Fundamental Mechanisms in the Extreme UV Resistance of Adenovirus

by

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Dissertation submitted in partial fulfillment of
the requirements for the degree of Doctor
of Philosophy in the Department of
Civil and Environmental Engineering in the Graduate School
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ABSTRACT

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Abstract

The adenoviruses are nonenveloped double stranded DNA viruses, which cause enteric dysentery and respiratory infection. Adenovirus has become a focus of the water treatment community because of its apparent resistance to ultraviolet disinfection; it is the basis for stringent new EPA regulations regarding UV disinfection of all viruses in both surface and ground waters. Most of the data generated for disinfection studies has involved the use of monochromatic (254 nm) low pressure (LP) UV sources and subsequent assay of viral infectivity in cell culture models. LP UV lamps primarily damage DNA, while polychromatic UV sources may damage other parts of the virus as well. Recent research has shown that these newer, polychromatic UV sources—such as medium pressure (MP) UV—are more effective than monochromatic LP UV for disinfection of adenovirus; however, the reasons for this increased effectiveness are not known. The objectives of this work were to study adenoviral response to UV using both LP and MP UV as well as using both standard cell culture infectivity assays and more direct methods of assessment based on molecular biology. These include quantitative long PCR for assessment of DNA damage and SDS-PAGE for assessment of protein damage; transmission electron microscopy was used to examine the structure of UV treated viral particles. This work was only the second significant study to show the response of adenoviruses to medium pressure UV and the first to thoroughly examine the response of adenoviruses to both LP and MP UV using cell culture-independent methods. Results confirm that adenovirus is sensitive to MP UV when assayed in cell culture; they show that LP and MP UV are equally effective at inducing damage to the adenoviral genome and that MP UV is more effective than LP UV at damaging the viral proteins. This work helps deepen our understanding of UV disinfection of adenovirus.
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1. INTRODUCTION

This chapter provides a review of the relevant literature on adenoviruses and UV disinfection; it describes the underlying rationale for the research project and why the project is important. The work described in this dissertation was done in conjunction with work on animal infectivity assays carried out at the United States Department of Agriculture (USDA). Much of the information included in this Introduction—as well as relevant information regarding animal infectivity assays of adenovirus—is being prepared for publication as a literature review. More details on animal infectivity assays and the collaboration with the USDA are included in Appendix 1.

1.1 Adenoviruses in the Environment and Effects on Human Health

Viruses are a leading cause of illness and death in humans, and viral infections burden both the healthcare system and the economy. Respiratory and enteric viruses are the most common human viruses worldwide (Sattar et al., 2002). Adenoviruses are a diverse group of viruses which infect a variety of host animals; in humans they cause eye and respiratory infections, potentially severe enteric dysentery, infections of the urinary tract, and have occasionally spread to the central nervous system (Rux and Burnett, 1999; Wadell, 1984). Adenovirus can be transmitted via the fecal-oral and respiratory routes (Wadell, 1984; Strauss and Strauss, 2002). Some species can cause persistent infection and shedding of virus for as long as several years; this can be especially problematic since healthy individuals may have only mild infection but shed viruses which can cause severe disease in more susceptible populations (Sattar et al., 2002).
Infants, young children, and the immunocompromised are most vulnerable to
disease caused by adenoviruses; they are second only to rotaviruses as a major
cause of gastroenteritis in children (Crabtree et al., 1997; Rux and Burnett,
1999), and infection is fatal in 50% of immunocompromised individuals (Wadell,
1984). Epidemics are likely in crowded populations and recently, repeated
epidemics of respiratory adenovirus infection have occurred in military recruits as
a result of vaccine shortages (Gray et al., 2000; Kolavic-Gray et al., 2002; Ryan
et al., 2002). These outbreaks show the highly communicable nature of some
adenoviruses. Adenoviruses show increased stability in the environment
compared to other viruses; estimated annual risks of contracting illness from
waterborne adenovirus are higher than EPA recommendations and they are
considered to be emerging human pathogens (Crabtree et al., 1997; Nwachcuku
and Gerba, 2004; Yates et al., 2006).

Adenoviruses are named for the adenoid tissue from which they were first
isolated in 1953 (Ginsberg, 1999). Human adenoviruses are classified into six
subgroups, A-F, based primarily on G-C content of their DNA and agglutination
properties (Ginsberg, 1999; Wadell, 1984). More than fifty individual serotypes
within these subgroups have been identified (Strauss and Strauss, 2002). The
serotypes in subgroups C and F are of most interest in the work described here.
Subgroup C includes adenovirus type 2 and adenovirus type 5. These are
considered to be endemic and account for over half of adenoviral infections; they
mainly infect children and result in both gastrointestinal and respiratory disease.
Ad2 and Ad5 are less virulent than other strains, rarely affect adults, and can be
grown to high titer in the laboratory; thus these two serotypes have been widely investigated in the medical community as potential vectors for gene therapy (Seth, 1999b; Strauss and Strauss, 2002). Subgenus F includes Ad40 and Ad41, which are considered to be the most important adenoviral species with respect to infantile dysentery and are shed in high concentrations by infected children. The diarrhea caused by Ad40 and Ad41 can be protracted and is a leading cause of infant death in the third world; Ad40 and Ad41 account for up to 20% of hospitalizations for childhood diarrhea even in developed countries (Sattar et al., 2002; Wadell, 1984). Adenovirus types 40 and 41 are most likely to become waterborne and have been studied most by the water treatment community (Baxter et al., 2007; Jothikumar et al., 2005; Ko et al., 2003; Ko et al., 2005a; Ko et al., 2005b; Thurston-Enriquez et al., 2003a; Thurston-Enriquez et al., 2003b) but research on these species is somewhat limited by the difficulties associated with propagating them in vitro (Mautner, 1999; Wadell, 1984).

1.2 Current Interest in UV Disinfection of Adenoviruses

Since viruses are significantly smaller than other pathogens, they often get through the filtration systems used in water treatment plants and our primary defense against them is through disinfection processes such as UV treatment (Nwachcuku and Gerba, 2004). Adenovirus has recently become a focus of the water treatment community because of its emerging role as a significant human pathogen and its apparent resistance to UV disinfection (Ballester and Malley, 2004; Gerba et al., 2002; Ko et al., 2003; Ko et al., 2005a; Ko et al., 2005b; Nwachcuku and Gerba, 2004). The response of adenoviruses to UV is of such
concern that a special workshop was recently convened on the science of adenoviruses and the impact of their apparent UV resistance on UV disinfection requirements (Yates et al., 2006). In the Long-Term 2 Enhanced Surface Water Treatment Rule (LT2ESWTR), the USEPA established that a delivered UV dose of 186 mJ/cm$^2$ is required for 4-log inactivation of all viruses; prior to the promulgation of the LT2ESWTR in January 2006, a UV dose of 40 mJ/cm$^2$ was considered sufficient (USEPA, 2003; USEPA, 2006a). The Groundwater Rule was promulgated in January 2007 and states that UV is not sufficient as a stand-alone treatment for 4-log inactivation of any viruses (USEPA, 2006b). Both of these rules are based on adenoviruses, which are currently thought to be the most UV resistant class of viruses and are therefore used as a standard for viral inactivation requirements. The additional costs involved in delivering a UV dose to surface waters that is five times higher than what has been required in the past are significant and may render UV disinfection technology out of reach for some water utilities. Adenovirus has been listed twice listed on the USEPA's Contaminant Candidate List (CCL), a listing of drinking water contaminants that are given priority in the EPA's research and data collection. Adenoviruses were listed on the CCL's first release in 1998 and relisted on the second version, released in February 2005 (USEPA, 2005); they are not listed on CCL 3, most likely because adenoviruses are now considered to be regulated by the LT2ESWTR.

Since UV has proven to be highly effective against most human pathogens (Chang et al., 1985; Gerba et al., 2002; Mofidi et al., 2001; Roberts
and Hope, 2003; USEPA, 2003) and adenoviruses have a different response to UV than other viruses, it is important that current research efforts be focused on the effects of UV irradiation on adenoviruses and why their response might differ from that of other viruses. Further investigation of ultraviolet disinfection of adenovirus is clearly necessary for a more thorough understanding of the mechanisms of UV disinfection—especially as they relate to viral inactivation—and to provide a clearer basis for determination of UV dose requirements.

1.3 Molecular Biology of Adenovirus and Infectious Cycle

Adenoviruses are nonenveloped, icosahedral particles consisting of a protein coat, or capsid, surrounding a DNA-protein core; they range in size from 70-100 nm (Strauss and Strauss, 2002). The adenoviral genome is linear double-stranded DNA (dsDNA) like that of its mammalian hosts and varies in length from approximately 30-40 kilobase pairs (kb) depending on the serotype. The protein coat contains several different types of proteins, the vast majority of which is hexon; at each vertex of the virus’s icosahedral coat is a penton complex from which a fiber protein protrudes (Rux and Burnett, 1999). Each adenovirus particle has 12 molecules of fiber protein extending outward from its surface; the fiber protein is primarily responsible for the attachment of viral particles to their host cells (Seth, 1999b). Adenovirus attaches to the coxsackie and adenovirus receptor (CAR) on the surface of host cells (Bergelson et al., 1997). Seth (1999a) has reviewed the infectious cycle: adenovirus enters cells via receptor-mediated endocytosis, during which the portion of the cell membrane containing the CAR and bound virus invaginates and becomes a membrane-bound vesicle,
or endosome, within the cell. Within 5 minutes of binding to the cell surface, viral particles are in endosomes within the host cell. When the endosomes lyse, viral particles are released to the cytosol and travel along microtubules to the host cell’s nucleus, where adenoviral DNA is replicated by the host cell’s DNA replication machinery; from there new viral particles are formed and released from the cell. The virus travels from its attachment site on the surface of the host cell to the nucleus in about 30 minutes; coat proteins and core proteins are gradually shed along the way (Greber et al., 1993). Coat proteins facilitate not only binding of viral particles to the cell surface but also lysis of the endosomes and release of virus to the cytosol; core proteins help the virus eject its DNA into the host cell nucleus. Viral proteins are therefore an integral part of every step in the infectious process, and they enable adenoviruses to infect host cells and lyse endosomes even if their DNA is damaged (Seth, 1999b). The nature of its infectious cycle highlights the fact that optimal disinfection of adenovirus requires not only damage to the viral genome, but also interference with its ability to infect host cells in the first place, which likely involves protein damage.

Attachment of adenovirus to the cell surface involves more than simply the CAR and the fiber protein. There may be a second receptor on host cells to which adenoviruses attach with less affinity than they do to the CAR (Fender, 1995; Strauss and Strauss, 2002). While binding to lower-affinity receptors likely helps localize adenovirus to the surface of host cells and accelerate rates of attachment and uptake, low-affinity receptors are not required for viral entry (Strauss and Strauss, 2002). Some portions of the viral coat are
also thought to bind to integrins, transmembrane proteins on the host cell which
mediate host cell interactions with each other and adhesion to the extracellular
matrix *in vivo*. Interaction of adenoviral particles with host cell integrins interferes
with this adhesion, causing the cell rounding and detachment that lead to early
cytopathic effects (CPEs) (Seth, 1999b). The binding of viral particles to the cell
surface and some aspects of their internalization into host cells may vary for
different serotypes of adenovirus (Defer et al., 1990; Fender et al., 1995).

When adenovirus reaches the host cell nucleus, viral DNA is transcribed
and replicated there by a combination of viral and host factors. The specifics of
adenoviral DNA transcription and replication have been reviewed (Ramachandra
and Padmanabhan, 1999). Initially, RNA is transcribed from the E1A
transcription unit—the only viral transcription unit that can be efficiently
processed by cellular factors alone. This activates the transcription of a second
viral RNA which codes for a polymerase, a DNA-binding protein, and a precursor
to the terminal protein which is covalently bound to the 5’ end of each strand of
the genome in a mature viral particle. Together, the viral polymerase and
terminal protein precursor form a complex which participates with the viral DNA
binding protein and some cellular nuclear factors to initiate replication. The viral
DNA-binding protein destabilizes the double helix and helps unwind the dsDNA
genome before replication, while the partner of the strand being copied is
displaced as single-stranded DNA and replicated later. Replication can be
initiated at each end of the double-stranded genome, and proceeds in linear
fashion from one end of the strand being copied to the other (Phillipson, 1983;
Strauss and Strauss, 2002). The terminal protein precursor serves as a primer for DNA replication in a novel protein priming mechanism; while both the precursor and the mature terminal protein are covalently attached to the ends of the viral genome, only the precursor binds DNA (Ramachandra and Padmanabhan, 1999). Replication of the viral DNA begins 6-8 hours post-infection and continues for up to about 25 hours (Strauss and Strauss, 2002). Replication occurs at sites within the host cell nucleus distinct from those at which transcription is carried out; the terminal protein precursor is believed to help properly localize viral DNA within the nucleus for replication (Ramachandra and Padmanabhan, 1999). As do most viral genomes, the genome of adenovirus has a very high density of coding sequences. Transcription of RNA occurs in both directions and alternative splicing of primary transcripts makes several gene products available from one coding sequence in the DNA. While there are only about 12 proteins in adenovirus particles, the genome also codes for numerous other proteins which participate in the infectious cycle (Flint, 1999).

The formation of mature adenovirions occurs in stages, and numerous incomplete particles are also formed. While there is some debate over the exact mechanism of virion maturation, the process can be broken down into approximately four stages (Phillipson, 1983; Schmid and Hearing, 1999): first, the capsid protein structure is assembled into what are called light intermediate particles which have very little to no viral DNA and no core proteins. The insertion of viral DNA results in the formation of heavy intermediate particles which contain the full complement of viral DNA inside the capsid structure, but no
core proteins. Addition of core proteins results in a third intermediate—the young virus particles—which contain all the components of mature adenovirions and all proteins in their precursor forms. Cleavage of these precursor proteins into their final forms by the adenovirus protease results in mature viral particles (Rosenwirth et al., 1974; Schmid and Hearing, 1999). Incomplete particles have a morphology which resembles that of mature adenovirions, but they are weakly or non-infectious (Schmid and Hearing, 1999). Incomplete adenovirus particles contain a range of DNA sizes, all of which include the left end of the genome; this has led researchers to conclude that adenoviral DNA is packaged based on signals located on the left end of the genome (Phillipson, 1983). While the incomplete particles often simply do not contain the full complement of DNA—and may therefore represent stages in the development of mature infectious particles—they have also been shown to contain more than the full complement of viral DNA, which indicates that at least some incomplete particles represent errors—not stages—in assembly (Burlingham et al., 1974). The fraction of incomplete particles formed by adenovirus is characteristic of serotype but independent of cell line and culture conditions (Schmid and Hearing, 1999); with adenovirus type 2—the focus of this work—approximately 5-15% of viral particles formed are incomplete, and this is fairly low compared to other serotypes (Rosenwirth et al., 1974). Infection can result in production of up to 100,000 viral particles from a given host cell, and during infection with adenovirus and production of viral particles, large excess pools of some virion components are formed. It is estimated that only 20% of hexons produced in the host cell, only 1-
5% of pentons, and only 10% of the viral DNA replicated are ultimately packaged into viral particles (Phillipson, 1983). Total amounts of adenoviral DNA and protein can equal total amounts of host cell DNA and protein during the height of infection (Phillipson, 1983).

### 1.4 UV Treatment of Adenovirus and Other Viruses

Wide variation in viral sensitivity to UV has long been recognized and is attributed to differences in virion size, genome composition, capsid structure, and host cell characteristics (Battigelli et al., 1993; Harris et al., 1987; Rauth, 1965; Shin et al., 2005). Larger virions have a smaller surface area-to-volume ratio which might make them more resistant to UV light, while smaller viruses are more susceptible because UV is better able to reach internal components (Harm, 1980; Harris et al., 1987). Relevant aspects of viral genome include whether the nucleic acid is DNA or RNA, whether it is single-stranded or double stranded, the extent of its association with proteins, its nucleotide composition, and its molecular weight. DNA which is associated with proteins may be more susceptible to UV damage than isolated DNA (Hegedus et al., 2003); however, the manner in which all of these factors interact to contribute to UV sensitivity of individual virus types is not well understood (Rauth, 1965; Shin et al., 2005). Whether or not a virus is enveloped is another structural feature that may affect its UV sensitivity. In addition to the protein coat, or capsid, which is a part of all viruses, enveloped viruses have a lipid bilayer envelope surrounding their core. Specific characteristics of individual viruses are also important: for example,
adenoviruses can successfully infect hosts even when their DNA is damaged and may be more robust than other viruses in this respect (Seth, 1999).

A significant amount of data has been published on UV inactivation of adenovirus and other viruses using monochromatic low pressure UV (LP UV) followed by assays of infectivity using cell culture (Ballester and Malley, 2004; Battigelli et al., 1993; Gerba et al., 2002; Harris et al., 1987; Ko et al., 2003; Ko et al., 2005a; Ko et al., 2005b; Meng and Gerba, 1996; Nwachuku et al., 2005; Shin et al., 2005; Thompson et al., 2003; Thurston-Enriquez et al., 2003a; Thurston-Enriquez et al., 2003b). Meng and Gerba (1996) found 3-log inactivation of adenovirus type 40 at a UV dose of 90 mJ/cm$^2$ and 4-log at 120 mJ/cm$^2$, while Thurston-Enriquez et al. (2003a) found that Ad40 requires over 150 mJ/cm$^2$ for 3-log and over 200 mJ/cm$^2$ for 4-log inactivation. Ad1, Ad2, and Ad6 require 120 mJ/cm$^2$ for 3-log inactivation (Nwachuku et al., 2005). Variation between studies can occur as a result of viral preparation methods, complexity of the adenovirus capsid, or the source of water used for irradiation: differences in adenoviral response to UV have been found for buffered demand-free, ground-, and waste-water (Thurston-Enriquez et al., 2003a; Thompson et al., 2003). While there is some variation in UV sensitivity among the adenoviruses, they all appear to be much more resistant to UV than other viruses: 4-log inactivation of other viruses can be achieved at UV doses of 30-40 mJ/cm$^2$ (Gerba et al., 2002; Meng and Gerba, 1996; Shin et al., 2005).

Most likely, though, adenovirus is not truly resistant to LP UV damage but is damaged by the UV and subsequently repaired in host cells during infectivity.
assays. The 254 nm UV irradiation used in these studies is well known to
damage only DNA, and does not interfere with adenovirus adsorption to host
cells during initial stages of infection (Rainbow and Mak, 1973). Host cells have
mechanisms to repair damaged DNA, and host cell reactivation of viruses
inactivated using 254 nm radiation is a well-established phenomenon (Harm,
1980). Formation of pyrimidine dimers in adenoviral DNA after irradiation is
similar to that found in mammalian cells and bacteria (Eischeid and Linden, 2007;
Rainbow and Mak, 1973); reports from the medical literature in which UV-
irradiated adenoviruses are assayed for infectivity in host cells which are known
to be deficient in DNA repair clearly indicate that adenovirus is sensitive to UV in
these cells (Day, 1974; Rainbow, 1980; Rainbow, 1989). More recently, Boszko
and Rainbow (1999) have shown decreased removal of UV photoproducts from
an adenoviral vector in these same repair-deficient cells as compared to normal
human cells.

Among cell lines which are capable of normal levels of DNA repair, there
may not be a significant difference in the extent to which adenoviral DNA is
repaired after damage by LP UV (Nwachuku et al., 2005). At the high LP UV
doses used in more recent studies of UV disinfection, DNA damage may be
sufficient to overwhelm host cell repair capabilities, which is why some level of
inactivation can be achieved despite repair of the viral DNA. The potential for
viral mutagenesis which might result from errors in repair is a further concern in
treatment of adenoviruses using LP UV, and this highlights the need for a more
thorough understanding of UV’s action on viruses and how it can be applied in a manner which ensures complete inactivation.

Damage to the genome of adenovirus is likely repaired by host cells more efficiently than damage to the genomes of other viruses because the adenoviral genome is double-stranded DNA like that of its human hosts, so host repair systems can effectively recognize and repair damage to the adenoviral genome. Studies on LP UV inactivation of other mammalian and closely related viruses, which are more susceptible to UV, have been carried out on viruses whose genomes are single-stranded or composed of RNA instead of DNA (Battigelli et al., 1993; Chang et al., 1985; Gerba et al., 2002; Harris et al., 1987; Meng and Gerba, 1996; Roberts and Hope, 2003; Thurston-Enriquez et al., 2003a). Any viral genome that is single-stranded cannot be repaired in host cells because there is no second strand to serve as a template for replication of the nucleic acid. Viral genomes made of RNA are not repaired efficiently because mammalian hosts do not have sufficient repair mechanisms for RNA. There are five families of double-stranded DNA viruses, including the adenoviruses, whose members can infect human hosts. These families are listed in Table 1, which shows the particular viruses of interest, their modes of transmission, the diseases they cause, and whether or not they are enveloped.
<table>
<thead>
<tr>
<th>Family</th>
<th>Name</th>
<th>Abbr.</th>
<th>Envelope</th>
<th>Transmission</th>
<th>Disease</th>
</tr>
</thead>
<tbody>
<tr>
<td>Adenoviridae</td>
<td>subgroups A-F</td>
<td>Ad</td>
<td>no</td>
<td>fecal-oral and</td>
<td>enteritis, diarrhea, respiratory infection</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td>aerosols</td>
<td></td>
</tr>
<tr>
<td>Papillomaviridae</td>
<td>human papillomavirus</td>
<td>HPV</td>
<td>no</td>
<td>aerosols,contact, urine, sexual</td>
<td>genital warts, cervical cancer</td>
</tr>
<tr>
<td>Polyomaviridae</td>
<td>BK polyomavirus</td>
<td>BKPyV</td>
<td>no</td>
<td>aerosols,contact, urine, sexual</td>
<td>tumors in the immuno-compromised</td>
</tr>
</tbody>
</table>
|                     | JC polyomavirus                              | JCPyV | no       | aerosols,contact, urine, sexual | severe nervous system disease in the immuno-
|                     |                                               |       |          |                 | Compromised                                  |
| Herpesviridae       | herpes simplex 1                             | HHV-1, HSV-1 | yes | contact | cold sores                                         |
|                     | herpes simplex 2                             | HHV-2, HSV-2 | yes | contact | genital ulcers                                     |
|                     | human herpesvirus 3 (varicella-zoster)       | HHV-3, VSV | yes | aerosols | chicken pox, shingles                             |
|                     | cytomegalovirus                              | CMV   | yes      | bodily fluids  | nervous system                                |
|                     | human herpesvirus 4 (Epstein-Barr)           | HHV-4, EBV | yes | contact | mononucleosis, lymphoma                          |
|                     | human herpesvirus 6                          | HHV-6 | yes      | contact, bodily fluids | "sixth disease"                                |
|                     | human herpesvirus 7                          | HHV-7 | yes      | contact     | unknown                                       |
|                     | human herpesvirus 8                          | HHV-8 | yes      | bodily fluids | Kaposi's sarcoma                               |
| Poxviridae          | smallpox (variola virus)                     | VARV  | yes      | contact     | smallpox                                      |
|                     | molluscum contagiosum                        | MOCV  | yes      | contact     | lesions                                       |
With the exception of the adenoviruses, investigation of UV inactivation of these viruses has been minimal. An early study of herpes simplex virus 1 suggests that herpes viruses may be somewhat susceptible to UV disinfection (Cameron, 1973); however, the manner in which these experiments were conducted makes it difficult to compare the UV dose used with that of more recent work and, unlike the adenoviruses, herpesviruses have envelopes. In addition to the adenoviruses, human papillomavirus, BK polyomavirus, and JC polyomavirus are potentially waterborne pathogens, because they can be shed in urine. In general, the diseases caused by the papilloma- and polyomaviruses are less severe and less acute than those caused by adenoviruses, and water is not their major means of transmission. The JC polyomavirus, however, can be associated with serious neurological disease in severely immunocompromised individuals and is considered an emerging human pathogen like adenovirus (Nwachcuku and Gerba, 2004). Since papilloma- and polyomaviruses are the only other families of human viruses which share the major structural features of a double-stranded DNA genome and nonenveloped coat with the adenoviruses, studies of their response to UV disinfection conducted in conjunction with work on the adenoviruses would provide further useful insight into UV disinfection of human viruses.

Work on viral inactivation has also been conducted using various phage, viruses that infect bacteria. Double-stranded DNA bacteriophage such as T4 and T7 have been shown to be sensitive to UV inactivation (Hegedus et al., 2003); however, there are numerous important differences between phage infection of
bacteria and adenovirus infection of humans. Phage particles inject their DNA into host cells through the bacterial cell wall, whereas adenoviruses are endocytosed by the plasma membrane. Bacteria are smaller than human cells by three orders of magnitude; they have no nuclei and a much simpler intracellular organization, so the transport and replication of viruses and viral DNA within the host is substantially different in bacteria and human cells. Bacteriophage T4 has 5-hydroxymethylcytosine rather than cytosine in its DNA; the presence of a modified nucleotide base makes T4 DNA somewhat different in structure from that of its host and less likely to be repaired by host cell mechanisms. Nonetheless, some repair of UV damage does occur in phage T4 using phage-encoded repair systems (Smith and Drake, 1998). Another divergence of bacteriophage and human virus systems is that prokaryotic hosts for bacteriophage are single-celled organisms, and each individual cell has mechanisms for defense against pathogens such as phage (Grabow, 2001). Humans, however, rely primarily on our more complex immune systems to recognize and destroy pathogenic microorganisms, and individual virus-infected cells may not respond to infection like their bacterial counterparts. The response of dsDNA bacteriophage to UV inactivation may provide limited insight into UV disinfection of dsDNA viruses which infect humans, and the differences described above suggest that caution should be used in the adoption of bacteriophage as surrogates for human viruses in studies of UV disinfection.

That adenoviral DNA is apparently being repaired in cell culture assays \textit{in vitro} indicates that it may also be repaired \textit{in vivo} in people who drink
contaminated water; this highlights the need for work which will lead the water
treatment community to a better understanding of how adenoviruses respond to
UV disinfection technologies. An optimally effective disinfection mechanism
should not only damage the viral DNA, but also eliminate the virus’s ability to
infect a host cell in the first place. Newer UV disinfection technologies are well
suited to this task.

1.5 Ultraviolet Disinfection Technologies

UV treatment has become a widely accepted method of water disinfection
in recent years (USEPA, 2003). UV is highly effective at inactivating most
pathogens and, unlike conventional chemical disinfection methods, it is not
considered to be a source of potentially harmful disinfection byproducts. Most
studies and applications of UV disinfection have been focused on low pressure
UV sources. In addition to low pressure UV, medium pressure (MP) and pulsed
UV lamps are also currently being investigated for disinfection of drinking water.
Differences in the source lamp characteristics of different types of UV result in
radiation with different spectral outputs and photon densities which vary in their
action on microorganisms. LP UV lamps used in disinfection contain mercury
vapor at relatively low pressures of 0.001 to 0.01 mm Hg (1.31x10^{-6} –1.31x10^{-5}
atm) and produce essentially monochromatic UV light at 253.7 nm. MP UV
lamps contain mercury vapor at relatively higher pressures ranging from 100-
10,000 mm Hg (0.131—13.15 atm) and emit polychromatic UV light ranging from
200 nm to as high as 1400 nm with several peaks between 185 nm and 300 nm,
which is considered to be the germicidal range (Marshall, 1999; Mofidi et al.,
Standard pulsed UV lamps differ from both LP and MP lamps in that they contain xenon vapor rather than mercury and emit intense pulses of light at high photon densities rather than the continuous, lower-density wave of LP and MP UV. Pulsed UV lamps emit polychromatic UV ranging from 185 nm to about 800 nm; again the germicidal range is approximately 200 nm-300 nm (Wang et al., 2005). The work described here will focus on LP and MP UV.

The differences in emission spectra between LP and MP UV (see Figure 1) are thought to be primarily responsible for the differences in their action on microorganisms. Since LP UV emits very near the 260 nm absorbance maximum for DNA, it inactivates pathogens by damaging their DNA and rendering them incapable of replicating in hosts (Harm, 1980; Jagger, 1967; USEPA, 2003). The primary DNA photoproduct formed during LP UV irradiation of microorganisms is the cyclobutane pyrimidine dimer (CPD), and it may constitute up to 90% of DNA photoproducts (Harm, 1980). MP UV emits other wavelengths—primarily those between 250 nm and 300 nm—which can damage DNA as well as additional cellular components such as protein amino acids, lipids, and small molecules such as carboxylic acid and ketone compounds (Harm, 1980). Wavelengths

![Figure 1: LP and MP UV lamp spectra](image)

Figure 1: LP and MP UV lamp spectra
below approximately 210 nm are absorbed by the phosphate backbone of DNA, and those below 240 nm are absorbed by the peptide bonds of proteins. While these lower wavelengths are highly effective at inactivating viruses, they have been studied less with respect to UV disinfection of drinking water.

Numerous studies have suggested that when UV dose is weighted for germicidal effectiveness, there is little difference between LP and MP UV for inactivation of various types of pathogens, including protozoans, bacteria, and spores (Eischeid and Linden, 2007; Mofidi et al., 2001; Upadhyaya et al., 2004). However, the additional wavelengths emitted by MP UV systems do seem to be advantageous in that they prevent reactivation of microbes; damage to photorepair capabilities has been shown for *E. coli* and *C. parvum* (Oguma et al., 2001; Oguma et al., 2002; Zimmer and Slawson, 2002). This is likely because damage to DNA caused by LP lamps can be repaired but the more widespread cellular damage inflicted by MP UV cannot; such damage can be expected to prevent host cell reactivation of adenovirus as well. Indeed, Linden et al. (2007) have shown that greater inactivation of adenovirus is achieved by MP UV than by LP UV; over 4-log inactivation of adenovirus can be achieved using approximately 40 mJ/cm$^2$ of MP UV. This data is in much closer agreement with the doses required to inactivate other viruses and likely reflects the induction of extragenomic damage which is not repaired in host cells.

1.6 UV Disinfection Studies of Adenovirus: the Role of Molecular Biology

Most of the work done so far has used cell culture infectivity assays to examine the effects of UV on adenovirus. Host cells used for infectivity assays
can differ in their responses to infection with adenovirus and in their ability to repair it (Day, 1974; Rainbow, 1989). The introduction of a second biological system (the host cells) during measurement has obfuscated how the adenoviruses themselves respond to UV, leading to confusion in the field of water treatment, UV dose requirements in LT2ESWTR and the Groundwater Rule which do not have a clear basis, and even the convening of a special conference on the science of adenoviruses to address these issues (Yates et al., 2006). While it is important that a mammalian host system be introduced at some point as an indicator of what treated adenoviruses may do when they infect humans, it is imperative that there also be independent means of assessing the effects of UV on adenoviruses, including both the genome and the coat. This will allow the effects of UV treatment to be assessed more directly and help clarify adenoviral response to UV treatment. Techniques of molecular biology are particularly well suited for this purpose.

Very little research has been published to date which uses molecular biology techniques to investigate the effects of UV disinfection on adenoviral DNA and proteins directly—without introduction of host cell systems. Polymerase chain reaction (PCR) technologies for investigation of DNA have been applied to studies of adenovirus; however, these studies have involved either combinations of PCR and cell culture for tests of viral infectivity after UV treatment or simple detection of adenoviral DNA in untreated environmental samples (Choi and Jiang, 2005; Jothikumar et al., 2005; Ko et al., 2003; Ko et al., 2005a; Ko et al., 2005b). Methods which combine RT-PCR with cell culture
techniques are more sensitive than previous methods but still involve introduction of a second biological system and are more time consuming than direct molecular methods might be. PCR assays for detection of adenovirus in environmental samples indicate prevalence of the adenoviral genome but not of infectious viral particles (Abbaszadegan et al., 2003; Choi and Jiang, 2005; Ko et al., 2003). Since PCR-based methods can detect nucleic acid which is not associated with any risk of infection, these methods can lead to false positive results with respect to public health. This is especially true when short segments of the genome are amplified: short stretches of intact viral DNA are more likely to survive in the environment than complete, intact viral particles. Sobsey et al. (1998) have shown that PCR-based methods can underestimate UV inactivation of adenovirus by as much as 4 logs. Some improvement in accuracy can be achieved by amplifying longer stretches of DNA, which are more likely to represent intact viral particles than short stretches.

While the potential for polychromatic UV to affect proteins is well accepted (Harm, 1980) and has been suggested as a factor in prevention of photoreactivation (Oguma et al., 2002; Zimmer and Slawson, 2002), there have been few direct investigations of protein structure in microorganisms subjected to UV disinfection. Studies of viruses have focused on stability of the viral capsid with respect to temperature, pH, ionic strength, and ionic composition of buffer (Chen et al., 2001; Grande et al., 2002; Rexroad et al., 2003; Rexroad et al., 2006; Volkin et al., 1997). Available data on the coat proteins in adenoviral particles at increasing temperatures indicate that between 40°C and 50°C,
adenovirus undergoes a structural transition which correlates with both disruption of the capsid and a decrease in infectivity. During this transition, initial loss of proteins III and IIIa leads to release of the penton bases and associated fiber proteins, degradation of the capsid, aggregation of proteins, and swelling of the viral particles (Rexroad et al., 2003; Rexroad et al., 2006). Thermal stability is influenced by pH, and pH alone can also affect capsid stability (Rexroad et al., 2006; Wiethoff et al., 2005). No significant decrease in levels of hexon or penton proteins has been demonstrated at temperatures sufficient to induce the loss of proteins III and IIIa which ultimately leads to degradation of the viral particle (Rexroad et al., 2003).

Taken together, these studies demonstrate that the adenovirus capsid functions as a tightly integrated whole, and that the structural integrity of all coat proteins is needed for the viral particle to remain intact and infectious. They further support the idea that polychromatic UV sources may be advantageous in viral disinfection through their potential to damage proteins. Harm (1980) notes that damage to proteins is less likely to inactivate microbes than DNA damage because proteins are present in cells and viruses in relatively high copy numbers, so that damage to a portion of the molecules can be compensated for by their counterparts. The highly interdependent nature of the adenoviral capsid suggests that damage to even a small fraction of its proteins is, in fact, sufficient to lead to particle degradation. That viral capsid stabilities are affected by pH, chemicals, ionic strength, and ionic composition (Grande et al., 2002; Rexroad et al., 2006) indicates that UV disinfection processes for adenovirus are likely to be
influenced by characteristics of the source water, and studies have shown this (Thompson et al., 2003; Thurston-Enriquez et al., 2003a). Direct methods of examining the integrity of viral proteins and whole particles in addition to their ability to infect host cells will be useful not only in UV disinfection studies, but also in detecting potentially infectious adenovirus in the environment.

1.7 Need for this Research

In summary, previous work on UV disinfection of adenovirus has been performed using low pressure (LP) UV and cell culture infectivity assays. Available data suggest that adenoviruses are not truly resistant to UV and that DNA damage induced by LP UV gets repaired in cell culture assays. In contrast to LP UV, polychromatic MP UV is expected to cause more widespread damage to viral structures which may not be repaired. Treatment of adenovirus with MP UV can be expected to damage proteins in the viral coat and core which are integral to both the stability of the viral particle and the process of infection, resulting in greater viral inactivation than LP UV. Numerous authors have called for work which leads to an increased understanding of the molecular mechanisms involved in viral response to UV as well as molecular methods for accurate pathogen detection (Nwachcuku and Gerba, 2004; Shin et al., 2005; Yates et al., 2006), however, the particular molecular assays which have been applied to UV inactivation of adenovirus are not designed for direct assessment of UV damage to genome and coat structures. In light of the current state of knowledge in the field, its impact on UV dose requirements, and the effect of those dose requirements on the cost and feasibility of implementing UV
disinfection, these issues need to be addressed. Newer UV technologies should be tested for their ability to inactivate adenovirus, and treatment results should be assessed in a manner which allows more direct observation of UV action on adenovirus than methods currently employed.

1.8 Research Objectives and Hypotheses

The primary objectives of the proposed research are 1) to apply molecular techniques to investigate the effects of LP and MP UV on adenoviral DNA and proteins, and 2) to compare the results obtained using molecular methods to those obtained using classical cell culture infectivity assays. Each method used to investigate adenoviral response to UV is described in a separate chapter of this document as follows: Chapter 2 describes use of standard cell culture assays to examine infectivity; Chapter 3 describes use of a PCR-based assay to examine DNA damage; Chapter 4 describes assessment of damage to the viral proteins using SDS-PAGE, and Chapter 5 describes assessment of viral particles and capsid integrity using transmission electron microscopy. A preliminary proposal for assessment of capsid integrity using flow cytometry was not carried out as planned and is described in Appendix 2. The hypotheses proposed for this work are that 1) LP and MP UV will be similar in their induction of DNA damage at a given UV dose, 2) MP UV will cause significant damage to adenoviral proteins and loss of capsid integrity at lower doses than LP UV and 3) this increased damage will correlate with decreased infectivity in cell culture assays.
2. ASSESSMENT OF INFECTIVITY

This chapter describes determination of viral infectivity after LP and MP UV irradiation using cell culture infectivity assays. The work described in this chapter and the PCR work described in Chapter 3 have been previously published (Eischeid, A.C., Meyer, J.N., and Linden, K.G. 2009. UV disinfection of adenovirus: Molecular indications of DNA damage efficiency. *Applied and Environmental Microbiology*. 75 (1): 23-28).

2.1 Introduction

A significant amount of data has been published on UV inactivation of adenovirus and other viruses using monochromatic low pressure UV (LP UV) followed by assays of infectivity using cell culture (Ballester and Malley, 2004; Battigelli at el., 1993; Baxter et al., 2007; Gerba et al., 2002; Harris et al., 1987; Ko et al., 2003; Ko et al., 2005a; Meng and Gerba, 1996; Nwachuku et al., 2005; Shin et al., 2005; Thompson et al., 2003; Thurston-Enriquez et al., 2003a). These studies have shown that 4-log inactivation of adenovirus requires a low pressure UV dose of up to 200 mJ/cm², while 30-40 mJ/cm² is sufficient to cause 4-log inactivation of other viruses (Gerba et al., 2002; Meng and Gerba, 1996; Shin et al., 2005). It is possible that the higher dose requirement for low pressure UV inactivation of adenovirus reflects not true resistance, but rather repair of damaged adenoviral DNA in host cells during the cell culture infectivity assays. Low pressure UV used in the studies described above is nearly monochromatic at 253.7 nm—very near the 260 nm absorbance maximum of nucleic acids such as DNA and RNA that make up the genomes of viruses and
other pathogens. It is widely accepted that low pressure UV inactivates microorganisms by damaging their genomes. Because adenovirus can infect host cells even when its genome is damaged (Seth, 1999a), and because that genome is double-stranded DNA like the genome of the host cell, it follows that the DNA repair machinery of the host cell might recognize and repair damage to the adenoviral genome during standard cell culture infectivity assays. Similar effects in cell culture have likely not been seen in other waterborne viruses because their genomes are single-stranded or composed of RNA (Battigelli et al., 1993; Chang et al., 1985; Gerba et al., 2002; Harris et al., 1987; Meng and Gerba, 1996; Roberts and Hope, 2003; Thurston-Enriquez et al., 2003a) and are therefore not recognized by host cell DNA repair machinery. Furthermore, when irradiated with medium-pressure (MP) UV, adenoviruses have been shown to be as susceptible to UV inactivation as other viruses, even in standard cell culture infectivity assays (Linden et al., 2007). Medium pressure UV is polychromatic—it emits a range of wavelengths including those which are absorbed by both DNA and proteins, so it has the potential to damage the viral coat and core proteins in addition to the genome. Such extragenomeric damage appears to play an important role in viral inactivation.

The purpose of the cell culture infectivity assays described in this chapter was to provide more data on MP UV inactivation of adenovirus infectivity and to provide a basis for comparison with previous work as well as with the molecular work described in subsequent chapters.
2.2 Materials and Methods

2.2.1 UV Irradiation and Experimental Design

Both LP and MP UV lamps were housed in separate bench scale "collimated beam" apparati. UV dose was calculated as the average irradiance of the wavelengths in the completely mixed batch irradiation vessel multiplied by the time of exposure as described by Bolton and Linden (2003). For the MP UV source, irradiance was weighted using the DNA absorbance spectrum to provide a "germicidal" dose between 200-300 nm. The absorbance spectrum of each sample across the entire germicidal range was accounted for in the dose calculations, and a Petri factor was applied in all cases to account for variation in UV intensity across the surface of the Petri dish. Such variation in intensity was minimal: the Petri factors for LP and MP UV were, respectively, 0.98 and 1.00. Average UV irradiance, exposure times for the 50 mJ/cm\(^2\) dose, and UV absorbance at 254 nm are given for each experiment in Table 2.

Table 2: UV irradiation conditions

<table>
<thead>
<tr>
<th>UV lamp/Experiment</th>
<th>Average irradiance (mW/cm(^2))</th>
<th>Exposure time (sec) for 50 mJ/cm(^2) dose</th>
<th>UV absorbance at 254 nm</th>
</tr>
</thead>
<tbody>
<tr>
<td>LP, experiment 1</td>
<td>0.3935</td>
<td>127</td>
<td>0.33</td>
</tr>
<tr>
<td>LP, experiment 2</td>
<td>0.3602</td>
<td>139</td>
<td>0.20</td>
</tr>
<tr>
<td>LP, experiment 3</td>
<td>0.3617</td>
<td>138</td>
<td>0.18</td>
</tr>
<tr>
<td>MP, experiment 1</td>
<td>0.4415</td>
<td>113</td>
<td>0.33</td>
</tr>
<tr>
<td>MP, experiment 2</td>
<td>0.4291</td>
<td>116</td>
<td>0.20</td>
</tr>
<tr>
<td>MP, experiment 3</td>
<td>0.4500</td>
<td>111</td>
<td>0.18</td>
</tr>
</tbody>
</table>

Five ml of viral suspension in phosphate- buffered saline (PBS) was irradiated for each sample; sample depth in the Petri dish was 0.23 cm for all samples and all samples were stirred constantly during irradiation. Three independent UV
irradiation experiments were performed for this work and one set of cell culture infectivity data was obtained for each independent UV irradiation experiment.

2.2.2 Culture of Stock Cells, Preparation of Virus, and Infectivity Assays

The A549 cell line (American Type Culture Collection, Manassas, VA) was used to propagate the virus and for all cell culture infectivity assays. The cell line was carried in Ham’s F12K medium supplemented with 10% fetal bovine serum (FBS), 100 units/ml penicillin, and 100 ug/ml streptomycin. Replating of stock cells was done by rinsing with PBS, trypsinization with 0.25% trypsin/EDTA, resuspension, and dilution into new flasks. Cell stocks were split once per week and medium was changed twice per week. Media and solutions for cell culture and viral propagation were obtained from Invitrogen (Carlsbad, CA).

Adenovirus type 2 was a gift from Dr. Gwy-Am Shin (University of Washington, Seattle, WA); the virus was propagated in host cells, then concentrated and purified via polyethylene glycol (PEG) precipitation as previously described (Thurston-Enriquez et al., 2003a). Briefly, A549 host cell monolayers in 150 cm$^2$ flasks were inoculated with 1 ml of DPBS (Invitrogen, Carlsbad, CA) containing $10^6$ most probable number per milliliter (MPN/ml) of virus. MPN is explained below in more detail. Viruses were allowed to adsorb to host cells for 1-1.5 hours at 37°C and were rocked gently by hand once every 15 minutes before addition of 60 ml per flask viral propagation medium (high-glucose DMEM, 2 mM L glutamine, 100 units/ml penicillin, 100 ug/ml streptomycin, and 0.25 ug/ml amphotericin B). Flasks were incubated at 37°C for 9-10 days to allow viral replication, then frozen and thawed twice to break open
host cells and release intracellular virus. The resulting solution was centrifuged at 6000g for 15 minutes at 4°C to remove cellular debris, and supernatant containing viruses was stirred with polyethylene glycol, molecular weight 8000 (9g/100 ml supernatant) and NaCl (5.8g /100 ml supernatant) for 2.5 hours at room temperature. Viruses were then pelleted by centrifugation at 6000g for 30 minutes at 4°C, resuspended in PBS, and extracted once with chloroform to disperse the virus. Viral stocks were kept at 4°C for short-term storage or at -80°C for longer term storage.

Cell culture infectivity assays and MPN enumeration were used both to determine the titer of viral stocks and to assay UV-irradiated virus. A549 cells were plated into 25 cm² flasks at a density of 3-3.5 x 10⁵ cells per flask in complete DMEM (high-glucose DMEM, 10% FBS, 2 mM L-glutamine, 100 units/ml penicillin, 100 ug/ml streptomycin and either 0.25 or 2.5 ug/ml amphotericin B) and allowed to grow for 2 days at 37°C and 5% CO₂. For the MPN calculations, at least three different dilutions of virus, and at least three different flasks per dilution, were inoculated onto cells; cells and viruses were incubated at 37°C in a 5% CO₂ incubator for up to 3 weeks before being scored. Flasks were scored as positive or negative for cytopathic effects and scoring data were entered into a computer program which calculates most probable number (MPN/ml) as described by Hurley and Roscoe (1983). Concentration of amphotericin B did not appear to affect the number of positive and negative flasks in cell culture infectivity assays (data not shown). Log survival for UV
irradiation studies was calculated as \( \log_{10}(\text{MPN/ml treated sample} / \text{MPN/ml untreated control}) \).

2.2.3 Statistical Analysis

Cell culture infectivity data were analyzed by 2-factor Analysis of Variance (ANOVA), and post-hoc analyses were carried out where appropriate using Fisher’s Protected Least Significant Differences (FPLSD) test. Statistical analyses were carried out using Statview® for Windows (Version 5.0.1, SAS Institute Inc., Cary, NC).

2.3 Results

Results of the cell culture infectivity assays are shown in Figure 2, with UV dose on the x axis and log survival on the y axis. Points on the plot are an average of the data from three independent experiments, one set of data per experiment; error bars show one standard error of the mean above and below the average. These data show that the UV dose requirement for a given level of inactivation using low pressure UV is consistently higher than the dose required for the same level of inactivation using medium pressure UV. Analysis of variance shows highly significant main effects for both

![Figure 2: LP and MP UV inactivation of adenovirus in cell culture](image-url)
lamp type (p<0.0001) and UV dose (p<0.0001) as well as a highly significant interaction for lamp*dose (p=0.0004). Pairwise post hoc comparisons (FPLSD) indicate that the difference in inactivation between LP and MP UV is highly significant at 25 mJ/cm$^2$ (p=0.005) and 50 mJ/cm$^2$ (p=0.0003); using p=0.05 as a cutoff, differences were not significant at 10 mJ/cm$^2$ (p=0.08) or 125 mJ/cm$^2$ (p=0.37). Four log inactivation requires a low pressure UV dose of approximately 95-100 mJ/cm$^2$ and only 25 mJ/cm$^2$ of medium pressure UV.

2.4 Discussion

Figure 2 shows that 2, 3, and 4 log inactivation of adenovirus type 2 can be achieved using LP UV doses of approximately 30, 50, and 80 mJ/cm$^2$, respectively. With MP UV, 2.5 and 4.5 log reductions can be reached with only 10 and 25 mJ/cm$^2$, and an MP UV dose of 50 mJ/cm$^2$ yields greater than 5 log reduction in viral infectivity. Some previous studies have shown adenovirus to be more resistant to LP UV than the current one, requiring above 100 mJ/cm$^2$ for 3 log inactivation and from 120-200 mJ/cm$^2$ for 4 log inactivation (Baxter et al., 2007; Gerba et al., 2002; Linden et al., 2007; Meng and Gerba, 1996; Nwachuku et al., 2005; Thurston-Enriquez et al., 2003a). Factors that might account for some of the differences between studies include differences in viral preparation method—specifically the number of freeze-thaw cycles—as well as storage time and temperature of the virus, cell line used for the infectivity assay, virus serotype, and water used for irradiation (Thompson et al., 2003; Thurston-Enriquez et al., 2003a). Other factors not usually cited but which may also account for differences between studies are the cell line used for viral
propagation and the history of the viral stock itself. It is worth noting that the data presented here are in close agreement with Shin et al. (2005); in both cases the virus was propagated and assayed in A549 cells, and the original viral stock used to propagate all adenovirus used in the current study was obtained from Dr. Shin. In the other studies cited above, adenovirus was propagated in PLC/PRF/5, HeLa, or HEK 293 cells. However, in previous studies as well as the current one, it has been shown that all serotypes of adenovirus are more resistant to LP UV than other waterborne human viruses studied so far.

The only other study published to date using MP UV to inactivate adenovirus (Linden et al., 2007) indicates that MP UV doses of 10 and 25 mJ/cm² achieve 1 and 2 log inactivation of adenovirus type 40; here these doses caused 2 and 4 log inactivation of adenovirus type 2. This is likely due to factors cited above or differences in the method used to calculate inactivation; Linden et al. (2007) used TCID₅₀ while the current study uses MPN. In both cases, MP UV is more effective at inactivating adenovirus than LP UV. This is likely because the polychromatic MP UV is capable of causing more widespread damage to the viral particle than monochromatic LP UV, which essentially only damages the DNA. The enhanced inactivation seen with MP UV could be a direct result of such extragenomic damage, or could be because of damage to the viral proteins which prevents repair of genomic damage. The dose-response for MP UV inactivation of adenovirus is similar to the dose-response for LP UV inactivation of other waterborne and enteric viruses, including echoviruses, coxsackieviruses, and rotavirus (Chang et al., 1985; Gerba et al., 2002). Since damage to the
genome is the primary mechanism of inactivation following LP UV treatment of these other viruses, it may also be the most important factor for MP UV inactivation of adenovirus. This could be the case if the more widespread damage caused by MP UV—e.g. to viral proteins—were significant less in its own right and more in that it prevents or interferes with repair of the damaged DNA.
3. ASSESSMENT OF DNA DAMAGE

This chapter describes assessment of damage to adenoviral DNA using a PCR-based assay. Direct assessment of DNA damage to irradiated adenoviruses not introduced into cell culture was carried out using two different sets of PCR primers which allowed determination of damage levels at two different sites in the viral genome. In order to investigate potential repair of the viral DNA in host cells, repair experiments were conducted in which viral DNA was examined after irradiated viruses were incubated with host cells. Some of the work described in this chapter and the cell culture infectivity work described in Chapter 2 have been previously published (Eischeid, A.C., Meyer, J.N., and Linden, K.G. 2009. UV disinfection of adenovirus: Molecular indications of DNA damage efficiency. Applied and Environmental Microbiology. 75 (1): 23-28).

3.1 Introduction

The PCR assays developed for use on adenovirus so far have involved the amplification of short stretches of DNA and are not designed to help one assess the structural integrity of the genome as a whole (Choi and Chiang, 2005; Jothikumar et al., 2005; Ko et al., 2003; Ko et al., 2005a; Ko et al., 2005b). Amplification of long stretches of DNA combined with PCR is powerful with respect to both its sensitivity and its target specificity in detection of DNA damage, and it has been extensively investigated by the van Houten group, which refers to it as the “quantitative PCR assay for DNA damage” (Ayala-Torres et al., 2000; Cheng et al., 1995; Santos et al., 2006; van Houten et al., 2000; also see Barnes, 1994). The use of PCR to determine DNA damage levels is based
on the fact that progression of the polymerases used to amplify DNA in PCR reactions is inhibited by DNA damage. Equal amounts of DNA from samples containing different amounts of DNA damage therefore differ in the extent to which they can be amplified; samples with less damage undergo greater amplification. When amplification is stopped while the PCR is still in the exponential phase, the yield is directly proportional to damage level, and amount of PCR product can be used for accurate quantitation of DNA damage. This assay is not conducted using real time PCR: rather, the range of cycle numbers in which amplification is exponential is determined using cycle tests which are carried out on DNA from untreated virus prior to PCR on experimental samples. Subsequent PCR on experimental samples is carried out using a standard thermocycler rather than a real time PCR machine; PCR is stopped after the number of cycles identified as producing quantitative results in the preliminary cycle tests (van Houten et al., 2000).

The high sensitivity of the method is achieved through the amplification of long segments of DNA, typically ranging from approximately 10,000-25,000 base pairs in length, with longer amplicons resulting in more sensitive DNA damage detection (Hegedus et al., 2003; Jenkins et al., 2000; Santos et al., 2005; van Houten et al., 2000). Another significant advantage of this PCR-based technique is that it can be used to investigate repair in specific stretches of DNA (van Houten et al., 2000); for example, primers can be designed to detect damage and repair in specific parts of the adenoviral genome. QPCR can be done on very small (nanogram) quantities of DNA (van Houten et al., 2000) so it may be
useful in studies of environmental samples where the amount of material available is often limited. The QPCR assay allows detection of all lesions which inhibit DNA polymerase, including nearly all UV induced lesions (Hegedus et al., 2003), but not differentiation of specific types of lesions.

This method has been used on mammalian cells and bacteriophage (Barnes, 1994; Hegedus et al., 2003; Santos et al., 2006) and was employed here for use on adenovirus. While it can be adapted to determine relative quantities of viral DNA in different samples, this PCR assay is primarily used not for detection or quantitation of the virus itself but for quantitation of damage to the viral genome. It allows direct assessment of damage to the adenoviral DNA after irradiation without introducing the virus into host cells. Assessment of DNA repair in cell culture can also be carried out when this PCR assay is done using viral DNA that has been isolated after incubation of irradiated viruses with host cells. The experiments described in this chapter were designed to 1) directly assess DNA damage in irradiated adenoviruses not introduced into cell culture, and 2) to assess repair of viral DNA damage in UV treated adenoviruses after incubation with host cells.

3.2 Materials and Methods

3.2.1 UV Irradiation and Experimental Design

UV irradiation and experimental design were carried out as described in Chapter 2. Three independent UV irradiation experiments were conducted as described, and two sets of PCR data were obtained for each independent UV
irradiation experiment using primer set #2; one set of PCR data per independent experiment was obtained using primer set #3.

3.2.2 Preparation of Viral Stocks

Viral stocks were prepared as described in Chapter 2. For the initial set of PCR experiments, viral stocks were irradiated and DNA was isolated directly; for the repair experiments, viral stocks were irradiated and used to infect host cells and viral DNA was isolated from infected host cells as described below.

3.2.3 Extraction of DNA from Viral Stocks for Direct Assessment of DNA Damage

DNA was extracted from viral stocks using the QIAamp DNA Blood Mini Kit (Qiagen Valencia, CA; catalog # 51104) according to manufacturer’s instructions with minor alterations.

3.2.4 Infection of Host Cells and Total DNA Extraction for Repair Experiments

A549 host cells were plated and infected with irradiated virus as described for the cell culture infectivity assays in Chapter 2. Cells and virus were incubated as described; incubation times for the repair experiments were significantly shorter than those of the cell culture infectivity assays and ranged from 12 hours to a few days. DNA extraction was carried out using a Qiagen Blood and Cell Culture DNA Kit (Qiagen Valencia, CA; catalog # 13323) according to manufacturer’s instructions with modifications to ensure collection of viral DNA in the cell cytosols and culture medium. At the desired timepoint, both cells and medium were harvested, vortexed briefly, and centrifuged at 1500g, 4°C for 10 minutes to pellet cells. The supernatant—possibly containing free viral particles
not attached to host cells—was kept on ice until further processing. The cell pellet was resuspended in PBS and processed into a nuclear pellet according to instructions for the kit. The cell cytosol fraction—isolated during collection of the nuclear pellet—was also kept on ice for further processing. Each sample was therefore separated into three fractions: 1) the cell nuclei (fraction N), 2) the cell cytosols (fraction C), and 3) the cell culture medium—referred to here as the free virus fraction (fraction V). The cell nuclei fraction was resuspended in Buffer G2 and digested with Qiagen protease (25 ul/ml) as per the kit instructions. Both the cell cytosols and the free virus fraction were incubated with Qiagen protease (25 ul/ml) and RNAse A (20ug/ml). All incubations were for 1 hour at 50°C. After incubation, the cell cytosol and free virus fractions were adjusted to a final concentration of 750 mM NaCl, vortexed briefly and loaded onto separate Genomic-tip columns (Qiagen) provided with the kit. The cell nuclei fraction was simply vortexed and added to a third Genomic-tip column. The protocols for washing columns, eluting DNA, and further processing of the DNA pellet were carried out according to kit instructions. DNA from each sample fraction was concentrated to a final volume of 20 ul in TE buffer prior to electrophoresis (section 3.2.5 below).

3.2.5 Separation and Isolation of Viral DNA for Repair Experiments

DNA samples extracted as described in the preceding section (3.2.4) contain both viral and cellular DNA. In order to separate the viral DNA—which is smaller than the cellular DNA—all DNA samples were electrophoresed in agarose gels, and viral DNA was recovered from the gels: gels (0.4% low
melting agarose in 1 X TAE buffer containing 0.7 ug/ml ethidium bromide) were run at 20 volts for approximately 20 hours. While viewing under a UV lamp, bands thought to contain primarily viral DNA were cut out of the gel. Agarose digestion and DNA precipitation were carried out using beta-agarase (New England Biolabs, Ipswich, MA) according to manufacturer’s instructions with minor alterations. Detailed protocols are included in Appendix 3.

3.2.6 QPCR Assay for DNA Damage

Preliminary work for establishing the long QPCR method was done in three parts: selection of primer pairs, optimization of PCR reaction conditions, and cycle tests. 1) Selection of primers: Previously published studies in which adenoviral DNA was amplified via PCR used primer sets which resulted in amplicons of approximately 200-500 bp; since "long PCR" for detection of DNA damage requires longer amplicons for optimal sensitivity, primers were designed prior to the PCR experiments. Table 3 shows sequences, product sizes, and location in the adenoviral genome for all six of these primer pairs, including those used for this study. Primer sets 2 and 3 were used to determine DNA damage in this work; as the table indicates, they resulted in PCR products of approximately 1000 bp. Though this product size is smaller than those typically used for long PCR detection of DNA damage, the results reported below show that this product size was suitable for detection of DNA damage levels caused by the UV doses used in these experiments. Use of two different primer sets amplifying two different parts of the adenoviral genome enabled determination of whether DNA damage induction differs in different parts of the viral genome. Early optimization
tests using each of the primer sets listed in Table 3 on untreated adenoviral DNA demonstrated the selective amplification of a single PCR band of the expected size, as detected on ethidium bromide-stained agarose gels (data not shown). While only sets 2 and 3 were used for detection of DNA damage in the work described here, all primer sets in the table worked well for the PCR conditions used.

Table 3: Adenovirus type 2 primer sets

<table>
<thead>
<tr>
<th>#</th>
<th>left sequence</th>
<th>right sequence</th>
<th>genome region</th>
<th>prod. (bp)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>CCCGTTTTTCGCTCGTACATCC</td>
<td>CGCCCGACTTTGTCCTCGGTTTTG</td>
<td>23162-24352</td>
<td>1190</td>
</tr>
<tr>
<td>2</td>
<td>CAGGAATCGCCCCCATCATCGTC</td>
<td>CGCCCGACTTTGTCCTCGGTTTTG</td>
<td>23336-24352</td>
<td>1016</td>
</tr>
<tr>
<td>3</td>
<td>CGGTTTCCTGTCGAGCAAAAGG</td>
<td>CCCGCACCTGTTTTGTCCTAG</td>
<td>33045-34104</td>
<td>1059</td>
</tr>
<tr>
<td>4</td>
<td>CGGCCGTGAAACCCCGAGATTATT</td>
<td>TGGCCTGCGGAAGCCTTTCTTT</td>
<td>13428-13530</td>
<td>102</td>
</tr>
<tr>
<td>5</td>
<td>GACAGCGTGTTTTCCCCGGAAC</td>
<td>TGGCCTGCGGAAGCCTTTCTTT</td>
<td>13556-13651</td>
<td>95</td>
</tr>
<tr>
<td>6</td>
<td>CGGCCGTGAAACCCGAGATTATT</td>
<td>TGGCCTGCGGAAGCCTTTCTTT</td>
<td>13428-13651</td>
<td>223</td>
</tr>
</tbody>
</table>

2) Optimization of PCR Reaction Conditions: This included optimization of both buffer components and thermal parameters, and it was carried out according to the guidelines set forth previously (Ayala-Torres et al., 2000; Santos et al., 2006; van Houten et al., 2000). Briefly, PCR buffer and components were used as supplied in the GeneAmp XL PCR kit (PE Applied Biosystems, Norwalk, Conn.). The GeneAmp XL kit contains an enzyme mix which is optimized for long PCR; Taq DNA polymerase which is often used in standard PCR applications has been shown to be unsuitable for long PCR (Barnes, 1994). Bovine serum albumin (BSA) was added to help stabilize the reaction components and products (van
3) Cycle Tests: Cycle tests were performed to determine the range of cycle numbers in which amplification was exponential; this was necessary because quantitation of DNA amplified by PCR is only valid during the exponential phase of the reaction. A semilog plot of DNA concentration as a function of cycle number was used to determine the range of cycle numbers in which the amplification was exponential (van Houten et al., 2000). According to the cycle test data in Figure 3 for primer set #2, 18-19 cycles were deemed appropriate. Similar data (not shown) were obtained for primer set #3, and 18-19 cycles were used for all PCR work described here.

**Method** Quality of the genomic DNA used as a template in long PCR applications is an essential aspect of obtaining good results; for the work described here, DNA was extracted from 200 ul of irradiated adenovirus using the QiaAmp DNA Blood Mini Kit (Qiagen, Valencia, CA) according to manufacturer's instructions. Concentration of this viral template DNA (in ng/ul) was determined using PicoGreen from Molecular Probes (Invitrogen, Carlsbad, CA) in a 96-well microplate according to manufacturer's instructions. All PCR reactions were set up using 0.5 ng of
adenoviral template DNA in a total of 10 ul Buffer AE as supplied with the DNA extraction kit (Qiagen, Valencia, CA).

PCR was performed using the GeneAmp XL PCR Kit (Applied Biosystems, Foster City, CA); final concentrations in 50 ul total reaction volume were: 1X PCR buffer, 800uM dNTPs (200 uM of each dNTP), 1.2 mM MgO(Ac)$_2$, and 1 unit $rTth$ DNA polymerase, all supplied with the kit; BSA was added to a final concentration of 0.1 mg/ml, left and right primers to a final concentration of 0.40 uM each, and each reaction had 0.5 ng of template DNA as stated above. PCR reactions were run in a Biometra thermocycler (Biometra, Goettingen, Germany). Thermal conditions for the PCR were: 72°C 2 minutes, 94°C 1 minute, 94°C 15 seconds, 65°C 3 minutes, 72°C 5 minutes, 8°C hold. Enzyme was added to each reaction after 1.5 minutes at 72°C. Conditions in italics represent one cycle; 18-19 cycles were performed for all PCR reactions as described above and 50% controls, containing half as much template as experimental samples, were used to confirm quantitative conditions (Santos et al., 2006; van Houten et al., 2000). PCR products were quantified using PicoGreen in the same manner as the template DNA. Amplification of UV irradiated samples relative to an untreated (undamaged) control—relative amplification—was determined using a simple ratio of the quantity of DNA in the treated sample to the quantity of DNA in the untreated control (Santos et al., 2006). UV-induced lesions per kb of viral DNA (lesions/kb) were calculated using the negative log (-ln) of the relative amplification according to Ayala-Torres et al. (2000).
Statistical Analysis

Data for primer set #2 were analyzed by 2-factor Analysis of Variance (ANOVA) as described in Chapter 2.

3.3 Results

3.3.1 Direct Assessment of DNA Damage Using Primer Set #2

![Figure 4: Lesions/kb viral DNA as calculated from PCR results for primer set #2](image)

Figure 4 shows lesions/kb of adenoviral DNA as a function of UV dose for both low pressure and medium pressure UV—these data were obtained using primer set #2. Lesions induced per kb of viral DNA were calculated as \([-\ln(\text{relative amplification}) / \text{amplicon size}]\) (Ayala-Torres et al, 2000). Two PCR replicates were run for each of the three independent experiments; lesions/kb were calculated for each PCR replicate, and these two replicates were averaged to determine lesions/kb for each of the three independent experiments. The mean and standard error of these three averages for each UV dose is shown in Figure 4. The figure shows an increase in lesions/kb with increasing UV dose. Figure 5 shows an image of the bands obtained when representative PCR products are run on a gel; as UV dose increases, the bands get narrower and more faint because there is greater DNA damage and fewer PCR products.
Again, the results are very similar for both LP and MP UV. Analysis of variance on the lesion data indicates that the main effect for UV dose is highly significant ($p<0.0001$), while the main effect for lamp is barely significant ($p=0.045$). The ANOVA interaction term for dose*lamp was not significant ($p=0.24$), so pairwise post hoc comparisons for each dose were not conducted.

UV dose (mJ/cm$^2$):

<table>
<thead>
<tr>
<th>0</th>
<th>10</th>
<th>25</th>
<th>50</th>
<th>125</th>
<th>blank</th>
</tr>
</thead>
</table>

LP

| 0 | 10 | 25 | 50 | 125 | blank |

MP

Figure 5: Agarose gel images of PCR products from irradiated adenovirus

3.3.2 Comparison of DNA Damage Assessment Using Primer Sets #2 and #3

As stated above, PCR was done using two different primer sets. Results for primer set #2 are discussed above; these data for primer set #2 are shown in Figure 6 with data from primer set #3 for purposes of comparison.

Figure 6: Comparison of lesions/kb viral DNA as calculated from primer set #2 and primer set #3
While DNA damage induction estimates by both types of UV lamp may be slightly higher using primer set 3 at the highest UV doses, overall there does not appear to be a significant difference in lesion estimates obtained using the two different primer sets.

3.3.3 Assessment of DNA Repair in UV Treated Adenoviruses

Results obtained for the repair experiments are shown in Figures 7-9. Figures 7 and 8 show agarose gel images of DNA extracted from cells infected with UV treated and untreated adenovirus 2 days (Figure 7) and 4 days (Figure 8) after infection. The figures suggest that at these timepoints, a majority of the DNA seen in the nuclear fraction of infected cells is viral DNA, as very little DNA is seen in samples from uninfected cells. The ladder shown in these gels contains molecular weight size standards ranging from approximately 1,500 bp to 48,000 bp.
Figure 7: Agarose gel image of total DNA isolated from A549 cells—2 days. Cells were uninfected or infected with UV treated and untreated adenovirus and collected 2 days after infection. N: nuclear fraction, C: cytosolic fraction, V: free viral fraction, L: ladder

Figure 8: Agarose gel image of total DNA isolated from A549 cells—4 days. Cells were uninfected or infected with untreated adenovirus. N: nuclear fraction, C: cytosolic fraction, V: free viral fraction, L: ladder
Numerous bands corresponding to different molecular weights were cut from the large smear of DNA seen in the nuclear fraction of infected cells in Figure 8. DNA from these bands as well as bands from the uninfected cells was isolated from the gel using beta-agarase digestion. PCR of a short (102 bp) amplicon using primer set #4 (Table 3) was performed to confirm the presence of viral DNA. DNA isolated from the gels worked well in PCR reactions; Figure 9 shows an agarose gel image of the 102 bp PCR products; all of the DNA isolated from infected cells contains viral DNA, while DNA isolated from uninfected cells did not result in amplification. These results further support the conclusion that a substantial portion of the DNA isolated from infected cells several days after infection is viral.

![Figure 9: Agarose gel image of PCR products for identification of viral DNA. PCR amplification was done on DNA isolated from gel in Figure 8. lane 1: ladder, lane 2: empty, lanes 3-8: DNA isolated from smear of infected nuclear fractions; lanes 9 and 10: DNA isolated from uninfected cells.](image)
3.4 Discussion

3.4.1 Direct Assessment of DNA Damage in UV Treated Adenovirus

PCR technologies have been applied in several studies of adenovirus; however, these studies have involved either combinations of PCR and cell culture for tests of viral infectivity after UV treatment or simple detection of adenoviral DNA in untreated environmental samples (Choi and Chiang, 2005; Jothikumar et al., 2005; Ko et al., 2003; Ko et al., 2005a; Ko et al., 2005b). Methods that combine PCR with cell culture techniques are more sensitive than previous methods but still involve introduction of a second biological system (the cultured cells) which can obfuscate interpretation of results, and they are more time consuming than direct molecular methods might be. The PCR assay described here was used directly on irradiated adenoviruses that were exposed to UV but were not introduced into cell culture. Figure 4 shows lesions/kb of DNA from irradiated adenoviruses as calculated using data from primer set #2. It shows that LP and MP UV are equally effective at damaging the viral DNA at these doses. The data shown suggest that LP UV may even be slightly more efficient than MP UV at causing DNA damage; this may be because nearly all of the emission from a low pressure lamp targets DNA, while MP UV has emission at other wavelengths which are not absorbed as efficiently by DNA.

As indicated in Table 3, primer set #2 spans the adenoviral genome from base pairs 23336 to 24352. In addition to the data obtained from primer set 2, data were also obtained using another primer set: primer set 3 spans the adenoviral genome from base pairs 33045 to 34104. Since the Ad2 genome is
linear and approximately 35 kb in length, primer set 3 gives estimates of DNA
damage at one end of the genome, while primer set 2 gives DNA damage
estimates based on a more internal location. Overall there does not appear to be
a significant difference between DNA damage estimates obtained from the two
primer sets. These results indicate that UV-induced DNA damage likely does not
vary significantly from one location in the genome to another at the UV doses
used in these experiments.

The lesion data for this study are in agreement with lesion data for previous
studies: Figure 10 shows the number of lesions (per 10 kb) introduced into adenoviral DNA in this study (using primer set 2) as compared to lesions induced in cultured cells, *E. coli*, and naked DNA in other studies, using both LP and MP UV, as a function of UV dose (in J/m²). In general, the relationship between lesions and UV dose remains linear across organisms and UV lamp type, even when different methods are used to measure the DNA damage: Eischeid and Linden (2007) used an enzyme-sensitive site
(ESS) assay, while the other studies used a PCR assay like the one described here (van Houten et al., 2000).

3.4.2 Assessment of DNA Repair in UV Treated Adenovirus

Plans for the repair experiments as described here were based on extraction of total (cellular and viral) DNA from infected cells, followed by separation of (smaller) viral and (larger) cellular DNA on agarose gels and isolation of the viral DNA via excision of 35 kb (viral) DNA bands from the gel. The results shown here indicate that the viral DNA from infected cells does not appear as a distinct band, so that its separation from cellular DNA is not readily achieved using electrophoresis. These results do indicate, however, that the majority of DNA isolated from infected cells after 2-4 days is viral, as shown in Figures 7 and 8, and PCR identification results in Figure 9 confirm this. Taken together, these experiments indicate that gel electrophoresis may be neither effective nor necessary for isolation of viral DNA. Since most DNA from infected cells is viral, some method in which PCR is carried out directly on DNA extracted from infected cells may be more suitable. In this case, cycle tests on each individual sample would likely be required, and measures must be taken to determine relative quantities of viral DNA in each sample.
4. ASSESSMENT OF PROTEIN DAMAGE

This chapter describes assessment of damage to adenoviral proteins using SDS-PAGE.

4.1 Introduction

Adenoviruses are icosahedral particles which contain approximately 12 different types of proteins. Most of the protein mass is in the capsid, the vast majority of which is hexon protein; at each vertex of the icosahedron is a penton base or penton complex from which a fiber protein protrudes (Rux and Burnett, 1999). The hexon and fiber exist as trimers in the mature viral particle, while the penton base is a pentamer; each is composed of identical subunits (Phillipson, 1983; Rux and Burnett, 1999). Hexon is the dominant capsid protein and there are 240 copies of hexon trimer per virion. Each adenovirus particle has 12 penton bases and 12 molecules of fiber protein extending outward from its surface; the fiber protein is primarily responsible for the attachment of viral particles to their host cells (Seth, 1999b). Table 4 lists the 12 proteins present in a mature adenoviral particle with some important features. The roman numerals given to some adenoviral proteins correspond to the order in which they appear in an SDS-PAGE gel from top to bottom, with higher numbers corresponding to lower molecular weights. Table 4 lists name, number, location in a mature viral particle, function, molecular weight in kilodaltons, subunit structure, and numbers of cysteine (cys) and tryptophan (trp) residues for each protein. Cysteine and tryptophan residues can play important roles in UV damage to proteins, as discussed below and in section 4.4.
Table 4: Proteins found in mature adenoviral particles

<table>
<thead>
<tr>
<th>Name</th>
<th>#</th>
<th>Location</th>
<th>Function</th>
<th>mol. wt. (kD)</th>
<th>Monomer copy #</th>
<th>Subunits</th>
<th>cys</th>
<th>trp</th>
</tr>
</thead>
<tbody>
<tr>
<td>hexon</td>
<td>II</td>
<td>major capsid</td>
<td>major structural</td>
<td>108</td>
<td>720</td>
<td>trimer</td>
<td>7</td>
<td>13</td>
</tr>
<tr>
<td>penton</td>
<td>III</td>
<td>major capsid</td>
<td>structural, anchor fiber</td>
<td>85</td>
<td>60</td>
<td>pentamer</td>
<td>3</td>
<td>5</td>
</tr>
<tr>
<td>fiber</td>
<td>IV</td>
<td>major capsid</td>
<td>attachment to host cell receptor</td>
<td>62</td>
<td>36</td>
<td>trimer</td>
<td>3</td>
<td>4</td>
</tr>
<tr>
<td>IIIa</td>
<td>IIIa</td>
<td>minor capsid</td>
<td>structural?, maturation</td>
<td>63.5</td>
<td>60</td>
<td>monomer</td>
<td>0</td>
<td>6</td>
</tr>
<tr>
<td>VI</td>
<td>VI</td>
<td>minor capsid</td>
<td>structural?, connect to core, protease cofactor</td>
<td>22</td>
<td>360</td>
<td>hexamer</td>
<td>0</td>
<td>5</td>
</tr>
<tr>
<td>VIII</td>
<td>VIII</td>
<td>minor capsid</td>
<td>structural?</td>
<td>12.1</td>
<td>120</td>
<td>monomer</td>
<td>0</td>
<td>2</td>
</tr>
<tr>
<td>IX</td>
<td>IX</td>
<td>minor capsid</td>
<td>“capsid cement”, stabilize GON hexons</td>
<td>14.4</td>
<td>240</td>
<td>trimer</td>
<td>0</td>
<td>1</td>
</tr>
<tr>
<td>major core</td>
<td>VII</td>
<td>core</td>
<td>forms “nucleosome” with DNA</td>
<td>18.5</td>
<td>1100</td>
<td>monomer/tetramer</td>
<td>0</td>
<td>3</td>
</tr>
<tr>
<td>minor core</td>
<td>V</td>
<td>core</td>
<td>forms shell around “nucleosome”</td>
<td>41.6</td>
<td>157</td>
<td>monomer</td>
<td>0</td>
<td>2</td>
</tr>
<tr>
<td>mu</td>
<td>X?</td>
<td>core</td>
<td>aid nuclear localization</td>
<td>4</td>
<td>104</td>
<td>?</td>
<td>?</td>
<td>?</td>
</tr>
<tr>
<td>protease</td>
<td></td>
<td>core</td>
<td>cleaves precursors: pIIIa, pVI, pVIII, pVII, pTP</td>
<td>23</td>
<td>10-40</td>
<td>?</td>
<td>8</td>
<td>3</td>
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<tr>
<td>terminal protein</td>
<td></td>
<td>core</td>
<td>DNA replication</td>
<td>55</td>
<td>2</td>
<td>?</td>
<td>6</td>
<td>8</td>
</tr>
</tbody>
</table>

In addition to the major capsid proteins discussed above, there are minor capsid proteins whose structures and locations in the mature virion are less well-understood. These include protein IIIa, protein VI, protein VIII and protein IX (Vellinga et al., 2005). Protein IIIa is likely on the inner capsid surface, associated with the penton bases, though part of it may also extend to the outer surface of the capsid. Protein IIIa also interacts with the major core protein and is important to proper maturation of viral particles, as empty capsids are formed without it. Protein VI is thought to be just inside the capsid surface associated with hexon and has a copy number of 370 per viral particle; it interacts with the minor core protein and binds DNA to connect the capsid to the core (Rux and
Burnett, 1999; Vellinga et al., 2005). Protein VIII is localized to the inner capsid surface near the hexons, as is protein IX. Protein IX helps stabilize the group-of-nine hexons in the center of each face of the viral surface, and therefore serves as “capsid cement.” In the viral core, the DNA is associated with the major and minor core proteins. The major core protein is by far dominant and is present in over 1100 copies per virion, while there are thought to be approximately 180 copies of the minor core protein (Phillipson, 1983; Rux and Burnett, 1999). Both of the core proteins are rich in positively-charged amino acids which facilitate their association with the negatively-charged phosphate backbone of the viral DNA. The viral core also contains two copies of a terminal protein bound to each end of the DNA, several copies of a viral protease, and a protein called mu. The viral protease cleaves several viral proteins from precursor to final form during the maturation of viral particles. As discussed in Chapter 1, viral proteins are an integral part of every step in the process of infection, and enable adenoviruses to successfully infect host cells even if their DNA is damaged (Seth, 1999b). Thus optimal disinfection of adenovirus requires not only damage to the viral genome, but also interference with its ability to infect host cells in the first place—damage to the viral proteins.

UV-induced damage to proteins can take several forms: these include oxidation of amino acids, crosslinking of the protein with itself or with DNA, breakage or formation of disulfide bridges, or breakdown of the polypeptide chain. Alterations in stereochemistry have also been reported (Fujii et al., 2004). Of the 20 amino acids which usually make up proteins, there are 5 aromatic
amino acids which have absorbance maxima at or very near 280 nm and are the primary UV absorbers in a protein. They are tryptophan, tyrosine, phenylalanine, cysteine, and cystine. While MP UV has emissions at and around 280 nm and is most easily absorbed, low pressure UV can also affect these amino acids, most notably tryptophan, cysteine, and cystine. Tryptophan is by far the best absorber and is oxidized by UV to N-formylkynurenine (NFK) (Borkman, 1977; Walrant and Santus, 1974). Breakage of disulfide bonds—which converts cystine amino acid residues to cysteine—also has a high quantum yield at the 254 nm wavelength emitted by LP UV lamps (Jagger 1967). The effect that different types of damage have on protein function varies. For example, because of their crucial importance to the tertiary structure of proteins, breakage of disulfide bonds can have a much more significant effect on proteins than alteration of the primary or secondary structures, such as changes in individual amino acids.

Specific investigations of the major types of UV-induced protein damage come from both in and outside of the water treatment community. One of the most pronounced effects of protein damage may be destruction of enzyme activity. In terms of protein damage to pathogens after UV disinfection, activity of the enzyme photolyase is by far the most-studied effect. Several authors have found that activity of this enzyme is decreased by MP UV but not by LP UV (Oguma et al., 2002; Zimmer and Slawson, 2002). Wang et al. (2004) report that 90% activity of a spiked protein is recovered after LP UV doses sufficient to cause 4 log inactivation of adenovirus. For proteins in aqueous solution, both oxidation of tryptophan to NFK and breakdown of the polypeptide chain appear to
require an LP UV dose beyond the range relevant to disinfection (Fujii et al., 2004; Kato et al., 1992). While the studies above suggest that LP UV is not likely to cause significant levels of enzyme inactivation, oxidation, or peptide bond breakage at disinfection-relevant doses, crosslinks may be an exception: several authors have studied formation of nucleic acid-protein crosslinks induced by LP UV in *in vitro* systems (Takahashi et al., 2005; Woppmann et al., 1988) and in human cells (Lai and Rosenstein, 1990). Interaction or binding of the nucleic acid and protein *in vivo* is a major factor in the formation of both DNA-protein and RNA-protein crosslinks on irradiation, and this has been shown to occur at disinfection –relevant UV doses below 20 mJ/cm². Using 302 nm UV, Sionkowska (2005) showed that at lower UV doses, intramolecular (protein-protein) crosslinks lead to increased stabilization of proteins, while at higher UV doses, chain breakdown predominates and proteins are destabilized. DNA-protein crosslinks predominate at the 254 nm wavelength emitted by LP UV lamps, while protein-protein crosslinks are dominant at 280 nm –emitted by MP UV. Both conformation and proximity are important in UV induction of crosslinks (Martinson et al., 1976).

The purpose of the experiments described here was to examine the major adenoviral proteins after LP and MP UV irradiation in order to determine whether or not MP UV is more effective at causing protein damage; this was done using SDS-PAGE. The structural stability of capsid proteins from several viruses has been investigated using gel electrophoresis. SDS-PAGE has been used to examine the dissociation of the adenovirus particle upon entry into host cells.
(Greber et al., 1993), the role of disulfide bonds in protein oligomerization of JC polyomavirus (Chen et al., 2001) and avian reovirus (Grande et al., 2002), and how capsid proteins are affected by temperature, pH, chemicals, ionic strength and composition, and crosslinking agents (Grande et al., 2002; Rexroad et al., 2003). Silver stain and Coomassie Brilliant Blue are commonly used to stain proteins in gels, and silver stain has been used to stain adenoviral proteins after SDS-PAGE (Rexroad et al., 2003). In the work described here, SDS-PAGE was followed by staining with a newer protein stain, SYPRO Ruby, to examine adenoviral proteins after UV treatment. SYPRO Ruby has greater sensitivity and specificity for proteins in one-dimensional SDS-PAGE than both silver stain and Coomassie Blue; the staining method is faster and simpler, the dye can be excited by several light sources, and results are linear over three orders of magnitude (Berggren et al., 2000). SDS-PAGE and gel staining can provide information about differences in levels of all the major coat and core proteins as well as their degradation into polypeptides of lower molecular weights, and it allows identification of specific proteins based on their molecular weights (Rux and Burnett, 1999; Rexroad et al., 2003).

4.2 Materials and Methods

4.2.1 UV Irradiation and Experimental Design

UV irradiation and dose calculation were carried out as described in Chapter 2. The experiments to examine protein damage used UV doses of 0, 25, 50, 186, 300, and 600 mJ/cm². Three independent UV irradiation
experiments were conducted for each UV dose; protein precipitation and SDS-PAGE were done twice for each independent experiment.

4.2.2 Culture of Stock Cells and Preparation of Virus

Culture of A549 cells and viral preparation were carried out as described in Chapter 2.

4.2.3 Protein Precipitation

For protein precipitation, 1 ml of irradiated virus was treated with 0.05% sodium deoxycholate for 5-10 minutes at room temperature and then precipitated with 10% trichloroacetic acid (TCA) for 2 hours on ice (Jiang et al., 2004; Rexroad et al., 2003). Precipitated proteins were pelleted at 20,000g for 20 minutes at 4°C, washed with 1 ml ice-cold acetone (Jiang et al., 2004), and spun again using the same conditions. Acetone was carefully removed with a pipette to avoid disturbing the protein pellets; pellets were allowed to air dry at room temperature for 20 minutes and then resuspended directly in Laemmli sample buffer (Bio-Rad, Hercules, CA) with 5% beta-mercaptoethanol for SDS-PAGE. Prior to precipitation, each irradiated virus sample was spiked with 1 ug aprotinin as an internal standard; all data were normalized to the aprotinin bands on SDS-PAGE gels. Sodium deoxycholate was used to increase protein recovery and ensure more efficient precipitation of smaller proteins (Bensadoun and Weinstein, 1976), while TCA precipitation ensured prompt inactivation of any proteases which might be present in the sample (Granier, 1988; Wu and Wang, 1984).
4.2.4 SDS-PAGE and Image Analysis

SDS-PAGE For SDS-PAGE, protein samples and molecular weight standards in Laemmlie buffer with 5% beta-mercaptoethanol were boiled for 10 minutes prior to being loaded onto 4-20% gradient Tris-HCl ReadyGel minigels (Bio-Rad, Hercules, CA). Gels were run at 200 volts for 45 minutes, fixed for 30 minutes in 10% methanol / 7% acetic acid, and stained overnight using 50 ml SYPRO Ruby protein gel stain (Bio-Rad, Hercules, CA) according to manufacturer's instructions. Gels were destained, also in 10% methanol / 7% acetic acid, for 30-45 minutes prior to imaging and data analysis. All materials used for SDS-PAGE were obtained from Bio-Rad (Hercules, CA), including those cited above as well as gel running assemblies, Tris-glycine-SDS running buffer, and broad-range SDS-PAGE molecular weight standards.

Imaging and data analysis Gels stained with SYRPO Ruby (Berggren et al., 2000) were imaged using a GelDoc (Bio-Rad, Hercules, CA). Bands in each image were analyzed for both molecular weight and protein quantity using QuantityOne software (Bio-Rad, Hercules, CA). Adenoviral proteins were identified based on molecular weight (Phillipson, 1983; Rux and Burnett, 1999) and quantity of protein in each sample was determined relative to an untreated control.

4.3 Results

Results are shown in Figures 11-14. Figure 11 shows an image of a representative gel for UV doses up to 186 mJ/cm². Seven bands with molecular weights of known adenoviral proteins were consistently identified—three of these
are the major capsid proteins—hexon, penton, and fiber (Figure 12). Two are minor capsid proteins—protein IIIa and protein VI (Figure 13), and two are the core proteins—the major core and minor core (Figure 14). The figures show LP and MP UV dose-response curves for each protein identified. An unidentified band (U1) noted in Figure 11 may represent the terminal protein of the virus, but its identity is uncertain and data for this band are not included in the analysis presented here.

Figure 11: SDS-PAGE gel image. Lanes are labeled with lamp type and UV dose (in mJ/cm²). The seven bands identified and the corresponding adenoviral protein are noted in the first sample lane (left side of gel). The sizes of molecular weight standards are noted in the standard lane on the far right.
Figure 12: SDS-PAGE data for major coat proteins from irradiated adenoviruses

Figure 13: SDS-PAGE data for minor coat proteins from irradiated adenoviruses
Figure 14: SDS-PAGE data for core proteins from irradiated adenovirus

The data in Figures 12, 13, and 14 show that MP UV causes more protein damage than LP UV at UV doses above 186 mJ/cm², while there appears to be relatively little difference at lower doses. The major coat proteins—hexon, penton, and fiber—are particularly vulnerable to MP UV damage at high doses, while LP UV causes substantially less damage at higher UV doses. At the highest UV dose used, MP UV decreased levels of the major capsid proteins by 80-90%, minor capsid proteins by 70-80%, and major core by approximately 50%. The minor core protein appears to be as susceptible to MP UV as capsid proteins. The highest dose of LP UV caused decreases of 40-60% in major capsid proteins, 40-50% in minor capsid proteins, and 10% and 40%, respectively, for the major and minor core proteins. There are steep declines in the levels of the major capsid proteins and—to a slightly lesser extent—the minor capsid proteins—for MP UV between 186 and 300 mJ/cm²; no such pronounced effect is seen for the core proteins.
4.4 Discussion

In the work described here, SDS-PAGE was used to investigate protein damage in adenovirus after irradiation with LP and MP UV. The data shown here indicate that, as expected, MP UV is more effective at damaging adenoviral proteins than LP UV; this is true for all seven viral proteins studied, primarily at UV doses greater than 186 mJ/cm$^2$. In general, the major coat proteins are the most susceptible to UV damage, followed by the minor coat proteins; the major core protein is least susceptible to damage by MP UV and almost entirely unaffected by LP UV. These relative levels of UV susceptibility are consistent with a physical shielding of internal proteins by the surface proteins: the major coat proteins are on the outer surface of the virus and are the most vulnerable to UV, the minor capsid proteins IIIa and VI are both primarily thought to be located on the internal surface of the capsid (Saban et al., 2006), giving them an intermediate level of protection. While the major core protein—located inside the virus—is physically protected by all the capsid proteins, the minor core is susceptible to UV. This is consistent with proposed models of the structure of the viral core, in which the DNA is wound around the major core protein in a tightly ordered structure and this core “nucleosome” is covered by a shell comprised of the minor core protein (Nermut, 1979; Sato and Hosokawa, 1981). Thus the minor core protein is nearly as susceptible to UV as the capsid because it is relatively exposed, while the major core is highly protected—especially from LP UV—not only by the other viral proteins but also to some extent by the viral DNA.
For medium pressure UV, the relative quantities of hexon, penton, and fiber at 300 mJ/cm\(^2\) are significantly less than they are at 186 mJ/cm\(^2\); there is a sharp drop in protein levels between these two doses, despite the fact that doses up to 186 mJ/cm\(^2\) have relatively little effect. Thus the kinetics of capsid protein damage appear to change between these two doses for viruses irradiated with MP UV. This may occur as a result of structural changes in the virus that occur with protein breakdown. The proteins of the adenoviral coat are part of a tightly integrated whole. It has been shown that damage to a relatively small fraction of these disrupts the capsid architecture, and that when a critical level of damage has been reached, the virus undergoes a distinct structural transition (Rexroad et al., 2003). It is possible that MP UV-induced damage to proteins causes such a structural transition, increasing exposure of the proteins to UV and allowing them to be more readily damaged. This effect is likely not seen for the major or minor core proteins because structural changes in the viral capsid have less effect on the viral core; capsid proteins may protect the viral core from UV even after a structural transition. Like the major coat proteins, the minor coat proteins IIIa and VI also appear to undergo a distinct decline between 186 and 300 mJ/cm\(^2\). For LP UV, there is a much less pronounced decline in levels of hexon, penton and protein VI between 186 and 300 mJ/cm\(^2\), and there is no such sharp decline for the fiber, protein IIIa, or the core proteins. While LP UV does cause some protein damage, that damage may not reach the critical level necessary to cause a significant structural transition in the virus.
It was expected that MP UV would be efficient at causing damage to proteins, however, LP UV was more effective at damaging the viral proteins than anticipated. As discussed above, LP UV can be effective at causing DNA-protein crosslinks even at doses lower than those used here (Lai and Rosenstein, 1990); this is primarily through absorption of UV by the DNA (Martinson et al., 1976). Since crosslinking with DNA would interfere with the mobility of proteins in SDS-PAGE gels—and might even interfere with their precipitation prior to SDS-PAGE—the formation of these crosslinks is a plausible explanation for declines in protein levels seen in the LP UV treated samples. Furthermore, while LP UV is not absorbed well by proteins in a general sense, some of the individual amino acids that make up proteins are susceptible to LP UV damage. As mentioned above, tryptophan is a very efficient absorber of UV; even upon LP UV irradiation, tryptophan is oxidized effectively to N-formylkyurenine (NFK). While required UV doses may be high, the action spectrum of tryptophan oxidation is of particular interest. Borkman (1977) found that while UV absorbance of tryptophan is maximal at 280 nm (emitted by MP UV), both the rate and quantum yield for its oxidation are higher at 254 nm (LP UV). In this case, LP UV appears to have an advantage over MP UV in causing protein damage. The advantage in causing further damage via activation of the oxidation product NFK, however, lies with MP UV: upon activation by UVA (wavelengths above 320 nm), NFK becomes a photosensitizer which reacts with other amino acids in proteins to cause further oxidation. This has been shown to occur in free amino acids as well as in whole protein; activated NFK can also affect nucleotides and may play
a role in DNA-protein crosslinks (Walrant and Santus, 1974). Analysis of the genetic sequence for adenovirus type 2 indicates that all of the proteins analyzed here do contain tryptophan—while the core proteins contain only 2-3 tryptophan residues each, the hexon protein contains up to 13, and the other capsid proteins each contain 4-6 tryptophan residues prior to any cleavages by the Ad protease. As mentioned above, MP UV also has greater potential to affect proteins via direct absorption at 280 nm, which leads to the formation of protein-protein crosslinks. Finally, while UV is capable of affecting disulfide bonds which form between cysteine residues, such effects would not be seen here because all disulfide bonds are broken when proteins are denatured prior to SDS-PAGE. The major adenoviral capsid proteins do contain cysteine residues, but no disulfide linkages (Phillipson, 1983).

The UV doses used here are higher than those typically used in disinfection. Current USEPA regulations for UV disinfection of viruses in surface water require 186 mJ/cm\(^2\) (USEPA, 2006a) while much lower doses are used for other pathogens. At these lower doses our data do not indicate that LP and MP UV differ significantly in their abilities to cause protein damage. This work used SDS-PAGE, which was chosen in part because it has the advantage of allowing examination of all the viral proteins simultaneously. In SDS-PAGE, however, the proteins are completely denatured prior to and during electrophoresis; the type of damage detected here is therefore breakdown of the polypeptide chain. This work with SDS-PAGE clearly shows that MP UV is more effective than LP UV at breaking down protein chains at high doses, and it suggests that MP UV may
also be better at causing more subtle forms of protein damage at lower doses.

According to some reports, breakdown of higher-order structures in the viral coat proteins can also be studied using SDS-PAGE. The hexon, penton, and fiber all exist as multimeric forms in the mature viral particle, and some authors report that these multimeric forms are left intact when SDS-PAGE is carried out without boiling samples prior to electrophoresis (Fortsas et al., 1994; Hong and Engler, 1996). Brief tests conducted as part of this work indicate that the multimers are not intact even in unboiled samples (data not shown). In addition, UV treated and untreated adenoviruses were incubated with Ellman’s reagent (Boyne and Ellman, 1972; Voss et al., 2007) to detect changes in sulfhydryl oxidation state, but it appears there is too little protein material in the viral samples for adequate detection using this reagent (data not shown). Other authors using Ellman’s reagent likely had samples with 10 times as much protein as the viral samples used here (Voss et al., 2007).

A few bands that do not correspond directly to major known adenoviral proteins were also identified. Other researchers conducting SDS-PAGE on adenovirus have had similar results (Everitt et al., 1973; Rexroad et al., 2003; van Oostrum and Burnett, 1985). Unknown bands might result from crosslinks or aggregation, from minor breakdown of proteins which occurs during freezing and thawing of the viral stocks, from differences caused by translation at alternative start codons (van Oostrum and Burnett, 1985), or from natural variation in the viral proteins. For example, Everitt et al. (1973) report what appear to be different isoforms of the fiber protein in a single type of adenovirus.
Furthermore, intermediate and incomplete viral particles contain polypeptides not found in mature virions (Rosenwirth et al., 1974), and since their morphology is similar to that of complete adenoviruses, it is likely that some incomplete viral particles are isolated using the viral preparation methods used for this work. Some of the smallest bands in these gels likely represent protein VIII and protein IX of adenovirus, but the intensity of these bands was very light and they were not used for analysis.
5. **ASSESSMENT OF CAPSID INTEGRITY**

   This chapter describes the use of transmission electron microscopy to assess the integrity of the viral capsid and of the viral particles as a whole after UV treatment.

5.1 **Introduction**

   Transmission electron microscopy (TEM) was used to examine the structure of adenovirus particles after UV treatment and as an aid in assessing the effects of UV on the viral capsid integrity. Initial plans for assessment of capsid integrity were based on the use of selectively permeable fluorescent dyes and flow cytometry, described in Appendix 2. This line of experiments did not prove fruitful and electron microscopy was used instead. Surprisingly few studies have used TEM for direct, simple examination of damage to adenovirus particles. Rexroad et al. (2003) used TEM of negatively stained particles to examine the effects of heat on adenovirus; here a very similar method was used to examine the effects of UV on adenovirus structure.

5.2 **Materials and Methods**

   Grids for TEM were 300 mesh copper, coated with formvar/carbon (Electron Microscopy Sciences, Hatfield, PA). Irradiated adenovirus stocks were fixed directly on the grids. Viral samples were applied to grids in 10 ul aliquots, allowed to settle for 60-75 minutes, and excess liquid was blotted off the grid using filter paper. This process was done three times so that a total of 30 ul virus sample was applied to each grid. Fixation was carried out for 15 minutes at room temperature using 5 ul of 4% paraformaldehyde applied directly to each grid.
Fixation solution was blotted off with filter paper and grids were stained using 1% uranyl acetate for 1 minute before viewing on the electron microscope. Microscopy was carried out using standard procedures on an FEI Tecnai G² Twin electron microscope.
5.3 Results

Results are shown in Figures 15 and 16. These include images of untreated, fresh adenovirus stock (Figure 15, left), and adenovirus stock after one cycle of freeze thaw with no UV treatment, (Figure 15, right), LP UV at 300 mJ/cm$^2$ (Figure 16, left), and MP UV at 300 mJ/cm$^2$ (Figure 16, right).

![Figure 15: TEM images of untreated adenovirus. left, fresh stock; right, frozen stock](image1)

![Figure 16: TEM images of UV treated adenovirus: left, 300 mJ/cm$^2$ LP UV; right, 300 mJ/cm$^2$ MP UV](image2)
5.4 Discussion

Figure 15 shows that the freezing and thawing of viral stocks—in the absence of UV treatment—causes some visible damage to the virus. Figure 16 shows that at the high UV dose used here, both LP and MP UV cause significant damage to the virus. The UV treated Ads are misshapen, and the particles are highly enlarged, aggregated, and more darkly stained. The increased staining in UV treated particles may represent staining of the interior of the virus, which is exposed as a result of the capsid damage; this has also been seen in TEM of adenoviral particles treated with heat (Rexroad et al., 2003). Striations seen in the background of Figure 16 (right) likely represent damage to the formvar/carbon coating on the EM grid from exposure to paraformaldehyde during fixation or from handling. There are fewer viruses in the MP UV than in the LP UV treated sample, and this may be because the very high dose of MP UV caused severe breakdown and degradation of the viral particles.

Based on these results, it appears that UV does damage adenovirus structure in a manner that is readily studied using the TEM method described in this chapter. Further investigation of UV treated adenovirus using TEM should include 1) tests of less stringent fixation procedures that may cause less damage to the grid, and 2) use of lower UV doses—for example, 25 mJ/cm² and 50 mJ/cm²—at which the difference between LP and MP UV is expected to be greater.
6. SIGNIFICANCE AND CONCLUSIONS

6.1 Significance

Adenovirus’ apparent resistance to UV disinfection was just discovered in recent years, yet it has already had a significant impact on EPA regulations for UV disinfection of both surface and ground waters. Most emerging pathogens now are viruses, and since they can get through the filter systems used in water treatment plants, disinfection through treatments such as UV irradiation is an important defense against them. Recent work on UV treatment of adenoviruses and its impact on UV disinfection requirements has indicated that a more thorough understanding of UV disinfection of viruses is needed. The work described here is the first major study to thoroughly investigate the response of adenoviruses to polychromatic UV light and to employ significant use of molecular biology to examine pathogen response to UV disinfection.

6.2 Conclusions and Suggestions for Future Work

The cell culture infectivity data presented here show that MP UV is better at inactivating adenovirus than LP UV, while the PCR data indicate that LP and MP UV are equally efficient at damaging the viral DNA. Taken together, these data support the hypothesis that LP UV-induced DNA damage is repaired in cell culture while MP UV causes more irreparable damage to the viral particle. The SDS-PAGE work presented here shows that both LP and MP UV are effective at damaging adenoviral proteins; however, MP UV is much more effective at high UV doses. Generally, the viral coat proteins appear to be more susceptible to UV damage than the internal proteins.
Future research should build on the work begun here investigating repair of adenoviral DNA in cell culture assays and damage to the viral particles using TEM. Investigation of more subtle forms of protein damage is also needed to determine whether there are significant differences between LP and MP UV lamps at lower UV doses. Pulsed UV is another polychromatic UV source, and carrying out the studies described here using pulsed UV would help to further enhance knowledge of monochromatic vs. polychromatic UV disinfection. Finally, investigations of protein damage using specific wavelengths or wavelength ranges from the medium pressure emission spectrum may help clarify which UV wavelengths are most important in causing capsid damage.
APPENDIX 1: IRRADIATION OF ADENOVIRUS FOR ANIMAL INFECTIVITY ASSAYS

This appendix describes work conducted to help with animal infectivity studies carried out in conjunction with the USDA.

Introduction

As discussed in Chapter 1, previous work on UV disinfection of adenovirus has been carried out on LP UV irradiated viruses assayed using cell culture infectivity. Adenoviruses appear to be highly UV resistant based on these studies, and there are two major directions in which further research can go to clarify the effects of UV on adenovirus. One of these is to investigate different types of UV lamp, and the other is to address assay method. The main body of this dissertation describes work using both LP and MP UV with molecular biology assays; this appendix will describe work done using both LP and MP UV as part of a project involving animal infectivity assays.

The objectives of this study were to use LP and MP UV to inactivate adenovirus, then to assay inactivation using both cell culture infectivity and animal infectivity assays so that data for the two types of assay—*in vivo* animal assays and *in vitro* cell culture assays—can be compared. The studies were conducted in treated wastewater effluents so that outcomes might better reflect what would happen in water reuse scenarios. Data obtained so far during UV irradiation of human adenovirus are presented below; data on animal infectivity assays are pending.
Methods

Irradiation of adenoviruses received from the USDA was carried out at Duke; irradiated samples were returned to the USDA for assay. Waters from three utilities (Pinellas County, FL; Orange County, CA; L.A. County, CA) were sterile-filtered and irradiated with high doses (1200 mJ/cm$^2$) of LP UV to break down chlorine compounds; removal was confirmed using the DPD method. Filtered, irradiated waters were used for 100-fold dilution of adenovirus types 2, 4, and 5. Since UV absorbance is the most important water quality parameter affecting UV disinfection, absorbance scans of each water were taken a) after filtration and before high-dose UV treatment (Figure 1), b) after high-dose LP UV and filtration but before addition of virus (Figure 2), and c) after addition of virus. In one experiment, one type of adenovirus (Ad2) was irradiated in all three different reuse waters (Figure 3), and in another experiment all three types of adenovirus (2, 4, and 5) were irradiated in the same water (Figure 4).

![Absorbance of reuse waters after sterile-filtration](image_url)
Figure 2: Absorbance of reuse waters after high-dose LP UV used for destruction of chlorine compounds

Figure 3: Absorbance of reuse waters containing Ad2

Figure 4: Absorbance of LA County reuse water containing Ad2, Ad4, and Ad5
APPENDIX 2: FLOW CYTOMETRY FOR ASSESSMENT OF UV TREATED ADENOVIRUS

The original proposal for this dissertation research included plans to assess viral capsid integrity after UV treatment using a method based on fluorescent dye staining followed by flow cytometry. These plans were not carried out because the dyes did not appear to behave as needed in the viral particles, and because a lack of significant light scatter by viral particles makes the execution of properly controlled flow cytometry experiments extremely difficult. In lieu of this flow cytometry work, damage to the structure of UV treated adenovirus has been examined using electron microscopy (Chapter 5). A review of previous literature on flow cytometry of viruses and original plans for this work are discussed in this appendix.

**Literature Review and Proposal for Flow Cytometry**

Flow cytometry is a widely available method which allows rapid analysis of a large number of cells or particles flowing past a light source based on their inherent scattering of its light or the excitation of fluorophores added in the laboratory. It has been used extensively in the literature to investigate mammalian cells, bacteria, and protozoans, as well as the interactions of infectious viruses or viral vectors with mammalian cells, but has been used much less frequently to study viral particles themselves. Their small size can make detection of viruses by flow cytometry difficult (Brussaard et al., 2000; Shapiro, 1995). However, it is feasible to detect viruses in a modified flow cytometer by light scatter alone (Hercher et al., 1979), and recent investigations using highly
fluorescent dyes have allowed direct detection and enumeration of individual viral particles using commercially available flow cytometers (Brussaard et al., 2000; Marie et al., 1999; Shen et al., 2002). Viruses lend themselves well to these studies because their low autofluorescence yields low background (Shapiro, 1995). The lower limit of detection for distinguishing particles based on light scatter alone is approximately 100 nm (Hercher et al., 1979; Shapiro, 1995); however, highly reproducible counts of viruses on the order of 30 nm to 60 nm are possible with the use of fluorescent dyes, and side scatter profiles of viruses stained with the DNA dyes SYBR Green I and SYBR Gold can be used to distinguish different populations of viruses in the same sample (Brussaard et al., 2000; Chen et al., 2001; Marie et al., 1999). Even individual DNA fragments can be detected using flow cytometry in a modified instrument (Goodwin et al., 1993; Yan et al., 2000). Quantitative analysis of viruses using flow cytometry and fluorescent dyes is robust and reproducible, and results are in good agreement with those obtained using microscopy (Brussaard et al., 2000; Chen et al., 2001; Marie et al., 1999; Shen et al., 2002).

A number of high quality fluorescent dyes are currently available (Invitrogen Molecular Probes, Eugene, OR) which enable highly sensitive detection based on both RNA and DNA. These probes can be excited with wavelengths from common light sources, and the range of available emission wavelengths allows dual labeling. Dyes are available for both live and dead cells. Membrane-impermeant dyes such as propidium iodide have been used to differentiate intact from damaged adenovirus particles (Rexroad et al., 2006).
Flow cytometry on viruses has so far used dyes for DNA; however, stains for protein and potentiometric probes are also available. The wide variety of probes available gives researchers flexibility in designing experiments, and using combinations of such probes in a single experiment expands the range of information which can be obtained (Shapiro, 1995). Since probes are available for both DNA and RNA, a method based on their detection can be used with a wide variety of viruses.

The SYTO and SYTOX families of dyes, available from Invitrogen Molecular Probes (Eugene, OR) are DNA-intercalating dyes which were used in dual staining experiments to examine the effects of UV on adenovirus particles. The SYTO family of dyes includes approximately 40 members, differing mainly in their excitation and emission spectra, which emit blue, green, orange, and red fluorescence. These dyes enter cells via passive diffusion and can therefore be used to stain either damaged or intact cells. While they do have a lower affinity for nucleic acids than other available dyes (Biggerstaff et al., 2006), the ease with which SYTO dyes enter cells makes them most suitable for use without detergents or other aids to permeability; this is integral to the work described here because nothing should be done to the viruses during analysis which might alter the integrity of the capsid and obscure the effects of UV treatment. This lower affinity is also beneficial in dual staining protocols like the one proposed here. The SYTOX family of dyes has 3 members which emit green, blue, and orange fluorescence. Like propidium iodide, the SYTOX dyes stain only dead or damaged cells. Since they exhibit 500-1000 fold increases in fluorescence on
nucleic acid binding, whereas propidium iodide exhibits a 10-30 fold increase, the SYTOX dyes are much more sensitive; SYTOX orange has been highly effective in the size determination of individual DNA fragments by flow cytometry (Yan et al., 2000). For the work proposed here, dual staining with SYTO and SYTOX dyes was attempted for simultaneous detection of intact and damaged adenovirus particles.

Dual staining with SYTO and SYTOX dyes allows simultaneous enumeration of live and dead cells. During dual staining, lower-affinity SYTO dyes bound to DNA in all cells are displaced and quenched by the higher-affinity SYTOX dyes in dead cells, yielding two distinctly stained populations. Since these two families of dyes exhibit minimal fluorescence in the unbound state, washing and filtration are not needed prior to analysis, and the ratio of dye concentration to viral particles should not have a significant effect on results. Methods for optimization of a dual staining protocol using SYTO 13 and SYTOX orange for analysis of cultured bacteria have been described (Biggerstaff et al., 2006) and were to be adapted here for adenovirus.

While the literature discussed above indicates that flow cytometry of viruses might be feasible despite their small size, the experiments attempted here did not prove fruitful for two primary reasons: the first is that—because viruses do not cause detectable light scatter signals—fluorescent signals from the dyes must be used as a trigger for the cytometer’s detectors, and the researcher is forced to choose an arbitrary cutoff for this signal. Thus it is difficult to determine exactly what is being detected. Second, the SYTO and SYTOX
dyes discussed in the above proposal were designed for use on true cells—which have membranes—rather than viruses. Based on the data collected for this work, it does not appear that these dyes behave as live/dead (damaged/undamaged) stains in viruses as they do in cells.
APPENDIX 3: DETAILED PROTOCOLS

I. Culture of Stock Cells

1. Grow A549 cells to confluence.

2. When confluent, remove medium.

3. Rinse 1x with PBS (no Ca^{2+}, no Mg^{2+}).

4. Add 0.25% trypsin/EDTA (approximately 0.5 ml for T25 flask, 1 ml for T75 flask, 1.5 ml for T150 flask)

5. Incubate with trypsin 3-5 minutes until cells are rounded and detached.

6. Resuspend cells in complete growth medium (Ham’s F12 K with 10% FBS and 1/100 dilution of antibiotic/antimycotic containing penicillin, streptomycin, and amphotericin B).

7. Add desired volume of cell suspension to a new flask containing complete growth medium.
II. Propagation, Purification, and Concentration of Viral Stocks

1. Several days before infection: plate host cells into 8-10 T150 flasks (with phenolic caps) and allow to reach approximately 80-90% confluence. Host cells used for this work were A549 cells.

2. Day of infection: prepare virus for infection at $10^6$ MPN/ml in PBS (with Ca$^{2+}$ and Mg$^{2+}$). Adenovirus type 2 was used for this work.

3. Day of infection: rinse all flasks 2x with DPBS (with Ca$^{2+}$ and Mg$^{2+}$)

4. Add 1 ml per flask virus at $10^6$ MPN/ml from step 2.

5. Incubate at room temperature or in 37°C cell culture incubator for 1-1.5 hours; rock by hand every 15 minutes to distribute virus over cells.

6. Add 60 ml per flask viral propagation medium (high-glucose DMEM with 2 mM L-glutamine and antibiotic/antimycotic, NO FBS).

7. Cap tight and return to cell culture incubator for 10 days or until cell monolayer is destroyed. *

*Note: Destruction of the cell monolayer is observed through the microscope; it is used as an indication that viral infection is at an advanced stage and therefore the concentration of viruses in the culture is optimally high. However, since the viral propagation medium used for this protocol is serum-free, some degradation of the cell monolayer will occur as a result of the lack of serum. This will cause the monolayer to look damaged well before 10 days, that is, before the viral infection has reached the desired advanced stage. There are two controls which can help the researcher distinguish the effects of the virus vs. the effects of serum-free medium:
Control 1: with FBS, with virus: On the same day viral infection for stock propagation is carried out, infect a flask of cells with the same amount of virus relative to host cells, but in this flask use medium with 10% FBS (the same medium used for cell culture infectivity assays). This control helps determine that the viral stock used for infection is in fact infectious—since the only degradation of cells in this flask should be the result of viral infection. Furthermore, true cytopathic effects are much more readily distinguished in cell cultures containing a full complement of serum.

Control 2: no FBS, no virus: On the same day viral infection for stock propagation is carried out, prepare a flask with no virus. This flask of cells should be rinsed with PBS like the viral stock flasks, and the same serum-free medium used in viral stock flasks should be added. The only difference is that no virus is added to this flask. This control will help the researcher determine how much the cell monolayer gets degraded as a result of being exposed to serum-free medium—even when no virus is present.

8. Shake flasks to dislodge cells and freeze at -80°C (first freeze; either transfer to conical tubes or freeze in flasks).

9. Thaw frozen virus at 37°C (first thaw), then return to -80°C freezer (2nd freeze).

10. Thaw again at 37°C (2nd thaw) and proceed with purification as below. Here there were 2 freeze thaw cycles; more can be used if desired but that may affect UV sensitivity of the viral stock obtained. The freeze-thaw cycles are used to break open the host cells and release intracellular virus. Virus should be handled
as sterile as possible and in a biological safety cabinet whenever possible. This is especially important for viral stocks that will later be used in cell culture infectivity assays; as the cell culture infectivity assays require long incubation times, they have a strict sterility requirement.

11. Vortex briefly and centrifuge at 6000g, 15 min, 4°C to pellet cellular debris.
12. Discard cell pellet and stir supernatant for 2.5 hours at room temperature with 9g/100 ml polyethylene glycol (PEG, mw 8000) and 5.8 g/100ml NaCl. This precipitates the virus. Do in sterile biological safety cabinet.
13. Centrifuge at 6000g, 4°C, 30 minutes. Will likely require use of several centrifuge tubes since volume of medium is relatively large.
14. Discard supernatant and resuspend viral pellet in PBS. The total volume of PBS used to resuspend the viral pellets should be equal to approximately ¼ the original total volume of viral propagation medium added in step 6.
15. Add an equal volume of chloroform; vortex or shake vigorously for 5 minutes. Chloroform extraction is used to unclump and disperse the viruses.
16. Centrifuge at 4500g, 20 minutes, 4°C. Top (aqueous) layer is viral stock.
17. Viral stocks can be kept at 4°C for short term (days – couple weeks). Longer term storage should be at -80°C.
III. UV Irradiation Procedures

1. Aliquot 5 ml of undiluted viral stock to a disposable Petri dish containing a miniature stir bar. Falcon #35-1007 dishes were used for the work described here; this results in a sample depth of 0.2267 cm.

2. Take absorbance spectrum of sample to be irradiated from 200 nm -400nm.

3. Measure incident irradiance at surface of sample using radiometer.

4. Calculate required exposure time for desired UV dose using spreadsheet and see Bolton and Linden (2003). Dose calculation accounts for a) absorbance spectrum, b) Petri factors (0.98 for LP, 1 for MP), c) sample depth, and d) incident irradiance.

5. Irradiate for required time while stirring. Keep cover over Petri dish as much as possible to maintain sterility. Remove cover quickly before UV exposure and replace quickly after exposure.
IV. Cell Culture Infectivity Assays

This method is used for both stock titer and assay of UV treated viruses.

1. Plate required number of T25 flasks (with phenolic caps) with $3 - 3.5 \times 10^5$ cells per flask using MPN medium (high-glucose DMEM, 10% FBS, 2 mM L-glutamine, and antibiotic/antimycotic). Required number of flasks is at least 9 per sample: this is enough for 3 different dilutions of virus, 3 flasks per dilution.

2. Incubate all flasks in cell culture incubator at 37°C with caps loose for 2 days. Caps are kept loose at this point so that air inside the flask can equilibrate with air in the CO$_2$ incubator.

3. Make desired dilutions of virus and plate 1 ml of diluted virus per flask. Use at least three different dilutions and at least 3 flasks per dilution. The table below shows typical dilutions used for a given UV dose. Dilutions used must span an appropriate range so that flasks containing the most dilute virus are negative for cytopathic effects while flasks with the least diluted virus are positive for cytopathic effects. At higher UV doses, there are fewer infectious viruses remaining and samples are diluted less than they are at lower UV doses. The required dilution is obviously dependent on the titer of the initial stock. Titers of viral stocks used in the work described here were approximately $10^7$-$10^8$ MPN per ml.
<table>
<thead>
<tr>
<th>UV lamp</th>
<th>UV dose (mJ/cm²)</th>
<th>dilutions</th>
</tr>
</thead>
<tbody>
<tr>
<td>No UV</td>
<td>0</td>
<td>$10^{-6}$, $10^{-7}$, $10^{-9}$</td>
</tr>
<tr>
<td>LP</td>
<td>10</td>
<td>$10^{-6}$, $10^{-7}$, $10^{-9}$</td>
</tr>
<tr>
<td>LP</td>
<td>25</td>
<td>$10^{-6}$, $10^{-7}$, $10^{-9}$</td>
</tr>
<tr>
<td>LP</td>
<td>50</td>
<td>$10^{-3}$, $10^{-5}$, $10^{-7}$</td>
</tr>
<tr>
<td>LP</td>
<td>125</td>
<td>$10^{-1}$, $10^{-3}$, $10^{-5}$</td>
</tr>
<tr>
<td>MP</td>
<td>10</td>
<td>$10^{-3}$, $10^{-5}$, $10^{-7}$</td>
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<tr>
<td>MP</td>
<td>25</td>
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<tr>
<td>MP</td>
<td>50</td>
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</tr>
<tr>
<td>MP</td>
<td>125</td>
<td>$10^{-1}$, $10^{-3}$, $10^{-5}$</td>
</tr>
</tbody>
</table>

4. Cap flasks tight after adding virus and incubate in cell culture incubator for up to three weeks. Check for cytopathic effects every few days and score each flask as positive or negative for cytopathic effects.

5. Calculate most probable number per milliliter (MPN/ml) of infectious virus in each sample based on the computer program developed by Hurley and Roscoe (1983).
V. QPCR Assay for DNA Damage

A. Extraction of DNA Directly from Irradiated Viral Stocks

This protocol uses the QIAamp DNA Blood Mini Kit (Qiagen Valencia, CA; catalog # 51104) according to manufacturer’s instructions with minor alterations.

1. Aliquot 200 ul of irradiated viral stock to microcentrifuge tube.
2. Add 20 ul Qiagen protease.
3. Add 200 ul Buffer AL
4. Vortex 15 seconds.
5. Incubate at 56°C for 15 minutes
6. Spin briefly to settle condensation and add 230 ul 100% ethanol.
7. Vortex 15 seconds and load sample onto mini spin column.
8. Spin at 6000g for 2 minutes and discard column effluent.
9. Add 500 ul Buffer AW1 to column and spin at 6000 g for 2 minutes; discard effluent.
10. Add 500 ul Buffer AW2 to column; spin at 20,000 g for 3 minutes and discard effluent.
11. Spin again at 20,000 g for 2 minutes to remove all traces of buffer.
12. Transfer column to a clean collection tube for elution of sample DNA.
13. Add 60 ul Buffer AE to column and incubate at room temperature 5 minutes.
14. Spin at 10,000 g for 3 minutes; keep eluate which contains sample DNA.
15. Store DNA in -20°C freezer.
B. Isolation of Viral DNA from Infected Cells for Repair Experiments

Extraction of Total Viral and Cellular DNA from Infected Cells

This procedure uses the Blood and Cell Culture DNA Kit (Qiagen, Valencia, CA; catalog # 13323). Procedure is written for cells and viruses in T25 flasks with 4 ml culture medium and 1 ml virus added.

1. After desired incubation time, use a cell scraper to dislodge cells and transfer both cells and medium to a 15 ml conical centrifuge tube; vortex briefly.
2. Centrifuge at 4°C for 10 minutes at 1500g to pellet cells.
3. Transfer supernatant (which is cell culture medium containing any viruses that did not adhere to cells during incubation) to a new tube and keep on ice. This is the free virus fraction (V).
4. Resuspend the cell pellet in 500 ul PBS.
5. Add 500 ul ice-cold Buffer C1
6. Add 1.5 ml ice-cold ddH2O.
7. Invert and incubate on ice 10 minutes. This breaks open cells without destroying the nuclei.
8. Centrifuge at 4°C for 15 minutes at 1300g to pellet cell nuclei.
9. Save supernatant to a new tube and keep on ice—this is the cytosolic fraction (C).
10. Resuspend the pellet in 1 ml Buffer G2. The is the nuclei fraction (N).
11b. Fraction V (should be 5 ml): Add RNAse A (50 ul of 2 mg/ml) and Qiagen protease (125 ul).
11c. Fraction C (should be 2.5 ml): add RNAse A (25 ul of 2 mg/ml) and Qiagen protease (62.5 ul).

12. Incubate all fractions at 50°C for 1 hour.

13a. Fraction N: Vortex 5-10 seconds and apply to equilibrated genomic tip column.

13b. Fraction V: Add 625 ul of 5M NaCl (final =750 mM); vortex 5-10 seconds and apply to equilibrated genomic tip column.

13c. Fraction C: Add 262.5 ul of 5M NaCl (final = 750 mM); vortex 5-10 seconds and apply to equilibrated genomic tip column.

14. Wash columns, elute, and collect DNA according to instructions provided with the kit. Final DNA pellet will be in a 15 ml conical centrifuge tube.

Resuspend in 300 ul TE buffer and let sit at room temperature overnight to dissolve DNA. In steps 15-19 the DNA will be concentrated to a smaller volume suitable for loading on to an agarose gel.

15. Transfer DNA to a microcentrifuge tube and add 30 ul 5M NaCl and 600 ul 100% ethanol.

16. Incubate on ice or in freezer for 30-60 minutes to precipitate DNA.

17. Centrifuge at 15,000g for 20 minutes and 4°C to pellet DNA.

18. Wash DNA pellet with 70% ethanol and repeat centrifugation.

19. Air dry pellet and resuspend in 20 ul TE buffer. It is important that all ethanol be evaporated during this step because it is a PCR inhibitor.

20. Let DNA sit at room temperature overnight or keep in freezer for longer term storage.
Separation and Isolation of Viral DNA from Cellular DNA

Viral and cellular DNA are separated on an agarose gel and viral DNA is then recovered from the gel.

1. Prepare gel: 0.4% low melting agarose in 1x TAE buffer with 0.7 ug/ml (final concentration) ethidium bromide.

2. Load samples (all 20 ul plus 2 ul 10x DNA gel loading dye) and DNA size standards (lambda DNA monocut mix, New England Biolabs, Ipswich, MA) into lanes of gel; electrophorese in 1 x TAE buffer, room temperature, at 20 volts for 20-22 hours.

3. View gel and cut out desired bands.

4. Wash gel slices twice in 1x beta-agarase buffer (New England Biolabs, Ipswich, MA) on ice for 30 minutes per wash.

5. Incubate gel slices with an equal volume of 1x beta-agarase buffer at 65°C for 10 minutes to melt the gel.

6. Equilibrate to 42°C for 20 minutes and then digest with beta-agarase (New England Biolabs, Ipswich, MA; 2 units per sample) at 42°C for 2 hours.

7. Precipitate DNA from digested slices using NaCl (final concentration 0.5 M in aqueous volume) and ethanol (twice the original aqueous volume) followed by incubation on ice for 30-60 minutes. For example, if the volume digested in step 6 was 500 ul, add 50 ul 5M NaCl and 1 ml 100% ethanol.

8. Pellet DNA by centrifugation at 15,000g, 20 minutes, 4°C.
9. Wash pellet with 70% ethanol, air dry, and resuspended in 60 ul TE buffer.

10. Viral DNA isolated from agarose gels in this way can be quantitated and used for the QPCR assay as described below.

C. PicoGreen Quantitation of Viral Template DNA

This protocol uses PicoGreen (Invitrogen Molecular Probes, Carlsbad, CA; catalog # P7581) according to manufacturer’s instructions with minor alterations.

1. Prepare PicoGreen dilution using 5 ul of PicoGreen per 1 ml of 1x TE Buffer. 10 ml of this dilution is enough for 1 96-well microplate.

2. Prepare DNA concentration standards from lambda HindIII digest (Invitrogen, Carlsbad, CA; catalog # 15612-013.). Standard curve used for assay can range in concentration from 20 ng/ul to 0.31 ng/ul.

3. Aliquot 95 ul/well 1x TE buffer.

4. Aliquot 5 ul/well sample or standard. Each sample and standard should be plated in duplicate or triplicate. Include a standard with no DNA to use for background values.

5. Add 100 ul/well PicoGreen dilution prepared in step 1.

6. Incubate at room temperature 10 minutes and read fluorescence on a 96-well plate reader. Use 485 nm excitation and 530 nm emission.

7. Subtract the zero-standard background from all values; a plot of background-subtracted fluorescence vs. standard concentration gives a straight line, the equation for which can be used to calculate concentrations of the unknown samples.
D. PCR Reaction Setup and Thermal Conditions

This protocol uses the GeneAmp XL PCR Kit (Applied Biosystems, Foster City, CA; catalog # N808-0193).

1. Prepare a master mix for the desired number of reactions. Master mix for one reaction contains: 4.6 ul PCR-grade water, 15 ul 3.3 x PCR buffer, 5 ul BSA (1 mg/ml), 4 ul dNTP mix, 2.4 ul magnesium acetate, 2 ul left primer, and 2 ul right primer.

2. For each reaction, aliquot sample volume that gives 0.5 ng template DNA plus Buffer AE (from QiaAmp kit used in DNA extraction) to a total of 10 ul. Add 35 ul master mix and put in thermal cycler. Cycle conditions for thermal cycler are as follows: 72°C 2 minutes, 94°C 1 minute, 94°C 15 seconds (step 1 of cycle), 65°C 3 minutes (step 2 of cycle), 72°C 5 minutes, 8°C hold (optional).

3. Prepare diluted polymerase using 4.5 ul 1x PCR buffer and 0.5 ul polymerase per reaction.

4. Add polymerase to reaction tubes in PCR machine after 1.5 minutes at 72°C.

5. Allow PCR to proceed for desired number of cycles and remove samples.

E. PCR Cycle Tests

1. Prepare and run PCR reactions as described above in section D.

2. Remove a sample from PCR machine every 3-5 cycles for up to 30 cycles.

3. Perform PicoGreen quantitation on PCR products as described above for template in section C. Samples at higher cycle numbers may need to be diluted because of the high concentration of amplified DNA.
4. Plot log DNA concentration vs. cycle number to determine the range of cycle numbers in which amplification is exponential—a linear increase on this semi-log plot. Use a cycle number in this range for quantitative PCR on UV-treated samples.

F. Quantitative PCR on UV Treated Samples

1. Set up PCR reactions and perform cycle tests as described above. Set up 50% control samples using undamaged template at half the quantity used for experimental samples. For more detail, see Santos et al. (2006).

2. Stop PCR after the number of cycles deemed appropriate in cycle tests.

3. Quantitate PCR products using PicoGreen as described above.

4. Calculate relative amplification using a simple ratio of (DNA concentration of UV treated sample) / (DNA concentration of untreated control).
VI. Protein Precipitation and SDS-PAGE

A. Precipitation of Protein Directly from Irradiated Viral Stocks

1. Aliquot 1 ml of viral stock to microcentrifuge tube.

2. Add 2 ul of 0.5 ug/ul aprotinin (1 ug total) for internal control.

3. Mix and add above sample to a new tube containing 32 ul of 2% sodium
deoxycholate; pipette up and down to mix.

4. Incubate at room temperature 5-10 minutes.

5. Add above mixture to a new tube containing 260 ul of 50% trichloroacetic
acid; pipette to mix.

6. Incubate on ice for 2 hours to precipitate proteins.

7. Centrifuge at 20,000g for 20 minutes at 4°C to pellet proteins.

8. Discard supernatant and invert on paper towel to absorb excess
trichloroacetic acid.

9. Add 1 ml ice-cold acetone to wash pellet and centrifuge again as in step 7.

10. Carefully remove supernatant with pipette to avoid disturbing protein pellet.

11. Air dry pellet for 10 minutes and resuspend in 10 ul Laemmli buffer (BioRad,
Hercules, CA) with 5% beta-mercaptoethanol.

B. SDS-PAGE of Precipitated Proteins

1. Prepare protein standards using 3 ul of a 1:30 dilution of broad-range protein
standards (BioRad, Hercules, CA) and 7 ul of Laemmli buffer with 5% beta-
mercaptoethanol.

2. Incubate protein samples and standards at 95°C for 10 minutes.
3. Load onto 4-20% gradient ReadyGel (BioRad, Hercules, CA) and electrophorese at 200 volts for 45 minutes-1 hour.

4. Remove gel from electrophoresis apparatus and fix in a solution of 10% methanol / 7% acetic acid for 30 minutes. Use gentle agitation and room temperature.

5. Remove fixing solution and incubate gel with 50 ml SYPRO Ruby protein gel stain (BioRad, Hercules, CA) overnight. Use gentle agitation at room temperature.

6. Remove staining solution and destain gel for 30 minutes in 10% methanol / 7% acetic acid.

7. Image and analyze gel using GelDoc and Quantity One software (both from Biorad, Hercules, CA).
CITED REFERENCES


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