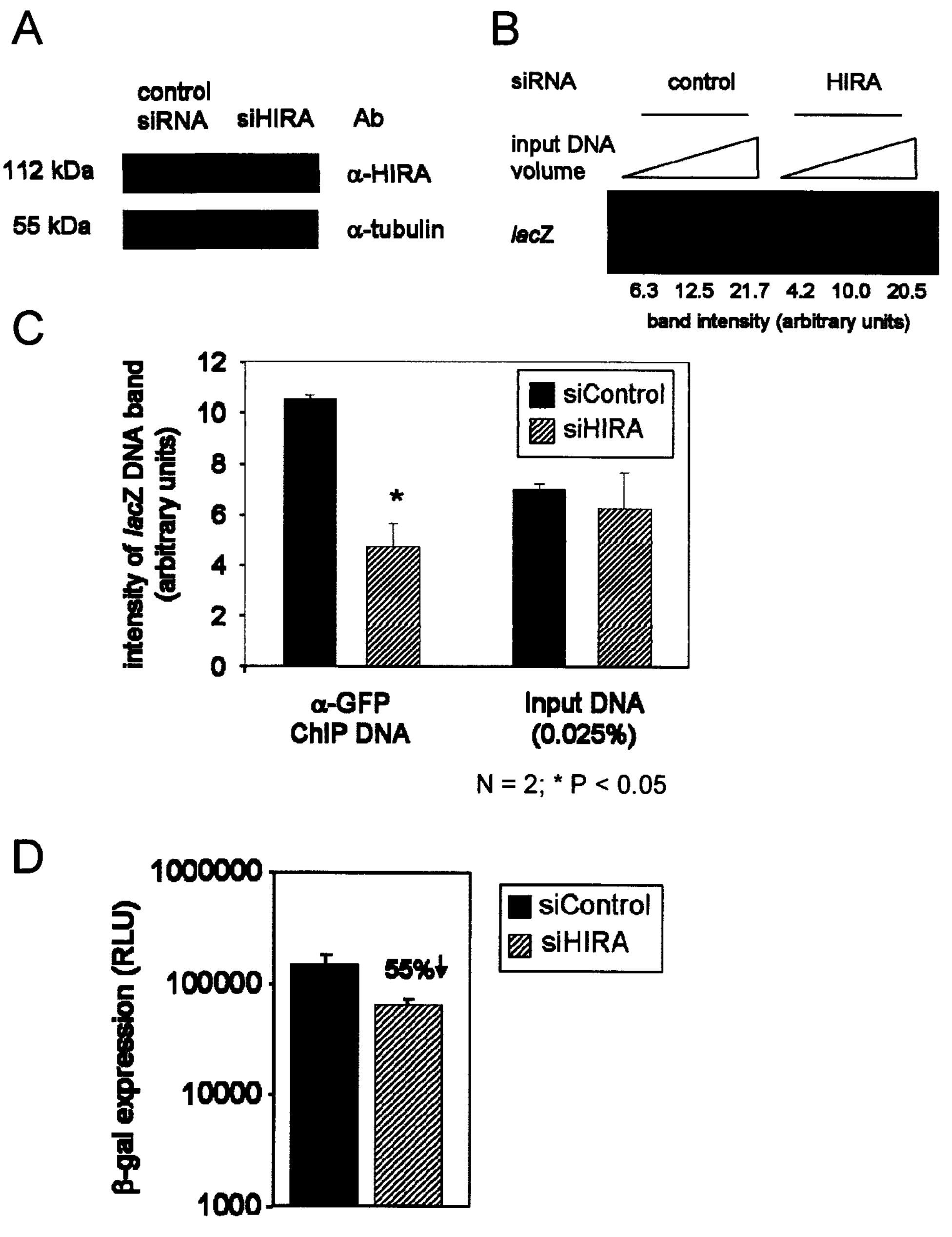

A
10⁸
10⁷
10⁶
10⁵
β-Gal expression (RLU)
DMSO
250 ng/m TSA
3
6
hours post-infection
B
Ab
mock
H2A
AcK5 H2A
H3
AcK9 H3
Ac H3
Ac H4
Input
TSA
- + - + - + - + - + - + - + - +
IFN-β
lacZ

H3.3 exclusively associates with HIRA (285). However, the role of HIRA in *de novo* replication-independent chromatin assembly in mammalian cells has yet do be determined. We used small-interfering (siRNA) RNA to deplete cells of HIRA in order to examine its role in deposition of H3.3 on hdAd vector DNA. Treatment with siRNA targeting HIRA (siHIRA) resulted in a ca. 75%-depletion of HIRA (Fig. 17A). To test whether HIRA depletion abrogates H3.3 deposition, we co-transfected cells with siHIRA and H3.3-YFP and used ChIP to detect association of H3.3-YFP with incoming hdAd vector DNA. Control experiments using increasing volumes of input DNA confirmed that our semi-quantitative PCR reaction conditions were within the linear range of amplification (Fig. 17B). Densitometry analysis of vector-specific PCR products from ChIP DNA revealed that depletion of HIRA resulted in a ca. 65% decrease in association of hdAd DNA with H3.3-YFP (Fig. 17C). Therefore, we conclude that HIRA is necessary for efficient deposition of H3.3 on hdAd vector DNA.

Assembly into chromatin promotes hdAd vector transgene expression

In vitro studies have revealed that assembly of bare DNA into chromatin drastically decreases transcription (163), which has led to the widely held belief that chromatin is an inherently repressive structure that blocks access to DNA. *In vivo*, modified histones serve as docking sites for the recruitment of factors that promote gene expression (reviewed in (137)), suggesting that assembly into chromatin may be beneficial for gene expression. Thus, assembly into chromatin may be important for rapid and efficient initiation of hdAd gene expression.

Figure 17. Deposition of H3.3 promotes efficient hdAd vector transgene expression. (A) HeLa cells were transfected with control siRNA (siControl) or pooled siRNA targeting sequences in the HIRA mRNA (siHIRA); 48 hpt, cell lysate was harvested and analyzed by immunoblot with the indicated antibodies. (B and C) HeLa cells were co-transfected with a plasmid encoding H3.3-YFP along with siControl or siHIRA; 48 hpi, cells were infected with hdAd-*lac*Z (MOI = 25 IU/cell); 6 hpi, cells were processed for ChIP with α-GFP. ChIP DNA (1, 2, and 4 ml; B) or (2 μl; C) was analyzed by sqPCR with primers targeting *lac*Z DNA. PCR products were resolved on agarose gels and visualized by EtBr staining. (B) Band intensity, as determined by densitometry, is displayed below each DNA band to confirm that the reaction conditions used in (C) were within the linear range of amplification. (C) Mean values obtained from densitometry analysis (ImageJ) of PCR products from two independent ChIP experiments are displayed; error bars depict standard deviation of the mean. Means were compared by student's T-test (* $p < 0.05$ versus siControl). (D) HeLa cells were transfected with siControl or siHIRA; 48 hpt, cells were infected with hdAd-euk (MOI = 1 IU/cell); 6 hpi, cell lysate was harvested for analysis of β-gal activity, which is displayed in RLU. Representative data from two similar experiments are displayed; error bars represent range of expression from duplicate samples. Note that the transgene expression data are also presented in Fig. 26.


A
control siRNA
siHIRA
Ab
112 kDa
α-HIRA
55 kDa
α-tubulin
B
siRNA
control
HIRA
input DNA volume
lacZ
6.3 12.5 21.7 4.2 10.0 20.5
band intensity (arbitrary units)
C
intensity of lacZ DNA band (arbitrary units)
0
2
4
6
8
10
12
siControl
siHIRA
*
α-GFP ChIP DNA
Input DNA (0.025%)
N = 2; * P < 0.05
D
β-gal expression (RLU)
1000
10000
100000
1000000
siControl
siHIRA
55%↓

The H3/H4 tetramer forms the core of the nucleosome (175); therefore, disruption of H3 deposition should preclude nucleosome formation and chromatin assembly. We hypothesized that siRNA-mediated depletion of HIRA, and resultant abrogation of H3.3 deposition, could be used to test whether assembly into chromatin affects gene expression *in vivo.* We found that HIRA-knockdown resulted in a 55% reduction of vector transgene expression (Fig. 17D). These data suggest that *de novo* assembly into chromatin promotes gene expression *in vivo*.

hdAd DNA assembles into physiologically-spaced nucleosomes

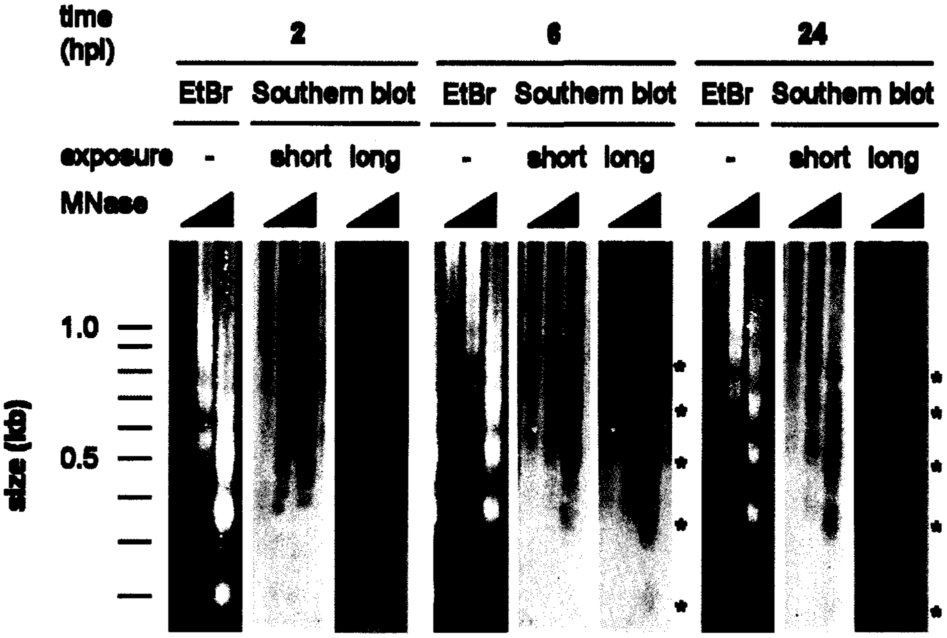
As a final step in the characterization of hdAd vector chromatin assembly, we examined whether histone deposition resulted in the assembly of physiologically-spaced nucleosomes. Southern analysis of micrococcal nuclease (MNase)-digested nuclei isolated from infected cells revealed that at the early time-point of 2 hpi, vector DNA exhibited no evidence of a periodic oligomeric array (Fig. 18). However, by 6 hpi and continuing until 24 hpi, hdAd vector DNA exhibited MNase-protection that was similar to bulk cellular chromatin (Fig. 18). We therefore conclude that Ad DNA packaging proteins are replaced with cellular histones, which results in assembly of hdAd DNA into physiologically-spaced nucleosomes.

3.3 - Discussion

hdAd vectors swiftly evict core VII and assemble into chromatin

We found that hdAd vectors evict core protein VII (Fig. 10A), associate with cellular histones (Fig. 14C), initiate transgene expression (Fig. 10B) and assemble into

Figure 18. hdAd vector DNA assembles into physiologically-spaced chromatin after host cell transduction. A549 cells were infected with hdAd-PGK-mSEAP (MOI = 500 IU/cell). At the indicated timepoints, nuclei were harvested and treated with 5, 10, or 25 units MNase per ~5 x 10^6 nuclei. After digestion, genomic DNA was purified and 10 μg was resolved on a 1.2% agarose gel, which was stained with EtBr. DNA was transferred to a nylon membrane and analyzed by Southern blot with a DIG-labelled probe encoding hdAd vector expression cassette; short (s) and long (l) exposures of the blot are displayed. (*) Indicates bands representing DNA that was protected from MNase cleavage.


time (hpi)
2
6
24
EtBr
Southern blot
EtBr
Southern blot
EtBr
Southern blot
exposure
-
short
long
-
short
long
-
short
long
MNase
1.0
0.5
size (kb)
*

physiologically-spaced nucleosomes (Fig. 18) within only a few hours of nuclear translocation. The swift assembly of hdAd DNA into chromatin was surprising. Studies of transfected plasmid DNA suggested that replication-independent chromatin assembly in mammalian somatic cells is inefficient and slow: detection of MNase-protected DNA requires 3 to 4 days (43) and results in aberrantly-spaced nucleosomes (271). Our observation that assembly into chromatin was essential for efficient expression of hdAd vector-encoded transgenes (Fig. 17) suggests that Ad may have evolved a mechanism to promote assembly into chromatin after entry into the host cell.

How do hdAd vectors assemble into chromatin so swiftly? Core protein VII is similar to the protamines that package sperm DNA (282). Several acidic chaperones that remove VII from Ad DNA *in vitro* and *in vivo*, such as B23/nucleophosmin (208), pp32 (318), the product of the SET oncogene (183), and NAP-1 (148), have also been implicated in the removal of protamines from incoming sperm DNA and in assembly of sperm DNA into chromatin (reviewed in (187)). Recruitment of acidic chaperones by protamines is thought to couple sperm decondensation to assembly of sperm DNA into chromatin (187, 221, 236). The similarities between the packaging and chromatinization of sperm and Ad DNA raise the intriguing possibility that Ad has co-opted this highly efficient cellular process, and now utilizes it to its own ends. In light of our data suggesting that H3.3 deposition promotes efficient hdAd vector gene expression, swift assembly of Ad DNA into chromatin may represent an important step in the early phase of infection that "kickstarts" the virus lifecycle.

Unexpected effects of TSA

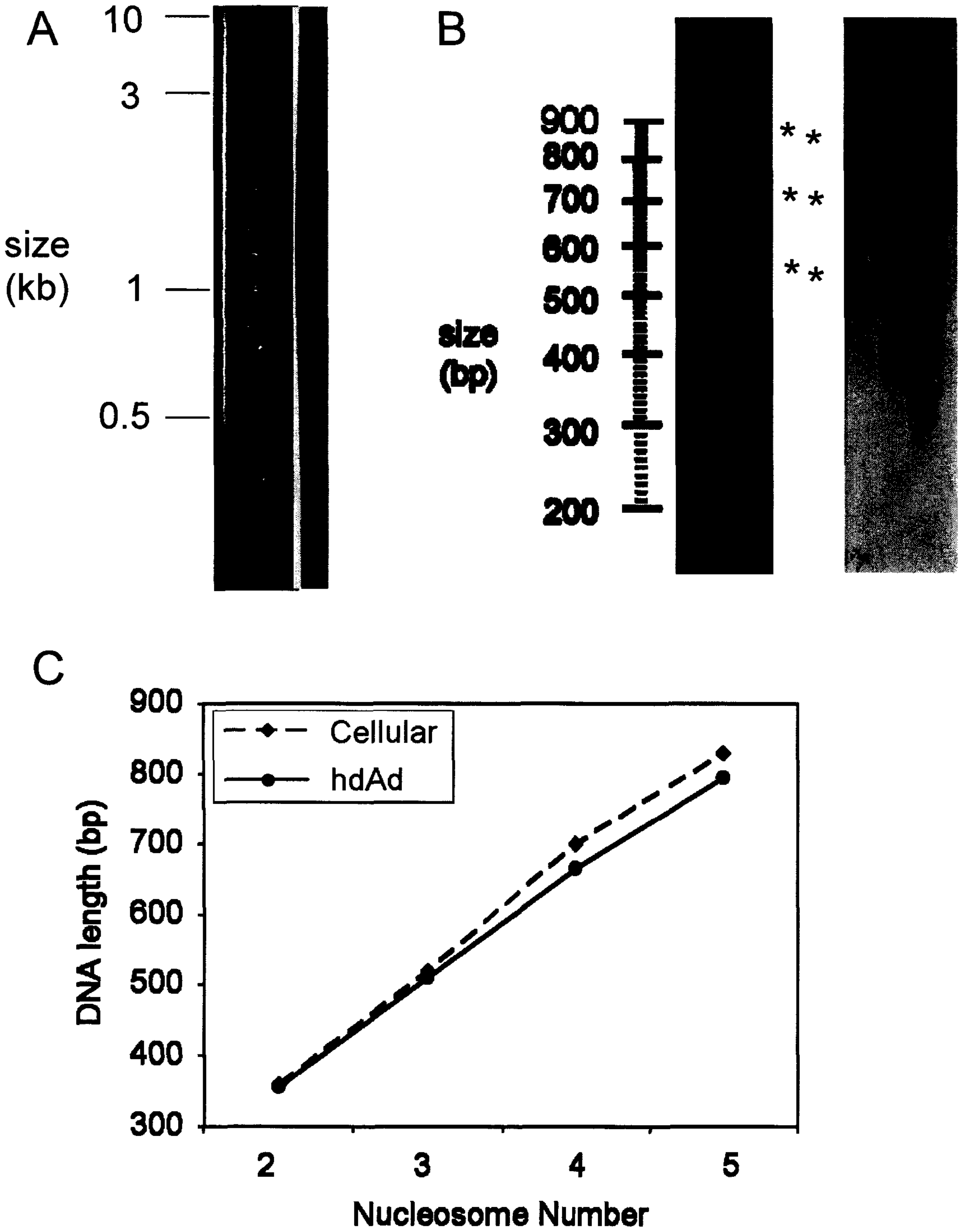
Our data revealed surprising effects of TSA treatment (Fig. 16). Specifically, we found that inhibition of HDACs resulted in decreased acetylation of histone H4. The antibody used for analysis of acetylated H4 was raised against an H4 peptide acetylated at K5, 8, 12, and 16. Newly-deposited H4 is di-acetylated at K5 and K12 (274); TSA should block deacetylation of these residues, in addition to promoting acetylation of the other two residues. However, hdAd vector DNA exhibited reduced association with acetylated H4 in the presence of TSA (Fig. 4B), this suggests that acetylation of K5 and/or K12 inhibits acetylation of K8 and/or K16. Similarly, TSA failed to increase association of hdAd DNA with acetylated H2A K5. Therefore, deacetylation of newly-deposited H4 may be necessary for recruitment of a histone acetyltransferase, such as the NuA4 complex, which acetylates both H2A and H4, but not H3 (207). Another possible mechanism is steric hindrance: as discussed above, TBP-associated factor 1 has a double bromodomain that binds H4 acetylated at K5 and K12 (134). Perhaps binding of diacetylated H4 by TBP-associated factor 1 occludes access to K8 and K16, resulting in reduced histone acetylation.

hdAd vector chromatin is unlikely to include the linker histone H1

We have shown that assembly of hdAd vector DNA into chromatin requires the histone chaperone HIRA (Fig. 17B). Genetic studies of *Drosophila* have shown that HIRA is necessary for assembly of sperm DNA into chromatin within oocytes (174). Similar studies showed that the ATP-dependent chromatin remodelling protein Chd1 is also necessary for assembly of sperm DNA into chromatin (155). Chd1 is one of only two eukaryotic proteins (in addition to ISWI) that are able to promote assembly of DNA into physiologically-spaced nucleosomes (178). Furthermore, Chd1 - unlike ISWI, which is involved-with replication-coupled chromatin assembly (224) - is unable to assemble chromatin that contains histone H1 (178). In the absence of H1 the nucleosome repeat length (i.e., the amount of DNA protected from MNase digestion) decreases by 5-10 bp, both *in vitro* (178) and *in vivo* (78). Although we did not perform experiments directed towards determining the repeat length of hdAd chromatin, *a posteriori* analyses of the Southern blot shown in Figure 18 suggested that hdAd vector DNA had a somewhat shorter nucleosome repeat length than that of bulk cellular chromatin (i.e., 168 ± 4 bp and 174 ± 3 bp, respectively) (Fig. 19). These values are similar to the nucleosome repeat length at the H19 gene in embryonic stem cells that are depleted of H1, compared to wild-type embryonic stem cells (i.e., 165 ± 5 bp and 171 ± 5 bp, respectively (78)). Therefore, hdAd vector chromatin likely does not incorporate H1.

H1 is an important constituent of transcriptionally-repressed chromatin (258). Furthermore, addition of H1 to chromatin assembly reactions decreases *in vitro* transcription by ~95% (163). Our results suggest that assembly into chromatin promotes gene expression *in vivo* (Fig. 17). However, this is presumably due to the fact that replication-independent chromatin assembly does not result in deposition of H1 (178). The fact that replication-coupled chromatin assembly is necessary for repression of basal transcription *in vivo* (8) also supports the notion that chromatin assembled independently of DNA replication does not incorporate H1.

Figure 19. hdAd chromatin exhibits a shorter repeat length than bulk cellular chromatin. (A) Shown is the DNA ladder that was resolved with the DNA that was analyzed in Figure 18. On the left is ladder DNA stained with EtBr; on the right is the signal that was obtained in Southern analysis due to cross-reaction between the probe and the ladder DNA. This was cross-reaction was essential for ascertaining the exact size of DNA in Southern analysis. (B) Data from Fig. 18 (i.e., the median concentration of MNase from the 24 hpi timepoint) are displayed at with ~2x magnification to emphasize the difference in migration of MNase-protected bands. Asterixes (*) denote the center of DNA bands generated from digestion with the median concentration of MNase from bulk cellular chromatin and hdAd DNA, respectively. (C) The size of indicated bands of oligonucleosomes for bulk cellular chromatin and hdAd chromatin displayed in (B) are plotted against the number of nucleosomes present in the band.


A
10
3
size
(kb)
1
0.5
B
900
800
700
600
500
400
300
200
size
(bp)
* *
* *
* *
C
Cellular
hdAd
900
800
700
600
500
400
300
DNA length (bp)
2
3
4
5
Nucleosome Number

hdAd vectors as a model system of replication-independent chromatin assembly

Aside from transfected plasmid DNA, the only existing *in vivo* models of *de novo* replication-independent chromatin assembly are *Xenopus* oocytes (228) and *Drosophila* (155, 174), which, like hdAd in mammalian cells, both swiftly-assemble exogenous DNA into physiologically-spaced chromatin in a HIRA-dependent manner. However, these models are both of limited-scalability for *in vivo* analyses. hdAd entry and trafficking to host-cell nuclei occurs swiftly (i.e., 1 h) via a well-characterized process (102, 164), and infection of host cells is simple, synchronizable, and scalable (102, 234). Thus, hdAd represent a unique model for experimental analysis of replication-independent chromatin assembly in mammalian cells.

Summary

We have characterized the assembly of hdAd DNA into chromatin. Core VII is swiftly evicted from vector DNA and is replaced by histones, which package vector DNA into physiologically-spaced chromatin. These data reveal that replication-independent chromatin assembly is not inherently inefficient in mammalian somatic cells. Furthermore, we have shown that assembly into chromatin is integral for efficient expression of ectopically-expressed transgenes in mammalian cells. Our data also show that hdAd vectors represent a novel and useful model for exploring the mechanism of replication-independent chromatin assembly.

Chapter 4 - Identification of Sp100 and Daxx as nuclear defense factors that drive epigenetic repression of invading DNA

4.1 - Introduction

Eukaryotic transgenes that are associated with prokaryotic DNA exhibit low level expression that is swiftly silenced. Thorough analyses have shown that prokaryotic DNA must be covalently attached to the transgene for repression to be observed (51). Having determined that hdAd vectors assemble into chromatin, and that chromatin plays an important role in the regulation of hdAd-encoded transgenes (Chapter 3), I tested the hypothesis that the repressive effects of prokaryotic DNA are mediated at the level of chromatin. Specifically, I hypothesized that prokaryotic DNA serves as a nucleation site by the formation of chromatin structures that are refractory to transcription, which spread from the bacterial DNA to the transgene.

In addition to examining the role of chromatin in mediating repression of genes associated with prokaryotic DNA, a second major goal of this research was to determine what cellular factors were responsible for this phenomenon. I hypothesized that repression may result from methylation of abundant CpG motifs in prokaryotic DNA and assembly of heterochromatin that spreads to the transgene. An alternative hypothesis is that prokaryotic DNA is recognized as foreign by innate nuclear defense factors, which repress associated genes for the safety of the cell. In this chapter, I describe experiments that tested these alternative hypotheses. My results suggest that prokaryotic DNA represses gene expression in cis via DNA methylation-independent nucleation of hypoacetylated chromatin, which then spreads to the transgene, and that the nuclear defense factors Sp100B/HMG and Daxx are crucial for this repression.

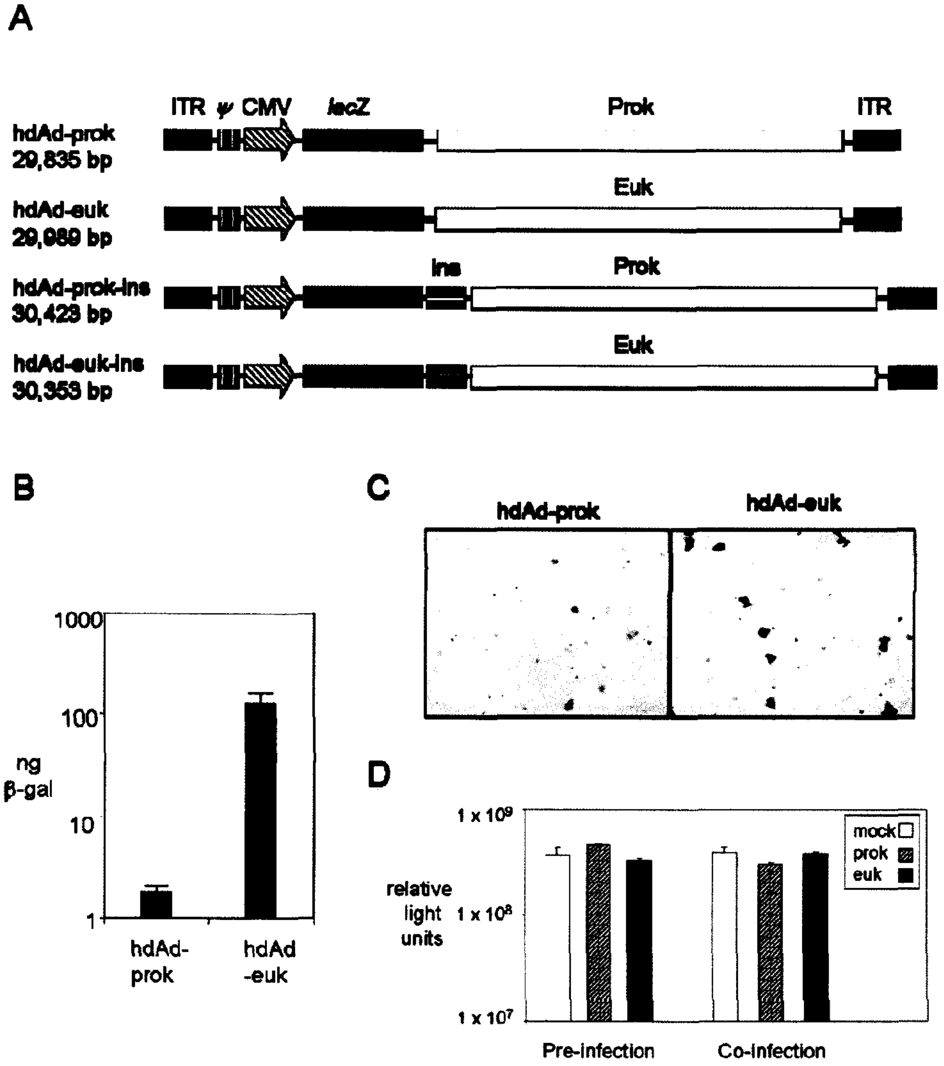
4.2 - Results

Prokaryotic DNA represses hdAd vector transgene expression

A major difference between prokaryotic and eukaryotic DNA is that prokaryotic DNA contains more CpG motifs (28). CpG motifs contained in prokaryotic DNA may inhibit expression of eukaryotic genes via two possible mechanisms: 1) in *cis* by CpG methylation-mediated formation of chromatin structures that actively repress transcription (27, 137) or 2) *in trans* through inflammatory responses to abundant CpG motifs may lead to down-regulation of transgene expression via global effects on host cell gene expression (60).

We have used hdAd vectors with identical transgene expression cassettes, but encoding approximately 22 kb of DNA derived from either prokaryotic (hdAd-prok) or eukaryotic organisms (hdAd-euk) (Fig. 20A) to test these hypotheses (215). Please note that although the hdAd-prok stuffer DNA was technically derived from a bacteriophage, we decided that it was an appropriate model because of its repressive effects of transgene expression (215). hdAd-prok expresses its transgene approximately 60-fold less efficiently than hdAd-euk: the poor performance of hdAd-prok after host cell transduction is clear on a global level, as cell lysate shows reduced β-gal expression (Fig. 20B), and on a per-cell basis, as individual transduced cells stain less intensely with X-gal (Fig. 20C). Pre- or co-infection with hdAd-prok had no affect on the ability of another hdAd vector to

Figure 20. hdAd-prok expresses its transgene less efficiently than hdAd-euk. (A) Schematic representation of the hdAd vectors used in this study. All vectors encode the Ad serotype 5 inverted terminal repeats (ITR) and packaging signal (ψ), murine cytomegalovirus immediate early promoter and enhancer (CMV), *lacZ* open reading frame, and the simian virus 40 poly-adenylation sequence. hdAd-prok and hdAd-prok-ins encode 22 kb of prokaryotic DNA (Prok); whereas hdAd-euk and hdAd-euk-ins encode 22 kb of eukaryotic DNA (Euk). hdAd-prok-ins and hdAd-euk-ins each have the HS4 double core insulator element (ins) inserted between the eukaryotic expression cassette and the stuffer DNA. (B) HeLa cells were infected with hdAd-prok or -euk (MOI = 1 IU/cell); 24 hpi, cell lysate was harvested and assayed for β-gal activity (N=4). (C) HeLa cells were infected with hdAd-prok or –euk (MOI = 0.1 IU/cell); 24 hpi, cells were stained with X-gal to detect transduced cells (blue). (D) HeLa cells were mock infected, or infected with hdAd-prok or -euk (MOI = 10 IU/cell) and pre- (i.e., 24 h before) or co-infected with hdAd-PGK-mSEAP (MOI = 1 IU/cell). Lysate was harvested 24 h after infection with hdAd-PGK-mSEAP and assayed for SEAP activity. Error bars represent the range of values obtained from two duplicate samples. Representative data from 2 independent experiments are presented.


A
ITR
ψ
CMV
lacZ
Prok
ITR
hdAd-prok
29,835 bp
Euk
hdAd-euk
29,989 bp
Ins
Prok
hdAd-prok-Ins
30,428 bp
Euk
hdAd-euk-Ins
30,353 bp
B
1000
100
10
1
ng
β-gal
hdAd-
prok
hdAd
-euk
C
hdAd-prok
hdAd-euk
D
1 x 10⁹
1 x 10⁸
1 x 10⁷
relative
light
units
mock
prok
euk
Pre-infection
Co-infection

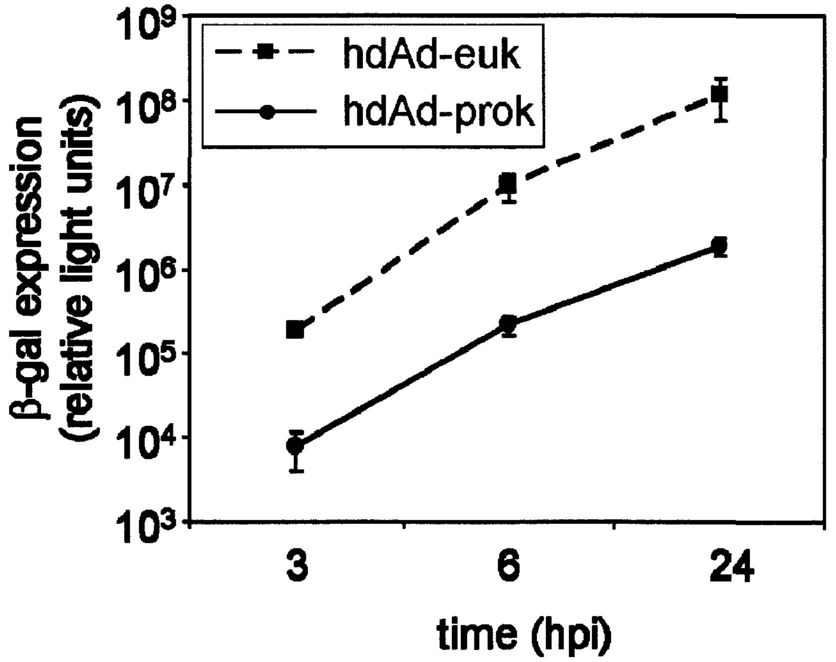
express its transgene, whether the reporter gene was identical (i.e., *lacZ*) (data not shown) or different (i.e., murine secreted alkaline phosphatase (mSEAP) (179, 199)) (Fig. 20D). Therefore, the prokaryotic DNA present in hdAd-prok represses transgene expression in *cis*.

Studies of plasmid DNA (232) and herpes amplicons (283) have shown that the repression of genes associated with prokaryotic DNA is time-dependent. In each case, prokaryotic DNA had little effect on transgene expression on the first day after gene delivery. However, over a period of days for herpes amplicons, or weeks for plasmids, the presence of prokaryotic DNA resulted in a pronounced repression of transgene expression. Contrary to these results, we observed nearly identical trends in the kinetics of transgene expression from both vectors, even at the earliest timepoint of 3 hpi (Fig. 21). Therefore, repression of hdAd-prok is not time-dependent.

Pharmacological abrogation of the repressive effects of prokaryotic DNA

Our observation that prokaryotic DNA represses gene expression in *cis* is consistent with previous observations (51) and supports our hypothesis that chromatin may play a role in mediating the repressive effects of prokaryotic DNA. As discussed in Chapter 1, two hallmarks of transcriptionally-repressed chromatin are methylated DNA and hypoacetylated histones. As a first step in analyzing the role of chromatin in repression of hdAd-prok transgene expression, we took a pharmacological approach to inhibit the enzymatic activity of either histone deacetylases (HDACs) or DNA methyltransferases (DNMTs).

Figure 21. Kinetics of transgene expression for hdAd-prok and -euk. HeLa cells were infected with hdAd-prok or -euk (MOI = 50 IU/cell). At the indicated times after infection, cells lysate was harvested and assayed for β-gal activity (N = 2).



hdAd-euk
hdAd-prok
β-gal expression (relative light units)
10^9
10^8
10^7
10^6
10^5
10^4
10^3
3
6
24
time (hpi)

HDACs were inhibited with TSA, a microbial small molecule that inhibits class I and II HDACs (291). TSA resulted in dose-dependent increases in transgene expression from both vectors, although hdAd-prok exhibited a larger increase in expression than hdAd-prok (i.e., ~40- and 10-fold, respectively) (Fig. 22A). Therefore, we conclude that hdAd-prok is more sensitive to the activity of HDACs than hdAd-euk. To examine the role of DNA methylation in repression of hdAd-prok, we treated cells with the DNA methyltransferase inhibitor 5-azacytidine (AZA) (143). We found that AZA treatment results in a dose-dependent increase in hdAd-prok transgene expression while having little effect on hdAd-euk (Fig. 22B). Therefore, histone deacetylation and DNA methylation may each contribute to the repressive effects of prokaryotic DNA.

The hdAd-prok transgene is associated with hypoacetylated histones

Results from the use of TSA (Figs. 16 and 22) suggested that covalent histone modifications, such as acetylation, may play important roles in regulating expression of hdAd vectors. Therefore, we employed chromatin immunoprecipitation (ChIP) to determine whether hdAd-prok and -euk differentially associated with histones bearing distinct post-translational modifications.

HeLa cells were infected with hdAd-prok or -euk, and 24 hpi were subjected to ChIP using antibodies raised against markers of transcriptionally-repressed (dimethyl K9 H3 and trimethyl K27 H3) or transcriptionally-active chromatin (acetylated histone H3 and acetylated histone H4 and RNA polymerase II) (Fig. 23). ChIP specificity and efficiency was confirmed by analyzing cellular loci. The myelin

Figure 22. Pharmacological inhibition of histone deacetylation or DNA methylation abrogates the repressive effects of prokaryotic DNA. HeLa cells were pre-treated 5 h with the indicated dose of TSA (A) or 18 h with the indicates dose of AZA (B) and subsequently infected with hdAd-prok or -euk (MOI = 1 IU/cell); 18 (A) or 24 (B) hpi, cells lysate was harvested and assayed for β-gal activity (N = 3 each).

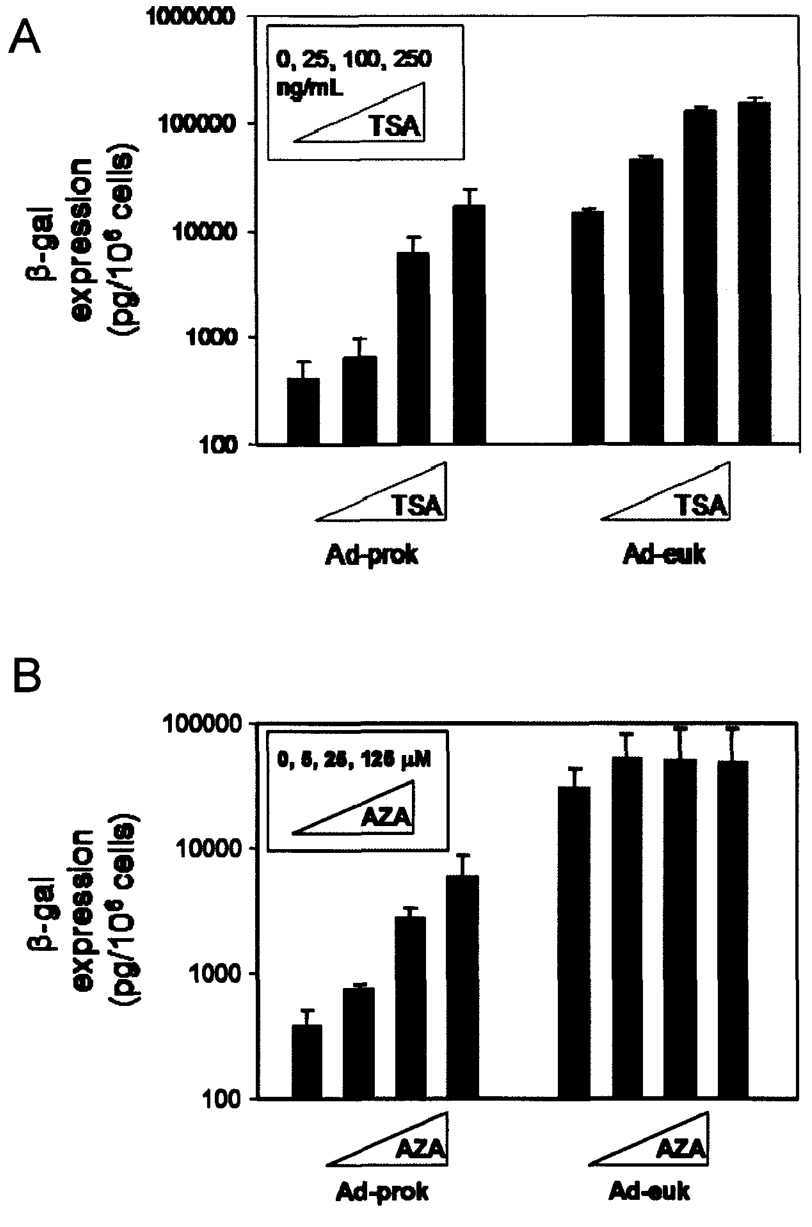
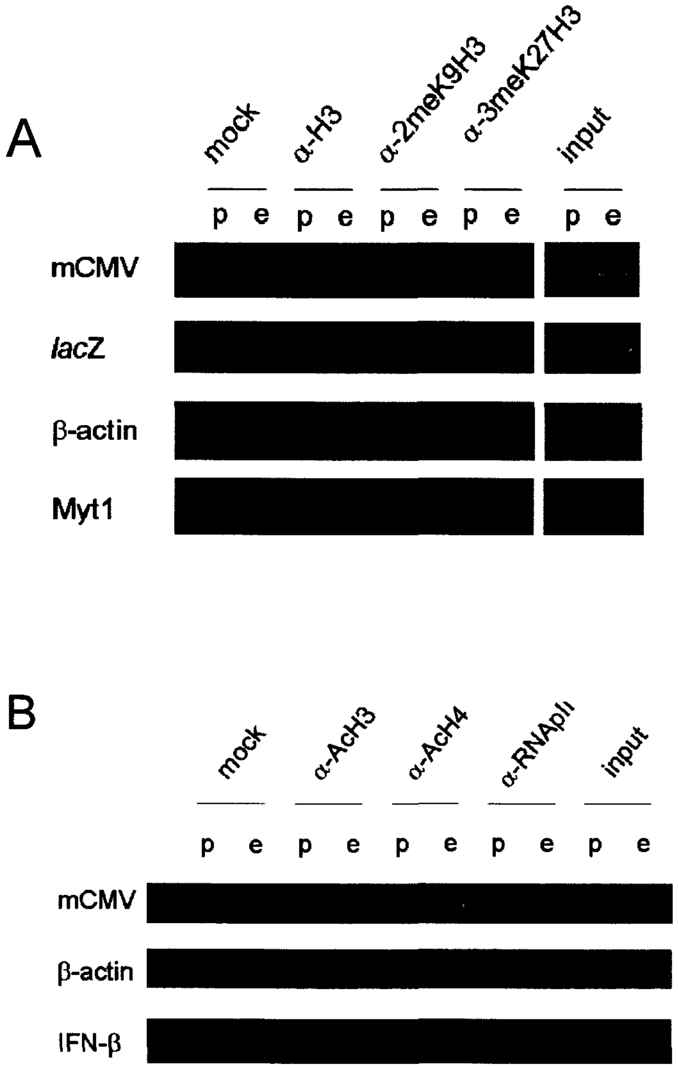

A
1000000
100000
10000
1000
100
β-gal expression (pg/10⁶ cells)
0, 25, 100, 250 ng/mL
TSA
TSA
TSA
Ad-prok
Ad-euk
B
100000
10000
1000
100
β-gal expression (pg/10⁶ cells)
0, 5, 25, 125 μM
AZA
AZA
AZA
Ad-prok
Ad-euk

Figure 23. hdAd-prok exhibits reduced association with markers of transcriptionally-active chromatin compared to hdAd-euk. HeLa cells were infected with hdAd-prok (p) or hdAd-euk (e) (MOI = 10 IU/cell); 24 hpi, cells were processed for ChIP with the indicated antibodies. (A) ChIP was performed with antibodies raised against heterochromatin-associated histone modifications. (B) ChIP was performed with antibodies raised against markers of transcriptionally active chromatin. ChIP DNA was analyzed by semi-quantitative PCR using vector-specific (mCMV promoter and *lac*Z open reading frame) or host-specific (β-actin, IFN- β and Myt1) primers. PCR products were resolved on agarose gels and visualized by EtBr staining.


A
mock
α-H3
α-2meK9H3
α-3meK27H3
input
p e p e p e p e p e
mCMV
lacZ
β-actin
Myt1
B
mock
α-AcH3
α-AcH4
α-RNApII
input
p e p e p e p e p e
mCMV
β-actin
IFN-β

transcription factor 1 (Myt1) promoter, which is transcriptionally-repressed in HeLa cells (153), was associated with H3 that was dimethylated at lysine 9 and trimethylated at lysine 27 (Fig. 23A). The constitutively-active β-actin promoter was associated with acetylated histones H3 and H4, but not with methylated H3 (Fig. 23A and B).

Both vectors exhibited similar association with unmodified histone H3 (Fig. 23A). Neither vector exhibited any significant association with histone H3 methylated at K9 or K27 within either the MCMV promoter or *lacZ* open reading frame (Fig. 23A). However, when compared to hdAd-euk, we observed a marked decrease in association of the hdAd-prok MCMV promoter with acetylated histones H3 and H4 (Fig. 23B). These data suggest that prokaryotic DNA represses gene expression independently of classical heterochromatin-associated histone modifications, (137, 156, 281), likely via recruitment of HDACs.

hdAd-prok vector DNA is not methylated

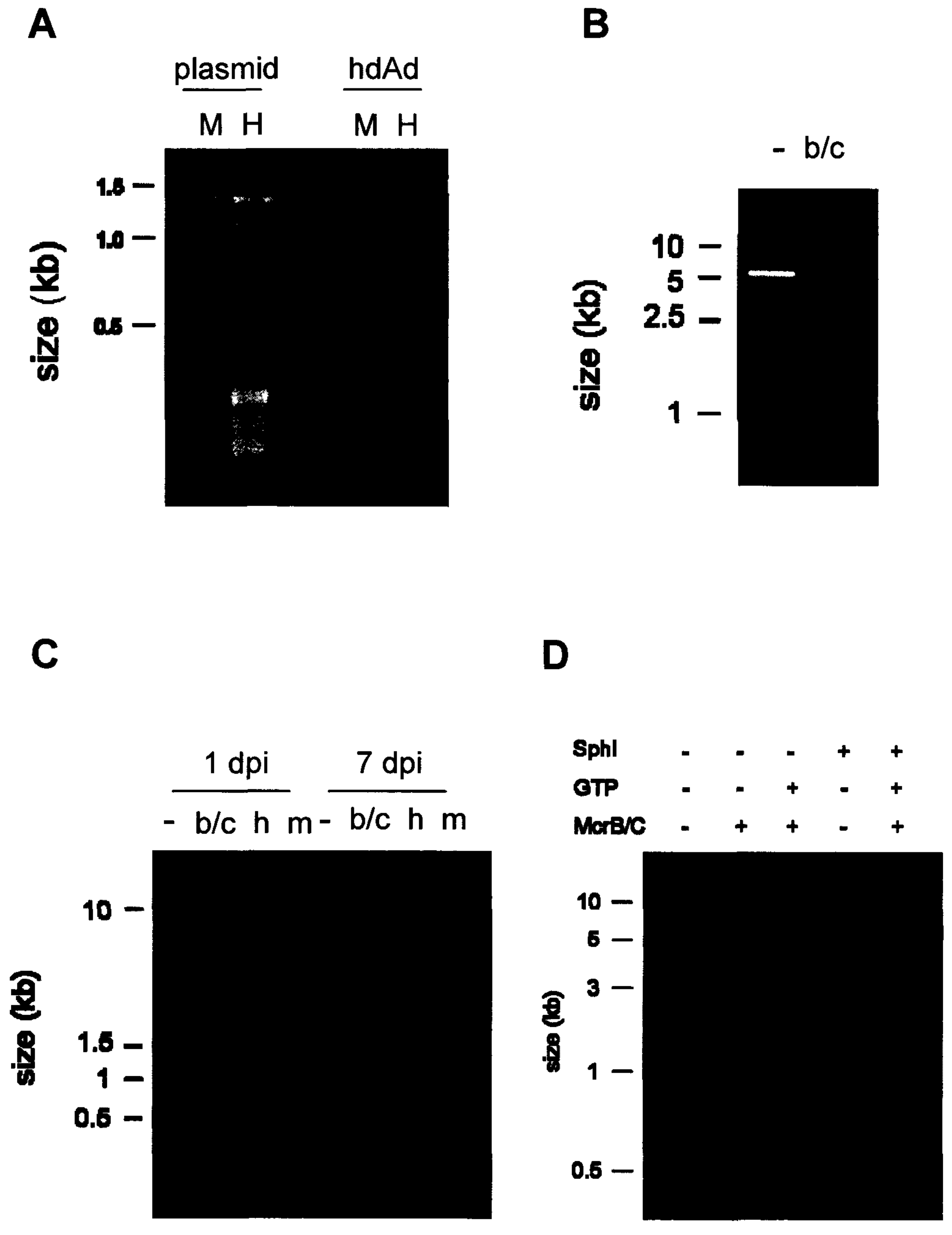
We hypothesized that the abundant CpG motifs present in prokaryotic DNA are methylated, leading to the formation of heterochromatin, which spreads from the stuffer DNA to the transgene. Although we did not detect markers of heterochromatin (Fig. 23A), our results from treatment with AZA (Fig. 22B) suggested that DNA methylation may play a role in repression of hdAd-prok. Therefore, we analyzed the methylation status of hdAd-prok DNA.

To determine whether hdAd-prok DNA is methylated prior to entering the host-cell, purified capsid DNA was digested with the isoschizomers MspI or HpaII.

MspI cuts the recoginition sequence CCGG in a methylation-independent manner, whereas HpaII digestion is blocked by CpG methylation. As expected, both enzymes were able to fully digest parental plasmid DNA (which is not CpG methylated, as *E. coli* do not encode a CpG methyltransferase (231)) (Fig. 24A). Similar results to the plasmid digestions were obtained upon digestion of capsid DNA, indicating that viral DNA is hypomethylated prior to host cell transduction (Fig. 24A). Therefore, if DNA methylation is indeed responsible for the inhibitory effects of prokaryotic stuffer DNA, hdAd-prok DNA must be subjected to de novo methylation after entry of the host cell.

We used two techniques to determine whether hdAd-prok DNA is methylated after host cell transduction. First, we isolated DNA from infected cells and digested with MspI, HpaII, or the endonuclease McrB/C (with or without co-digestion with SphI). McrB/C digests DNA between (A/G) methyl-C half sites (Fig. 24B) (therefore, excluding the MspI/ HpaII recognition sequences, CCGG). Southern analysis clearly showed that no significant McrB/C cleavage was observed at 1 or 7 days after infection (Fig. 24C). Also, no differences were observed in digestion with HpaII and MspI at either timepoint (Fig. 24C). The hdAd-prok genome is ~30 kb and modest digestion of a small number of methylated CpG motifs by McrB/C would be difficult to discern under the conditions of Figure 24C. Therefore, we digested genomic DNA from cells infected with hdAd-prok with SphI (which digests the hdAd-prok genome into three fragments that are easily resolved by conventional agarose gel electrophoresis) with or without MrcB/C. Results of this experiment clearly showed no difference in hdAd-prok banding pattern upon addition of McrB/C (Fig.

Figure 24. hdAd-prok DNA is unmethylated. (A) Plasmid DNA encoding hdAd-prok or DNA from purified hdAd-prok capsids was digested with MspI (M) or HpaII (H). (B) A linear, CpG-methylated plasmid with a single McrB/C recognition sequence was mock digested (-) or digested with McrB/C (b/c). (A) and (B) Digested DNA was resolved on an agarose gel and visualized by EtBR staining. (C) A549 cells were infected with hdAd-prok (MOI = 250 IU/cell) and genomic DNA was harvested at the indicated timepoints. Genomic DNA was undigested (-) or digested with McrB/C (b/c), H, or M. (D) A549 cells were infected with hdAd-prok (MOI = 250 IU/cell); 24 hpi genomic DNA was harvested. DNA was digested with the indicated enzymes with or without GTP. Digested DNA was analyzed by Southern blot with DIG-labelled hdAd-prok plasmid DNA.


A
plasmid
hdAd
M H
M H
size (kb)
1.5
1.0
0.5
B
- b/c
size (kb)
10
5
2.5
1
C
1 dpi
7 dpi
- b/c h m
- b/c h m
size (kb)
10
1.5
1
0.5
D
SphI - - - + +
GTP - - + - +
McrB/C - + + - +
size (kb)
10
5
3
1
0.5

24D). Therefore, hdAd-prok DNA is hypomethylated, even up to one week after infection (Fig. 24C).

The analyses described above excluded hypermethylation of hdAd-prok DNA via global analyses of the entire vector genome. However, to analyze specific regions of the viral genome in greater detail, we performed high resolution bisulfite sequencing (89) of DNA from infected cells. We bisulfite-converted genomic DNA and amplified and sequenced a 380 bp fragment of the MCMV promoter containing 22 CpG motifs and a 287 bp fragment from the central region of the prokaryotic stuffer DNA containing 20 CpG motifs. Sequence analysis of 6 clones from the MCMV promoter and 2 clones from the stuffer DNA detected no methylation of the 42 CpG motifs examined (data not shown). Together, our global and site-specific analyses failed to detect any evidence of hdAd-prok DNA methylation. Therefore, we conclude that DNA methylation does not mediate the repressive effects of prokaryotic DNA on eukaryotic expression cassettes.

An insulator element blocks the repressive effects of prokaryotic DNA

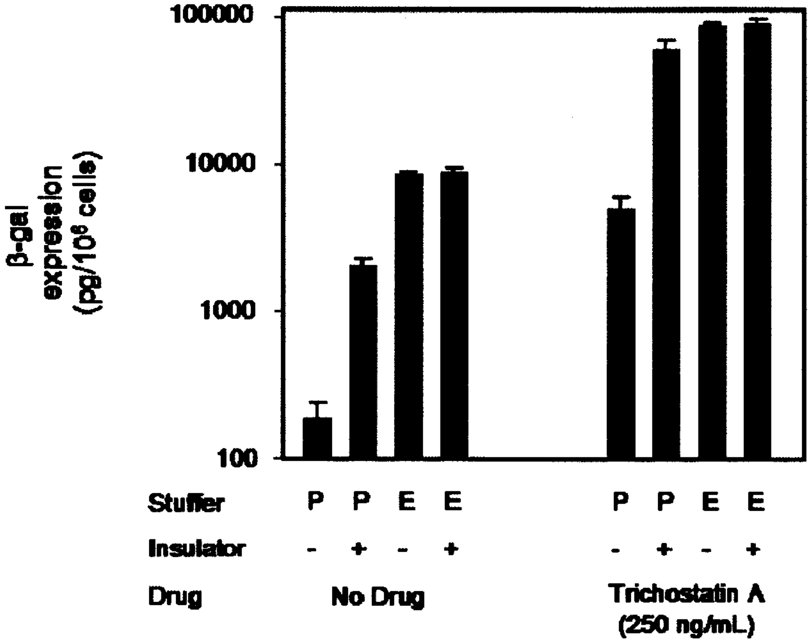
The results of the experiments described above indicate that prokaryotic DNA nucleates the formation of hypoacetylated chromatin, which results in the repression of transgene expression. Thus, insertion of an insulator element, which blocks the spread of transcriptionally repressive chromatin (200, 305), should alleviate the repressive effects of prokaryotic stuffer DNA. To test this hypothesis, we inserted the HS4 double core insulator element, which was derived from the chicken globin locus (229), between the transgene and stuffer DNA of hdAd-prok and hdAd-euk (producing hdAd-prok-ins and hdAd-euk-ins, respectively) (Fig. 20A).

Insertion of the insulator element had no effect on hdAd-euk transgene expression (Fig. 25). However, hdAd-prok-ins exhibited 33-fold greater expression than hdAd-prok (Fig. 25). Although insertion of the insulator element specifically increased expression of hdAd-prok, we were surprised to see that the insulator element did not completely abrogate the repressive effects of prokaryotic stuffer DNA. Recent evidence suggests that regions of the chicken globin insulator element not included in the double core insulator used here were required for efficient blocking of position-effect variegation in retroviral vectors (6). Thus, the minimal core element of HS4 may not completely block the spread of repressive chromatin from the prokaryotic DNA to the expression cassette.

To test this hypothesis, we treated cells with TSA and infected with hdAd-prok, -euk, -prok-ins, or -euk-ins (Fig. 25). The results of this experiment clearly showed that inhibition of histone deacetylation (which did not fully abrogate the repressive effects of prokaryotic DNA (Fig. 22A)) resulted in equal expression of hdAd-prok-ins and hdAd-euk-ins (Fig. 25). These data revealed that hdAd-prok is not intrinsically limited in its ability to express its transgene: when the repressive effects of prokaryotic DNA are completely abrogated, vectors stuffed with prokaryotic and eukaryotic DNA exhibited identical transgene expression.

Chromatin-independent repression of hdAd-prok

Figure 25. Blocking the spread of chromatin modifications from the hdAd-prok stuffer DNA to the transgene abrogates the repressive effects of prokaryotic DNA. The HS4 insulator element was inserted between the transgene and stuffer DNA of hdAd-prok and hdAd-euk (-) to produce hdAd-pro-ins and hdAd-euk-ins (+), respectively. HeLa cells were pre-treated 24 h with 5 h with 250 ng/ml TSA and subsequently infected with the indicated virus; 18 hpi, cell lysate was harvested and assayed for β-gal activity (N = 3).

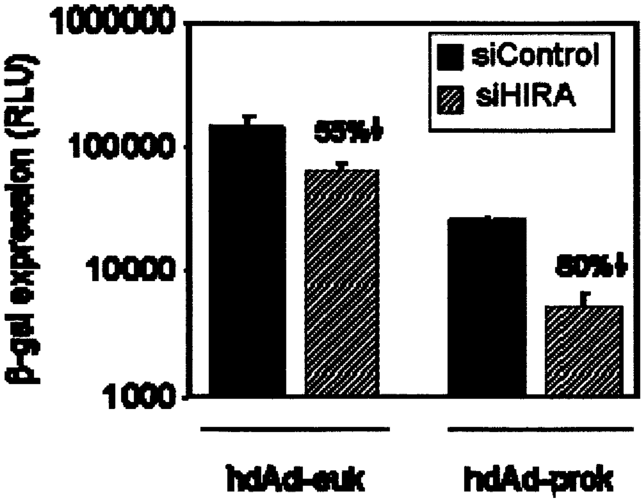

β-gal
expression
(pg/10^6 cells)
100000
10000
1000
100
Stuffer
P
P
E
E
P
P
E
E
Insulator
-
+
-
+
-
+
-
+
Drug
No Drug
Trichostatin A
(250 ng/mL)

Having found that the repressive effects of prokaryotic DNA are mediated primarily at the level of chromatin (Fig. 22A, 23B, and 25), we tested the hypothesis that abrogation of chromatin assembly would derepress hdAd-prok transgene expression. To this end, we depleted cells of HIRA using siRNA, which we have shown decreases both H3.3 deposition and expression of hdAd-euk (Fig. 17). Interestingly, abrogation of chromatin assembly decreased hdAd-prok transgene expression to a greater degree that of hdAd-euk (Fig. 26; i.e., 80 and 55% decreases, respectivley). Therefore, repression of hdAd-prok gene expression may be mediated both at the level of chromatin modifications (Fig. 23B) and via a chromatin-independent mechanism.

Heat stress results in increased expression of the hdAd-prok transgene

Having determined that methylation of abundant CpG motifs in prokaryotic stuffer DNA was not required for repression of hdAd-prok (Fig. 24), we sought to identify cellular factors that recognize and repress transgenes associated with prokaryotic DNA. The promyelocytic leukemia (PML) protein nucleates the formation of subnuclear structures called PML bodies (24). PML bodies assemble *de novo* near the genomes of large DNA viruses soon after host cell transduction (75). These viruses, which include CMV, herpes simplex virus-1, and Ad encode genes that reorganize PML bodies, which are necessary for productive infection (74). PML bodies also contain the cellular proteins Sp100 and DAXX, which have been implicated in repressing viral gene expression (204, 241). Therefore, we

Figure 26. Chromatin-independent repression of hdAd-prok transgene expression. HeLa cells were transfected with control siRNA or siRNA that targeted sequences in HIRA mRNA. Forty-eight hours later, cells were infected with hdAd-prok or -euk (MOI = 1 IU/cell); 6 hpi, cell lysate was harvested for analysis of β-gal activity, which is displayed in RLU. Representative data from two similar experiments are displayed; error bars represent range of expression from duplicate samples. Note that the hdAd-euk data was also displayed in Figure 17.


β-gal expression (RLU)
1000000
100000
10000
1000
siControl
siHIRA
55%↓
80%↓
hdAd-euk
hdAd-prok

hypothesized that PML bodies may repress expression of DNA associated with prokaryotic DNA.

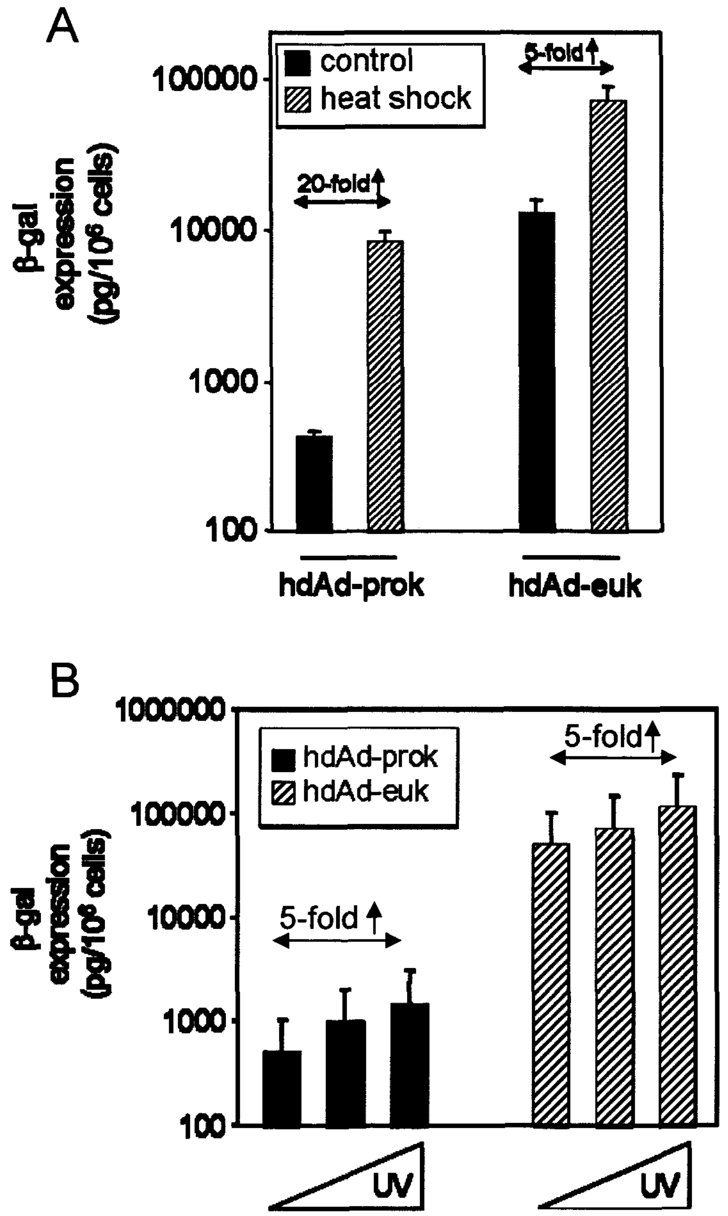
To test this hypothesis, we subjected cells to heat stress, which disrupts PML bodies (203), prior to infection with hdAd-prok or -euk. We observed that heat stress prior to infection resulted in increased expression of both hdAd-prok and -euk 24 hpi (Fig. 27A). However, heat stress resulted in a greater increase in expression of hdAd-prok than -euk (i.e., ~20-fold versus 5-fold, respectively). Therefore, heat stress derepresses hdAd-prok.

As a control, we examined the ability of another stress-inducer, ultra-violet radiation (UV), to derepress hdAd-prok. UVR, like heat stress, activates JNK signaling, and activation of AP-1 target genes (2). However, UVR does not result in the disruption of PML bodies; rather, PML bodies play an important role in DNA repair after UVR stress (66). To test the specificity of heat stress-induced derepression of hdAd-prok, we pre-treated cells with increasing doses of UVR stress and then infected with hdAd-prok or -euk. UVR stress resulted in equal, dose-dependent, increases in expression of both vectors (i.e., 5-fold with the highest dose tested) (Fig. 27B). Therefore, heat shock-induced derepression of hdAd-prok expression is not due to a generalized cellular stress response.

PML bodies and PML are unnecessary for repression of hdAd-prok

Heat stress specifically derepressed expression of hdAd-prok (Fig. 27A). This observation supports our hypothesis that PML bodies contribute to repression expression of hdAd-prok. To further examine the role of PML bodies in repression

Figure 27. Heat stress derepresses hdAd-prok transgene expression. HeLa cells were subjected to (A) heat stress (2 h 43°C) or (B) Cells were subjected to UV stress (100 J/m^2 for 0, 30, or 60 min) and then allowed to recover for 30 min at 37°C and 5% CO_2. Cells were subsequently infected with hdAd-prok or -euk (MOI = 1 IU/cell); 24 hpi, cells lysate was harvested and assayed for β-gal activity (N = 3) (A); (B) displayed is mean ± range of values from duplicate samples.


A
β-gal expression (pg/10^6 cells)
100000
10000
1000
100
control
heat shock
20-fold ↑
5-fold ↑
hdAd-prok
hdAd-euk
B
β-gal expression (pg/10^6 cells)
1000000
100000
10000
1000
100
hdAd-prok
hdAd-euk
5-fold ↑
5-fold ↑
UV
UV

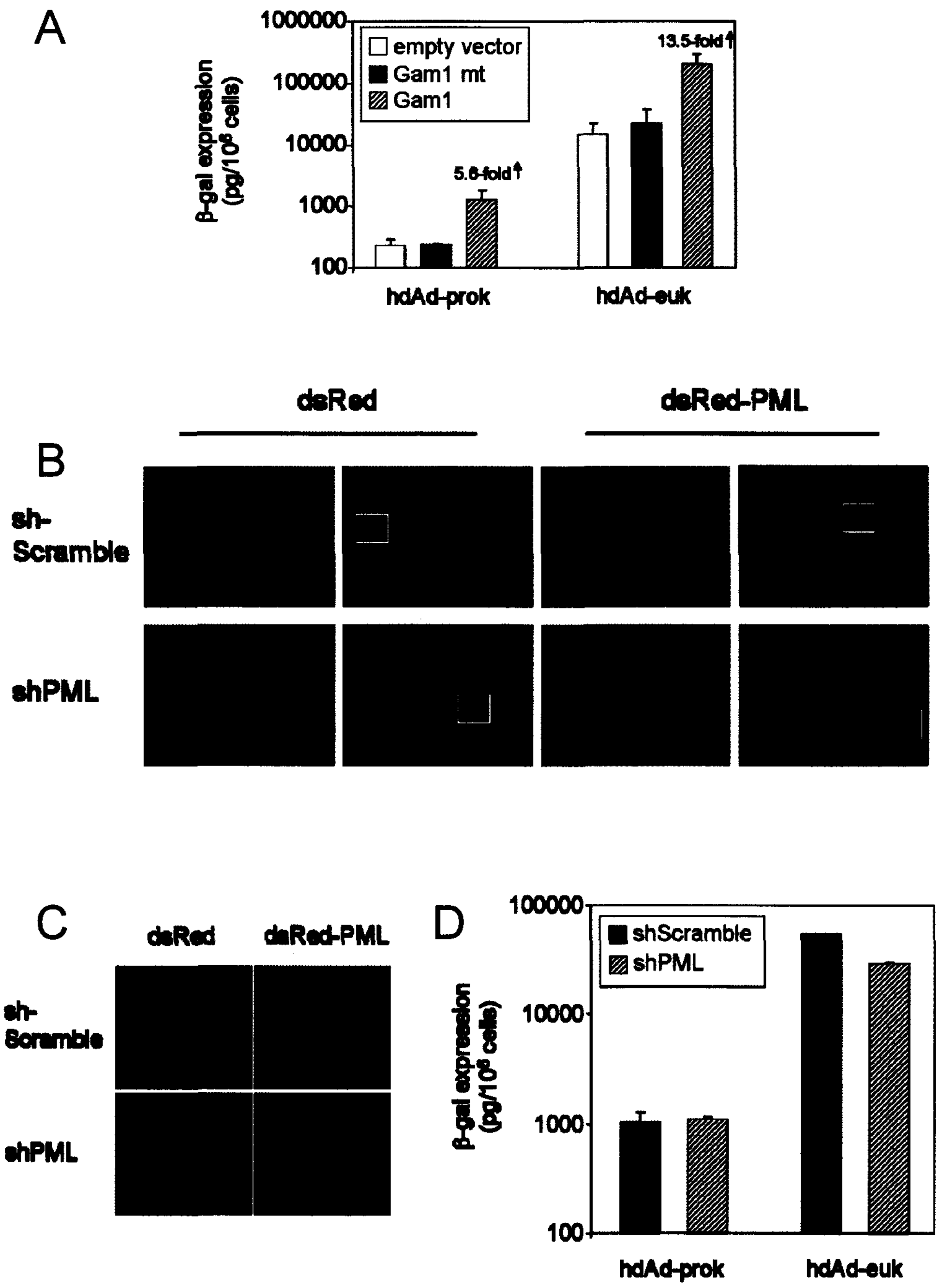
of hdAd-prok, we disrupted PML bodies via a second, independent process. Covalent attachment of SUMO to PML is necessary for PML body formation (324). The chicken Ad protein Gam1 blocks SUMOylation of cellular proteins and disrupts PML bodies (56). Transfection of cells with a mutant Gam1 that is unable to abrogate SUMOylation of host proteins (56) had no effect on expression of either vector. However, expression of wild-type Gam1 derepressed both hdAd-prok and -euk (Fig. 28A). Contrary to what was observed with heat shock, Gam1 derepressed hdAd-euk to a greater degree than hdAd-prok (i.e., 13.5 and 5.6-fold, respectively) (Fig. 28A). Therefore, PML disruption does not specifically derepress hdAd-prok.

To directly examine the role of PML in repression of hdAd-prok, we generated a plasmid encoding a short-hairpin RNA that was shown previously to knock-down expression of PML (77). Transfection of cells with this plasmid blocked ectopic expression of dsRed-PML, indicating its efficacy in my hands (Fig. 28B and C). However, depletion of PML had no effect on expression of hdAd-prok, and modestly decreased expression of hdAd-euk (Fig. 28D). Therefore, PML plays no significant role in repression of hdAd-prok transgene expression.

Sp100B and Sp100HMG repress hdAd-prok transgene expression

Although PML is essential for the formation of PML bodies, several constituents of PML bodies, Sp100, Daxx, and ATRX are able to accumulate upon incoming HSV genomes in the absence of PML, where they contribute to repression of gene expression (76). The major Sp100 isoform, Sp100A, is constitutively localized to PML bodies *in vivo*; Sp100B and Sp100 HMG, on the other hand, exhibit a diffuse

Figure 28. PML bodies are not necessary for repression of hdAd-prok gene expression. HeLa cells were transiently transfected with empty vector, or with the indicated plasmids; 24 h (A) or 48 h (D) post-transfection, cells were infected with hdAd-prok or -euk (MOI = 1 IU/cell). Twenty-four hpi, cell lysate was harvested and assayed for β-gal activity (A, N = 2); (B) displayed is mean ± range of values from duplicate samples (representative data from two similar experiments are shown) (D). (B) Twenty-four hours after the transfection described for (D), cells were transfected with plasmids encoding dsRed or dsRed-PML. Next day, the cells were visualized using fluorescence microscopy and photographed either in bright field or in the red channel using a 10x objective. (C) Higher resolution photographs of boxed portions of pictures in (B)


A
β-gal expression (pg/10⁶ cells)
1000000
100000
10000
1000
100
□ empty vector
■ Gam1 mt
▨ Gam1
5.6-fold↑
13.5-fold↑
hdAd-prok
hdAd-euk
B
dsRed
dsRed-PML
sh-Scramble
shPML
C
dsRed
dsRed-PML
sh-Scramble
shPML
D
β-gal expression (pg/10⁶ cells)
100000
10000
1000
100
■ shScramble
▨ shPML
hdAd-prok
hdAd-euk

nuclear distribution, have a DNA binding domain the specifically binds unmethylated CpG motifs (i.e., the SAND (Sp100, autoimmune regulator 1, NucP41/P75, deformed epidermal autoregulatory factor 1 domain) (127), and repress HSV-1 gene expression and replication (204). To examine the role of Sp100 in the regulation of hdAd-prok expression, we transfected cells with a pool of 4 siRNA that anneal to sequences common to mRNA encoding all four Sp100 isoforms. siRNA transfection resulted in a ~65% knockdown of Sp100A, the only isoform that we could detect at steady state levels in HeLa cells by immunoblot (Fig. 29A). Sp100 knockdown resulted in a 25% decrease of hdAd-euk expression, while up-regulating hdAd-prok expression by 2.6 -fold (Fig. 29B). Therefore, Sp100 repressor isoforms may contribute to repression of hdAd-prok transgene expression.

To determine whether distinct Sp100 isoforms exhibited differential effects of hdAd-prok gene expression, we transiently over-expressed Sp100A, Sp100B, or Sp100HMG and subsequently infected with hdAd-prok or -euk (Fig. 30A and B). Interestingly, over-expression of Sp100A resulted in ~2-fold increases in expression of both vectors (Fig. 30B). However, over-expression of Sp100B had no effect on hdAd-euk, but decreased hdAd-prok expression by 20%. Sp100HMG decreased expression of both vectors, although hdAd-prok was repressed by a greater degree than hdAd-euk (i.e., 50% and 30%, respectively) (Fig. 6D). Therefore, Sp100 isoforms that have previously been implicated in innate defense against viral intruders repress expression of hdAd-prok.

Daxx mediates repression hdAd-prok

Figure 29. Depletion of all Sp100 isoforms differentially-affects hdAd-prok and -euk transgene expression. HeLa cells were transfected with control siRNA or pooled siRNA targeting sequences common to mRNA encoding all four Sp100 isoforms. Forty-eight hours after transfection, cells were harvested for immunoblot with the indicated antibodies (A) or infected with hdAd-prok or -euk (B). (A) The membrane was probed with anti-Sp100, stripped, and reprobed with anti-α-tubulin. (B) Twenty-four hours after infection cells lysate was harvested and assayed for β-gal activity ($N = 2$).

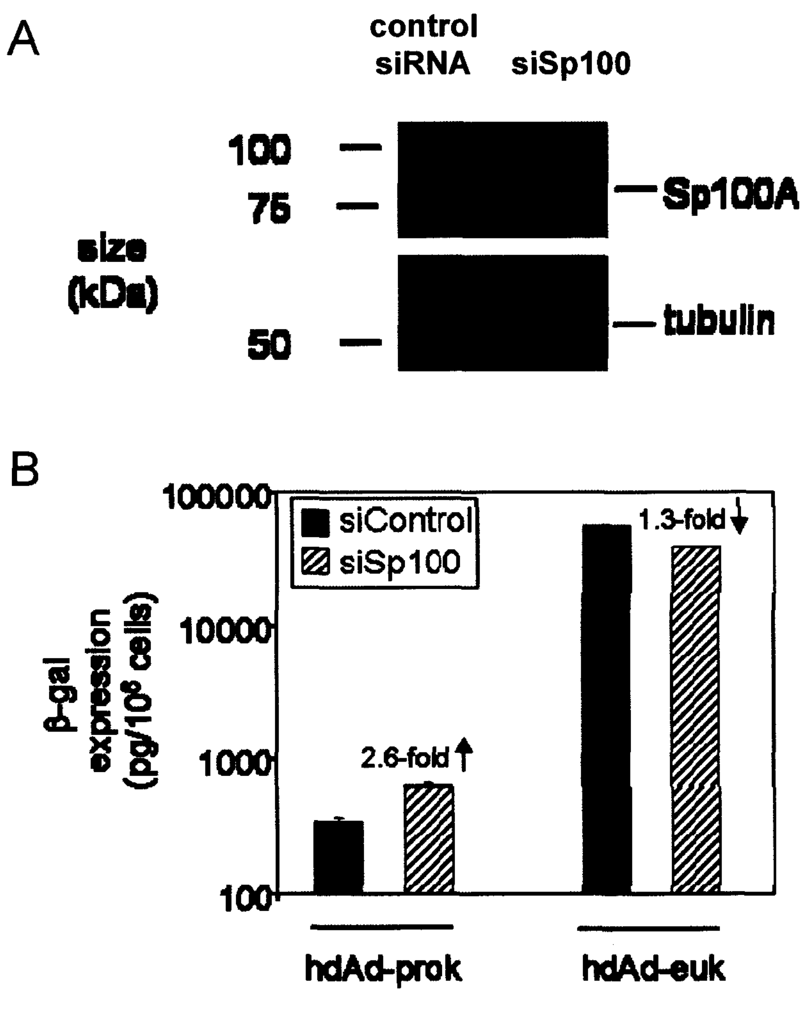
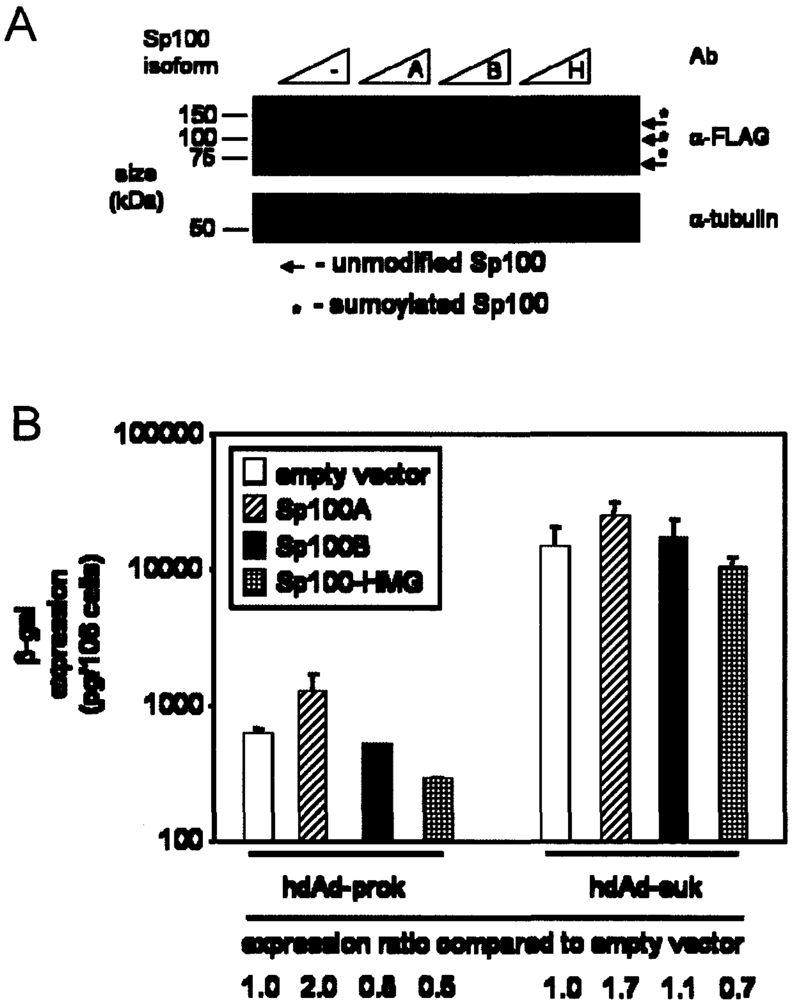

A
control siRNA
siSp100
100
75
50
size (kDa)
Sp100A
tubulin
B
100000
10000
1000
100
β-gal expression (pg/10⁶ cells)
siControl
siSp100
2.6-fold ↑
1.3-fold ↓
hdAd-prok
hdAd-euk

Figure 30. Sp100B and Sp100HMG repress hdAd-prok transgene expression. HeLa cells were transfected with empty vector or plasmids encoding Sp100A, Sp100B, or Sp100HMG. Twenty-four hours after transfection, cells were harvested for immunoblot with the indicated antibodies (A) or infected with hdAd-prok or -euk (B). (B) Twenty-four hours after infection cells lysate was harvested and assayed for β-gal activity (N = 2).

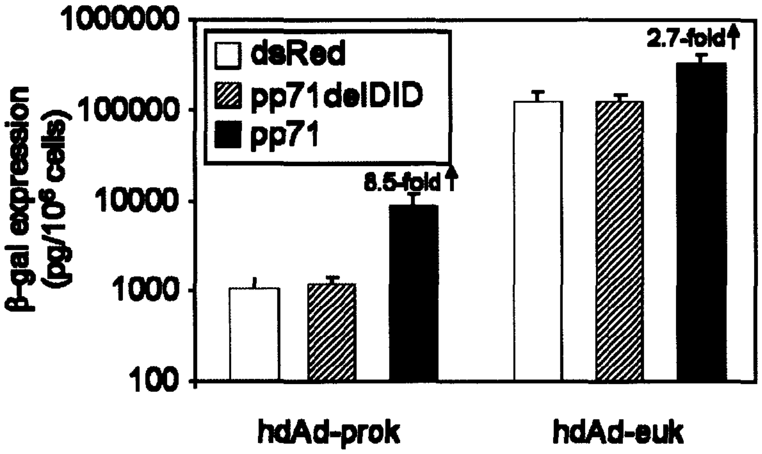

A
Sp100 isoform
-
A
B
H
Ab
150
100
75
size (kDa)
50
α-FLAG
α-tubulin
← - unmodified Sp100
* - sumoylated Sp100
B
100000
10000
1000
100
β-gal expression (pg/106 cells)
empty vector
Sp100A
Sp100B
Sp100-HMG
hdAd-prok
hdAd-euk
expression ratio compared to empty vector
1.0 2.0 0.8 0.5
1.0 1.7 1.1 0.7

Repressive isoforms of Sp100 appear to modestly affect hdAd-prok expression. However, such small differences in hdAd-prok expression upon depletion or over-expression of Sp100 isoforms suggests that more factors may repress expression of hdAd-prok. Therefore, we focused on a third PML body constituent, Daxx. Daxx is a transcriptional co-repressor that binds to HDAC1 and HDAC2 (122) and represses expression of CMV genes (241, 289). Heat shock releases Daxx from PML bodies (203). Furthermore, upon heat shock, Daxx binds to heat shock factor 1 and mediates target gene activation (31). Our observations of TSA- (Fig. 22A) and heat shock-induced (Fig. 27A) expression of hdAd-prok are consistent with a role for Daxx in repression of hdAd-prok.

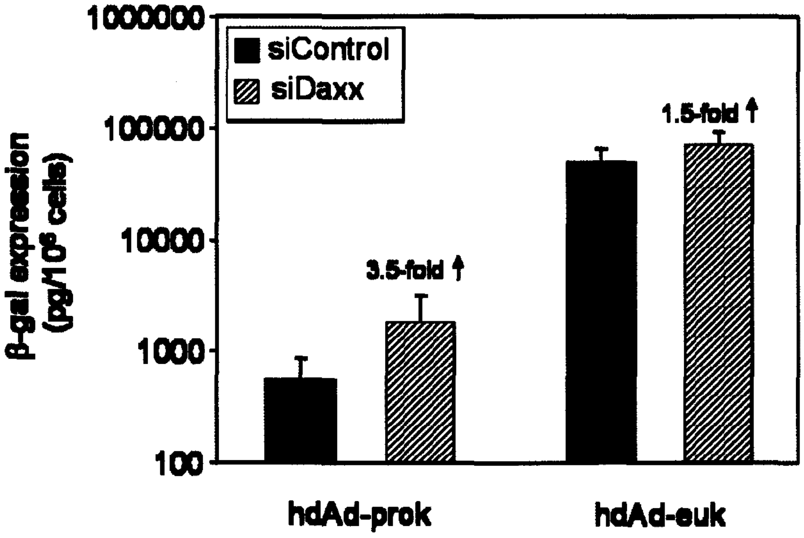
To determine whether Daxx plays a role in repression of hdAd-prok, we transfected cells with pp71, a cytomegalovirus immediate early gene product that induces proteosomal degradation of Daxx (126, 241). As a control, cells were transfected with a mutated form of pp71 unable to bind Daxx (pp71delDID2/3) (120). Compared to empty vector-transfected cells, transgene expression by hdAd-prok and -euk was unaffected by pp71delDID2/3. However, pp71 resulted in an 8.5 and 2.7-fold increases in transgene expression by hdAd-prok and -euk, respectively (Fig. 31A). These data mirror those obtained in HDAC-inhibition (Fig. 22A) and heat shock (Fig. 27A) experiments. To confirm that Daxx mediates repression of hdAd-prok, we employed siRNA to knock-down expression of Daxx. Although siRNA-mediated depletion of Daxx was not as effective as pp71-mediated degradation in relieving the repressive effects of prokaryotic DNA (Fig. 31B), similar trends were observed (i.e., 3.5- and 1.5-fold induction of hdAd-prok and -euk, respectively).

Figure 31. Daxx contributes to repression of hdAd-prok transgene expression. HeLa cells were transfected with empty vector or plasmids encoding mutant or wild-type pp71 (A) or with control siRNA or pooled siRNA targeting sequences in the Daxx mRNA (B). Twenty-four (A) or 48 (B) hours post-transfection, cells were infected with hdAd-prok or -euk. Twenty-four hours after infection, cell lysate was harvested and assayed for β-gal activity (N=2).

A


β-gal expression (pg/10^6 cells)
1000000
100000
10000
1000
100
dsRed
pp71delDID
pp71
8.5-fold ↑
2.7-fold ↑
hdAd-prok
hdAd-euk

B


β-gal expression (pg/10^6 cells)
1000000
100000
10000
1000
100
siControl
siDaxx
3.5-fold ↑
1.5-fold ↑
hdAd-prok
hdAd-euk

Therefore, in addition to Sp100 repressor isoforms, Daxx plays a role in repressing expression of genes associated with prokaryotic DNA.

4.3 - Discussion

Mechanistic basis of the repressive effects of repression of hdAd-prok

We have used hdAd vectors stuffed with prokaryotic DNA to address the mechanistic basis of how the covalent linkage of prokaryotic DNA represses eukaryotic gene expression. The repressive effects of prokaryotic DNA were abrogated by inhibition of histone deacetylation (Fig. 22A) or by insertion of an insulator element between the transgene and stuffer DNA (Fig. 25B). Although we saw no evidence of heterochromatin (i.e., methylation of H3 K9 or K27) (Fig. 23A), we observed reduced association of the hdAd-prok expression cassette with acetylated histones H3 and H4 (Fig. 23B). Finally, we did not detect methylation of prokaryotic stuffer DNA or the hdAd-prok chromatin (Fig. 24). These data suggest that prokaryotic DNA recruits HDACs, which induce local histone hypoacetylation that spreads to the expression cassette, resulting in reduced transgene expression.

Our results are partially supported by research on other gene delivery systems. Mark Kay and colleagues found that prokaryotic DNA sequences in plasmid DNA exclusively repressed gene expression in *cis* (51). Furthermore, their recent results support my conclusion that DNA methylation is unnecessary for repression of genes linked to prokaryotic DNA (52). However, results from this group (232) and another analyzing HSV amplicon vectors (283) suggested that prokaryotic DNA results in the assembly of associated transgenes into heterochromatin. In contrast to both of these studies, we detected no evidence of association with methylated H3 K9 (Fig. 23A). However, we believe that this is due primarily to differences in the timepoints chosen for ChIP analyses. Specifically, we performed ChIP analyses within 1 day of infection, whereas these groups analyzed the chromatin state of HSV amplicons (283) or plasmids (232) on days six and thirty-five post-gene delivery, respectively. In each case the authors chose timepoints at which the difference in expression between transgenes was maximal, despite obvious differences in expression at much earlier timepoints. Our results suggest that soon after gene delivery, the repressive effects of prokaryotic DNA are mediated by histone deacetylation. Assembly into heterochromatin at later timepoints is likely a consequence of transcriptional inactivation, not its cause.

Derepression of hdAd-prok by 5-azacytidine

We found that treatment with the DNA methyltransferase inhibitor AZA derepressed hdAd-prok (Fig. 22B). This initially led us to believe that hdAd-prok DNA was methylated. However, we found no evidence of a single methylated CpG in the hdAd-prok stuffer DNA or promoter (Fig. 24 and data not shown). How did AZA induce expression of hdAd-prok? As suggested above, Sp100 repressor isoforms may recruit HP1 to repress expression of genes associated with foreign DNA. Recent studies have shown that treatment with AZA results in mobilization of HP1 from heterochromatin to pericentric heterochromatin, where it contributes to stress-recovery (321). Therefore, AZA may have derepressed hdAd-prok by stimulating release of HP1 from Sp100 repressor isoforms.

PML is unnecessary for repression of hdAd-prok

We initially chose to examine the role of PML bodies in repression of hdAd-prok because of their known role in the repression of DNA virus replication (72, 74, 77, 198, 317). However, we found that neither PML bodies, nor PML protein were necessary for repression of hdAd-prok (Fig. 28). Recent studies of Ad (297) and HSV (77), which confirmed that mutant viruses that are unable to reorganize PML bodies are replication-defective, also showed that PML bodies had no impact on transcription of Ad genes and that expression of HSV genes was only slightly-delayed. Rather, PML bodies were required for inhibition of steps of viral lifecycles that were downstream of early gene transcription. In light of these data, it is unsurprising that PML played no significant role in the repression of hdAd-prok gene expression.

Identification of cellular factors that mediate repression of hdAd-prok

Having excluded DNA methylation as the cause of the repressive effects of prokaryotic DNA (Fig. 24), we searched for cellular factors that may recognize and repress expression of genes associated with foreign DNA. We targeted PML bodies for analysis because of their known role in repression of DNA virus gene expression (74). Heat stress, which disrupts PML bodies (203), specifically derepressed hdAd-prok (Fig. 27A). However, neither Gam1-mediated PML body disruption, nor depletion of PML specifically derepressed hdAd-prok (Fig. 27). Therefore, heat shock-induced derepression of hdAd-prok was likely independent of PML body disruption.

The Sp100 gene encodes four isoforms, of which three (B, C, and HMG) have been implicated in transcriptional repression and antiviral defense (127, 204). Depletion of all Sp100 isoforms decreased expression of hdAd-euk and increased expression of hdAd-prok (Fig. 29B). Also, over-expression of repressive Sp100 isoforms specifically repressed hdAd-prok (Fig. 30B). Interestingly, over-expression of Sp100A resulted in increased expression of both vectors (Fig. 30B). This may explain why depletion of Sp100 resulted in a decrease of hdAd-euk gene expression and suggests that depletion all Sp100 isoforms may have masked induction of hdAd-prok transgene expression in response to depletion of the repressive isoforms.

Identification of Daxx as a repressor of hdAd-prok gene expression reveals a mechanism for recruitment of HDACs to the hdAd-prok genome. Daxx is transcriptional co-repressor that binds to HDAC2 (122) and represses expression of CMV genes (241, 289). Heat shock releases Daxx from PML bodies (203). Furthermore, upon heat shock, Daxx binds to heat shock factor 1 and mediates target gene activation (31). Our observations of TSA- (Fig. 22A) and heat shock-induced (Fig. 27A) expression of hdAd-prok are consistent with a role for Daxx in repression of hdAd-prok.

How does Sp100 repress hdAd-prok?

Although some previous studies have suggested that Sp100B and HMG repress gene expression (127, 204, 310), the mechanistic basis of repression is unknown. The Sp100 repressor isoforms each contain a DNA binding domain that binds unmethylated CpG motifs (which are prevalent in the hdAd-prok backbone) in a cooperative manner (127). All Sp100 isoforms interact with heterochromatin protein 1 (HP1) (252, 253). When artificially tethered to a transgene, HP1 is a potent repressor of gene expression (298). This repression is relieved by the HS4 insulator, which is consistent with our data (Fig. 25). However, transcriptional repression by HP1 is thought to be mediated by recruitment of the H3K9-specific histone methyltransferase Suv39H1 and DNA methytransferases (93). We observed no evidence of recruitment of these enzymes (Fig. 23A and 24), indicating that repression must be via a distinct mechanism. Interestingly, transcriptionally-silent, quiescent HSV genomes are enveloped by enlarged PML structures that also contain Sp100 and HP1, but no evidence of methylated H3K9 (76). These data are consistent with a role for HP1 and Sp100-mediated repression.

We found that inhibition of chromatin assembly by depletion of HIRA resulted in greater reduction in transgene expression from hdAd-prok than -euk (i.e., 80% and 55% decrease, respectively) (Fig. 26). In light of our results that suggested that repression of hdAd-prok is mediated at the level of chromatin (Fig. 22A, 23B, and 25), we found this surprising. However, Sp100-mediated recruitment of HP1 may represent a chromatin-independent mechanism of repression of genes associated with foreign DNA, perhaps by chromatin-independent DNA compaction (322).

How is Daxx recruited to hdAd-prok DNA?

Daxx does not have a DNA binding domain (169), which suggests that another factor must recruit Daxx to the hdAd-prok genome. Daxx binds a number of transcription factors, including androgen and gucocorticoid receptors, and Smad4, and mediates repression of their target genes (265). However, these interactions are mediated by covalent attachment of SUMO to the target protein and the C-terminal SUMO-interacting domain of Daxx (168, 265), and we found that the repressive effects of prokaryotic DNA are SUMOylation- independent (Fig. 28B). Daxx may be recruited by an unknown protein that binds GC-rich DNA or CpGs. One of few known cellular target of Daxx-mediated repression is c-Met (193, 196). The region of the mouse c-Met promoter that recruits Daxx is a 65 bp fragment that is 78% GC and contains 10 CpG motifs (196). This fragment is poorly conserved between humans and mice, although the 88 bp human fragment is 74% GC and contains 9 CpG motifs (only 3 of which are conserved from mice to humans) (Fig. 32). Therefore, Daxx may be recruited to DNA with high GC-content and/or CpG ratios, in a sequence-independent manner.

Like large DNA viruses (discussed above), small DNA viruses associate with PML bodies. However, small DNA viruses spend most of their lifecycles near PML bodies (65, 266, 284), suggesting that their genes are not repressed by PML body constituents. Small DNA viruses have CpG and GC content that is similar to that of host DNA, whereas large DNA viruses have high GC content and encode the statistically-expected number of CpG motifs (256). Interestingly, hdAd-prok stuffer DNA has GC and CpG content that are similar to those of large DNA viruses (215, 256). Daxx may be recruited to DNA with abnormally high GC or CpG content, where it represses gene expression.

Figure 32. Alignment of sequences from the c-Met promoters from rat, human, and cow, that are homologous to the mouse fragment that recruits Daxx (188). The sequence is numbered according to the mouse genome sequence, with +1 representing the transcription start site. Well-conserved fragments are boxed. The box labelled AP1 matches the consensus AP1 binding site (15).

```
       -206                                        -172
        |                                   unknown|
mouse   GTCTCTTC----------GCCTCCAGCCCGGACGCGCCCGGGC-
rat     ..T.....----------.........................
human   ....G...TCCTGGCCGT..T...T-..T.....T........T
cow     ....G...TCCTGGCCGT......G....CA............C

       -171                                         -142
                             unknown   AP1
        |                                             |
mouse   GCCGCTGT-------------TGCCCGGCTGAGTCACGGGCCGG
rat     .....C..T------------..G.....................
human   ...C.G..CCGCGCGCGCCGG................T...A..
cow     .....G..CCGCGCGCGCCGA................T...A..
```

Limitations of our approach

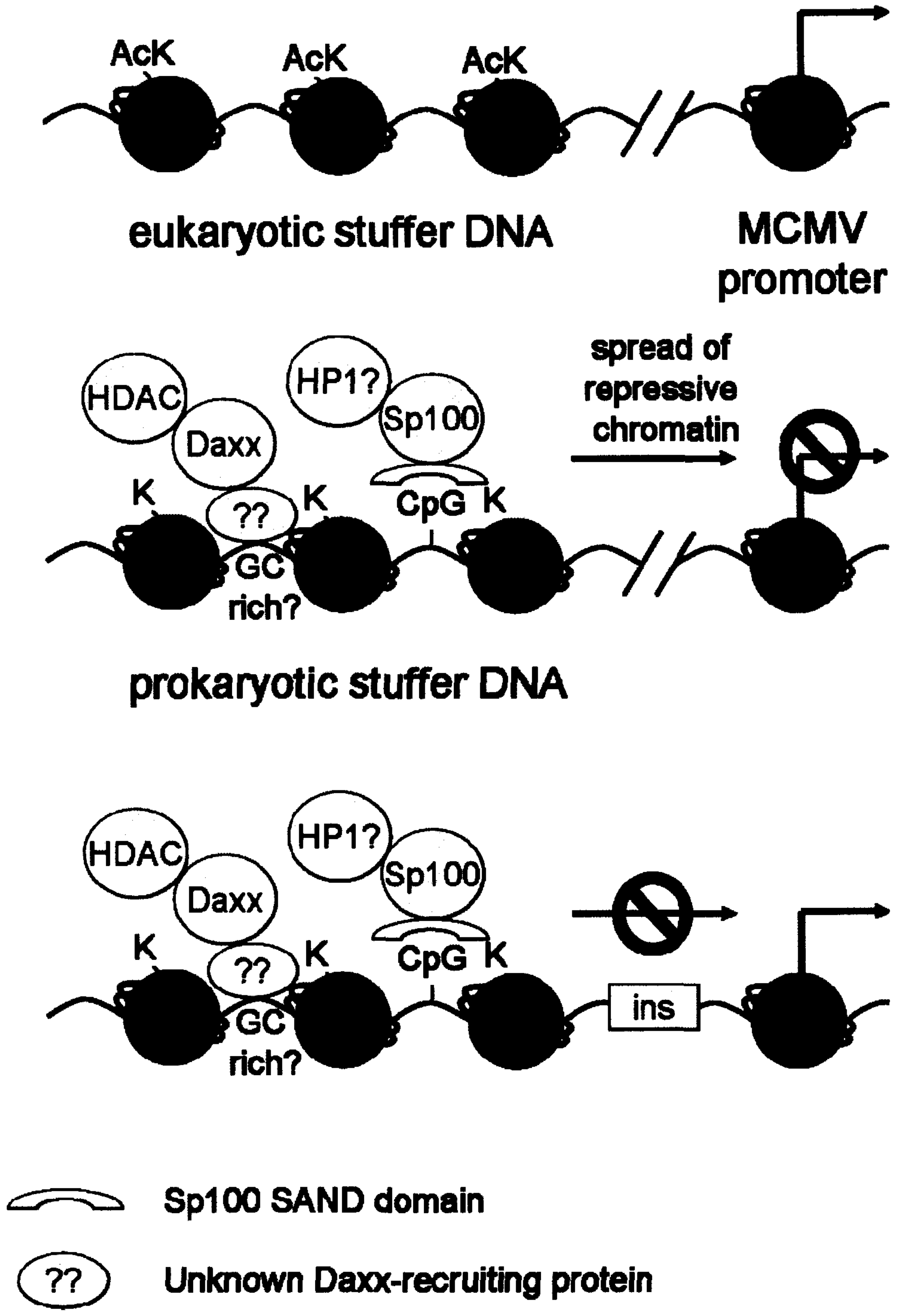
Our goal was to identify the mechanism of repression of genes associated with foreign DNA. Unfortunately, our model system was limited in some respects. Firstly, hdAd-euk encodes ~6 kbp of foreign DNA. Early hdAd vectors, such as hdAd-prok and -euk, encode both a bacterial origin of replication and antibiotic resistance gene for propagation as a plasmid (215, 216). Plasmids encoding newer generations of hdAd vectors, such as hdAd-*lac*Z (210) have bacterial elements between the Ad ITRs and flanked by restriction sites that are absent from the hdAd genome (e.g., PmeI), such that digestion releases that hdAd genome from the bacterial sequences. Furthermore, the *lac*Z gene is also of prokaryotic origin (296). Therefore, factors that recognize and repress expression of genes associated with foreign DNA were likely recruited to hdAd-euk, although presumably to a lesser degree than hdAd-prok

Our experiments using global perturbations, such as TSA (Fig. 22A), AZA (Fig. 22B), and heat shock (Fig. 27) resulted in dramatic derepression of hdAd-prok. However, treatments that involved transient transfection to deliver siRNA or plasmids resulted in more modest changes in hdAd-prok gene expression (Figs. 26, and 28-31). In a typical experiment, transfection efficiencies were ~30-50%. Furthermore, cells must (1) express the exogenous gene product and (2) become infected by the hdAd vector for any effect to be observed. Therefore, we suspect that the less dramatic changes in hdAd-prok gene expression that were observed in experiments that relied upon transient transfection were likely due to the smaller population of infected cells in which the desired experimental perturbation was achieved.

Summary

Our results suggest that Daxx and Sp100B/HMG represent nuclear sensors that are recruited to foreign DNA, where they repress expression of associated genes (Fig. 33). This is consistent with the observation that Sp100 and Daxx swiftly accumulate near viral genomes - even in the absence of PML - and with numerous studies that have shown that Sp100 repressors and Daxx inhibit expression of genes encoded by large DNA viruses. Our results are consistent with a model in which abundant unmethylated CpG motifs and relatively-high GC content that are both present in foreign DNA results in recruitment of Sp100 repressor isoforms and Daxx, respectively, which then repress expression of associated-genes (Fig. 33). Our results provide insight into the mechanism of identification of self and foreign DNA, and how foreign DNA can be swiftly and efficiently repressed, so that potentially-harmful genes are not expressed.

Figure 33. Model for repressive effects of prokaryotic DNA. Prokaryotic DNA is GC rich and encodes an abundance of unmethylated CpG motifs. Daxx is recruited by an unknown DNA binding protein, which likely exhibits an affinity for GC-rich DNA. HDAC bound to Daxx then deacetylate hdAd-prok chromatin. Sp100 repressor isoforms are likely recruited to hdAd-prok DNA by unmethylated CpG motifs. Sp100 likely represses hdAd-prok gene expression by recruitment of HP1. The transcriptionally-repressed chromatin spreads from the prokaryotic stuffer DNA to the transgenes. Insertion of an insulator element (ins) between the stuffer DNA and transgene blocks the spread of repressed chromatin. Adapted by permission from Macmillan Publishers Ltd: Nature (79), copyright 2001.

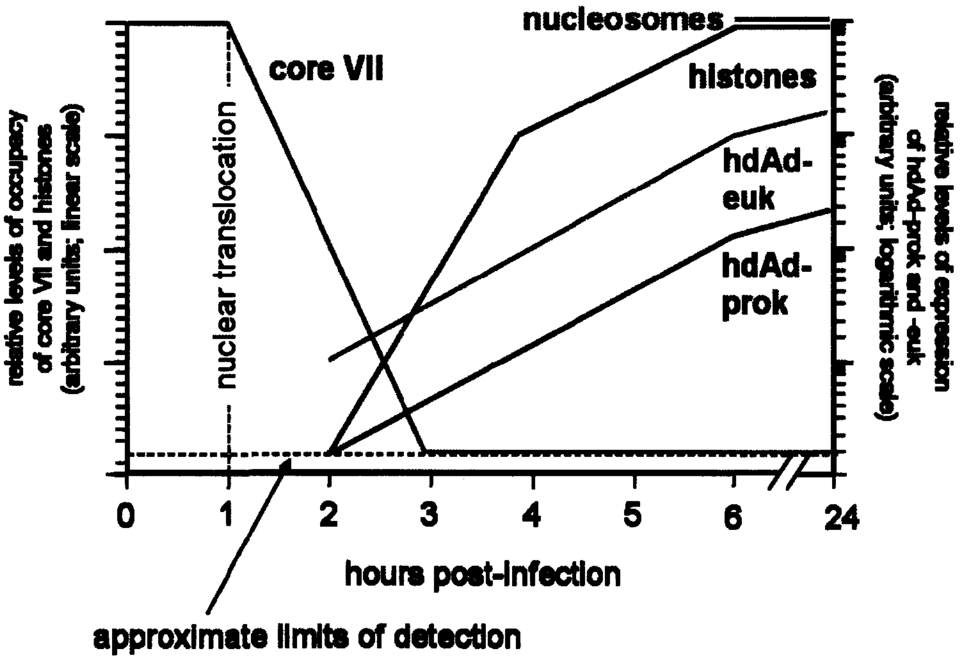

AcK
AcK
AcK
eukaryotic stuffer DNA
MCMV promoter
HDAC
Daxx
HP1?
Sp100
spread of repressive chromatin
K
??
K
CpG
K
GC rich?
prokaryotic stuffer DNA
HDAC
Daxx
HP1?
Sp100
K
??
K
CpG
K
GC rich?
ins
Sp100 SAND domain
?? Unknown Daxx-recruiting protein

Chapter 5 - General Discussion

Together, the data presented herein show that epigenetic factors play a major role in the regulation of Ad vector-encoded transgenes. The data presented in Chapter 3 show that (1) hdAd vector DNA packaging proteins are evicted and replaced by cellular histones, (2) hdAd vectors assemble into chromatin, and (3) chromatin assembly promotes expression of hdAd-encoded genes (Fig. 34). The data presented in Chapter 4 identified a mechanism for repression of genes associated with foreign DNA, and revealed that chromatin can also play a role in limiting the expression of hdAd transgenes (Fig. 34). Therefore, chromatin can have both positive and negative influences on hdAd vector transgene expression, and must be an important consideration in the design of vectors for gene therapy. Furthermore, in light of our results that revealed that hdAd vectors swiftly assemble into nucleosomes, we conclude that the template of early gene expression during the Ad lifecycle is chromatin. This has important implications for development of a complete understanding of the earliest steps of Ad infection.

Implications for design of gene therapy vectors

Our results revealed that chromatin is the template of hdAd vector transcription. As such, epigenetic factors must be considered for the design of safe and efficient gene therapy vectors. For example, transcriptional targeting is an approach that many groups are using to limit the expression of Ad vector-encoded transgenes to specific cell types (239). However, the technology relies upon cellular promoters to repress expression of transgenes in non-target cells. Our results suggest that assembly into


nucleosomes
core VII
histones
hdAd-euk
hdAd-prok
nuclear translocation
relative levels of occupancy of core VII and histones (arbitrary units; linear scale)
relative levels of expression of hdAd-prok and -euk (arbitrary units; logarithmic scale)
0
1
2
3
4
5
6
24
hours post-infection
approximate limits of detection

chromatin promotes expression of promoters that exhibit broad cell-type specificity and strong expression (i.e., the MCMV promoter (1)). However, this may also apply to cell-type specific promoters.

Eukaryotic transcriptional regulatory elements have evolved to function in the context of a chromatinized DNA template. As discussed above, DNA in eukaryotic cells is assembled into chromatin during DNA replication, which is necessary for repression of basal transcription (8). Our results (Fig. 19) and those of others (155, 178) suggest that the linker histone H1 is not deposited during replication-independent chromatin assembly. Therefore, transcriptional-targeting of hdAd vectors may require the design of promoters that do not require H1 for repression of basal gene expression.

The IFN-β promoter is repressed in basal conditions, even when encoded by plasmids that have undergone replication-independent chromatin assembly (172). This is because of a nucleosome-positioning signal that promotes assembly of chromatin that obscures the TATA-box, thus occluding the basal transcription machinery from the core promoter (172, 173). In response to viral infection the transcription factors nuclear factor kappa B (NFκB), interferon-regulatory factor 3, and activating transcription factor 2 bind the IFN-β enhancer and form an enhancesosome (190, 292). The enhanceosome then recruits HATs and chromatin remodelling enzymes, which modify the promoter-proximal nucleosome (3, 173). Chromatin remodelling allows TBP to bind of the TATA-box, which repositions the promoter-proximal nucleosome and promotes transcription (173). The IFN-β core promoter could therefore be used to repress basal transcription of hdAd-encoded transgenes, despite the fact that H1 may not be deposited in hdAd vector chromatin.

Previous studies have shown that a "simple" enhancer that activates transcription by recruitment of a single transcription factor (i.e., the NFκB-responsive interleukin-8 enhancer) is unable promote expression from the IFN-β core promoter (172), as NFκB alone cannot recruit the chromatin remodelling proteins that promote access to the TATA-box. As discussed above, the MCK enhancer/promoter has been used to target Ad vector transgene expression to muscle (112). Upon myogenic differentiation, activation of the MCK promoter requires recruitment of HATs and chromatin remodelling proteins (269). Therefore, a heterologous genetic regulatory element consisting of the MCK enhancer and the IFN-β core promoter may be repressed in non-target tissues, and become specifically-activated in response to myogenic differentiation, or perhaps even in differentiated-myotubes. This heterologous promoter could then be modified to promote stronger expression in the induced state. This activation could be accomplished by addition of NFκB binding sites, which would be unable to induce remodelling of the INF-β core promoter chromatin (172), but which may promote increased gene expression in the inflammatory environment of dystrophic muscle (158, 159, 191). This approach would use chromatin as an asset, in order to target transgene expression to cells that produce that transcription factors that recruit chromatin-remodelling proteins capable of inducing transcription, regardless of whether the DNA template has undergone replication-dependant or -independent chromatin assembly.

Another consideration for production of transcriptionally-targeted Ad vectors is nucleosome positioning. A number of inducible genes in yeast and humans have stably positioned nucleosomes that are remodelled during transcriptional activation (reviewed in (59)). Due to technical challenges, nucleosome positioning has been mapped in very few human promoters. Furthermore, to our knowledge, no promoters that exhibit potential for transcriptional targeting have been subjected to such analyses. Analyses of budding yeast have revealed the existence of a genomic code that can account for the positioning of ~50% of nucleosomes (254). However, whether the promoter fragments used for transcriptional targeting of hdAd vectors encode the positioning sequences necessary for formation of properly phased nucleosomes is unknown. Future experiments should determine the positioning of nucleosomes in endogenous promoters to be used for gene therapy applications and whether these nucleosomes are mobilized during gene activation. After obtaining these data, experiments can be performed to determining whether positioning and mobilization are recapitulated when promoters are encoded by hdAd vectors.

Implications of swift assembly into chromatin for wild-type Ad

The Ad E1A protein is the first Ad gene expressed during infection and is a transcriptional activator that is necessary for efficient expression of Ad early genes (260). E1A interacts with a number of cellular HATs, including p300 (109), CBP (cyclic AMP response element binding protein (CREB)-binding protein) (20), p300/CBP associated factor (PCAF) (319), p400 (90), and general control of amino acid synthesis 5 (GCN5) (161). Interactions between the N-terminus of E1A and these proteins confer considerable HAT activity to E1A isolated from infected cells (161); early analyses of E1A mutants revealed that efficient activation of early genes required the N-terminus (312); and recruitment of HAT activity to the E2A promoter is necessary for its transcription (80). E1A from all six subgroups of human Ad interact with CBP (267), and the binding motifs that mediate recruitment are well conserved between human and simian Ad (18). These observations suggest that Ad has evolved to exploit efficient assembly into chromatin, and uses host HATs to create a local chromatin environment that promotes expression of Ad early genes.

Genomes of large DNA viruses are similar in GC and CpG content to hdAd-prok stuffer DNA. Although we found that the repressive effects of prokaryotic DNA were mediated primarily at the level of chromatin (i.e., histone deacetylation), we found that abrogation of chromatin assembly by depletion of HIRA resulted in a greater fold-reduction in gene expression from hdAd-prok than hdAd-euk. As discussed above, Sp100 repressor isoforms may repress gene expression independently of chromatin modifications by recruitment of HP1. Swift assembly into chromatin makes ca. 146 of every 200 bp of the genome relatively-inaccessible to sequence-specific DNA binding factors (311). Perhaps reduced expression of hdAd-prok in response to HIRA depletion was mediated in part through improved accessibility of non-nucleosomal DNA to Sp100 repressor factors. If this is the case, CpG-rich Ad DNA may be partially-protected from Sp100 repressor isoforms by assembly into nucleosomes.

Upon accumulation of Ad early gene products, the viral genome is replicated, resulting in transition to the late phase of the Ad life cycle (259). How does the Ad DNA polymerase evict nucleosomes during DNA replication? Does it, like cellular DNA polymerase, coordinate with histone chaperones and chromatin remodelling proteins (87, 224, 264)? Furthermore, what is the structure of Ad DNA after DNA replication? Ad actively inhibits expression of host genes, including histones (85), suggesting that chromatin may only play an important role in the Ad lifecyle during the early phase and the earliest rounds of replication. However, many questions remain: are histones evicted and swiftly re-deposited on both daughter strands? Do new nucleosomes assemble? Does post-replication Ad chromatin incorporate H3.1 or H1? A whole new level of the complexities of interactions between Ad and its host cells may be revealed by analyses of Ad chromatin.

Conclusions

The body of work presented here represents a step-forward in our understanding of interactions between human nuclei and hdAd vectors. We have established that hdAd assemble into chromatin, and that chromatin plays an important role in the regulation of vector-encoded transgenes. Our results indicate that development of safe and effective hdAd vectors for gene therapy will require consideration of not just the genetic information that the vector encodes, but also the role of epigenetic factors in regulating transcription of these genes.

Chapter 6 - References

1. **Addison, C. L., M. Hitt, D. Kunsken, and F. L. Graham.** 1997. Comparison of the human versus murine cytomegalovirus immediate early gene promoters for transgene expression by adenoviral vectors. J Gen Virol **78:**1653-1661.

2. **Adler, V., A. Schaffer, J. Kim, L. Dolan, and Z. Ronai.** 1995. UV irradiation and heat shock mediate JNK activation via alternate pathways. J Biol Chem **270:**26071-7.

3. **Agalioti, T., S. Lomvardas, B. Parekh, J. Yie, T. Maniatis, and D. Thanos.** 2000. Ordered recruitment of chromatin modifying and general transcription factors to the IFN-beta promoter. Cell **103:**667-78.

4. **Ahmad, K., and S. Henikoff.** 2002. Histone H3 variants specify modes of chromatin assembly. Proc Natl Acad Sci U S A **99 Suppl 4:**16477-84.

5. **Ahmad, K., and S. Henikoff.** 2002. The histone variant H3.3 marks active chromatin by replication-independent nucleosome assembly. Mol Cell **9:**1191-200.

6. **Aker, M., J. Tubb, A. C. Groth, A. A. Bukovsky, A. C. Bell, G. Felsenfeld, H.-P. Kiem, G. Stamatoyannopoulos, and D. W. Emery.** 2007. Extended Core Sequences from the cHS4 Insulator Are Necessary for Protecting Retroviral Vectors from Silencing Position Effects. Human Gene Therapy **18:**333-343.

7. **Akey, C. W., and K. Luger.** 2003. Histone chaperones and nucleosome assembly. Curr Opin Struct Biol **13:**6-14.

8. **Almouzni, G., and A. P. Wolffe.** 1993. Replication-coupled chromatin assembly is required for the repression of basal transcription in vivo. Genes Dev **7:**2033-47.

9. **Amalfitano, A., C. R. Begy, and J. S. Chamberlain.** 1996. Improved adenovirus packaging cell lines to support the growth of replication-defective gene-delivery vectors. Proc Natl Acad Sci U S A **93:**3352-3356.

10. **Amalfitano, A., and J. S. Chamberlain.** 1997. Isolation and characterization of packaging cell lines that coexpress the adenovirus E1, DNA polymerase, and preterminal proteins: implications for gene therapy. Gene Ther **4:**258-263.

11. **Amalfitano, A., A. J. McVie-Wylie, H. Hu, T. L. Dawson, N. Raben, P. Plotz, and Y. T. Chen.** 1999. Systemic correction of the muscle disorder

glycogen storage disease type II after hepatic targeting of a modified adenovirus vector encoding human acid-alpha-glucosidase. Proc Natl Acad Sci U S A **96:**8861-6.

12. **Amalfitano, A., and R. J. Parks.** 2002. Separating fact from fiction: assessing the potential of modified adenovirus vectors for use in human gene therapy. Curr Gene Ther **2:**111-33.

13. **Anderson, C. W., P. R. Baum, and R. F. Gesteland.** 1973. Processing of adenovirus 2-induced proteins. J Virol **12:**241-52.

14. **Andersson, M. G., P. C. Haasnoot, N. Xu, S. Berenjian, B. Berkhout, and G. Akusjarvi.** 2005. Suppression of RNA interference by adenovirus virus-associated RNA. J Virol **79:**9556-65.

15. **Archer, T. K., P. Lefebvre, R. G. Wolford, and G. L. Hager.** 1992. Transcription factor loading on the MMTV promoter: a bimodal mechanism for promoter activation. Science **255:**1573-6.

16. **Armentano, D., C. C. Sookdeo, K. M. Hehir, R. J. Gregory, St, G. A. Prince, S. C. Wadsworth, and A. E. Smith.** 1995. Characterization of an adenovirus gene transfer vector containing an E4 deletion. Hum Gene Ther **6:**1343-1353.

17. **Ausio, J.** 2006. Histone variants--the structure behind the function. Brief Funct Genomic Proteomic **5:**228-43.

18. **Avvakumov, N., A. E. Kajon, R. C. Hoeben, and J. S. Mymryk.** 2004. Comprehensive sequence analysis of the E1A proteins of human and simian adenoviruses. Virology **329:**477-92.

19. **Axel, R., W. Melchior, Jr., B. Sollner-Webb, and G. Felsenfeld.** 1974. Specific sites of interaction between histones and DNA in chromatin. Proc Natl Acad Sci U S A **71:**4101-5.

20. **Bannister, A. J., and T. Kouzarides.** 1996. The CBP co-activator is a histone acetyltransferase. Nature **384:**641-643.

21. **Bell, A. C., A. G. West, and G. Felsenfeld.** 1999. The protein CTCF is required for the enhancer blocking activity of vertebrate insulators. Cell **98:**387-96.

22. **Belotserkovskaya, R., S. Oh, V. A. Bondarenko, G. Orphanides, V. M. Studitsky, and D. Reinberg.** 2003. FACT facilitates transcription-dependent nucleosome alteration. Science **301:**1090-3.

23. **Bergelson, J. M., J. A. Cunningham, G. Droguett, E. A. Kurt-Jones, A. Krithivas, J. S. Hong, M. S. Horwitz, R. L. Crowell, and R. W. Finberg.** 1997. Isolation of a common receptor for Coxsackie B viruses and adenoviruses 2 and 5. Science **275:**1320-1323.

24. **Bernardi, R., and P. P. Pandolfi.** 2007. Structure, dynamics and functions of promyelocytic leukaemia nuclear bodies. Nat Rev Mol Cell Biol **8:**1006-16.

25. **Bernt, K. M., S. Ni, Z. Y. Li, D. M. Shayakhmetov, and A. Lieber.** 2003. The effect of sequestration by nontarget tissues on anti-tumor efficacy of systemically applied, conditionally replicating adenovirus vectors. Mol Ther **8:**746-55.

26. **Bett, A. J., L. Prevec, and F. L. Graham.** 1993. Packaging capacity and stability of human adenovirus type 5 vectors. Journal of Virology **67:**5911-5921.

27. **Bird, A.** 2002. DNA methylation patterns and epigenetic memory. Genes & Development **16:**6-21.

28. **Bird, A. P.** 1986. CpG-rich islands and the function of DNA methylation. Nature **321:**209-13.

29. **Bird, A. P., and A. P. Wolffe.** 1999. Methylation-induced repression--belts, braces, and chromatin. Cell **99:**451-4.

30. **Block, G. J., C. H. Eskiw, G. Dellaire, and D. P. Bazett-Jones.** 2006. Transcriptional regulation is affected by subnuclear targeting of reporter plasmids to PML nuclear bodies. Mol Cell Biol **26:**8814-25.

31. **Boellmann, F., T. Guettouche, Y. Guo, M. Fenna, L. Mnayer, and R. Voellmy.** 2004. DAXX interacts with heat shock factor 1 during stress activation and enhances its transcriptional activity. Proceedings of the National Academy of Sciences, USA **101:**4100-5.

32. **Bonnefoy, E., G. A. Orsi, P. Couble, and B. Loppin.** 2007. The essential role of Drosophila HIRA for de novo assembly of paternal chromatin at fertilization. PLoS Genet **3:**1991-2006.

33. **Bramson, J. L., N. Grinshtein, R. A. Meulenbroek, J. Lunde, D. Kottachchi, I. A. Lorimer, B. J. Jasmin, and R. J. Parks.** 2004. Helper-dependent adenoviral vectors containing modified fibre for improved transduction of developing and mature muscle cells. Human Gene Therapy **15:**179-188.

34. **Bresnick, E. H., C. Rories, and G. L. Hager.** 1992. Evidence that nucleosomes on the mouse mammary tumor virus promoter adopt specific translational positions. Nucleic Acids Res **20:**865-70.

35. **Brooks, A. R., R. N. Harkins, P. Wang, H. S. Qian, P. Liu, and G. M. Rubanyi.** 2004. Transcriptional silencing is associated with extensive methylation of the CMV promoter following adenoviral gene delivery to muscle. J Gene Med **6:**395-404.

36. **Brown, D. T., M. Westphal, B. T. Burlingham, U. Winterhoff, and W. Doerfler.** 1975. Structure and composition of the adenovirus type 2 core. J Virol **16:**366-87.

37. **Brown, M., and J. Weber.** 1980. Virion core-like organization of intranuclear adenovirus chromatin late in infection. Virology **107:**306-10.

38. **Brownell, J. E., J. Zhou, T. Ranalli, R. Kobayashi, D. G. Edmondson, S. Y. Roth, and C. D. Allis.** 1996. Tetrahymena histone acetyltransferase A: a homolog to yeast Gcn5p linking histone acetylation to gene activation. Cell **84:**843-51.

39. **Bruno, M., A. Flaus, C. Stockdale, C. Rencurel, H. Ferreira, and T. Owen-Hughes.** 2003. Histone H2A/H2B dimer exchange by ATP-dependent chromatin remodeling activities. Mol Cell **12:**1599-606.

40. **Cantrell, S. R., and W. A. Bresnahan.** 2006. Human cytomegalovirus (HCMV) UL82 gene product (pp71) relieves hDaxx-mediated repression of HCMV replication. J Virol **80:**6188-91.

41. **Cantrell, S. R., and W. A. Bresnahan.** 2005. Interaction between the human cytomegalovirus UL82 gene product (pp71) and hDaxx regulates immediate-early gene expression and viral replication. J Virol **79:**7792-802.

42. **Cao, T. M., and M. T. Sung.** 1982. A protamine-like domain in basic adenovirus core protein. Biochem Biophys Res Commun **108:**1061-6.

43. **Cereghini, S., and M. Yaniv.** 1984. Assembly of transfected DNA into chromatin: structural changes in the origin-promoter-enhancer region upon replication. Embo J **3:**1243-53.

44. **Chakkalakal, J. V., J. Thompson, R. J. Parks, and B. J. Jasmin.** 2005. Molecular, cellular, and pharmacological therapies for Duchenne/Becker muscular dystrophies. FASEB J. **19:**880-891.

45. **Chatterjee, P. K., M. E. Vayda, and S. J. Flint.** 1986. Adenoviral protein VII packages intracellular viral DNA throughout the early phase of infection. EMBO Journal **5:**1633-44.

46. **Chatterjee, P. K., M. E. Vayda, and S. J. Flint.** 1986. Identification of proteins and protein domains that contact DNA within adenovirus nucleoprotein cores by ultraviolet light crosslinking of oligonucleotides 32P-labelled in vivo. Journal of Molecular Biology **188:**23-37.

47. **Chelbi-Alix, M. K., and H. de The.** 1999. Herpes virus induced proteasome-dependent degradation of the nuclear bodies-associated PML and Sp100 proteins. Oncogene **18:**935-41.

48. **Chen, J., N. Morral, and D. A. Engel.** 2007. Transcription releases protein VII from adenovirus chromatin. Virology.

49. **Chen, Z. Y., C. Y. He, A. Ehrhardt, and M. A. Kay.** 2003. Minicircle DNA vectors devoid of bacterial DNA result in persistent and high-level transgene expression in vivo. Molecular Therapy **8:**495-500.

50. **Chen, Z. Y., C. Y. He, and M. A. Kay.** 2005. Improved production and purification of minicircle DNA vector free of plasmid bacterial sequences and capable of persistent transgene expression in vivo. Hum Gene Ther **16:**126-31.

51. **Chen, Z. Y., C. Y. He, L. Meuse, and M. A. Kay.** 2004. Silencing of episomal transgene expression by plasmid bacterial DNA elements in vivo. Gene Therapy **11:**856-64.

52. **Chen, Z. Y., E. Riu, C. Y. He, H. Xu, and M. A. Kay.** 2008. Silencing of episomal transgene expression in liver by plasmid bacterial backbone DNA is independent of CpG methylation. Molecular Therapy **16:**548-56.

53. **Chiocca, S.** 2007. Viral control of the SUMO pathway: Gam1, a model system. Biochem Soc Trans **35:**1419-21.

54. **Clark, R. J., and G. Felsenfeld.** 1974. Chemical probes of chromatin structure. Biochemistry **13:**3622-8.

55. **Clarke, P. A., and M. B. Mathews.** 1995. Interactions between the double-stranded RNA binding motif and RNA: definition of the binding site for the interferon-induced protein kinase DAI (PKR) on adenovirus VA RNA. Rna **1:**7-20.

56. **Colombo, R., R. Boggio, C. Seiser, G. F. Draetta, and S. Chiocca.** 2002. The adenovirus protein Gam1 interferes with sumoylation of histone deacetylase 1. EMBO Reports **3:**1062-8.

57. **Compton, J. L., M. Bellard, and P. Chambon.** 1976. Biochemical evidence of variability in the DNA repeat length in the chromatin of higher eukaryotes. Proc Natl Acad Sci U S A **73:**4382-6.

58. **Cordingley, M. G., A. T. Riegel, and G. L. Hager.** 1987. Steroid-dependent interaction of transcription factors with the inducible promoter of mouse mammary tumor virus in vivo. Cell **48:**261-70.

59. **Cosma, M. P.** 2002. Ordered recruitment: gene-specific mechanism of transcription activation. Mol Cell **10:**227-36.

60. **Cowdery, J. S., J. H. Chace, A. K. Yi, and A. M. Krieg.** 1996. Bacterial DNA induces NK cells to produce IFN-gamma in vivo and increases the toxicity of lipopolysaccharides. Journal of Immunology **156:**4570-5.

61. **Craig, J. M.** 2005. Heterochromatin--many flavours, common themes. Bioessays **27:**17-28.

62. **Daganzo, S. M., J. P. Erzberger, W. M. Lam, E. Skordalakes, R. Zhang, A. A. Franco, S. J. Brill, P. D. Adams, J. M. Berger, and P. D. Kaufman.** 2003. Structure and function of the conserved core of histone deposition protein Asf1. Curr Biol **13:**2148-58.

63. **Daniell, E., D. E. Groff, and M. J. Fedor.** 1981. Adenovirus chromatin structure at different stages of infection. Mol Cell Biol **1:**1094-105.

64. **Davis, J. J., L. Wang, F. Dong, L. Zhang, W. Guo, F. Teraishi, K. Xu, L. Ji, and B. Fang.** 2006. Oncolysis and suppression of tumor growth by a GFP-expressing oncolytic adenovirus controlled by an hTERT and CMV hybrid promoter. Cancer Gene Ther **13:**720-3.

65. **Day, P. M., C. C. Baker, D. R. Lowy, and J. T. Schiller.** 2004. Establishment of papillomavirus infection is enhanced by promyelocytic leukemia protein (PML) expression. Proceedings of the National Academy of Sciences, USA **101:**14252-7.

66. **Dellaire, G., and D. P. Bazett-Jones.** 2004. PML nuclear bodies: dynamic sensors of DNA damage and cellular stress. BioEssays **26:**963-977.

67. **Dery, C. V., M. Toth, M. Brown, J. Horvath, S. Allaire, and J. M. Weber.** 1985. The structure of adenovirus chromatin in infected cells. Journal of General Virology **66 (Pt 12):**2671-84.

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

69. **Earnshaw, W. C., B. M. Honda, R. A. Laskey, and J. O. Thomas.** 1980. Assembly of nucleosomes: the reaction involving X. laevis nucleoplasmin. Cell **21:**373-83.

70. **Edelstein, M. L., M. R. Abedi, J. Wixon, and R. M. Edelstein.** 2004. Gene therapy clinical trials worldwide 1989-2004-an overview. J Gene Med **6:**597-602.

71. **Engelhardt, J. F., X. Ye, B. Doranz, and J. M. Wilson.** 1994. Ablation of E2A in recombinant adenoviruses improves transgene persistence and decreases inflammatory response in mouse liver. Proc Natl Acad Sci U S A **91:**6196-200.

72. **Everett, R. D.** 2001. DNA viruses and viral proteins that interact with PML nuclear bodies. Oncogene **20:**7266-73.

73. **Everett, R. D.** 2000. ICP0 induces the accumulation of colocalizing conjugated ubiquitin. J Virol **74:**9994-10005.

74. **Everett, R. D., and M. K. Chelbi-Alix.** 2007. PML and PML nuclear bodies: implications in antiviral defence. Biochimie **89:**819-30.

75. **Everett, R. D., and J. Murray.** 2005. ND10 components relocate to sites associated with herpes simplex virus type 1 nucleoprotein complexes during virus infection. J Virol **79:**5078-89.

76. **Everett, R. D., J. Murray, A. Orr, and C. M. Preston.** 2007. Herpes simplex virus type 1 genomes are associated with ND10 nuclear substructures in quiescently infected human fibroblasts. Journal of Virology **81:**10991-1004.

77. **Everett, R. D., S. Rechter, P. Papior, N. Tavalai, T. Stamminger, and A. Orr.** 2006. PML contributes to a cellular mechanism of repression of herpes simplex virus type 1 infection that is inactivated by ICP0. Journal of Virology **80:**7995-8005.

78. **Fan, Y., T. Nikitina, E. M. Morin-Kensicki, J. Zhao, T. R. Magnuson, C. L. Woodcock, and A. I. Skoultchi.** 2003. H1 linker histones are essential for mouse development and affect nucleosome spacing in vivo. Mol Cell Biol **23:**4559-72.

79. **Fang, B., H. Wang, G. Gordon, D. A. Bellinger, M. S. Read, K. M. Brinkhous, S. L. Woo, and R. C. Eisensmith.** 1996. Lack of persistence of E1- recombinant adenoviral vectors containing a temperature-sensitive E2A mutation in immunocompetent mice and hemophilia B dogs. Gene Ther **3:**217-222.

80. **Fax, P., O. Lehmkuhler, C. Kuhn, H. Esche, and D. Brockmann.** 2000. E1A12S-mediated activation of the adenovirus type 12 E2 promoter depends on the histone acetyltransferase activity of p300/CBP. J Biol Chem **275:**40554-60.

81. **Felsenfeld, G., and M. Groudine.** 2003. Controlling the double helix. Nature **421:**448-53.

82. **Fields-Berry, S. C., A. L. Halliday, and C. L. Cepko.** 1992. A recombinant retrovirus encoding alkaline phosphatase confirms clonal boundary assignment in lineage analysis of murine retina. Proc Natl Acad Sci U S A **89:**693-7.

83. **Fischle, W., Y. Wang, S. A. Jacobs, Y. Kim, C. D. Allis, and S. Khorasanizadeh.** 2003. Molecular basis for the discrimination of repressive methyl-lysine marks in histone H3 by Polycomb and HP1 chromodomains. Genes Dev **17:**1870-81.

84. **Fletcher, T. M., N. Xiao, G. Mautino, C. T. Baumann, R. Wolford, B. S. Warren, and G. L. Hager.** 2002. ATP-dependent mobilization of the glucocorticoid receptor during chromatin remodeling. Mol Cell Biol **22:**3255-63.

85. **Flint, S. J., M. A. Plumb, U. C. Yang, G. S. Stein, and J. L. Stein.** 1984. Effect of adenovirus infection on expression of human histone genes. Molecular and Cellular Biology **4:**1363-71.

86. **Fragoso, G., S. John, M. S. Roberts, and G. L. Hager.** 1995. Nucleosome positioning on the MMTV LTR results from the frequency-biased occupancy of multiple frames. Genes Dev **9:**1933-47.

87. **Franco, A. A., W. M. Lam, P. M. Burgers, and P. D. Kaufman.** 2005. Histone deposition protein Asf1 maintains DNA replisome integrity and interacts with replication factor C. Genes Dev **19:**1365-75.

88. **Fridman, J. S., and S. W. Lowe.** 2003. Control of apoptosis by p53. Oncogene **22:**9030-40.

89. **Frommer, M., L. E. McDonald, D. S. Millar, C. M. Collis, F. Watt, G. W. Grigg, P. L. Molloy, and C. L. Paul.** 1992. A genomic sequencing protocol

that yields a positive display of 5-methylcytosine residues in individual DNA strands. Proc Natl Acad Sci U S A **89:**1827-31.

90. **Fuchs, M., J. Gerber, R. Drapkin, S. Sif, T. Ikura, V. Ogryzko, W. S. Lane, Y. Nakatani, and D. M. Livingston.** 2001. The p400 complex is an essential E1A transformation target. Cell **106:**297-307.

91. **Fujita, N., S. Watanabe, T. Ichimura, S. Tsuruzoe, Y. Shinkai, M. Tachibana, T. Chiba, and M. Nakao.** 2003. Methyl-CpG binding domain 1 (MBD1) interacts with the Suv39h1-HP1 heterochromatic complex for DNA methylation-based transcriptional repression. J Biol Chem **278:**24132-8.

92. **Fujiwara, T., Y. Urata, and N. Tanaka.** 2007. Telomerase-specific oncolytic virotherapy for human cancer with the hTERT promoter. Curr Cancer Drug Targets **7:**191-201.

93. **Fuks, F., P. J. Hurd, R. Deplus, and T. Kouzarides.** 2003. The DNA methyltransferases associate with HP1 and the SUV39H1 histone methyltransferase. Nucleic Acids Research **31:**2305-12.

94. **Fuks, F., P. J. Hurd, D. Wolf, X. Nan, A. P. Bird, and T. Kouzarides.** 2003. The methyl-CpG-binding protein MeCP2 links DNA methylation to histone methylation. Journal of Biological Chemistry **278:**4035-40.

95. **Gaszner, M., and G. Felsenfeld.** 2006. Insulators: exploiting transcriptional and epigenetic mechanisms. Nature Reviews: Genetics **7:**703-13.

96. **Georgakopoulos, T., and G. Thireos.** 1992. Two distinct yeast transcriptional activators require the function of the GCN5 protein to promote normal levels of transcription. Embo J **11:**4145-52.

97. **Glasgow, J. N., M. Everts, and D. T. Curiel.** 2006. Transductional targeting of adenovirus vectors for gene therapy. Cancer Gene Ther **13:**830-44.

98. **Glover, D. J., H. J. Lipps, and D. A. Jans.** 2005. Towards safe, non-viral therapeutic gene expression in humans. Nat Rev Genet **6:**299-310.

99. **Goverdhana, S., M. Puntel, W. Xiong, J. M. Zirger, C. Barcia, J. F. Curtin, E. B. Soffer, S. Mondkar, G. D. King, J. Hu, S. A. Sciascia, M. Candolfi, D. S. Greengold, P. R. Lowenstein, and M. G. Castro.** 2005. Regulatable gene expression systems for gene therapy applications: progress and future challenges. Mol Ther **12:**189-211.

100. **Graham, F. L.** 1987. Growth of 293 cells in suspension culture. J Gen Virol **68:**937-940.

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

102. **Greber, U. F., M. Willetts, P. Webster, and A. Helenius.** 1993. Stepwise dismantling of adenovirus 2 during entry into cells. Cell **75:**477-486.

103. **Grigoryev, S. A., Y. A. Bulynko, and E. Y. Popova.** 2006. The end adjusts the means: heterochromatin remodelling during terminal cell differentiation. Chromosome Res **14:**53-69.

104. **Grozinger, C. M., and S. L. Schreiber.** 2002. Deacetylase enzymes: biological functions and the use of small-molecule inhibitors. Chem Biol **9:**3-16.

105. **Gu, J., and B. Fang.** 2003. Telomerase promoter-driven cancer gene therapy. Cancer Biol Ther **2:**S64-70.

106. **Guldner, H. H., C. Szostecki, P. Schroder, U. Matschl, K. Jensen, C. Luders, H. Will, and T. Sternsdorf.** 1999. Splice variants of the nuclear dot-associated Sp100 protein contain homologies to HMG-1 and a human nuclear phosphoprotein-box motif. J Cell Sci **112 (Pt 5):**733-47.

107. **Hake, S. B., and C. D. Allis.** 2006. Histone H3 variants and their potential role in indexing mammalian genomes: the "H3 barcode hypothesis". Proc Natl Acad Sci U S A **103:**6428-35.

108. **Hardy, S., M. Kitamura, T. Harris-Stansil, Y. Dai, and M. L. Phipps.** 1997. Construction of adenovirus vectors through Cre-lox recombination. J Virol **71:**1842-1849.

109. **Harlow, E., P. Whyte, B. R. Franza, Jr., and C. Schley.** 1986. Association of adenovirus early-region 1A proteins with cellular polypeptides. Molecular and Cellular Biology **6:**1579-89.

110. **Harui, A., S. Suzuki, S. Kochanek, and K. Mitani.** 1999. Frequency and stability of chromosomal integration of adenovirus vectors. J Virol **73:**6141-6.

111. **Hatfield, L., and P. Hearing.** 1991. Redundant elements in the adenovirus type 5 inverted terminal repeat promote bidirectional transcription in vitro and are important for virus growth in vivo. Virology **184:**265-76.

112. **Hauser, M. A., A. Robinson, D. Hartigan-O'Connor, D. Williams-Gregory, J. N. Buskin, S. Apone, C. J. Kirk, S. Hardy, S. D. Hauschka, and J. S. Chamberlain.** 2000. Analysis of Muscle Creatine Kinase Regulatory Elements in Recombinant Adenoviral Vectors. Molecular Therapy **2:**16-25.

113. **Heins, J. N., J. R. Suriano, H. Taniuchi, and C. B. Anfinsen.** 1967. Characterization of a nuclease produced by Staphylococcus aureus. J Biol Chem **242:**1016-20.

114. **Heitz, E.** 1928. Das heterochromatin der moose. Jahrb Wiss Bot **69:**762-818.

115. **Hendrich, B., C. Abbott, H. McQueen, D. Chambers, S. Cross, and A. Bird.** 1999. Genomic structure and chromosomal mapping of the murine and human Mbd1, Mbd2, Mbd3, and Mbd4 genes. Mamm Genome **10:**906-12.

116. **Henikoff, S., and K. Ahmad.** 2005. Assembly of variant histones into chromatin. Annual Review of Cell and Developmental Biology **21:**133-53.

117. **Hensel, G. M., H. H. Meyer, I. Buchmann, D. Pommerehne, S. Schmolke, B. Plachter, K. Radsak, and H. F. Kern.** 1996. Intracellular localization and expression of the human cytomegalovirus matrix phosphoprotein pp71 (ppUL82): evidence for its translocation into the nucleus. J Gen Virol **77 (Pt 12):**3087-97.

118. **Herman, J. G., J. R. Graff, S. Myohanen, B. D. Nelkin, and S. B. Baylin.** 1996. Methylation-specific PCR: a novel PCR assay for methylation status of CpG islands. Proc Natl Acad Sci U S A **93:**9821-6.

119. **Hinman, A. R.** 1998. Global progress in infectious disease control. Vaccine **16:**1116-21.

120. **Hofmann, H., H. Sindre, and T. Stamminger.** 2002. Functional interaction between the pp71 protein of human cytomegalovirus and the PML-interacting protein human Daxx. Journal of Virology **76:**5769-83.

121. **Holcik, M., H. Gibson, and R. G. Korneluk.** 2001. XIAP: apoptotic brake and promising therapeutic target. Apoptosis **6:**253-61.

122. **Hollenbach, A. D., C. J. McPherson, E. J. Mientjes, R. Iyengar, and G. Grosveld.** 2002. Daxx and histone deacetylase II associate with chromatin through an interaction with core histones and the chromatin-associated protein Dek. Journal of Cell Science **115:**3319-30.

123. **Huisinga, K. L., B. Brower-Toland, and S. C. Elgin.** 2006. The contradictory definitions of heterochromatin: transcription and silencing. Chromosoma **115:**110-22.

124. **Hurtado Pico, A., X. Wang, I. Sipo, U. Siemetzki, J. Eberle, W. Poller, and H. Fechner.** 2005. Viral and nonviral factors causing nonspecific replication

of tumor- and tissue-specific promoter-dependent oncolytic adenoviruses. Mol Ther **11:**563-77.

125. **Hwang, D. Y., W. A. Carlezon, Jr., O. Isacson, and K. S. Kim.** 2001. A high-efficiency synthetic promoter that drives transgene expression selectively in noradrenergic neurons. Hum Gene Ther **12:**1731-40.

126. **Hwang, J., and R. F. Kalejta.** 2007. Proteasome-dependent, ubiquitin-independent degradation of Daxx by the viral pp71 protein in human cytomegalovirus-infected cells. Virology **367:**334-8.

127. **Isaac, A., K. W. Wilcox, and J. L. Taylor.** 2006. SP100B, a repressor of gene expression preferentially binds to DNA with unmethylated CpGs. Journal of Cellular Biochemistry **98:**1106-22.

128. **Ishov, A. M., O. V. Vladimirova, and G. G. Maul.** 2002. Daxx-mediated accumulation of human cytomegalovirus tegument protein pp71 at ND10 facilitates initiation of viral infection at these nuclear domains. J Virol **76:**7705-12.

129. **Ito, T., M. Bulger, R. Kobayashi, and J. T. Kadonaga.** 1996. Drosophila NAP-1 is a core histone chaperone that functions in ATP-facilitated assembly of regularly spaced nucleosomal arrays. Mol Cell Biol **16:**3112-24.

130. **Ito, T., M. Bulger, M. J. Pazin, R. Kobayashi, and J. T. Kadonaga.** 1997. ACF, an ISWI-containing and ATP-utilizing chromatin assembly and remodeling factor. Cell **90:**145-55.

131. **Ito, T., M. E. Levenstein, D. V. Fyodorov, A. K. Kutach, R. Kobayashi, and J. T. Kadonaga.** 1999. ACF consists of two subunits, Acf1 and ISWI, that function cooperatively in the ATP-dependent catalysis of chromatin assembly. Genes Dev **13:**1529-39.

132. **Ito, T., J. K. Tyler, M. Bulger, R. Kobayashi, and J. T. Kadonaga.** 1996. ATP-facilitated chromatin assembly with a nucleoplasmin-like protein from Drosophila melanogaster. J Biol Chem **271:**25041-8.

133. **Jacobs, S. A., and S. Khorasanizadeh.** 2002. Structure of HP1 chromodomain bound to a lysine 9-methylated histone H3 tail. Science **295:**2080-3.

134. **Jacobson, R. H., A. G. Ladurner, D. S. King, and R. Tjian.** 2000. Structure and function of a human TAFII250 double bromodomain module. Science **288:**1422-5.

135. **Janicki, S. M., T. Tsukamoto, S. E. Salghetti, W. P. Tansey, R. Sachidanandam, K. V. Prasanth, T. Ried, Y. Shav-Tal, E. Bertrand, R. H. Singer, and D. L. Spector.** 2004. From silencing to gene expression: real-time analysis in single cells. Cell **116:**683-98.

136. **Janknecht, R.** 2004. On the road to immortality: hTERT upregulation in cancer cells. FEBS Lett **564:**9-13.

137. **Jenuwein, T., and C. D. Allis.** 2001. Translating the histone code. Science **293:**1074-80.

138. **Jeong, S., and A. Stein.** 1994. Micrococcal nuclease digestion of nuclei reveals extended nucleosome ladders having anomalous DNA lengths for chromatin assembled on non-replicating plasmids in transfected cells. Nucleic Acids Res **22:**370-5.

139. **Jin, C., and G. Felsenfeld.** 2006. Distribution of histone H3.3 in hematopoietic cell lineages. Proc Natl Acad Sci U S A **103:**574-9.

140. **Johnson, J. E., B. J. Wold, and S. D. Hauschka.** 1989. Muscle creatine kinase sequence elements regulating skeletal and cardiac muscle expression in transgenic mice. Mol Cell Biol **9:**3393-9.

141. **Johnson, T. A., C. Elbi, B. S. Parekh, G. L. Hager, and S. John.** 2008. Chromatin remodeling complexes interact dynamically with a glucocorticoid receptor-regulated promoter. Mol Biol Cell **19:**3308-22.

142. **Jones, M. S., 2nd, B. Harrach, R. D. Ganac, M. M. Gozum, W. P. Dela Cruz, B. Riedel, C. Pan, E. L. Delwart, and D. P. Schnurr.** 2007. New adenovirus species found in a patient presenting with gastroenteritis. J Virol **81:**5978-84.

143. **Jones, P. A., and S. M. Taylor.** 1980. Cellular differentiation, cytidine analogs and DNA methylation. Cell **20:**85-93.

144. **Jones, P. L., G. J. Veenstra, P. A. Wade, D. Vermaak, S. U. Kass, N. Landsberger, J. Strouboulis, and A. P. Wolffe.** 1998. Methylated DNA and MeCP2 recruit histone deacetylase to repress transcription. Nat Genet **19:**187-91.

145. **Jooss, K., Y. Yang, K. J. Fisher, and J. M. Wilson.** 1998. Transduction of dendritic cells by DNA viral vectors directs the immune response to transgene products in muscle fibers. Journal of Virology **72:**4212-4223.

146. **Kass, S. U., J. P. Goddard, and R. L. Adams.** 1993. Inactive chromatin spreads from a focus of methylation. Mol Cell Biol **13:**7372-9.

147. **Kass, S. U., N. Landsberger, and A. P. Wolffe.** 1997. DNA methylation directs a time-dependent repression of transcription initiation. Curr Biol **7:**157-65.

148. **Kawase, H., M. Okuwaki, M. Miyaji, R. Ohba, H. Handa, Y. Ishimi, T. Fujii-Nakata, A. Kikuchi, and K. Nagata.** 1996. NAP-I is a functional homologue of TAF-I that is required for replication and transcription of the adenovirus genome in a chromatin-like structure. Genes Cells **1:**1045-56.

149. **Kay, M. A., D. Liu, and P. M. Hoogerbrugge.** 1997. Gene therapy. Proc Natl Acad Sci U S A **94:**12744-6.

150. **Kelly, C., R. Van Driel, and G. W. Wilkinson.** 1995. Disruption of PML-associated nuclear bodies during human cytomegalovirus infection. J Gen Virol **76 (Pt 11):**2887-93.

151. **Kim, I. H., A. Jozkowicz, P. A. Piedra, K. Oka, and L. Chan.** 2001. Lifetime correction of genetic deficiency in mice with a single injection of helper-dependent adenoviral vector. Proc Natl Acad Sci U S A **98:**13282-7.

152. **Kim, N. W.** 1997. Clinical implications of telomerase in cancer. Eur J Cancer **33:**781-6.

153. **Kirmizis, A., S. M. Bartley, A. Kuzmichev, R. Margueron, D. Reinberg, R. Green, and P. J. Farnham.** 2004. Silencing of human polycomb target genes is associated with methylation of histone H3 Lys 27. Genes & Development **18:**1592-605.

154. **Klose, R. J., and A. P. Bird.** 2006. Genomic DNA methylation: the mark and its mediators. Trends Biochem Sci **31:**89-97.

155. **Konev, A. Y., M. Tribus, S. Y. Park, V. Podhraski, C. Y. Lim, A. V. Emelyanov, E. Vershilova, V. Pirrotta, J. T. Kadonaga, A. Lusser, and D. V. Fyodorov.** 2007. CHD1 motor protein is required for deposition of histone variant H3.3 into chromatin in vivo. Science **317:**1087-90.

156. **Kouzarides, T.** 2007. Chromatin Modifications and Their Function. Cell **128:**693-705.

157. **Krogan, N. J., M. Kim, S. H. Ahn, G. Zhong, M. S. Kobor, G. Cagney, A. Emili, A. Shilatifard, S. Buratowski, and J. F. Greenblatt.** 2002. RNA polymerase II elongation factors of Saccharomyces cerevisiae: a targeted proteomics approach. Mol Cell Biol **22:**6979-92.

158. **Kumar, A., and A. M. Boriek.** 2003. Mechanical stress activates the nuclear factor-kappaB pathway in skeletal muscle fibers: a possible role in Duchenne muscular dystrophy. Faseb J **17:**386-96.

159. **Kumar, A., S. Lnu, R. Malya, D. Barron, J. Moore, D. B. Corry, and A. M. Boriek.** 2003. Mechanical stretch activates nuclear factor-kappaB, activator protein-1, and mitogen-activated protein kinases in lung parenchyma: implications in asthma. Faseb J **17:**1800-11.

160. **Lall, S.** 2007. Primers on chromatin. Nat Struct Mol Biol **14:**1110-5.

161. **Lang, S. E., and P. Hearing.** 2003. The adenovirus E1A oncoprotein recruits the cellular TRRAP/GCN5 histone acetyltransferase complex. Oncogene **22:**2836-41.

162. **Larochelle, N., H. Lochmuller, J. Zhao, A. Jani, P. Hallauer, K. E. Hastings, B. Massie, S. Prescott, B. J. Petrof, G. Karpati, and J. Nalbantoglu.** 1997. Efficient muscle-specific transgene expression after adenovirus- mediated gene transfer in mice using a 1.35 kb muscle creatine kinase promoter/enhancer. Gene Therapy **4:**465-472.

163. **Laybourn, P. J., and J. T. Kadonaga.** 1991. Role of nucleosomal cores and histone H1 in regulation of transcription by RNA polymerase II. Science **254:**238-45.

164. **Leopold, P. L., and R. G. Crystal.** 2007. Intracellular trafficking of adenovirus: Many means to many ends. Advanced Drug Delivery Reviews **59:**810-821.

165. **Leopold, P. L., B. Ferris, I. Grinberg, S. Worgall, N. R. Hackett, and R. G. Crystal.** 1998. Fluorescent virions: dynamic tracking of the pathway of adenoviral gene transfer vectors in living cells. Hum Gene Ther **9:**367-378.

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

167. **Li, B., M. Carey, and J. L. Workman.** 2007. The Role of Chromatin during Transcription. Cell **128:**707-719.

168. **Lin, D. Y., Y. S. Huang, J. C. Jeng, H. Y. Kuo, C. C. Chang, T. T. Chao, C. C. Ho, Y. C. Chen, T. P. Lin, H. I. Fang, C. C. Hung, C. S. Suen, M. J. Hwang, K. S. Chang, G. G. Maul, and H. M. Shih.** 2006. Role of SUMO-interacting motif in Daxx SUMO modification, subnuclear localization, and repression of sumoylated transcription factors. Molecular Cell **24:**341-54.

169. **Lindsay, C. R., V. M. Morozov, and A. M. Ishov.** 2008. PML NBs (ND10) and Daxx: from nuclear structure to protein function. Frontiers in Bioscience **13:**7132-42.

170. **Ling, P. D., R. S. Peng, A. Nakajima, J. H. Yu, J. Tan, S. M. Moses, W. H. Yang, B. Zhao, E. Kieff, K. D. Bloch, and D. B. Bloch.** 2005. Mediation of Epstein-Barr virus EBNA-LP transcriptional coactivation by Sp100. Embo J **24:**3565-75.

171. **Lo, H. W., C. P. Day, and M. C. Hung.** 2005. Cancer-specific gene therapy. Advances in Genetics **54:**235-55.

172. **Lomvardas, S., and D. Thanos.** 2002. Modifying gene expression programs by altering core promoter chromatin architecture. Cell **110:**261-71.

173. **Lomvardas, S., and D. Thanos.** 2001. Nucleosome sliding via TBP DNA binding in vivo. Cell **106:**685-96.

174. **Loppin, B., E. Bonnefoy, C. Anselme, A. Laurencon, T. L. Karr, and P. Couble.** 2005. The histone H3.3 chaperone HIRA is essential for chromatin assembly in the male pronucleus. Nature **437:**1386-90.

175. **Luger, K., A. W. Mader, R. K. Richmond, D. F. Sargent, and T. J. Richmond.** 1997. Crystal structure of the nucleosome core particle at 2.8 A resolution. Nature **389:**251-60.

176. **Lunyak, V. V.** 2008. Boundaries. Boundaries...Boundaries??? Curr Opin Cell Biol **20:**281-7.

177. **Lusser, A., and J. T. Kadonaga.** 2004. Strategies for the reconstitution of chromatin. Nat Methods **1:**19-26.

178. **Lusser, A., D. L. Urwin, and J. T. Kadonaga.** 2005. Distinct activities of CHD1 and ACF in ATP-dependent chromatin assembly. Nat Struct Mol Biol **12:**160-6.

179. **Maelandsmo, G. M., P. J. Ross, M. Pavliv, R. A. Meulenbroek, C. Evelegh, D. A. Muruve, F. L. Graham, and R. J. Parks.** 2005. Use of a murine secreted alkaline phosphatase as a non-immunogenic reporter gene in mice. Journal of Gene Medicine **7:**307-315.

180. **Maizel, J. V., Jr., D. O. White, and M. D. Scharff.** 1968. The polypeptides of adenovirus. II. Soluble proteins, cores, top components and the structure of the virion. Virology **36:**126-36.

181. **Mason, P. B., and K. Struhl.** 2003. The FACT complex travels with elongating RNA polymerase II and is important for the fidelity of transcriptional initiation in vivo. Mol Cell Biol **23:**8323-33.

182. **Matsumoto, K., K. Nagata, M. Miyaji-Yamaguchi, A. Kikuchi, and M. Tsujimoto.** 1999. Sperm chromatin decondensation by template activating factor I through direct interaction with basic proteins. Mol Cell Biol **19:**6940-52.

183. **Matsumoto, K., K. Nagata, M. Ui, and F. Hanaoka.** 1993. Template activating factor I, a novel host factor required to stimulate the adenovirus core DNA replication. J Biol Chem **268:**10582-7.

184. **Maul, G. G., A. M. Ishov, and R. D. Everett.** 1996. Nuclear domain 10 as preexisting potential replication start sites of herpes simplex virus type-1. Virology **217:**67-75.

185. **McDaniel, I. E., J. M. Lee, M. S. Berger, C. K. Hanagami, and J. A. Armstrong.** 2008. Investigations of CHD1 function in transcription and development of Drosophila melanogaster. Genetics **178:**583-7.

186. **McKittrick, E., P. R. Gafken, K. Ahmad, and S. Henikoff.** 2004. Histone H3.3 is enriched in covalent modifications associated with active chromatin. Proc Natl Acad Sci U S A **101:**1525-30.

187. **McLay, D. W., and H. J. Clarke.** 2003. Remodelling the paternal chromatin at fertilization in mammals. Reproduction **125:**625-33.

188. **Medzhitov, R.** 2007. Recognition of microorganisms and activation of the immune response. Nature **449:**819-26.

189. **Meier, O., and U. F. Greber.** 2003. Adenovirus endocytosis. The Journal of Gene Medicine **5:**451-462.

190. **Merika, M., and D. Thanos.** 2001. Enhanceosomes. Curr Opin Genet Dev **11:**205-8.

191. **Messina, S., A. Bitto, M. Aguennouz, L. Minutoli, M. C. Monici, D. Altavilla, F. Squadrito, and G. Vita.** 2006. Nuclear factor kappa-B blockade reduces skeletal muscle degeneration and enhances muscle function in Mdx mice. Exp Neurol **198:**234-41.

192. **Meulenbroek, R. A., K. L. Sargent, J. Lunde, B. J. Jasmin, and R. J. Parks.** 2004. Use of adenovirus protein IX to display large polypeptides on the virion - generation of fluorescent virus through incorporation of pIX-GFP. Mol Ther **9:**617-624.

193. **Michaelson, J. S., and P. Leder.** 2003. RNAi reveals anti-apoptotic and transcriptionally repressive activities of DAXX. Journal of Cell Science **116:**345-52.

194. **Mito, Y., J. G. Henikoff, and S. Henikoff.** 2005. Genome-scale profiling of histone H3.3 replacement patterns. Nat Genet **37:**1090-7.

195. **Mizuguchi, G., X. Shen, J. Landry, W. H. Wu, S. Sen, and C. Wu.** 2004. ATP-driven exchange of histone H2AZ variant catalyzed by SWR1 chromatin remodeling complex. Science **303:**343-8.

196. **Morozov, V. M., N. A. Massoll, O. V. Vladimirova, G. G. Maul, and A. M. Ishov.** 2008. Regulation of c-met expression by transcription repressor Daxx. Oncogene **27:**2177-86.

197. **muller, H. J.** 1930. Types of visible variations induced by X-rays in *Drosophila*. Journal of Genetics **22**.

198. **Muller, S., and A. Dejean.** 1999. Viral immediate-early proteins abrogate the modification by SUMO-1 of PML and Sp100 proteins, correlating with nuclear body disruption. Journal of Virology **73:**5137-43.

199. **Muruve, D. A., M. J. Cotter, A. K. Zaiss, L. R. White, Q. Liu, T. Chan, S. A. Clark, P. J. Ross, R. A. Meulenbroek, G. M. Maelandsmo, and R. J. Parks.** 2004. Helper-Dependent adenovirus vectors elicit intact innate but attenuated adaptive host immune reponses. Journal of Virology **78**.

200. **Mutskov, V. J., C. M. Farrell, P. A. Wade, A. P. Wolffe, and G. Felsenfeld.** 2002. The barrier function of an insulator couples high histone acetylation levels with specific protection of promoter DNA from methylation. Genes Dev **16:**1540-54.

201. **Mymryk, J. S., C. J. Fryer, L. A. Jung, and T. K. Archer.** 1997. Analysis of chromatin structure in vivo. Methods **12:**105-14.

202. **Nan, X., R. R. Meehan, and A. Bird.** 1993. Dissection of the methyl-CpG binding domain from the chromosomal protein MeCP2. Nucleic Acids Res **21:**4886-92.

203. **Nefkens, I., D. G. Negorev, A. M. Ishov, J. S. Michaelson, E. T. Yeh, R. M. Tanguay, W. E. Muller, and G. G. Maul.** 2003. Heat shock and Cd2+ exposure regulate PML and Daxx release from ND10 by independent mechanisms that modify the induction of heat-shock proteins 70 and 25 differently. Journal of Cell Science **116:**513-24.

204. **Negorev, D. G., O. V. Vladimirova, A. Ivanov, F. Rauscher, 3rd, and G. G. Maul.** 2006. Differential role of Sp100 isoforms in interferon-mediated repression of herpes simplex virus type 1 immediate-early protein expression. Journal of Virology **80:**8019-29.

205. **Nelson, J. E., and M. A. Kay.** 1997. Persistence of recombinant adenovirus in vivo is not dependent on vector DNA replication. J Virol **71:**8902-8907.

206. **O'Neal, W. K., H. Zhou, N. Morral, E. Aguilar-Cordova, J. Pestaner, C. Langston, B. Mull, Y. Wang, A. L. Beaudet, and B. Lee.** 1998. Toxicological comparison of E2a-deleted and first-generation adenoviral vectors expressing alpha1-antitrypsin after systemic delivery. Hum Gene Ther **9:**1587-1598.

207. **Ohba, R., D. J. Steger, J. E. Brownell, C. A. Mizzen, R. G. Cook, J. Cote, J. L. Workman, and C. D. Allis.** 1999. A novel H2A/H4 nucleosomal histone acetyltransferase in Tetrahymena thermophila. Mol Cell Biol **19:**2061-8.

208. **Okuwaki, M., A. Iwamatsu, M. Tsujimoto, and K. Nagata.** 2001. Identification of nucleophosmin/B23, an acidic nucleolar protein, as a stimulatory factor for in vitro replication of adenovirus DNA complexed with viral basic core proteins. J Mol Biol **311:**41-55.

209. **Olins, D. E., and A. L. Olins.** 2003. Chromatin history: our view from the bridge. Nat Rev Mol Cell Biol **4:**809-14.

210. **Palmer, D., and P. Ng.** 2003. Improved system for helper-dependent adenoviral vector production. Molecular Therapy **8:**846-52.

211. **Palmer, D. J., and P. Ng.** 2005. Helper-dependent adenoviral vectors for gene therapy. Hum Gene Ther **16:**1-16.

212. **Palmer, D. J., and P. Ng.** 2007. Production and Characterization of Helper-dependent Adenoviral Vectors. *In* T. Friedmann and J. Rossi (ed.), Gene Transfer:Delivery and Expression of DNA and RNA. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY.

213. **Pardo-Mateos, A., and C. S. Young.** 2004. Adenovirus IVa2 protein plays an important role in transcription from the major late promoter in vivo. Virology **327:**50-9.

214. **Parks, R. J.** 2000. Improvements in adenoviral vector technology: overcoming barriers for gene therapy. Clin Genet **58:**1-11.

215. **Parks, R. J., J. L. Bramson, Y. Wan, C. L. Addison, and F. L. Graham.** 1999. Effects of stuffer DNA on transgene expression from helper-dependent adenovirus vectors. Journal of Virology **73:**8027-34.

216. **Parks, R. J., L. Chen, M. Anton, U. Sankar, M. A. Rudnicki, and F. L. Graham.** 1996. A helper-dependent adenovirus vector system: removal of helper virus by Cre-mediated excision of the viral packaging signal. Proceedings of the National Academy of Sciences, USA **93:**13565-13570.

217. **Parks, R. J., and F. L. Graham.** 1997. A helper-dependent system for adenovirus vector production helps define a lower limit for efficient DNA packaging. Journal of Virology **71:**3293-3298.

218. **Passarge, E.** 1979. Emil Heitz and the concept of heterochromatin: longitudinal chromosome differentiation was recognized fifty years ago. Am J Hum Genet **31:**106-15.

219. **Paull, T. T., E. P. Rogakou, V. Yamazaki, C. U. Kirchgessner, M. Gellert, and W. M. Bonner.** 2000. A critical role for histone H2AX in recruitment of repair factors to nuclear foci after DNA damage. Curr Biol **10:**886-95.

220. **Peng, Z.** 2005. Current status of gendicine in China: recombinant human Ad-p53 agent for treatment of cancers. Hum Gene Ther **16:**1016-27.

221. **Philpott, A., and G. H. Leno.** 1992. Nucleoplasmin remodels sperm chromatin in Xenopus egg extracts. Cell **69:**759-67.

222. **Philpott, A., G. H. Leno, and R. A. Laskey.** 1991. Sperm decondensation in Xenopus egg cytoplasm is mediated by nucleoplasmin. Cell **65:**569-78.

223. **Polo, S. E., and G. Almouzni.** 2006. Chromatin assembly: a basic recipe with various flavours. Curr Opin Genet Dev **16:**104-11.

224. **Poot, R. A., L. Bozhenok, D. L. van den Berg, N. Hawkes, and P. D. Varga-Weisz.** 2005. Chromatin remodeling by WSTF-ISWI at the replication site: opening a window of opportunity for epigenetic inheritance? Cell Cycle **4:**543-6.

225. **Pray-Grant, M. G., J. A. Daniel, D. Schieltz, J. R. Yates, 3rd, and P. A. Grant.** 2005. Chd1 chromodomain links histone H3 methylation with SAGA- and SLIK-dependent acetylation. Nature **433:**434-8.

226. **Preston, C. M., and M. J. Nicholl.** 2006. Role of the cellular protein hDaxx in human cytomegalovirus immediate-early gene expression. Journal of General Virology **87:**1113-21.

227. **Proesmans, M., F. Vermeulen, and K. De Boeck.** 2008. What's new in cystic fibrosis? From treating symptoms to correction of the basic defect. Eur J Pediatr.

228. **Ray-Gallet, D., J. P. Quivy, C. Scamps, E. M. Martini, M. Lipinski, and G. Almouzni.** 2002. HIRA is critical for a nucleosome assembly pathway independent of DNA synthesis. Mol Cell **9:**1091-100.

229. **Recillas-Targa, F., M. J. Pikaart, B. Burgess-Beusse, A. C. Bell, M. D. Litt, A. G. West, M. Gaszner, and G. Felsenfeld.** 2002. Position-effect protection and enhancer blocking by the chicken beta-globin insulator are separable activities. Proceedings of the National Academy of Sciences, USA **99:**6883-8.

230. **Regad, T., and M. K. Chelbi-Alix.** 2001. Role and fate of PML nuclear bodies in response to interferon and viral infections. Oncogene **20:**7274-86.

231. **Renbaum, P., D. Abrahamove, A. Fainsod, G. G. Wilson, S. Rottem, and A. Razin.** 1990. Cloning, characterization, and expression in Escherichia coli of the gene coding for the CpG DNA methylase from Spiroplasma sp. strain MQ1(M.SssI). Nucleic Acids Res **18:**1145-52.

232. **Riu, E., Z. Y. Chen, H. Xu, C. Y. He, and M. A. Kay.** 2007. Histone Modifications are Associated with the Persistence or Silencing of Vector-mediated Transgene Expression in vivo. Molecular Therapy **15:**1348-1355.

233. **Riu, E., D. Grimm, Z. Huang, and M. A. Kay.** 2005. Increased maintenance and persistence of transgenes by excision of expression cassettes from plasmid sequences in vivo. Human Gene Therapy **16:**558-70.

234. **Ross, P. J., and R. J. Parks.** 2007. Construction of first-generation adenoviral vectors, p. 149-166. *In* T. Friedmann and J. Rossi (ed.), Gene Transfer: Delivery and Expression of DNA and RNA. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, New York.

235. **Rubinchik, S., S. Lowe, Z. Jia, J. Norris, and J. Dong.** 2001. Creation of a new transgene cloning site near the right ITR of Ad5 results in reduced enhancer interference with tissue-specific and regulatable promoters. Gene Ther **8:**247-53.

236. **Ruiz-Lara, S. A., L. Cornudella, and A. Rodriguez-Campos.** 1996. Dissociation of protamine-DNA complexes by Xenopus nucleoplasmin and minichromosome assembly in vitro. Eur J Biochem **240:**186-94.

237. **Russell, W. C.** 2000. Update on adenovirus and its vectors. J Gen Virol **81:**2573-604.

238. **Rux, J. J., and R. M. Burnett.** 2004. Adenovirus structure. Hum Gene Ther **15:**1167-76.

239. **Sadeghi, H., and M. M. Hitt.** 2005. Transcriptionally targeted adenovirus vectors. Current Gene Therapy **5:**411-27.

240. **Saffert, R. T., and R. F. Kalejta.** 2007. Human cytomegalovirus gene expression is silenced by Daxx-mediated intrinsic immune defense in model latent infections established in vitro. Journal of Virology **81:**9109-20.

241. **Saffert, R. T., and R. F. Kalejta.** 2006. Inactivating a cellular intrinsic immune defense mediated by Daxx is the mechanism through which the human cytomegalovirus pp71 protein stimulates viral immediate-early gene expression. Journal of Virology **80:**3863-71.

242. **Saha, A., J. Wittmeyer, and B. R. Cairns.** 2006. Chromatin remodelling: the industrial revolution of DNA around histones. Nat Rev Mol Cell Biol **7:**437-47.

243. **Sambrook, J., E. F. Fritsch, and T. Maniatis.** 1989. Molecular Cloning: A Laboratory Manual, 2nd Ed. Cold Spring Harbor Press, Cold Spring Harbor, USA.

244. **Sandig, V., R. Youil, A. J. Bett, L. L. Franlin, M. Oshima, D. Maione, F. Wang, M. L. Metzker, R. Savino, and C. T. Caskey.** 2000. Optimization of the helper-dependent adenovirus system for production and potency in vivo. Proc Natl Acad Sci U S A **97:**1002-7.

245. **Sanematsu, F., Y. Takami, H. K. Barman, T. Fukagawa, T. Ono, K. Shibahara, and T. Nakayama.** 2006. Asf1 is required for viability and chromatin assembly during DNA replication in vertebrate cells. J Biol Chem **281:**13817-27.

246. **Sargent, K., P. Ng, C. Evelegh, F. L. Graham, and R. J. Parks.** 2004. Development of a size restricted pIX deleted helper virus for amplificaton of helper dependent adenovirus vectors. Gene Therapy **11:**504-511.

247. **Sarraf, S. A., and I. Stancheva.** 2004. Methyl-CpG binding protein MBD1 couples histone H3 methylation at lysine 9 by SETDB1 to DNA replication and chromatin assembly. Molecular Cell **15:**595-605.

248. **Schmidt-Zachmann, M. S., and W. W. Franke.** 1988. DNA cloning and amino acid sequence determination of a major constituent protein of mammalian nucleoli. Correspondence of the nucleoplasmin-related protein NO38 to mammalian protein B23. Chromosoma **96:**417-26.

249. **Schwabish, M. A., and K. Struhl.** 2006. Asf1 mediates histone eviction and deposition during elongation by RNA polymerase II. Molecular Cell **22:**415-22.

250. **Schwabish, M. A., and K. Struhl.** 2004. Evidence for eviction and rapid deposition of histones upon transcriptional elongation by RNA polymerase II. Mol Cell Biol **24:**10111-7.

251. **Schwartz, B. E., and K. Ahmad.** 2005. Transcriptional activation triggers deposition and removal of the histone variant H3.3. Genes Dev **19:**804-14.

252. **Seeler, J. S., A. Marchio, R. Losson, J. M. Desterro, R. T. Hay, P. Chambon, and A. Dejean.** 2001. Common properties of nuclear body protein SP100 and TIF1alpha chromatin factor: role of SUMO modification. Mol Cell Biol **21:**3314-24.

253. **Seeler, J. S., A. Marchio, D. Sitterlin, C. Transy, and A. Dejean.** 1998. Interaction of SP100 with HP1 proteins: a link between the promyelocytic leukemia-associated nuclear bodies and the chromatin compartment. Proc Natl Acad Sci U S A **95:**7316-21.

254. **Segal, E., Y. Fondufe-Mittendorf, L. Chen, A. Thastrom, Y. Field, I. K. Moore, J. P. Wang, and J. Widom.** 2006. A genomic code for nucleosome positioning. Nature **442:**772-8.

255. **Sergeant, A., M. A. Tigges, and H. J. Raskas.** 1979. Nucleosome-like structural subunits of intranuclear parental adenovirus type 2 DNA. Journal of Virology **29:**888-98.

256. **Shackelton, L. A., C. R. Parrish, and E. C. Holmes.** 2006. Evolutionary basis of codon usage and nucleotide composition bias in vertebrate DNA viruses. Journal of Molecular Evolution **62:**551-63.

257. **Shaw, B. R., T. M. Herman, R. T. Kovacic, G. S. Beaudreau, and K. E. Van Holde.** 1976. Analysis of subunit organization in chicken erythrocyte chromatin. Proc Natl Acad Sci U S A **73:**505-9.

258. **Shen, X., and M. A. Gorovsky.** 1996. Linker histone H1 regulates specific gene expression but not global transcription in vivo. Cell **86:**475-83.

259. **Shenk, T.** 2001. Adenoviridae: the viruses and their replication, p. 2265-2300. *In* B. N. Fields, P. M. Howley, D. E. Griffin, R. A. Lamb, M. A. Martin, B. Roizman, S. E. Strauss, and D. M. Knipe (ed.), Fields Virology, vol. 67. Lippincott-Raven Publishers, Philadelphia.

260. **Shenk, T.** 1996. Adenoviridae: the viruses and their replication, p. 2111-2148. *In* B. N. Fields, D. M. Knipe, and P. M. Howley (ed.), Fields Virology, vol. 67. Lippincott-Raven Publishers, Philadelphia.

261. **Shi, C. X., F. L. Graham, and M. M. Hitt.** 2006. A convenient plasmid system for construction of helper-dependent adenoviral vectors and its application for analysis of the breast-cancer-specific mammaglobin promoter. J Gene Med **8:**442-51.

262. **Shi, C. X., M. Hitt, P. Ng, and F. L. Graham.** 2002. Superior Tissue-Specific Expression from Tyrosinase and Prostate-Specific Antigen Promoters/Enhancers in Helper-Dependent Compared with First-Generation Adenoviral Vectors. Human Gene Therapy **13:**211-224.

263. **Shi, C. X., M. A. Long, L. Liu, F. L. Graham, J. Gauldie, and M. M. Hitt.** 2004. The human SCGB2A2 (mammaglobin-1) promoter/enhancer in a helper-dependent adenovirus vector directs high levels of transgene expression in mammary carcinoma cells but not in normal nonmammary cells. Mol Ther **10:**758-67.

264. **Shibahara, K., and B. Stillman.** 1999. Replication-dependent marking of DNA by PCNA facilitates CAF-1-coupled inheritance of chromatin. Cell **96:**575-85.

265. **Shih, H. M., C. C. Chang, H. Y. Kuo, and D. Y. Lin.** 2007. Daxx mediates SUMO-dependent transcriptional control and subnuclear compartmentalization. Biochemical Society Transactions **35:**1397-400.

266. **Shishido-Hara, Y., S. Ichinose, K. Higuchi, Y. Hara, and K. Yasui.** 2004. Major and minor capsid proteins of human polyomavirus JC cooperatively accumulate to nuclear domain 10 for assembly into virions. Journal of Virology **78:**9890-903.

267. **Shuen, M., N. Avvakumov, J. Torchia, and J. S. Mymryk.** 2003. The E1A proteins of all six human adenovirus subgroups target the p300/CBP acetyltransferases and the SAGA transcriptional regulatory complex. Virology **316:**75-83.

268. **Simic, R., D. L. Lindstrom, H. G. Tran, K. L. Roinick, P. J. Costa, A. D. Johnson, G. A. Hartzog, and K. M. Arndt.** 2003. Chromatin remodeling protein Chd1 interacts with transcription elongation factors and localizes to transcribed genes. Embo J **22:**1846-56.

269. **Simone, C., S. V. Forcales, D. A. Hill, A. N. Imbalzano, L. Latella, and P. L. Puri.** 2004. p38 pathway targets SWI-SNF chromatin-remodeling complex to muscle-specific loci. Nat Genet **36:**738-43.

270. **Singh, R. S.** 2003. Darwin to DNA, molecules to morphology: the end of classical population genetics and the road ahead. Genome **46:**938-42.

271. **Smith, C. L., and G. L. Hager.** 1997. Transcriptional regulation of mammalian genes in vivo. A tale of two templates. J Biol Chem **272:**27493-6.

272. **Smith, S., and B. Stillman.** 1989. Purification and characterization of CAF-I, a human cell factor required for chromatin assembly during DNA replication in vitro. Cell **58:**15-25.

273. **Smothers, J. F., and S. Henikoff.** 2000. The HP1 chromo shadow domain binds a consensus peptide pentamer. Curr Biol **10:**27-30.

274. **Sobel, R. E., R. G. Cook, C. A. Perry, A. T. Annunziato, and C. D. Allis.** 1995. Conservation of deposition-related acetylation sites in newly synthesized histones H3 and H4. Proc Natl Acad Sci U S A **92:**1237-41.

275. **Song, J. S.** 2005. Adenovirus-mediated suicide SCLC gene therapy using the increased activity of the hTERT promoter by the MMRE and SV40 enhancer. Biosci Biotechnol Biochem **69:**56-62.

276. **Southern, E. M.** 1975. Detection of specific sequences among DNA fragments separated by gel electrophoresis. J Mol Biol **98:**503-17.

277. **Spector, D. J.** 2007. Default assembly of early adenovirus chromatin. Virology **359:**116-25.

278. **Spector, D. J., J. S. Johnson, N. L. Baird, and D. A. Engel.** 2003. Adenovirus type 5 DNA-protein complexes from formaldehyde cross-linked cells early after infection. Virology **312:**204-12.

279. **Sternsdorf, T., K. Jensen, and H. Will.** 1997. Evidence for covalent modification of the nuclear dot-associated proteins PML and Sp100 by PIC1/SUMO-1. Journal of Cell Biology **139:**1621-34.

280. **Stracker, T. H., C. T. Carson, and M. D. Weitzman.** 2002. Adenovirus oncoproteins inactivate the Mre11-Rad50-NBS1 DNA repair complex. Nature **418:**348-52.

281. **Strahl, B. D., and C. D. Allis.** 2000. The language of covalent histone modifications. Nature **403:**41-5.

282. **Sung, M. T., T. M. Cao, R. T. Coleman, and K. A. Budelier.** 1983. Gene and protein sequences of adenovirus protein VII, a hybrid basic chromosomal protein. Proc Natl Acad Sci U S A **80:**2902-6.

283. **Suzuki, M., K. Kasai, and Y. Saeki.** 2006. Plasmid DNA sequences present in conventional herpes simplex virus amplicon vectors cause rapid transgene silencing by forming inactive chromatin. Journal of Virology **80:**3293-300.

284. **Swindle, C. S., N. Zou, B. A. Van Tine, G. M. Shaw, J. A. Engler, and L. T. Chow.** 1999. Human papillomavirus DNA replication compartments in a transient DNA replication system. Journal of Virology **73:**1001-9.

285. **Tagami, H., D. Ray-Gallet, G. Almouzni, and Y. Nakatani.** 2004. Histone H3.1 and H3.3 complexes mediate nucleosome assembly pathways dependent or independent of DNA synthesis. Cell **116:**51-61.

286. **Takahashi, Y., J. B. Rayman, and B. D. Dynlacht.** 2000. Analysis of promoter binding by the E2F and pRB families in vivo: distinct E2F proteins mediate activation and repression. Genes Dev **14:**804-16.

287. **Tang, J., S. Wu, H. Liu, R. Stratt, O. G. Barak, R. Shiekhattar, D. J. Picketts, and X. Yang.** 2004. A novel transcription regulatory complex containing death domain-associated protein and the ATR-X syndrome protein. J Biol Chem **279:**20369-77.

288. **Tang, Q., P. Bell, P. Tegtmeyer, and G. G. Maul.** 2000. Replication but not transcription of simian virus 40 DNA is dependent on nuclear domain 10. J Virol **74:**9694-700.

289. **Tang, Q., and G. G. Maul.** 2003. Mouse cytomegalovirus immediate-early protein 1 binds with host cell repressors to relieve suppressive effects on viral transcription and replication during lytic infection. Journal of Virology **77:**1357-67.

290. **Tate, V. E., and L. Philipson.** 1979. Parental adenovirus DNA accumulates in nucleosome-like structures in infected cells. Nucleic Acids Res **6:**2769-85.

291. **Taunton, J., C. A. Hassig, and S. L. Schreiber.** 1996. A mammalian histone deacetylase related to the yeast transcriptional regulator Rpd3p. Science **272:**408-11.

292. **Thanos, D., and T. Maniatis.** 1995. Virus induction of human IFN beta gene expression requires the assembly of an enhanceosome. Cell **83:**1091-100.

293. **Thoma, F., and T. Koller.** 1977. Influence of histone H1 on chromatin structure. Cell **12:**101-7.

294. **Trotman, L. C., N. Mosberger, M. Fornerod, R. P. Stidwill, and U. F. Greber.** 2001. Import of adenovirus DNA involves the nuclear pore complex receptor CAN/Nup214 and histone H1. Nat Cell Biol **3:**1092-100.

295. **Trotter, K. W., and T. K. Archer.** 2004. Reconstitution of glucocorticoid receptor-dependent transcription in vivo. Mol Cell Biol **24:**3347-58.

296. **Tsukamoto, T., N. Hashiguchi, S. M. Janicki, T. Tumbar, A. S. Belmont, and D. L. Spector.** 2000. Visualization of gene activity in living cells. Nat Cell Biol **2:**871-8.

297. **Ullman, A. J., and P. Hearing.** 2008. Cellular proteins PML and Daxx mediate an innate antiviral defense antagonized by the adenovirus E4 ORF3 protein. J Virol **82:**7325-35.

298. **van der Vlag, J., J. L. den Blaauwen, R. G. Sewalt, R. van Driel, and A. P. Otte.** 2000. Transcriptional repression mediated by polycomb group proteins and other chromatin-associated repressors is selectively blocked by insulators. Journal of Biological Chemistry **275:**697-704.

299. **Varga-Weisz, P.** 2005. Chromatin remodeling factors and DNA replication. Prog Mol Subcell Biol **38:**1-30.

300. **Vayda, M. E., and S. J. Flint.** 1987. Isolation and characterization of adenovirus core nucleoprotein subunits. J Virol **61:**3335-9.

301. **Verma, I. M., and M. D. Weitzman.** 2005. Gene therapy: twenty-first century medicine. Annu Rev Biochem **74:**711-38.

302. **von Lindern, M., S. van Baal, J. Wiegant, A. Raap, A. Hagemeijer, and G. Grosveld.** 1992. Can, a putative oncogene associated with myeloid leukemogenesis, may be activated by fusion of its 3' half to different genes: characterization of the set gene. Mol Cell Biol **12:**3346-55.

303. **Wallrath, L. L.** 1998. Unfolding the mysteries of heterochromatin. Current Opinion in Biotechnology **8:**147-53.

304. **Wang, K., S. Huang, A. Kapoor-Munshi, and G. Nemerow.** 1998. Adenovirus Internalization and Infection Require Dynamin. J. Virol. **72:**3455-3458.

305. **West, A. G., M. Gaszner, and G. Felsenfeld.** 2002. Insulators: many functions, many mechanisms. Genes Dev **16:**271-88.

306. **West, A. G., S. Huang, M. Gaszner, M. D. Litt, and G. Felsenfeld.** 2004. Recruitment of histone modifications by USF proteins at a vertebrate barrier element. Mol Cell **16:**453-63.

307. **Wickham, T. J., E. J. Filardo, D. A. Cheresh, and G. R. Nemerow.** 1994. Integrin alpha v beta 5 selectively promotes adenovirus mediated cell membrane permeabilization. J Cell Biol **127:**257-264.

308. **Wickham, T. J., P. Mathias, D. A. Cheresh, and G. R. Nemerow.** 1993. Integrins alpha v beta 3 and alpha v beta 5 promote adenovirus internalization but not virus attachment. Cell **73:**309-319.

309. **Wiethoff, C. M., H. Wodrich, L. Gerace, and G. R. Nemerow.** 2005. Adenovirus protein VI mediates membrane disruption following capsid disassembly. J Virol **79:**1992-2000.

310. **Wilcox, K. W., S. Sheriff, A. Isaac, and J. L. Taylor.** 2005. SP100B is a repressor of gene expression. Journal of Cellular Biochemistry **95:**352-65.

311. **Wolffe, A. P.** 1998. Chromatin: Structure and Function, 3rd ed. Academic Press.

312. **Wong, H. K., and E. B. Ziff.** 1994. Complementary functions of E1a conserved region 1 cooperate with conserved region 3 to activate adenovirus serotype 5 early promoters. Journal of Virology **68:**4910-20.

313. **Wong, M. L., and M. T. Hsu.** 1988. Psoralen-cross-linking study of the organization of intracellular adenovirus nucleoprotein complexes. J Virol **62:**1227-34.

314. **Woodcock, C. L.** 2006. Chromatin architecture. Curr Opin Struct Biol **16:**213-20.

315. **Woodcock, C. L., and S. Dimitrov.** 2001. Higher-order structure of chromatin and chromosomes. Curr Opin Genet Dev **11:**130-5.

316. **Woraratanadharm, J., S. Rubinchik, H. Yu, F. Fan, S. M. Morrow, and J. Y. Dong.** 2004. Highly specific transgene expression mediated by a complex adenovirus vector incorporating a prostate-specific amplification feedback loop. Gene Ther **11:**1399-407.

317. **Xu, Y., J. H. Ahn, M. Cheng, C. M. apRhys, C. J. Chiou, J. Zong, M. J. Matunis, and G. S. Hayward.** 2001. Proteasome-independent disruption of PML oncogenic domains (PODs), but not covalent modification by SUMO-1, is required for human cytomegalovirus immediate-early protein IE1 to inhibit PML-mediated transcriptional repression. J Virol **75:**10683-95.

318. **Xue, Y., J. S. Johnson, D. A. Ornelles, J. Lieberman, and D. A. Engel.** 2005. Adenovirus protein VII functions throughout early phase and interacts with cellular proteins SET and pp32. J Virol **79:**2474-83.

319. **Yang, X. J., V. V. Ogryzko, J. Nishikawa, B. H. Howard, and Y. Nakatani.** 1996. A p300/CBP-associated factor that competes with the adenoviral oncoprotein E1A. Nature **382:**319-24.

320. **Yang, Y., Q. Li, H. C. Ertl, and J. M. Wilson.** 1995. Cellular and humoral immune responses to viral antigens create barriers to lung-directed gene therapy with recombinant adenoviruses. J Virol **69:**2004-15.

321. **Zhang, R., S. T. Liu, W. Chen, M. Bonner, J. Pehrson, T. J. Yen, and P. D. Adams.** 2006. HP1 proteins are essential for a dynamic nuclear response that rescues the function of perturbed heterochromatin in primary human cells. Mol Cell Biol.

322. **Zhao, T., T. Heyduk, C. D. Allis, and J. C. Eissenberg.** 2000. Heterochromatin protein 1 binds to nucleosomes and DNA in vitro. Journal of Biological Chemistry **275:**28332-8.

323. **Zheng, X., X. M. Rao, C. Snodgrass, M. Wang, Y. Dong, K. M. McMasters, and H. S. Zhou.** 2005. Adenoviral E1a expression levels affect virus-selective replication in human cancer cells. Cancer Biol Ther **4:**1255-62.

324. **Zhong, S., S. Muller, S. Ronchetti, P. S. Freemont, A. Dejean, and P. P. Pandolfi.** 2000. Role of SUMO-1-modified PML in nuclear body formation. Blood **95:**2748-52.

Appendix 1 - Contribution of Collaborators

All of the data presented herein were generated by the author. Catherine Barrett provided technical assistance in the screening and propagation of plasmid DNA used for some of the experiments described herein. Kathy Poulin and Karen Powell provided valuable laboratory management support. My thesis supervisor Dr. Robin J. Parks generated hdAd-prok and hdAd-euk while he was a postdoctoral fellow in the laboratory of Dr. Frank Graham (McMaster University, Hamilton, ON). Dr. Parks also provided valuable guidance and feedback in all aspects of the research presented herein, including the editorial comments on the thesis itself. Some reagents were generous gifts of other scientists. Their contributions are acknowledged where appropriate.

Appenix 2 - Reagents

Note: unless otherwise indicated, all chemicals were purchased as solids and solutions were made using water as the solvent.

General Chemicals and Reagents

1 M tris(hydroxymethyl)aminomethane (Tris)-hydrogen chloride (HCl), pH 8.0

1 M Tris-HCl, pH 6.8

1 M Tris-HCl, pH 6.5

0.5 M (4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid) HEPES, pH 8.0

0.5 M piperazine-N,N'-bis(2-ethanesulfonic acid) (PIPES). pH 8.0

10 M sodium hydroxide (NaOH)

5 M sodium chloride (NaCl)

5 M lithium chloride (LiCl)

2.5 M calcium chloride ($CaCl_2$)

2 M magnesium chloride ($MgCl_2$)

20% (w/v) sodium dodecyl sulfate (SDS)

0.5 M ethylenediaminetetraacetic acid (EDTA), pH 8.0

1 x TE: 10 mM Tris-HCl (pH 8.0), 1mM EDTA (pH 8.0)

Chloroform:isoamyl alcohol (20:1, V/V)

10 mM phenylmethylsulphonyl in isopropanol

10% sodium deoxycholate

20x SSC: 3 NaCl, 300 mM sodium citrate

20 mg/ml proteinase K

20 mg/ml 5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside (X-gal) in dimethylformamide

Cell Culture

Minimum essential medium (MEM) and Dulbecco's modified Eagle medium (DMEM)

Growth medium: 10% FBS, 1% antibiotic/antimycotic, 1% Glutamax MEM for 293 and A549 cells; 10% FBS, 1% antibiotic/antimycotic, 1% Glutamax 100 mM hygromycin MEM for 116 cells; and 10% FBS, 1% antibiotic/antimycotic, 1% Glutamax DMEM for HeLa cells

Selection medium: 10% FBS, 1% antibiotic/antimycotic, 1% Glutamax DMEM supplemented with 800 μg/ml G418

40 mg/ml G418 (Invitrogen)

Maintenance medium: MEM, 5% FBS, 1% antibiotic/antimycotic, 1% Glutamax

1x PBS (pH 7.4): 1.06 mM KH_2PO_4, 155.17mM NaCl, 2.97 mM Na_2HPO_4-$7H_2O$

1x Citric Saline: 135 mM KCl, 15 mM $Na_3C_6H_5O_7$

1x trypsin-EDTA (Invitrogen) in PBS

SuperFect (Qiagen) and Lipofectamine 2000 (Invitrogen) transfection reagents

Overlay Medium: 1 x MEM, 5% FBS, 1% antibiotic/antimycotic, 0.5% agarose

4% and 40% sucrose (w/v) in 10 mM Tris (pH 8.0)

β-gal fix: 0.2% glutaraldehyde, 2% para-formaldehyde, 2 mM $MgCl_2$ in PBS

β-gal staining solution: 5 mM $K_4Fe(CN)_6$, 5 mM $K_3Fe(CN)_6$, 2 mM $MgCl_2$ in PBS supplement with 1 mg/ml X-gal immediately before staining

500 mg/ml trichostatin A (Upstate) in dimethyl sulfoxide (DMSO)

200 mM 5,6-dichlorobenzimidazole 1-β-D-ribofuranoside (DRB) (Sigma) in DMSO

50 mM 5-Azacytidine (Sigma)

Cloning and Plasmid DNA Preparation

Solution I: 50 mM Dextrose, 250mM Tris-HCl (pH 8.0), 10mM EDTA (pH 8.0)

Solution II: 200 mM NaOH, 1% SDS

Solution III: 3 M Potassium acetate ($KC_2H_3O_2$), 11.5% glacial acetic acid

10 x SSC: 1.5M NaCl, 50 mM $Na_3C_6H_5O_7$

0.1 x SSC: 15mM NaCl, 0.5 mM $Na_3C_6H_5O_7$

Loading Buffer 6x (Electrophoresis): 40% Sucrose, 0.125% bromophenol blue, 0.1M EDTA pH8.0

Loading Buffer + RNAse: 40% Sucrose, 0.125% bromophenol blue, 0.1M EDTA pH8.0, 0.33 mg/mL RNAse

50 x TAE: 2M Tris, 5.7% glacial acetic acid, 50mM EDTA

1 x TAE: 4mM Tris, 0.114% glacial acetic acid, 5mM EDTA

Restriction digests: DNA (2.5-15 μg), 1x reaction buffer (as recommended by the supplier), 1-2 μl restriction enzyme; total volume 20-100 μl (DNA in TE was never more than 1/3 of the reaction volume); when appropriate (i.e., DNA was intended to be the vector in a ligation reaction) 1 μl of calf intestinal alkaline phosphatase (NEB) was added to the reaction.

Repair of plasmid DNA ends: DNA from restriction digest, 100 μM dNTPs, 1 unit T4 DNA Polymerase per μg DNA, 1 x NEB2 buffer

Ligation reactions: vector and insert fragments, 1 μl ligase, 1x ligase buffer. Volume of vector and insert fragments to be used were determined by multiplying the size in kb of the fragment by 10 and 30, respectively and then dividing by the concentration of the DNA fragment (in ng/μl). The amount of DNA was doubled for blunt-ligations

PCR reaction for cloning: 1x High Fidelity PCR Buffer, 0.2mM dNTP's, 2mM $MgSO_4$, 100 μM Template DNA, 1 unit Platinum Taq High Fidelity (Invitrogen)

SDS-Proteinase K: 10 mM Tris-HCl pH 8.0, 10 mM EDTA pH 8.0, 1% SDS, 1 mg/mL Proteinase K

Isopropanol precipitation: 1.0 volume of DNA solution was mixed with 0.7-1.0 volumes of isopropanol, 0.2 volumes of isopropanol, and 1 μl glycogen (Roche).

Bacterial Amplification of Cloned DNA

Luria Broth (LB) medium

LB Broth with Agar: 500mL LB Broth, 7.5g Agar

Antibiotic selection: 500mL LB Broth or LB broth with agar supplemented with 500 µl 50 mg/ml ampicillin or 500-1000 µl 25 mg/ml kanamycin

2x YT: 8g Tryptone-peptone, 5g Yeast extract, 2.5g NaCl, up to 500mL H_2O; adjust to pH 7.0; filter sterilize

RF1: 100mM RbCl, 50mM $MnCl_2.4\ H_2O$, 30mM potassium acetate, 10mM $CaCl_2.2H_2O$, 15% w/v Glycerol; adjust to pH 5.8 with 0.2M acetic acid

RF2: 10mM MOPS, 10mM RbCl, 75mM $CaCl_2.2H_2O$, 15% w/v Glycerol; adjust to pH 6.8 with 2N NaOH

30% (w/v) glycerol (Sigma)

Immunoblot

2x Laemmli Sample Buffer: 62.5 mM Tris-HCl (pH 6.8), 25% glycerol, 2% SDS, 0.01g bromophenol blue, 0.715 M β-mercaptoethanol (BME; BME is added immediately before use: 50 µl/ml)

5x Laemmli Sample Buffer: 150 mM Tris-HCl (pH 6.8), 62.5% glycerol, 5% SDS, 0.025 g bromophenol blue, 1.79 M BME

10% (w/v) ammonium persulfate (APS)

2x Stacking Gel Buffer pH 6.8: 250mM Tris-HCl pH 6.8, 0.2% SDS

2x Separating Gel Buffer pH 8.8: 750 mM Tris-HCl pH 8.8, 0.2% SDS

Separating Gel Solution:

	volume (ml)		
Reagent	7.5% SDS-PAGE	10% SDS-PAGE	12% SDS-PAGE
ddH2O	2.5	2.0	1.0
2x Sep Gel Buffer	5.0	5.0	5.0
30% Acrylamide	2.5	3.0	4.0
10% APS	0.1	0.1	0.1
TEMED	0.01	0.01	0.01

Stacking Gel Solution: 2.2 ml H_2O, 3ml 2x Stacking Gel Buffer, 0.8 ml 30% Acrylamide, 100 µL 10% APS, and 10 µL TEMED.

Running Buffer: 50mM Tris-HCl (pH 8.0), 1.44% glycine, 0.1% SDS

Transfer Buffer: 48mM Tris-HCl (pH 8.0), 0.293% glycine, 0.0375% SDS, 20% MeOH

0.1% (v/v) TBST: 150mM NaCl, 10mM Tris-HCl pH8.0, 0.1% TWEEN-20

5% (w/v) Milk Blocking Solution: 5% skim milk powder in 0.1% TBST

Lysis buffer for acid extraction of histones: 10 mM HEPES (pH 8.0), 1.5 mM MgCl2, 10 mM KCl, 0.5 mM DTT, 1.5 mM PMSF, 4 mM butyrate

Antibodies:

- anti-FLAG M2 (Sigma), 1:10,000
- anti-α-tubulin (Oncogene), 1:5,000
- anti-HIRA (a kind gift of Dr. Peter Adams, Fox Chase Cancer Center, Philadelphia, PA), 1:50
- anti-core V (a kind gift of Dr. Jane Flint, Princeton University, Princeton NJ), 1:50
- anti-H1 AE-4 (Millipore), 1:1,000
- anti-unmodified H3 (Abcam), 1:2,000
- anti-GFP (Invitrogen), 1:5,000
- anti-Sp100 (Millipore), 1:2,000.

Micrococcal nuclease digestions

Micrococcal nuclease (Roche) 15,000 U/ml in 50 mM Tris, HCl (pH 8.0), 25 mM KCl, 4 mM $MgCl_2$, 1 mM $CaCl_2$, 12.5 % glycerol

Homogenization buffer: 10 mM Tris–HCl, pH 7.4, 15 mM NaCl, 60 mM KCl, 1 mM EDTA, 0.1% NP-40, 5% sucrose, 0.15 mM spermine, and 0.5 mM spermidine (spermine and spermidine were added immediately prior to buffer use)

Wash buffer: 10 mM Tris–HCl, pH 7.4, 15 mM NaCl, 60 mM KCl, 0.15 mM spermine, and 0.5 mM spermidine

10% sucrose wash buffer: 10 mM Tris–HCl, pH 7.4, 15 mM NaCl, 60 mM KCl, 10% sucrose 0.15 mM spermine, and 0.5 mM spermidine.

1 mM $CaCl_2$ wash buffer: 10 mM Tris–HCl, pH 7.4, 15 mM NaCl, 60 mM KCl, 1 mM $CaCl_2$, 0.15 mM spermine, and 0.5 mM spermidine

Stop solution: 10 mM Tris HCl, pH 7.6, 10 mM EDTA, 0.5% SDS, 0.4 mg/mL proteinase K

150 mM spermine (Sigma)

500 mM spermidine (Sigma)

Southern blot

Note: only reagents not supplied with the DIG High Prime DNA Labeling and Detection Starter Kit II (Roche) are listed

DNA probe wash buffer 1: 2 × SSC, 0.1% SDS

DNA probe wash buffer 2: 0.5 × SSC, 0.1% SDS

Maleic acid buffer: 0.1 M Maleic acid, 0.15 M NaCl, pH 7.5

Maleic acid wash buffer: 0.1 M maleic acid, 0.15 M NaCl; pH 7.5, 0.3% (v/v) Tween 20

Detection Buffer: 0.1 M Tris-HCl, 0.1 M NaCl, pH 9.5

Chromatin immunopreciptiation

1.25 M glycine

Cell lysis buffer: 5 mM PIPES (pH 8.0), 85 mM KCl, 0.5% NP-40

Nuclear lysis buffer: 50 mM Tris-HCl (pH 8.0), 1% SDS, 10 mM EDTA

100 mg/ml bovine serum albumin (Sigma) in TE

10 mg/ml herring sperm DNA (Invitrogen; purchased in solution)

1 mg/ml leupeptin (Roche)

1 mg/ml aproptinin (Roche) in 0.01 M HEPES, pH 8.0

200 mM NaCl TE

Antibodies:

- mouse anti-adenovirus E1A M58 (Neomarkers), 5 µl (isotype control)
- horseradish peroxidase-conjugated goat-anti mouse IgG (BioRad) 25 µl (isotype control)
- rabbit anti-chloramphenicol acetyltransferase (Sigma) 2.5 µl (isotype control)
- anti-H1, AE-4 (Millipore), 10 µl
- anti-unmodified H3 (Abcam), 2.5-3 µl
- anti-unmodified H2A acidic patch (Millipore), 5 µl
- anti-yeast acetyl-K7 H2A (Millipore), 5 µl
- anti-pan-acetyl-H3 (K9, K14) (Millipore), 5-7.5 µl
- anti-pan-acetyl-H4 (K5, K8, K12, K16) (Millipore), 2.5-3 µl;
- anti-acetyl-K9 H3 (Millipore), 5 µl
- anti-acetylK16 H4 (Abcam), 5 µl
- anti-RNA polymerase II (Santa Cruz), 50 µl
- anti-dimethyl-K9 H3 (Upstate), 5 µl
- anti-trimethyl-K27 H3 (Abcam), 5 µl
- anti-GFP (Invitrogen) (which cross-reacted with the YFP tag), 5-7.5 µl.

Low salt wash buffer: 20 mM Tris (pH 8.0), 150 mM NaCl, 2 mM EDTA, 0.1% SDS, 1% Triton X-100

High salt wash buffer: 20 mM Tris (pH 8.0), 500 mM NaCl, 2 mM EDTA, 0.1% SDS, 1% Triton X-100

LiCl wash buffer: 10 mM Tris (pH 8.0), 0.25 M LiCl, 1 mM EDTA 1% NP40, 1% deoxycholate

Immune complex elution buffer: 1% SDS, 0.1 M sodium bicarbonate

PCR reactions: 1x PCR buffer, 1.5 mM $MgCl_2$, 0.2 mM (each) dNTP, 0.5 μM (each) primer, 1 unit (0.2 μl) *taq* DNA polymerase (Invitrogen), 1-5 μl ChIP DNA.

Analysis of DNA methylation

Genomic DNA extraction buffer: 10 mM Tris-HCl (pH 8.0), 10 mM EDTA, 0.5 % SDS and 200 μg/ml proteinase K

2 M NaOH

3 M NaOH

10 mM hydroquinone (55 mg to 50 ml with H_2O) prepared immediately before use

3 M sodium bisulfite, pH 5.0 (1.88 g sodium bisulfite to 5 ml with H_2O; ~80 μl 10 M NaOH to reach proper pH) prepared immediately before use

CURRICULUM VITAE

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Born: 30 December, 1980 (Saint John, New Brunswick, Canada)

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Languages: English (primary) and French (fluent)

Education

2002 – 2008	University of Ottawa Ph.D., Microbiology and Immunology GPA: 10.0/10.0	Ottawa, ON

Thesis: On the role of chromatin in the regulation of adenovirus vector transgene expression

Thesis Supervisor: Robin J. Parks, Ph.D.

1998 – 2002	University of New Brunswick B.Sc. (First Class Honours), Biology GPA: 3.8/4.3	Fredericton, NB

Thesis: Molecular Investigation of *Mazzaella* morphologically intermediate to *M.*

splendens and *M. linearis.*

Thesis supervisor: Gary W. Saunders, Ph.D.

Professional Experience

2008-present	Post-Doctoral Research Fellow Laboratory of Sheena Josselyn, Ph.D. Neuroscience and Mental Health Progam The Hospital for Sick Children

References

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Honours, Scholarships, and Awards (National or provincial awards in bold)

Name of award	Value	Awarded by	Date
Post-Doctoral Fellowship	**$50,000 (over 24 months)**	**Ontario Ministry of Research and Innovation via the University of Toronto**	**2008**
Second prize for poster presentation by a Ph.D. student	$75	University of Ottawa, Department of Biochemistry, Microbiology, and Immunology (BMI)	2007
Gold Category Excellence Award at the Canadian Institutes of Health Research (CIHR) National Poster Competition	**$500**	**CIHR Institute of Infection and Immunity**	**2006**
Travel Award	$250	DNA Tumor Virus Meeting Awards Committee	2006
Selected to represent the University of Ottawa Microbiology Program at the CIHR National Poster Competition.	-	The University of Ottawa Graduate Program in Microbiology and Immunology	2006
Second Prize for oral presentation by a Ph.D. student	$75	University of Ottawa Department of BMI	2006
University of Ottawa National Excellence Scholarship	Tuition waiver (ca. $12,000)	University of Ottawa	2005-2007
Canada Graduate Scholarship (D2)	**$70,000 (over 24 months)**	**Natural Science and Engineering Research Council of Canada**	**2005-2007**
University of Ottawa National Excellence Scholarship	$2,000 plus tuition waiver (ca.	University of Ottawa	2003-2005

	$14,000 total value)		
NSERC Post-graduate scholarship A	**$34,600 (over 24 months)**	**Natural Science and Engineering Research Council of Canada**	**2003-2005**
Ontario Graduate Scholarship (Declined)	**$15,000**	**Ministry of Training, Government of Ontario**	**2003-2004**
Entrance Scholarship	$5209	University of Ottawa	2002
Microbiology and Immunology Entrance Award	$3000	University of Ottawa department of BMI	2002
Dean's list	-	University of New Brunswick	2002
Travel Award	$ 250	Northeast Algal Society	2002
Travel Award	$ 150	University of New Brunswick, Dept. of Biology	2002
Alumni Undergraduate Scholarship	$1000	University of New Brunswick	2001
Dean's list	-	University of New Brunswick	2001
George Frederick Boyer Memorial Bursary	$400	University of New Brunswick	2001
Dean's List	-	University of New Brunswick, Saint John Campus	1999
J. Fraser Gregory Scholarship	$600	University of New Brunswick, Saint John Campus	1999
Francis H. Premdas Prize in Biology	$150	University of New Brunswick, Saint John Campus	1999
Entrance Scholarship	$500	University of New Brunswick, Saint John Campus	1998

Mentoring and leadership experience

September 2006-August 2007 – Catherine Barrett, research technician

I was supervised the day-to-day activities of a technician whose responsibility was generating helper-dependent adenovirus vectors for collaborative studies with several laboratories in Canada and the United States. Catherine is currently a student in the MD program at the University of Ottawa (class of 2011).

Publications

Peer Reviewed

Muruve DA, Petrilli V, Zaiss AK, White LR, Clark SA, Ross PJ, Parks RJ, Tschopp J. 2008. The inflammasome recognizes cytosolic microbial and host DNA and triggers an innate immune response. **Nature**. 452:103-107.

Mælansdmo GM, Ross PJ, Pavliv M, Meulenbroek RA, Muruve DA, Graham FL, and Parks RJ. 2005. Use of a murine secreted alkaline phosphatase as a non-immunogenic reporter gene in mice. **Journal of Gene Medicine** 7(3):307-315.

Muruve DA, Cotter MJ, Zaiss AK, White LR, Liu Q, Chan T, Clark SA, Ross PJ, Meulenbroek RA, Maelandsmo GM, and Parks RJ. 2004. Helper-dependent adenovirus vectors elicit intact innate but attenuated adaptive host immune responses. **Journal of Virology** 78(11): 5966-5972.

Ross PJ, Donaldson SL, and Saunders GW. 2003. Molecular investigation of *Mazzaella* (Gigartinales, Rhodophyta) morphologically intermediate to *M. splendens* and *M. linearis*. **Botanica Marina** 46(2): 203-213.

Book Chapters

Ross PJ and Parks RJ. 2006. Construction of first-generation adenoviral vectors. In: *DNA Delivery and Gene Transfer* (T. Friedmann and J. Rossi, Editors). Cold Spring Harbor Laboratory Press. Cold Spring Harbor, New York, USA. pp 149-169.

Non-peer Reviewed

Ross PJ and Parks RJ. 2004. The future of gene therapy for the treatment of neuromuscular disease. Web-based publication: http://www.mdac.ottawa.on.ca/. 14 pp. (Patient- and family-directed tutorial written for the Ottawa Chapter of Muscular

Dystrophy Canada; subsequently posted by the Jesse's Journey Foundation for Gene and Cell Therapy on their website: http://www.jessesjourney.com/).

Ross PJ and Parks RJ. 2003. Oncolytic Adenovirus: Getting there is half the battle. **Molecular Therapy** 8(5): 707-708. (Solicited commentary).

Manuscripts Submitted for Publication

Ross PJ and Parks RJ. Identification of Sp100 and Daxx as nuclear defense factors that drive epigenetic repression of invading DNA. Manuscript submitted for publication (August 2008).

Ross PJ and Parks RJ. Rapid assembly of adenovirus DNA into chromatin promotes efficient gene expression *in vivo*. Manuscript submitted for publication (August, 2008).

Invited Presentations

Ross PJ and Parks RJ. 2008. Non-replicating adenovirus vectors: a model of of replication-independent chromatin assembly in mammalian cells. Department of Biochemistry, Microbiology, and Immunology Open House Day.

Ross PJ and Parks RJ. 2007. Prokaryotic DNA represses eukaryotic gene expression in cis via DNA methylation-independent induction of histone deacetylation. Department of Biochemistry, Microbiology, and Immunology Open House Day.

Oral Presentations

Ross PJ and Parks RJ. 2008. Swift assembly of adenovirus vector DNA into chromatin promotes efficient transgene expression. Annual BMI Research Symposium. Ottawa, ON, Canada.

Ross PJ and Parks RJ. 2006. Negative effects of prokaryotic stuffer DNA on helper-dependent adenovirus vector transgene expression. Annual BMI Research Symposium. Ottawa, ON, Canada.

Muruve DA, Cotter MJ, Zaiss AK, White LR, Liu Q, Chan T, Clark SA, Ross PJ, Meulenbroek RA, Maelandsmo GM, and Parks RJ. 2004. Helper-dependent adenovirus vectors elicit intact innate but attenuated adaptive host immune responses. 7th Annual Meeting of the American Society of Gene Therapy, Minneapolis, Minnesota, USA.

Ross PJ, Donaldson SL, and Saunders GW. 2002. Molecular investigation of individuals morphologically intermediate to *Mazzaella splendens* and *M. linearis* (Gigartinales, Rhodophyta). 41st Northeast Algal Symposium, Durham, New Hampshire, USA.

Ross PJ. 2002. Molecular investigation of *Mazzaella* morphologically intermediate to *M. splendens* and *M. linearis* (Gigartinales, Rhodophyta). 1st UNB Biology Honours Conference and Defence. Fredericton, New Brunswick, Canada.

Poster Presentations

Muruve DA, Petrilli V, Zaiss AK, White LR, Clark SA, Ross PJ, Parks RJ, and Tschopp J. 2007. 2007. The NALP3 inflammasome senses viral, bacterial and human DNA and triggers an innate immune response. 39th Annual Meeting of the Canadian Society of Nephrology. Halifax, Nova Scotia, Canada.

Muruve DA, Petrilli V, Zaiss AK, White LR, Clark SA, Ross PJ, Parks RJ, and Tschopp J. 2007. The NALP3 inflammasome senses viral, bacterial and human DNA and triggers an innate immune response. Canadian Society for Immunology, Annual Spring Conference. Lake Louise, Alberta, Canada.

Ross PJ and Parks RJ. 2006. Effects of prokaryotic stuffer DNA on helper-dependent adenovirus vector transgene expression. DNA Tumor Virus Meeting, San Diego, California, USA.

Ross PJ and Parks RJ. 2006. Effects of prokaryotic stuffer DNA on helper-dependent adenovirus vector transgene expression. Canadian Student Health Research Forum. Winnipeg, Manitoba, Canada.

Ross PJ and Parks RJ. 2005. Effects of prokaryotic stuffer DNA on helper-dependent adenovirus vector transgene expression. Annual Biochemistry, Microbiology, and Immunology Poster Conference. Ottawa, ON, Canada.

Ross PJ, Meulenbroek RA, Sargent KL, Picketts DJ, and Parks RJ. 2003. Cancer therapy utilizing an adenoviral vector expressing only E1A. Bionorth: Showcasing the Success Gene. 11th Annual Ottawa Life Sciences International Conference and Exhibition. Ottawa, Ontario, Canada

Ross PJ, Sargent KS, Ng P, Evelegh C, Graham FL, and Parks RJ. 2003. Development of a size-restricted, pIX-deleted helper virus for amplification of helper-dependent adenovirus vectors. 50 Years of Adenoviridae: Structure, Biology, and Vectorology. Montpellier, France.

Ross PJ, Picketts DJ, and Parks RJ. 2003. The SNF2L chromatin-remodeling protein interacts with the retinoblastoma protein. Annual BMI Poster Conference. Ottawa, ON, Canada.

Ross PJ, Meulenbroek RA, Sargent KL, Picketts DJ, and Parks RJ. 2003. Cancer therapy utilizing an adenoviral vector expressing only E1A. Gene Therapy: The Next Five Years. Banff, Alberta, Canada.

Parks RJ, Sargent KL, Meulenbroek RA, Ross PJ, and Hubberstey AV. 2002. Cancer therapy utilizing and adenoviral vector expressing only E1A. Annual Research Meeting of the Alberta Cancer Board, 2002. Banff, AL, Canada.