DNA DEGRADATION BY ADENOVIRUS IN PERMISSIVE
AND NON-PERMISSIVE INFECTIONS

by

BY JOHN W. GYSBERS, B.SC.

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AUTHOR: John W. Gysbers, B.Sc. (McMaster University)

SUPERVISOR: Professor S. Mak

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The adenovirus type 2 (Ad2) lp mutants: lp3, lp5, and the Ad2 deletion mutant, d1250, all have altered early region E1B sequences which affect the encoded 19K product. Alkaline sucrose gradient analysis shows that DNA degradation occurs in permissive human (KB) cells infected by lp3 at high temperatures (39°C), but not at lower temperatures. This indicates a thermolabile 19K product which is altered in its ability to prevent DNA degradation. The lp5 virus did not induce DNA degradation in KB cells at all temperatures tested, in contrast to the results from the 19K negative mutant d1250, in which extensive DNA degradation occurred. Results showed that the Ad2 lp3 or d1250 viral yields were not drastically reduced despite the DNA degradation phenotypes. Thus the 19K product is not essential for viral production although it does inhibit intracellular DNA degradation.

The DNA degradation phenotype was also observed in wildtype Ad12 infections of the non-permissive monkey cell line, CV1. Analysis of viral specific protein and RNA expression in the Ad12 infected CV1 cells suggest that the level of E1B expression was reduced in these non-permissive cells when compared to expression in permissive (KB) cells. Expression of the other early regions was slightly reduced in the CV1 cells compared to that in Ad12 infected KB cells.
Despite expression of most early regions and normal DNA replication, adenoviral late gene expression was very much reduced in CV1 cells. This may be a result of reduced amounts of E1B 55K and/or E4 products which are required for efficient transport and translation of late viral messages.

DNA degradation was also assayed in CV1 cells infected by one of two recombinant viruses: T1227 or T2743. These viruses had Ad12 E1A, or Ad12 FlA and E1B sequences, respectively, replacing the equivalent sequences in an Ad5 virus background. The latter virus codes for a complete Ad12 19K product, the former an Ad5 19K. Degradation induced by both viruses suggests that the Ad12 E1A has a nuclease-effector function which is not completely inhibited by the Ad5 19K in CV1 cells.
ACKNOWLEDGEMENTS

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LIST OF ABBREVIATIONS

Ad2  Adenovirus type 2
Ad5  Adenovirus type 5
Ad12 Adenovirus type 12
cpm  counts per minute
CV1  Monkey cell line
cyt  cytotoxic
DBP  DNA Binding Protein
DNase DNA nuclease
K, kDa Kilodaltons
19K  E1B 19 kDa protein
55K  E1B 55 kDa protein
KB  Human cell line
kb  kilobases
lp  large plaque
MH12-C2 human embryonic kidney cells constitutively expressing Ad12 E1 products
m.o.i. Multiplicity of Infection
m.u.  genome map units
MW  molecular weight
O.D. optical density
PBS phosphate buffered saline
p.f.u. particle forming units
p.i.  post infection
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<td>RNase</td>
<td>RNA nuclease</td>
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<tr>
<td>SDS</td>
<td>Sodium dodecyl sulfate</td>
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<td>SDS - PAGE</td>
<td>Sodium dodecyl sulfate - polyacrylamide gel electrophoresis</td>
</tr>
<tr>
<td>TBS</td>
<td>tris buffered saline</td>
</tr>
<tr>
<td>TCA</td>
<td>trichloroacetic acid</td>
</tr>
<tr>
<td>U.V.</td>
<td>ultra violet</td>
</tr>
<tr>
<td>w/o</td>
<td>without calcium or magnesium</td>
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<td>wt</td>
<td>wildtype</td>
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INTRODUCTION

Adenoviruses have been the object of much scrutiny since the early 1960s, when Trentin published a report showing that they could induce tumors when injected into newborn rats and hamsters (Trentin et. al., 1962). This was the first instance in which a human virus was shown to have oncogenic (tumour promoting) potential. Subsequent studies have shown that not all adenovirus serotypes are oncogenic, but all those tested could transform cultured rodent cells (Pope and Rowe, 1964; MacAllister et.al., 1969; Galimore, 1974). A lot is now known about the mechanisms of adenovirus infection due to intensive study. Adenovirus has provided an excellent model system for examining the molecular details of gene expression and regulation. For example, the phenomenon of RNA splicing was first reported from studies of adenoviral gene expression (Chow et. al.,1977,1978.;Berk and Sharp, 1977; Kitchingman et.al.,1977). Although the exact mechanism of transformation and tumorigenicity has yet to be elucidated the key adenoviral regions and proteins involved have been identified. Approximately 11% of the genome is all that is required for the transformation phenotype (Galimore,et.al.,1974; Graham et. al.,1974; revewied by Graham, 1984 and Tooze, 1981). By convention, this portion of the genome is referred to as the
"left" end and contains the gene regions E1A and E1B). Other regions of the genome, such as the early regions E2A and E2B, can also affect the rate of establishment of transformation by intact viruses (Ginsberg et.al., 1975; Williams et.al., 1975; Logan et.al., 1981).

Human adenoviruses are DNA containing, non-enveloped viruses which can cause upper respiratory tract infections and/or gastric enteritis (reviewed in Ginsberg, 1984). The 23 x 10^6 molecular weight (MW) or 35 kilobase, double-stranded, linear molecule of DNA (Green et. al. 1967) is enclosed in an icosahedral protein capsid. This capsid is unique among viruses in that it has a spike-like projection, the fibre protein (Pettersson et. al., 1968), protruding from each of its corners. These corners are comprised of a second capsid protein termed the penton base (Ginsberg et. al., 1966). The hexon proteins are a third major component and make up each side of the capsid (Ginsberg, et. al., 1966). Minor proteins, referred to as proteins VI, IIIa, and IX make up the rest of the capsid. The viral genome within the capsid is associated with three structural proteins (V, VII, and u), comprising the core of the virus (reviewed in Tooze, 1981). A schematic diagram of an adenovirus is shown in Fig. 1.
Figure 1. Schematic representation of an adenovirus.

The position of each polypeptide in the virion is designated by means of roman numerals. The bands to the right of the model indicate the mobilities and relative abundance of each protein after dissociation of the virus and analysis on a SDS - polyacrylamide gel. The capsid consists of subunits created by the proteins II, III, IV, IIIa, VI, VIII, and IX. The core contains the proteins V, VII, and u, in addition to the 55 kDa terminal proteins linked to each 5' end of the virion DNA. The configuration of the DNA shown here is not intended to represent the actual structure within the capsid. (This figure was obtained from the review article by Horwitz, 1985)
The ends of the adenoviral DNA molecule are unique because they contain a: an inverted terminal repeat sequences of about 100-200 basepairs (Garon et. al., 1972) and b: a 55,000 molecular weight terminal protein is covalently bound to the 5' end of each strand (Robinson et. al., 1973). Both of these features are important in the initiation of adenoviral DNA replication (reviewed in Challberg and Kelly, 1989).

Adenoviral gene expression is generally divided into two major phases: early and late, which are temporally separated by DNA replication. Viral expression is determined and regulated by both cellular functions and the ability of the virus to exploit these functions. Depending on both the type of host cell and the viral serotype, there may be several consequences of infection. The "normal" lytic response, in which large amounts of progeny virions are produced and released result from infection of permissive host cells. Abortive or non-permissive infections, in which no virus replication or production takes place can be another response. Human cells and cell lines are examples of permissive hosts for all human adenovirus serotypes. The result of an infection in cells derived from other species, such as monkey or rodent cells, depends primarily on the serotype of the infecting human adenovirus.

There are currently 49 identified human adenovirus
serotypes and these have been classified into four major groups based on oncogenicity and several other criteria. Group A viruses, which include human adenovirus type 12 (hAd12 or commonly referred to as Ad12), are highly oncogenic in newborn mice or hamsters. They have several other properties in common such as base composition (low G + C content) in their DNA genomes (48 - 49%), high DNA homology and similar hemagglutination abilities on rat or monkey erythrocytes (reviewed in Tooze, 1981). The more commonly studied human adenovirus types 2 and 5 (hAd2 or Ad2, and hAd5 or Ad5, respectively) are classified as group C adenoviruses and are non-oncogenic in newborn rodents, have a higher G + C content in their DNAs (55 - 60%) and have highly homologous DNA sequences (Tooze, 1981).

Ad12 cannot replicate in hamster cell lines such as baby hamster kidney (BHK) cells but these cells are permissive for the group C viruses, Ad2 and Ad5 (Rouse et. al., 1966; Strohl et.al.,1966; Doerfler, 1969). Many monkey cell lines (eg. AGMK or CV1) are nonpermissive for Ad2, Ad5, and Ad12 (Eron et. al., 1975; Klessig and Anderson, 1975; and the present study). There are reports, however, that another monkey cell line (VERO) may be permissive for Ad2 infection (Eron et. al.,1975; Eggerding and Pierce, 1986). The differences in these cell lines that account for the varying
permissivity are not known.

1. Adenovirus Lytic Infection

   1.a. Early Gene Expression: Structure and function of the early gene regions.

   After the initial steps of virus adsorption, internalization and uncoating, several distinct regions on the viral genome are first expressed. These are the early gene regions E1A, E1B, E2A, E2B, E3, and E4. These genes function to set up the infection for viral DNA replication, late gene expression. They also act to inhibit host DNA synthesis and cellular mRNA expression (reviewed in Horwitz, 1985). Two of these regions, E1A and E1B, have been more intensively studied than the others due to their roles in the transformation of cultured rodent cells. In addition, these regions in Ad12 have the potential for inducing tumour formation in rodents. These properties are described in section 3 of the Introduction (below). The Ad12 E1A and E1B regions will be now be described in detail as a major portion of this thesis deals with the Ad12 serotype. The various gene regions and directions of transcription on the Ad12 genome are shown on the map in Fig. 2. The E1 gene regions are shown in greater detail in Figure 3.
Figure 2. Genomic map of adenovirus type 12 mRNA coding regions. Early mRNA regions are bold arrows designated E. The five late mRNA regions, designated by L and light arrows are approximations, as Ad12 has not been mapped as completely as Ad2 or Ad5. The major late tripartite leader sequences are designated 1, 2, and 3. 1X is a late protein which is encoded in the E1B region but is expressed later from an independent promoter region (see figure 2). va refers to the viral associated 145 nucleotide RNA transcribed by RNA polymerase III. Ad12 has only one species of VA RNA, compared to two in group C adenoviruses. TP is the terminal protein covalently attached to the 5'end of each DNA strand. (adapted from Saito, et. al.,1981, Esche, et. al.,1984).
Figure 3. Detailed transcription and translation map of the adenovirus type 12 E1A and E1B regions. mRNAs are solid lines with the splicing pattern indicated by dotted lines. The first three mRNAs from each region, identified by their sizes (S), are the major detectable species. Open reading frames (ORF) are designated by open and closed boxes. The open boxes represent identified translation products but no proteins have been detected from the striped ORFs. The numbers in small print represent the protein size in amino acids. For E1B, the two major early products are identified as 55 kDa and 19 kDa based on their size in kilodaltons (K) as determined by SDS-PAGE. These are normally referred to as the 19K and 55K products in the literature and in this study. The 9S product has been identified as a 17 kDa protein in our gel system. This product has its own independent promoter. The promoter regions (TATA) and poly A sites (AATAAA) are indicated on the genomic map. (adapted from Saito et al., 1981; Grand, 1987).
Ad 12 - E1

RNA protein

13 S 266

12 S 235

9 S 53

protein RNA

487 (55 kd) 22 S

163 (19 kd)

163 13 S

79

163 13 S

ix

9 S

266

235
1. a. i. Region E1A: map units 1.3 to 4.5.

Structure: At least 4 different mRNA species are transcribed from the Ad12 E1A region. The proteins derived from these are all translated from the same reading frame (Sawada and Fujinaga, 1980; Saito et al., 1981) (Figure 3). The two major messages, the 13S and 12S, are similar to the major 13S and 12S transcripts of the more thoroughly studied Ad2 and Ad5 E1A regions. The smaller Ad12 E1A transcript encodes a 235 residue product (235R) which differs from the larger product (266R) of the 13S transcript only in the absence of 31 internal amino acids. This is due to alternative splicing of the primary E1A transcript, in which a common splice acceptor site is joined to one of two different splice donor sites (Perricaudet et al. 1980, Saito et al., 1981) (see Fig. 3). These additional amino acids constitute a domain that is highly conserved among human adenoviruses (Moran et al., 1987), and is responsible for the transactivation function of E1A (see below). These Ad12 products are equivalent to the 243R and 289R proteins, in the group C adenoviruses. In Ad12 infections, at least eight proteins ranging from molecular weights of 22 kDa to 45 kDa have been detected as originating from these messages (Esche and Seigmann, 1982; Jochemsen et al., 1980). Phosphorylation of
the primary protein products may account for the heterogenity and numbers of products detected for Ad12, as suggested for the E1A of the group C adenoviruses (Ad2 and Ad5) (Harlow et al., 1986; Dumont et al., 1989; Smith et al., 1989). These products all seem to be localized predominately in the nucleus of infected cells (Grand and Gallimore, 1984; Lucher et al., 1984).

The 9S message is a minor product in Ad2/Ad5 infected cells (Spector et al., 1978) and is only predicted in the Ad12 virus based on sequence comparisons (van Ormondt and Galibert, 1984). No 9S gene product has ever been detected in Ad12 infected cells.

Functions: The E1A gene region is also referred to as an immediate early gene because this is one of the first regions to be expressed upon infection. It is very important for infection because all other viral early genes, and also the major late transcription unit, are transcriptionally activated by the E1A gene products; a process known as transactivation (Berk et al., 1979; Jones and Shenk, 1979; Nevins, 1981; Ohshima and Shiroki, 1986; reviewed by Berk, 1986 and by Flint and Shenk, 1989). The product of the 13S mRNA, the 266R (Ad12) or 289R (Ad2/Ad5) protein is the primary transactivator (Montell et al., 1984; reviewed by Berk, 1986), although there are some reports that suggest limited
transactivation by the (Ad2/Ad5) 12S product (Leff et.al., 1984; Wineberg and Shenk, 1984; Ferguson et.al., 1985). The 12S product is not required for efficient viral growth in human cell cultures (Montell et.al., 1983; Lamberti and Williams, 1990).

E1A proteins are the only viral products required for early gene activation and based on the diversity of the early region promoter sequences, do not seem to bind to specific DNA sequences (see review by Berk, 1986). They are thought to modify or induce existing host cell transcription factors (Berk, 1986). Most of these factors are related to activation/enhancement of DNA polymerase II transcription, but several DNA polymerase III transcription units can also be activated (Berger and Folk, 1985; Gaynor et.al., 1985; Hoeffler and Roeder, 1985). Consistent with the idea of having a general transactivating effect, E1A (from group C adenoviruses, at least) can transactivate transcription from several host cell derived promoters in DNA transfection assays (Gaynor et.al., 1984; Green et.a., 1983; Imperiale et.al., 1983; Svensson and Akusjarvi, 1984).

In addition to the transactivation role, E1A also represses transcription in enhancer-mediated transient expression assays (Borelli et.al., 1984; Velmich and Ziff, 1985; Velmich et.al., 1986) and can repress the transcription
of cellular genes (Hen et al., 1985; Lillie et al., 1986).

E1A from both the A and C groups of adenovirus may induce host DNA synthesis in rodent cells (Stabel et al., 1985; Quinlan and Grodzicker, 1986; Oda et al., 1986).

The E1A of Ad12 is able to depress the expression of class I major histocompatibility antigens in transformed cells (Schrier et al., 1983), at the level of transcription (Friedman and Ricciardi, 1988; Meijer et al., 1989). The Ad5 E1A does not have the ability to do this (Schrier et al., 1983).

The E1A gene region is also absolutely required for the transformation of rodent cells and the induction of tumours in newborn rodents (see review by Graham, 1984). The requirements of E1A in these processes shall be discussed briefly, together with the role of E1B in oncogenesis, in a latter segment of the Introduction (section 3).

1.a.ii. Region E1B: map units 4.6 - 11.2.

Structure: In Ad12 there are 5 messages transcribed from this region, with the two major messages 22S and 13S, and additional minor species of 14.5S and 14S being initiated at the same cap site within the same promoter region (Saito et al., 1981, 1983; Virtanen et al., 1982; van Ormondt and Galibert, 1984). Another 9S mRNA is initiated from a second
distinct transcription unit (9.7 to 11.2 map units) and is active later in infection. This message encodes protein XI, a virion structural protein (Petterson and Matthews, 1977). The largest message (22S) encodes at least two products, a 55 kDa (or 482R) protein, and a 19 kDa (163R) protein from two separate and overlapping reading frames (Bos et al., 1981). These products are commonly referred to as the 55K and 19K. The 19 kDa protein initiates at the first (5' proximal) AUG and the 55 kDa protein at the next downstream AUG. Both proteins have been readily detected in infected and transformed cells (Byrd et al., 1988; Grand and Galimore, 1984) and identified from the in vitro translation of selected RNA (Jochenssen et al., 1980; Esche and Siegman, 1982; Saito et al., 1983; Mak and Mak, 1986). The 13S message also includes the complete 19 kDa protein reading frame, and its expression peaks slightly later in infection than the 22S, causing a relative increase in 19 kDa production later in infection (cited in Stillman, 1986). This protein is also encoded by the two minor 14S species, as is a putative 110R product. This product has not been identified yet but additional 17 kDa, 15 kDa and 14 kDa products have been translated from E1B mRNA, in vitro (Mak and Mak, 1986). An additional 79R open reading frame is present on the 13S mRNA, but no products have been identified from this region (van Ormondt and Galibert, 1984).
The organization of the corresponding E1B region from the group C adenoviruses is very similar to the Ad12 E1B and includes both the 55 kDa (495R) and 19 kDa (175R) coding regions on 22S and 13S mRNAs (reviewed by Petterson et.al., 1983).

The Ad12 19K product is non-glycosylated and acylated (Grand et. al. 1985) and perhaps due to this modification has been localized to nuclear, cytoplasmic and plasma membranes (Fohring et. al., 1983; Grand and Galimore, 1984; Grand et.al., 1985).

The Ad12 55 kDa product is phosphorylated and has been found in the nucleus and the cytoplasm at approximately the same concentrations (Grand and Galimore, 1984). The E1B proteins of the group C adenoviruses are similarly localized (reviewed by Stillman, 1986).

Studies have shown that the Ad12 55 kDa is not associated with a cellular 53 kDa (p53) protein (Mak et.al., 1988, Zantema et.al., 1985), although the equivalent product of group C adenoviruses is physically associated with this protein (Sarnow et.al., 1982; Zantema et.al., 1985). The 55 kDa protein from adenovirus type 2 or 5 also forms a physical complex with a virally encoded (region E4) 34 kDa product: immunoprecipitation of the 55 kDa by specific antisera also precipitates this 34 kDa product, although there
is no direct recognition of this protein by the antisera (Sarnow et. al., 1984). This complex is required for the efficient transport of viral late messages from the nucleus to the cytoplasm of the infected cell (Babiss et. al., 1985, Pilder et.al., 1986). Defects in either of these two proteins lead to similar reductions in the transport of late viral mRNAs and incomplete inhibition of cellular mRNA transport from the nucleus (Babiss et. al., 1985; Weinberg and Ketner, 1989). It has not been determined if the Ad12 55 kDa protein forms a similar complex, although the regulatory functions (of late gene expression) ascribed to the Ad12 55 kDa parallel that of the Ad5 E1B 55 kDa/E4 34 kDa protein complex (Breiding et. al., 1988; see below).

Functions: The large (55 kDa) protein of Ad12 E1B is required for viral DNA synthesis (Fukui et.al., 1984; Shiroki et.al., 1986; Breiding et.al. 1988; Mak and Mak, 1990) but the Ad5 55 kDa protein is not (Babiss and Ginsberg, 1984). It is, however, required for efficient viral growth. This has been determined to be a result of defective transport of viral mRNAs to the cytoplasm and a related defect in the inhibition of transport of host mRNAs to the cytoplasm (Babiss and Ginsberg, 1984; Babiss et.al., 1985; Pilder et.al., 1986; Williams et.al., 1986). In this role, the Ad5 55 kDa forms a physical complex with a 34 kDa protein encoded by the early
region E4 (Halbert et al., 1985; Sarnow et al., 1984; Bridge and Ketner, 1990). Ad12 55 kDa protein mutants also are defective in late protein synthesis (Breiding et al., 1988) and transport of late viral mRNA (Shiroki et al., 1986). For Ad12, the determination of the precise nature of the defective functions is complicated by the lack of DNA replication. A property unique to the 55 kDa protein of Ad12 is its ability to induce several non-random chromosomal breaks in human cell DNA (Schramayr et al., 1990). Although some chromosomal breaks are induced in Ad5 infections, these are apparently random events (McDougall, 1971).

The 19 kDa product is not absolutely required for growth on human cells (Barker and Berk, 1987; Edbauer et al., 1988; Herbst et al., 1988, White et al., 1984), but defects in this protein (or its absence) can reduce yields (Pilder et al., 1984; Subramanian et al., 1984a, b; Bernards et al., 1986). In some human cells, such as growth-arrested WI38 (White et al., 1986) or A549 (Edbauer et al., 1988), 19 kDa protein mutants can grow better than or equivalent to the wt virus.

The 19 kDa protein is required to prevent the degradation of intracellular DNA in infected cells and prevent the early and extensive destruction (cytoidal or "cyt" phenotype) of these cells (Lai Fatt and Mak, 1982; Chinnadurai, 1983; Subramanian et al., 1984a, b; Pilder
et.al.,1984, White et.al., 1984; Takemori et.al.,1984; Barker and Berk, 1987; Edbauer et.al.,1988). E1B 19 kDa protein mutants also exhibit a distinct, large-plaque phenotype on infected human cell monolayers, in contrast to the small fuzzy plaques produced by wt adenovirus infection. Although most of the above mutants were in the Ad2 or Ad5 serotypes, these phenotypes have been observed in the Ad12 cyt mutants (Takemori et.al., 1969; Ezoe and Mak, 1974; Lai Fatt and Mak,1982). The mutations in two of these Ad12 cyt mutants have recently been mapped to the E1B 19 kDa protein (Schaller, Ph.D. Thesis, 1990).

These different phenotypes may be the result of a single altered function, but there is some suggestion that these are separable functions. Edbauer et.al. ,(1988) showed that Ad12 19 kDa protein mutants display the cytocidal and lp phenotypes on human A549 cells, but do not degrade DNA. These mutants however do degrade DNA in human KB cells. The Ad2 19 kDa protein mutants lp3 and lp5 both form large plaques but neither causes an extensive cytopathic effect (Chinnadurai, 1983; Subramanian et.al.,1984b).

There are some discrepancies in the data regarding the role of the 19 kDa protein in gene regulation. Herbst et.al.,(1988) found that 19 kDa protein mutations did not affect the level of gene expression from several viral early
regions, including E1A. Data obtained by White, et al., (1986, 1988) suggests that the 19 kDa protein acts to repress E1A expression and therefore also represses expression of the other E1A-dependent early regions. More recently it has been shown that the 19 kDa product can act to stabilize transfected plasmid DNA (Hermann and Mathews, 1989). The increase in stability was suggested to increase the apparent gene expression from these plasmids due to the resultant extended lifetime of the plasmid. In this manner, perhaps the 19 kDa protein stabilizes viral DNA, protecting it from degradation and increasing the amount of available transcription templates. This role for the 19 kDa protein has yet to be determined.

The other early regions of Ad12 have not been as extensively analysed in regards to sequences, mRNAs and gene products. However a brief description of these early regions and their known functions will be given with some reference to Ad2 or Ad5. Their locations on the Ad12 genome are shown in Figure 2.

1.a.iii. Region E4: map units 90 to 98.

As indicated earlier, a 34 kDa product from the group C adenoviruses is involved in the regulation of transport of
mRNA to the cytoplasm late in infection, as part of a complex with the E1B 55 kDa protein (Sarnow et al., 1984). E4 mutants produce phenotypes very similar to that of the 55 kDa protein mutants (see Bridge and Ketner, 1990, and references therein). In vitro transcription of Ad2 E4 mRNA has produced at least 6 polypeptides (Matsuo et al., 1982). E4 has recently been shown to help stimulate E2 transcription by activating the cellular transcription factor, E2F (Babiss, 1989; Reichel et al., 1989; Marton et al., 1990). This activity has been determined to be the function of an E4 17 kDa product (Marton et al., 1990).

In Ad12 there are at least 5 messages from this region (Saito et al., 1981) and three identifiable products of 20 kDa, 18 kDa and 11.5 kDa (Esche et al., 1984). The role(s) of these proteins have not been studied. Recently, this region of the genome has been sequenced (Hogenkamp and Esche, 1990). From this sequence, open reading frames for a possible 8 proteins have been identified. The authors report a great similarity to the Ad2 and Ad5 E4 DNA sequences.

1.a.iv. Region E3: map units 76 to 87.

This region is dispensible for growth of group C adenoviruses in cell culture (Kvist et al., 1978; Paabo
et al., 1983). It encodes a 19 kDa glycoprotein that appears on the cell surface of infected human cells associated with transplantation antigens (Paabo et al., 1983). An E3 10.4 kDa product seems to regulate the expression of the epidermal growth factor (EDGF) receptor in infected cells (Carlin et al., 1990), and a 14.7 kDa product can protect virus-infected cells from cytolysis by the cytokine tumor necrosis factor (TNF) (Gooding et al., 1988). The Ad12 E3 encodes at least two products of 23 kDa and 16 kDa (Jochemsen et al., 1980), the functions of which have yet to be determined. The Ad12 equivalent to the Ad5 14.7 kDa protein has also been identified (Horton et al., 1990).

l.a.v. Regions E2A: 67 to 61 map units, and E2B: 30 to 11 map units.

These two regions have been greatly studied in the group C adenoviruses and encode the three viral products necessary for adenoviral DNA replication (reviewed extensively by Tooze, 1981; Frutterer and Winnacker, 1984; Kelly, 1984; Challberg and Kelly, 1989). They are the 72 kDa DNA binding protein (DBP) from the E2A region, the 55 kDa terminal protein and 140 kDa viral DNA polymerase (DNA-pol) encoded in region E2B (Challberg et al., 1980; Enomoto et al., 1981; Stillman...
et.al., 1981; van der Vliet and Levine, 1973). Although the coding regions for these two regions are separate, the mRNA transcripts use the same promoter region and leader sequences at about position 75 - 76 map units (Stillman et.al.,1981). At later times after infection the leader sequence for E2A starts at about 72 map units (Kruijer et.al.,1981).

In Ad12 the E2A region has been shown to encode a 61 kDa protein which is analogous to the Ad2 DBP (Jochemsen et.al.,1980; Esche and Seigmann, 1982; Rosenwirth et.al.,1975). The E2B region contains the sequences encoding a 69 to 73 kDa peptide which is considered to be the Ad12 version of the precursor to the terminal peptide (Esche et.al.,1984). A product analogous to the Ad2 viral DNA-pol has not been detected, but is presumed to be encoded in this area by virtue of the similar gene organization in these adenoviruses.

The 72 kDa DBP is an apparently multifunctional protein which binds to single stranded DNA molecules (van der Vleit and Levine, 1973). It can also bind to the ends of double stranded DNA (Fowlkes et.al., 1979) and RNA (Cleghon and Klessig, 1986). The functions attributed to this protein include: an important role in the elongation and initiation of viral DNA replication (reviewed in Kelly et.al, 1988); a role in transcriptional regulation (Carter and Blanton,
1978a,b) including inhibition of region E4 transcription (Nevins and Winkler, 1980) or stimulation of transcription from the E1A, E2A and major late promoters (Chang and Shenk, 1990).

The E2B products function primarily to replicate viral DNA, as described briefly below. The precursor of the terminal protein acts as a 5' primer for the initiation of replication and the DNA-pol synthesizes the nascent DNA strand in a strand-displacement mechanism.

1.b. DNA Replication

Adenoviral replication has been intensively studied using the group C adenoviruses and both in vitro and in vivo systems have been employed. This process will be described here very briefly (for extensive reviews see Challberg and Kelly, 1989; Frutterer and Winnacker, 1984; Kelly, 1984). References for the statements presented below can be found in Challberg and Kelly, (1989).

Replication is initiated at both ends of the genome by the covalent binding of dCMP to a hydroxyl group present on an 80 kDa precursor to the terminal protein (which is cleaved to a 55 kDa product during packaging of the virus). This dCMP is the first nucleotide in the nascent DNA strand.
This reaction is dependent on a DNA template, preferrably a double stranded molecule containing the 55 kDa terminal peptide at each 5' end, although linear and/or partially single stranded DNA molecules can be utilized. Initiation also requires the presence of the DNA polymerase and two host transcription factors (NF-I, and NF-II). A replication fork (containing the elongation complex of at least the DNA polymerase, DBP and NF-II) moves along the template strand, resulting in the synthesis of a nascent DNA strand to form the daughter duplex and the displacement of a single parental strand. This strand is thought to circularize and form a small DNA duplex "panhandle" structure by virtue of the inverted terminal repeat sequences at each end of the molecule. This duplex has the same structure as the ends of a double stranded viral genome and replication is initiated again, producing a second daughter duplex DNA molecule.

1.c. Late Gene Expression

The late gene regions, L1 - L5 (Figure 2), code primarily for structural proteins or proteins required for the assembly of the progeny virions (reviewed in Tooze, 1981 and Horwitz, 1985). All of these late gene "families" of mRNA are produced from identical primary transcripts which are
transcribed rightward from a major late promoter (MLP) and subsequently processed into the 5 families of mRNA, based on alternative splicing and utilization of poly (A) sites (see McGrogan and Raskas, 1978; Nevins and Darnell, 1978; reviewed by Tooze, 1981 and Horwitz, 1985). The basis for the switch from expression of primarily early regions to predominantly late gene expression is not clear but seems to require previous viral DNA replication. Some of the controls may be cis - acting (Thomas and Mathews, 1980) or could involve any or all of: an increase in template number, an alteration in the RNA polymerase by early gene factors, or a change in nucleosome structure (see Persson and Philipson, 1982). There has been a suggestion that E4 may be involved in the switch, as some mutants display delays in both DNA replication and late gene expression (Yoder and Berget, 1986).

Although not mapped as precisely as the Ad2 or Ad5 late mRNAs, the Ad12 late families of mRNA are also located and organized very similarly (Esche et.al.,1984).

One family of "late" mRNA, L1, is transcribed early in infection from the major late promoter (MLP), from which the bulk of the late gene products are initiated. The other regions are prevented from being transcribed by a combination of early transcriptional termination and preferential utilization of the first poly (A) site (Chow et.al.,1979;
Lewis and Mathews, 1980; Nevins and Wilson, 1981). Although expressed early, the primary functions of the encoded L1 proteins are structural (protein IIIa) or they play a role in the assembly of virions (52/55 kDa proteins in Ad2) (Hasson et.al., 1989).

In addition to the structural proteins encoded in the major late transcription unit, there are two more "late" gene regions expressed from separate promoters: Protein IX, in region E1B (described above) and IVa2 (mapping approximately from 16 to 10 map units and partially overlapping region E2B) (Petterson and Matthews, 1977; Wilson et.al., 1979; Chow et.al., 1979). Both promoters are somewhat active early but expression is greatly enhanced later in infection (Petterson and Matthews, 1977).

One additional gene region expressed by the adenoviruses encodes the virus associated (VA) RNAs, of which there are two in Ad2 or Ad5, but only one in Ad12 (Reich et.al., 1966; Mathews, 1975; Saito et.al., 1981). This is a 145 nucleotide (Ad12) or pair of approximately 155 nucleotide (Ad2) transcripts originating from 29 - 30 map units and are transcribed by RNA polymerase III (Weinmann et.al., 1976). These RNAs do not code for any polypeptides (Celma et.al., 1977a,b). Their role was first thought to be in the regulation of splicing of the late RNA messages (Mathews,
1980), but now has been shown to be in the regulation of viral mRNA translation (Thimmappaya et al., 1982; Schneider et al., 1985). VA RNA seems to act by preventing the phosphorylation of a translation initiation factor (eIF-2) (Reichel et al., 1985; Schneider et al., 1985). VA RNA acts by binding to double-stranded RNA activated inhibitor (DAI) (Katze et al., 1987), which is a cellular kinase (phosphorylates eIF-2) induced during viral infection (Schneider and Shenk, 1987). Interestingly, O'Malley et al., (1989) have suggested a model in which the interaction between VA RNA and DAI may play a role in both the selective translation of late viral mRNA and host protein shutoff. This may be related to the role of ElB 55 kDa in the selective transport of viral mRNAs to the cytoplasm and blockage of cellular mRNA transport.

2. Non-permissive or Abortive Infections

Unlike the infection of human cells, in which the Ad2, Ad5 and Ad12 virus can replicate there are cell systems in which the lytic cycle cannot go to completion, resulting in a non-permissive or abortive infection. In these cases there is a block in replication at some point due to some incompatibility between the viral and host cell functions. The two non-permissive adenovirus infections that have been
studied most intently are Ad12 infections of baby hamster kidney (BHK) cells and Ad2 or Ad5 infection of monkey cell lines such as African green monkey kidney (AGMK) or CV1. These are two examples which show that both viral serotype and host cell type determine the permissivity of infection. They also show that the block(s) in the replication cycle can occur at different steps in the formation of progeny virus.

2.a. Ad12 infection of BHK cells

Early experiments involving the infection of BHK cells with adenovirus have shown that virions are produced in Ad2 or Ad5 infections, but none are detected after infection by Ad12 (Strohl, 1966,1969; Doerfler,1969). Further study has shown that DNA replication does not occur in the Ad12 infections of BHK cells (Doerfler,1969; Klimkat and Doerfler,1985), which indicates that an early adenovirus function must be affected. Not surprisingly, given the dependence of late gene expression on DNA replication, no late mRNA or late protein synthesis has been detected in Ad12 infections of BHK cells (Ortin et.al.,1976, Esche et.al.,1979).

Assays of early gene expression have shown that early genes are expressed, but there are indications that it may be
incomplete (Raska and Strohl, 1972; Ortin and Doerfler, 1975; Ortin et.al., 1976). Raska and Strohl (1972), have shown that only approximately 60% of early viral mRNA is expressed in Ad12 infected BHK cells, compared to that in human (HEK) cells. Further analysis has shown that the E1 region may be affected. Ortin et.al.(1976) found that at least 30% of early Ad12 E1 mRNA seen in infected KB cells were not expressed in Ad12 infected BHK cells. Immunoprecipitation of E1 products from Ad12 infected BHK cells showed that levels of both E1A and E1B products were reduced to at least 10% of that seen in productively infected KB cells (Lucher, 1990).

Esche, et.al. (1976) have reported that a 34 kDa E1A protein was lacking from in vitro translation products from RNA obtained from Ad12 infected BHK cells. Another study indicated that the lack of expression from region E1B may be involved: Ad12 could not complement the infection of an Ad5 host range mutant (hr6) in BHK cells, but could in human (HeLa) cells (Rowe and Graham, 1981). This mutant, hr6, is defective in region E1B (Frost and Williams, 1978).

In a series of studies involving the coinfection of BHK cells with Ad2/Ad5 and Ad12, or the infection of Ad2 transformed hamster cells by Ad12, Klimkat and Doerfler showed that the E1B of Ad2 or Ad5 could complement, at least partially, the Ad12 defect of DNA replication and late gene
expression in BHK cells (Klimkat and Doerfler 1985; Klimkat and Doerfler, 1987). These experiments also indicated that the Ad12 major late promoter was defective in BHK cells. Transfection experiments utilizing plasmids containing the Ad2 or Ad12 E1 regions and a reporter plasmid containing the chloramphenicol acetyl transferase (CAT) gene driven by an adenovirus major late promoter (MLP) from either Ad12 or Ad2, confirmed this observation (Weyer and Doerfler, 1985). Recently, a DNA sequence just downstream of the Ad12 MLP sequence (a mitigator sequence at +249 to +435) has been shown to somehow block transcription from this promoter in BHK cells (Zock and Doerfler, 1990). The Ad12 L1 and VA RNA expression is also defective in BHK cells, but their contribution to the block in replication has not been determined (Juttermann et.al., 1989). VA RNA, as noted previously, does have a role in the regulation of viral mRNA translation (Thimmappaya et.al., 1982; Schneider et.al., 1985).

2.b. Ad2 or Ad5 infection of monkey cells

The monkey cell lines CV1 and AGMK are non-permissive for both group C adenoviruses (Ad2/Ad5) and for Ad12 (Rabson et.al., 1964). This is unlike the situation in BHK cells (discussed above), in which the group C adenoviruses are
productive. The block to replication in the monkey cells, at least for Ad2 and Ad5, is probably quite different from that in the above BHK system. Early gene expression and several early proteins (the E2A 72 kDa DBP and the T (tumor) antigens, which are essentially E1A and E1B products) have been assayed and seem to be expressed normally, when compared to infected human (HeLa) cells (Anderson and Klessig, 1982; Feldman et.al., 1966; van der Vliet and Levine, 1973). Adenoviral DNA replication does occur in the monkey cells, at a level equivalent to that in productive infection of KB cells (Freidman et.al., 1970; Hashimoto et.al., 1973). Most late genes are expressed, although at reduced levels (Farber and Baum, 1978; Klessig and Anderson, 1975). The major block to infection seems to be aberrant splicing, and reduced accumulation, of a late region (L5) mRNA encoding the virion structural protein known as the fibre protein (Klessig and Chow, 1980; Anderson and Klessig, 1983, 1984). Further analysis of late gene mRNA expression suggests that the rates of RNA transcription from most late regions are moderately reduced (4 to 10 fold) and premature termination of transcription is enhanced in CV1 infected cells (Johnston et.al., 1985). Cytoplasmic stability and transport of mRNA from the nucleus appear to be normal (Klessig and Anderson, 1975; Johnston et.al., 1985). However, the 10 to 40 fold reduction
in mature, and normally spliced, fibre message in CV1 cells may not completely account for the drastic reduction in late protein levels and subsequent virion production. Anderson and Klessig have noted a decrease in VA RNA in Ad2 infected CV1 cells, compared to normal KB or enhanced (see below) CV1 infections (Anderson and Klessig, 1982). This defect may result in a reduction of translation of late viral mRNAs in CV1 cells.

The defect(s) in CV1 infections can be partially complemented by coinfection with SV40, resulting in an 'enhanced' adenovirus infection. Studies with SV40-Ad2 recombinant viruses and coinfections of adenovirus with SV40 mutants have shown that the presence of the carboxy-terminal region of the SV40 large T antigen is sufficient to allow for productive adenoviral infection (reviewed by Tooze, 1981; Ginsberg, 1984). Interestingly, some other Ad2 mutants defective in the region encoding the DNA binding protein (E2A-DBP) also are able to express late proteins and produce virus (Klessig and Grodzicker, 1979). It has been shown that a single base pair substitution in the 72 kDa-protein coding region can confer permissivity in CV1 cells (Brough et al., 1985). The function(s) of these two factors and whether they act via the same mechanism(s) to allow full adenoviral expression in monkey cells is not known.
An additional note which stresses the importance of host cell factors for permissive infection of adenovirus is that although Ad2 cannot replicate fully in the monkey cell lines CV1 or AGMK (African green monkey kidney), they can productively infect the monkey cell line known as VERO (Eron et.al., 1975; Eggerding and Pierce, 1986). The crucial differences between these cell lines that are responsible for the different responses to adenoviral infection are not known.

A number of mutants of Ad12 have recently been isolated that have been adapted for growth in the VERO monkey cell line, which is normally nonpermissive for Ad12 infection (Salewski et.al., 1989). All four mutants characterized had identical 69 bp deletions in the first exon of the E1A gene and various insertions at the right end of the adenovirus genome (Salewski et.al., 1989). It is not known how these mutations allow growth in monkey cells but the deletion is in the region of E1A which has been defined as a domain necessary for the ability to transform, and not in the domain required for transactivation (Moran and Mathews, 1987). As cellular transformation and ability to suppress transcriptional enhancers are highly correlated in various mutants (Schneider et.al, 1987), the ability of these Ad12 mutants to productively infect VERO cells may be due to the lack of suppression of some cellular gene enhancers (Salewski
et.al., 1989).

3. Transformation

Infection of cells that are semi- or non-permissive for adenoviral replication, (such as rodent or monkey cells) can lead to the transformation of a small percentage of cells to a potentially oncogenic, or immortalized state (for reviews see: Tooze, 1980; Graham, 1984; Branton et.al., 1985; Mak and Mak, 1986). In all the stable transformants analysed there was integration of at least part of the viral genome into host DNA (for example: Doerfler et.al., 1974; Sambrook et.al., 1974; Gallimore et.al., 1974). Direct transfection of viral DNA, rather than infection, can also lead to transformation (Graham and van der Eb, 1973). The integration and expression of at least the left 12% of the adenoviral genome is required for the fully transformed phenotype (Gallimore et.al., 1974; Graham et.al., 1974; van der Eb et.al., 1979; Esche and Seigman, 1982). This section contains the E1A and E1B regions described earlier. Use of transfected viral DNA fragments has shown that the leftmost 5% (E1A) is necessary for immortalization (Graham et.al., 1974), and the left 8% (E1A and 5' end of E1B) for the "full" transformation phenotype (immortalization and morphological alterations) (Graham et.al., 1974; Jochemsen
et.al., 1982). Although both group A (such as Adenovirus type 12) and group C (e.g. Ad2, Ad5) adenoviruses can transform rodent cells in culture (see for example: McBride and Weiner, 1964; McAllister et.al., 1969), only group A viruses such as Ad12 can induce tumor formation when injected directly into rodents (Trentin et. al., 1962; Huebner et.al., 1962). In fact, this difference in oncogenicity is the major characteristic used to categorize human adenovirus serotypes into the various subgroups mentioned above: A - highly oncogenic, B - weakly oncogenic, and C/D - non-oncogenic (see Tooze, 1981, for classification details).

3.a. Roles of the ElA and ElB products in transformation

3.a.i. ElA

Both of the major ElA products are required for full transformation by the group C adenoviruses (Montell et.al., 1984; Hurwitz and Chinnadurai, 1985; Winberg and Shenk, 1984). Studies utilizing ElA plasmids have shown that both products are equally effective in transformation (Moran et.al., 1986; Roberts et.al., 1985). A recent study has suggested that only the larger, 13S, product is necessary for transformation by Ad12 (Lamberti and Williams, 1990),
although a couple of other studies have shown that mutations affecting only the Ad12 13S product can still transform, albeit at much reduced efficiencies (Ohshima and Shiroki, 1986; Byrd et.al., 1988). The precise role of E1A in transformation is not yet clear, but is most likely related to some of its functions in the lytic infection, such as transactivation, or enhancer repression of host cell functions. Several groups suggest that transformation may be related to the enhancer repression function of E1A (Lillie et.al.,1987; Schneider et.al., 1987). Binding of E1A to several cellular proteins, including a cellular anti-oncogene protein (RB), may indicate other mechanisms leading to transformation (Whyte et.al.,1989).

Lamberti and Williams (1990), showed that an Ad12 virus which could not express the 12S product could still induce tumors in rats at wild-type efficiency. The E1A of Ad12 is definitely required for tumor induction (Murphy et. al., 1987; Byrd et.al., 1988), and the use of Ad5/Ad12 recombinant viruses has shown that the serotype origin of the E1A region was a major factor in enabling established cell lines to induce tumors in syngeneic rats (Bernards et.al., 1983; Sawada et.al., 1988). Ad5 E1A-containing, transformed cells could not induce tumors. This difference is thought to result, at least in part, from the ability of the Ad12 E1A to reduce cell
surface expression of MHC class 1 antigen in transformed rat
cells, and the inability of the Ad5 E1A to do likewise
(Schrier et al., 1983; Eager et al., 1985). This function would
presumably enable Ad12 induced tumors to evade the host immune
response (Schrier et al., 1983).

3.a.ii. E1B

The E1B regions of both groups of adenovirus is also
required for full transformation, in addition to E1A (Byrd
et al., 1988; Graham et al., 1974; van der Eb et al., 1982;
Lamberti and Williams, 1990; Shiroki et al., 1979). However,
as with E1A, there are differences between the group A and
group C adenoviruses as to what is absolutely required.
Studies of Ad12 transformation indicate that while the larger
55 kDa product is required for this process (Byrd et al.,
1988; Edbauer et al., 1988; Mak and Mak, 1990; Shiroki et al.,
1986), the 19 kDa protein may not be (Edbauer et al., 1988;
Mak and Mak, 1986). The inability of 19 kDa protein mutants
to transform the rat cell line, 3Y1, may suggest that this
function may be cell type dependent (Fukui et al., 1984;
Edbauer et al., 1988). The 19 kDa protein of Ad2 or Ad5 is
definitely required for the efficient transformation of many
rodent cells (Chinnadurai, 1983; Babiss et al., 1984b; Pilder
et.al., 1984; Subramanian et.al., 1984b; Takemori et.al., 1984; Bernards et.al., 1986; Barker and Berk, 1987), as is the 55 kDa (Graham et.al., 1978; Ho et.al., 1982; Barker and Berk, 1987; Babiss et.al., 1984b; Bernards et.al., 1986). There are reports that the 55 kDa protein is not absolutely required for transformation via transfection of Ad5 (Rowe and Graham, 1983) or Ad12 (Shiroki et.al., 1986) DNA, leading these groups to suggest a role for the 55 kDa product in the integration of viral DNA sequences in virus mediated transformation. Transfection studies utilizing DNA restriction fragments indicate that the presence of the complete 55 kDa product may not be necessary, as long as the amino terminal region is present (Graham et.al., 1974; Jochemsen et.al., 1982). This region overlaps that of the 19 kDa coding region, however, and may indicate a requirement for this product (Graham et.al., 1974).

The E1B of Ad12 also is an important determinant in the tumorigenicity of this virus or Ad12 transformed cells. Again the 55 kDa product is definitely required (Shiroki et.al., 1986; Byrd et.al., 1988) but the role of the 19 kDa protein in tumorigenicity is still not clearly established. Tumor induction in hamsters required 19 kDa (Fukui et.al., 1984), whereas that in rats did not (Edbauer et.al., 1988). The E1B of Ad12 also has a greater tumorigenic potential than
that of Ad5, in conjunction with the Ad12 E1A (Bernards et al., 1983a,b; Sawada et al., 1988).

4. The present study

Viral mutants and non-permissive systems can and have been used to examine the details of adenoviral gene regulation. In the present study, both approaches have been utilized to analyze more fully the role of the adenovirus E1B 19 kDa product and how its expression may affect the outcome of infection.

First, three Ad2 mutants: lp3, lp5, and dl250 (Chinnadurai, 1983; Subramanian et al., 1984) were assayed for their ability to degrade intracellular DNA in permissive human KB cells. The effect that this may have on viral yields was also determined. All three mutants have mutations which affect the E1B 19 kDa product, resulting in altered (in the case of lp3 and lp5) or greatly truncated (dl250) 19 kDa products (see Fig. 4, and the Materials and Methods section).

These mutants were analyzed because they display the same large plaque (lp) phenotype, and low transforming ability of the Ad12 cyt mutants (see above section on function of E1B; Chinnadurai, 1983; Subramanian et al., 1984; Takemori et al., 1968, 1969). Observations in our laboratory has shown that the
Figure 4. a. Physical map of the Ad2 mutations in lp3, lp5 and dl250 E1B-19 kDa products.

The open boxes represent the 19 kDa product in the indicated mutants. The closed boxes represent additional (missense) amino acids added to the translation products due to mutation. The arrow indicate the nucleotides (small numbers), and thus codons, that have been affected by the mutations. The triangle indicates the region deleted in the creation of dl250. (adapted from Chinnadurai et. al., 1984)

b. Genomic map of the two Ad12/Ad5 recombinant viruses: T1227 and T2743.

The open boxes represent the open reading frames coded by Ad12 sequences and the closed boxes represent that part of the E1 proteins encoded on Ad5 sequences. All sequences to the right of the junction point are from Ad5, those to the left from Ad12. The numbers in fine print refer to the last nucleotide of the Ad12 sequences and the first nucleotide of the Ad5 sequences at the junction points.
a) Ad2-19K

b) Ad12/5 recombinants
cyt mutants also extensively degrade intracellular DNA in infected KB cells (Ezoe et al., 1981; Lai Fatt and Mak, 1981) and the defect maps to the E1B 19 kDa coding region (Lai Fatt and Mak, 1981; Schaller, 1990). As part of our laboratory's effort to determine if the 19 kDa product was multifunctional due to separate domains, these mutants were analyzed to determine if the various alterations within the 19 kDa protein also degrade intracellular DNA. Mutant viral yields were also assayed to determine if these mutations had an affect on viral productivity, with or without degradation.

Next, Ad12 infections of monkey (CV1) cells were analyzed to determine if lack of expression of adenoviral early regions, particularly E1B, contributes to non-permissivity in this system. Ad12 is non-permissive in these cells (Rabson et al., 1964), but little has been done to determine the defect. Although group C virus infection of monkey cells has been studied extensively (see section on non-permissive infections), Ad12 may act differently. As discussed earlier, Ad2/Ad5 infection of hamster cells (BHK) leads to productive infection whereas that of Ad12 does not. Preliminary data has indicated that intracellular DNA was degraded in CV1 cells infected by Ad12 (Results: Part B), an observation not reported for Ad2 or Ad5 infections of monkey cells. Lai Fatt and Mak (1982), had reported that the
infection of non-permissive hamster embryo cells by wild type Ad12 resulted in DNA degradation. It was suggested that this may be due to lack of E1B expression in these cells (Lai Fatt, Ph.D. Thesis, 1983; see section on non-permissive infections, above). Therefore, the expression of the Ad12 E1B gene region in CV1 cells was assayed to determine if it was defective. This would explain the degradation results. Concomitantly, the expression of the other early gene regions was also measured to determine if they, too, may be altered. Together these data may provide insights into the mechanisms of gene regulation in this cell system, leading to DNA degradation and non-permissive Ad12 infection. There have been suggestions that other Ad12 early regions, possibly encoding the nuclease (or its activator), may be overexpressed in the absence of E1B expression (H. Caussy, M.Sc. Thesis, 1984). White et al., (1986) suggests that E1A is overexpressed in the absence of E1B expression, leading to an accelerated infection process which culminates in excessive DNA degradation. Determination of the levels of expression of Ad12 early gene regions in CV1 cells may provide some support to these hypotheses.

In the last portion of this study, Ad12/Ad5 recombinant viruses were used to further study the regulation of E1B expression in CV1 cells, and how it relates to the DNA degradation phenotype. Two Ad12/5 recombinant viruses
(generous gifts from T. Jelenik, McMaster University) were used which have: a) either most of the Ad12 E1A (virus T1227) or b) all of the Ad12 E1A and part of Ad12 E1B (virus T2743) in an Ad5 genome (see Figure 4, and Materials and Methods). All of the Ad12 E1B 19 kDa coding region is present in the T2743 virus.

Altered E1B expression in Ad12 infected CV1 cells could be due several possibilities. Ad12 E1A may not act to transactivate early regions such as E1B because of subtle differences in E1A and host transcription factor interaction(s) or alternatively, CV1 transcription factors may not efficiently recognize the Ad12 E1B promotor elements properly. Post transcriptional regulation of E1B by other viral or host functions is another possibility. Ad5 E1B has been shown by others to express normally in CV1 cells upon infection with Ad5 as do the other early regions (Anderson and Klessig, 1982), so its promotor functions and transactivating activities are functional.

E1B expression from both recombinants was assayed at the protein level to determine if the serotype origin of the E1B (and its promotor) sequence was an important determinant for expression, in the presence of the Ad12 E1A and an Ad5 background. In addition, DNA degradation assays were performed to correlate E1B expression with protection of DNA degradation.
MATERIALS AND METHODS

1. Cells and Tissue Culture Methods

Human KB cells were maintained as monolayers or in suspension. The monolayer cultures were grown in 800 ml glass bottles with MEM (F11) supplemented with 10% calf serum, 0.225% sodium bicarbonate and contained 50 000 units of penicillin and 50 000 ug. of streptomycin per 800 mls. The cells were grown at 37°C in a humid atmosphere of 5% CO₂.

Suspension cultures were grown in Joklik (S-MEM) culture medium (Gibco Canada Ltd., Burlington, Ontario) plus 5% horse serum at 37°C with constant stirring provided by teflon magnetic bars over a magnetic stirrer. Cell concentrations were kept between $2.5 \times 10^5$ and $4 \times 10^4$ cells/ml by daily dilutions using prewarmed culture medium.

The monkey cell line CV-1R (referred to as CV1) was obtained from S. Bacchetti (Dept. of Pathology, McMaster University). These cells were maintained as monolayer cultures on plastic tissue culture dishes (Gibco) in minimal essential medium (MEM)-alpha supplemented with 10% calf serum, sodium bicarbonate and antibiotics (as above). Cells were subcultured
upon confluency by being washed with citrate saline (0.134M KCl and 15mM sodium citrate), lightly trypsinized (0.25% trypsin in 1X citrate saline) to remove them from the dish, and replated on fresh plates at a lower concentration. All techniques were aseptic. Media (powdered), sera and antibiotics were purchased from Gibco and trypsin was obtained from Difco through BDH Chemicals Canada Ltd. (Toronto, Ontario).

2. Viruses

Wild type human adenovirus type 12 (strain Huie or 3569) and type 2 were grown from stocks obtained from I. Mak (Dept. of Biology, McMaster University). The Ad12 cyt mutants cyt 68 and cyt 61 were also obtained from I. Mak. The Ad 2 mutants Ad2 lp3, Ad2 lp5 and Ad2 d1250 were obtained from G. Chinnadurai (Institute for Molecular Virology, St. Louis University Medical Center). The mutations are shown in Figure 4. Lp3 has a single C to T transition (at nucleotide 1718 from the extreme left end) near the N terminus sequence of the E1B 19 kDa protein which results in an amino acid change from alanine to valine. Lp5 has two alterations in the 19 kDa encoding sequence. One of these is a G to T transversion (nucleotide 1954) resulting in a tyrosine substitution for
aspartic acid, also near the N terminus. The other alters the termination codon to a leucine codon due to another G to T transversion at nucleotide 2237. This results in an increase of either 12 or 14 amino acids, depending on which of the two mRNAs it is translated from. Dl250 was created by cleaving out a 141 bp fragment (joining the basepair at 1771 to 1912) from the 19 kDa encoding region and does not affect any other reading frames (Chinnadurai, 1983). This results in a shortened 19K protein which has the normal first 19 amino acids of the 19 kDa protein and then 28 additional aminoacids encoded by the frameshifted sequence after the deletion. This protein has not been detectable by immunoprecipitation with sera specific for E1B proteins (Chinnadurai, 1983). Wildtype Ad5 was obtained from P. Branton (Dept. of Pathology, McMaster University). The two Ad12/5 recombinant viruses T1227 and T2743 were gifts from T. Jelinek (Dept. of Biology, McMaster University). T1227 contains the first exon of Ad12 E1A (from nucleotide 1 to 1142) attached to the rest of the Ad5 genome at nucleotide 1227, within Ad5 E1A. T2743 contains the entire Ad 12 E1A and part of E1B (up to nucleotide 2525) attached to the rest of Ad5 at nucleotide 2743, within the Ad5 E1B (T. Jelinek and F.L. Graham, unpublished data). This virus has an Ad12 E1B 19 kDa protein and a hybrid E1B 55 kDa protein. Both of these viruses have a wildtype phenotype in HeLa cells with
regards to gene expression and virus production (titre), (Jelinek and Graham, unpublished results) which suggests that the hybrid proteins are functional. These are shown in Figure 4.

3. Infection Procedures

KB cells were grown in monolayer cultures until confluent and then transferred into suspension cultures. After approximately 2 days in this culture the cells were pelleted by low speed centrifugation (12 minutes at 1200 rpm (280 x g)) and resuspended to a concentration of 3 x 10^6 to 1 x 10^7 cells/ml in warm Joklik media plus 1% calf serum in 15 ml tubes or in small flasks (25 cm² growth area). The viruses were added at a multiplicity of infection (m.o.i.) ranging from 40 to 1000 virions per cell and adsorbed at 37°C for 90 minutes with constant agitation (tubes were rotated on a rolling wheel and flasks were gently shaken). After adsorption, the infected cells were replated on plastic tissue culture dishes in alpha media plus 10% calf serum.

CVI cells were treated slightly differently. Cells were grown in monolayers, washed with 1x citrate saline (0.134 M KCl and 15 mM sodium citrate), trypsinized, suspended in Joklik and 1% calf serum and pelleted by low speed
centrifugation. Infection was done in a manner similar to that for KB cells except that tubes were not used for adsorption since these cells tended to clump. Viruses were usually added at a m.o.i. of 500-1000 virions per cell, or in the case of T1227 and T2743, 10 to 20 p.f.u. per cell (from titrated virus) or 1000 virions per cell (purified virus).

For the infection of large amounts of KB cells, to prepare viral stocks, the virus was adsorbed in large 250 ml centrifuge bottles in which a small teflon spinner bar was placed. The cells were then diluted to $3 \times 10^5$ cells per ml. in Joklik plus 5% horse serum. To prepare $[^{14}C]$-labeled Ad2 stocks, 5 mls of $[^{14}C]$-labeled thymidine (40-60mCi/mM) was added at 8 hours post infection. Cells were harvested 24-36 hours post infection, pelleted by low speed centrifugation and resuspended in 0.01 M Tris (pH 8.0) or 1x phosphate buffered saline (PBS) (w/o).

4. Purification of Virus

4.a. Viral stocks

Extraction and virus purification was done essentially by the procedure of Green and Pina (1963). Cells were resuspended in 10mM Tris-HCL (pH 8.1), disrupted by sonication
(Biosonic, 30% output) and homogenized with cold (4°C) Freon 113 (1,1,2-trichlorotrifluoroethane) (Matheson Gas Products Canada, Whitby, Ontario). The virus was then concentrated from the aqueous phase by sedimentation on to a cesium chloride cushion of density 1.43 g/ml. The virus at the interface was further purified by isopycnic banding in cesium chloride of density 1.34 g/ml, by centrifugation in a Beckman 50Ti rotor at 33,000 rpm for 20 hours at 0 C. The virion concentration was determined by U.V. absorption at a wavelength of 260 nm (O.D. 260), with one absorbance unit (O.D.) corresponding to 4 x 10^{11} virions (Mak, 1971). Virus was stored at a concentration of 1-2 O.D. per ml in 1x TBS (w/o) (137mM NaCl, 5mM KCl, 0.7mM Na 2 HPO 4, 5.6mM glucose and 30mM Tris pH 7.4) and 20% glycerol, at -70°C.

4.b. Yield analysis

Virus was extracted from infected cells with the sodium deoxycholate (NaDOC) method of Lawrence and Ginsberg (1967). Harvested cells were resuspended in the same volume of 0.01M Tris (pH 7.4). NaDOC was added to a final concentration of 0.5% (w/v). After 30 minutes of lysis at room temperature, MgCl2 was added to a final concentration of 0.02M together with DNase and RNase, each at a concentration of 10-
25 ug/ml. This was incubated at 37 C for 1.5 hours and then centrifuged at 1400g for 30 minutes at 5°C. The supernatant was then extracted with an equal volume of cold Freon-113, three times. For yield as determined by \(^{3}H\) -cpm, the supernatant was added (together with 1.0 O.D. of unlabeled virus (4 x 10\(^{11}\) virions) as a visible marker) on top of a pre-formed cesium chloride gradient of densities 1.32 g/ml to 1.36 g/ml in a SW40 nitrocellulose centrifuge tube. Centrifugation was done at 20,000 rpm for 5 hours at 5°C. The viral band was collected as a whole, or in fractions of 5 drops (0.5 ml) from the bottom of the tube. Each fraction, or aliquot of a fraction was precipitated in 4 mls of cold 10% trichloroacetic acid (TCA) for at least 20 minutes on ice. The samples were then collected onto a nitrocellulose membrane filter (pore size 0.45 um) by suction, rinsed with 5% cold TCA and dried (80°C for 1 hour). Radioactivity present on the filter was then measured by liquid scintillation counting in 5 mls of an Omnifluor-toluene mixture. The viral peak was taken as the total [3H]-cpm present in the visible band or the highest peak if the band was collected in fractions.

To determine virus yield by U.V. absorbance at 260 nm, the volume of extracted virus after NaDOC treatment was adjusted to 2.5 mls with 0.01M Tris (pH 8.1). This was layered over 2 mls of cesium chloride of density 1.43 g/ml in a SW50.1
polyallomer tube. After centrifugation for 60 minutes at 25,000 rpm the viral band was collected from the interface, adjusted to a density of 1.34 g/ml and rebanded in cesium chloride of density 1.34 g/ml in a SW65 rotor at 33,000 rpm for 24 hours. The resultant band was collected from the tube and the absorbance measured on a spectrophotometer to get an O.D. reading. 1 O.D. unit was taken to be equivalent to 4 x 10^{11} virions.

5. Viral Titres

The yield of Ad 12 in CV1 cells was also determined by plaque assay on MH12-C2 cells, a human embryonic kidney cell line constitutively expressing the E1 region of Ad12 (Mak and Mak, 1990). At 48 or 70 hrs p.i., infected CV1 or KB cells were harvested by scraping from monolayers and collected by centrifugation at 340 g for 10 minutes. The cells were resuspended in 1 ml. of 1 X tris buffered saline, TBS (w/o), and 20% glycerol. The samples were stored frozen at -70°C until used. The cells were then thawed and refrozen repeatedly (6 times) to release intracellular virus. Dilutions of each lysate were used to infect monolayers of MH12-2C cells (grown on 60 mm^2 dishes) which were about 80 - 90% confluent. After incubation for 1.5 hrs. at 37°C the monolayers were overlaid
with 5 ml. of overlay medium which contained 0.9% bactoagar and alpha media supplemented with 5% calf serum. The cells were overlaid with fresh overlay sera every 6 days, and visible plaques were counted at 18 days post infection.

6. Recombinant DNA Techniques

6.a. Plasmids containing Ad12 sequences

Most plasmids used in this study contained Ad12 DNA sequences representing the Ad12 'early' regions cloned into pBR322. The E1A containing plasmid, pHA5, consisted of the first (left end) 1400 bp of wt Ad12 inserted in the BamHI site of pBR322 (Mak et al., 1986) (Fig. 5). Two plasmids representing E1B were used: pHB14R, which contains an Ad12 fragment extending from nucleotide 1594 to 2317 taking the place of the pBR322 Acc1 (2246) to HindIII (29) restriction fragment (S. Mak, unpublished); and pHB15R, which has the Ad12 fragment of nucleotides 2317 to 3706 inserted into the BamHI site of pBR322 (S. Mak, unpublished) (Fig. 5).

Two 'late' gene regions were also cloned into pBR322. pHBF contains the Ad12 restriction fragment 'F' (51.2 map units to 59.6 map units (the total genome is 100 map units and the nucleotide numbers are not known as Ad12 has not
Figure 5. Ad12 DNA fragments used as radiolabeled probes for the various early and late gene regions.

A simple genomic map of Ad12 is depicted as are the various Ad12 fragments used to probe Ad12 gene expression. The first four probes were cloned into pBR322 and labeled by nick-translation (see Materials and Methods). The E1 probes were obtained from S. Mak and the L2 and L3 probes were cloned as described in the next figure (Figure 6).

The bulk of the probes used were cloned into pUC118 by S. Zhang (Thesis in preparation, McMaster University, 1991). The arrows indicate the relative position of the vector sequences complementary to the universal primer and the direction of synthesis of a radiolabeled probe generated by primer extension (Materials and Methods). A similar probe was generated from E4 sequences cloned into a M13 vector (M. Schaller, Ph.D. Thesis, McMaster University, 1990).
been totally sequenced yet) in the pBR322 BamHI site. This fragment covers most of the sequence coding for L3. A L2 containing plasmid was made by inserting the BamHI D fragment (from 34.0 map units to 47.2 m.u.) into the pBR322 BamHI site. Both of these were made using the basic recombinant techniques as described by Maniatis et. al., (1982). Briefly, the BamHI D and F fragments were isolated from a preparatory agarose gel containing Ad12 DNA digested with restriction enzyme BamHI, for the F fragment, or BamHI and EcoRI to obtain the D fragment. The extra enzyme was needed to cut the BamHI C fragment into two smaller fragments as it comigrates with the D fragment. These were ligated into BamHI-cut pBR322 plasmid DNA and used to transform competent bacterial cells (Le392). Tetracycline sensitive (BamHI cuts pBR322 within a sequence coding for a product that confers tetracycline resistance to colonies containing pBR322) colonies were screened for the presence of insert-containing plasmids by restriction enzyme analysis of extracted DNA (Birnboim and Doly, 1979). Large scale preparations were made from colonies containing the DNA of interest and this purified plasmid DNA was used to make labeled probes (Fig. 6).

Four plasmids, from which single stranded templates were obtained for labeling by primer extension, contained the other Ad12 early regions (E2A, E2B, E3, and E4) inserted into
Figure 6. a) Outline of procedures used to obtain the two plasmids used to provide radiolabeled probes for the detection of late region 1 and 2 (pHB D) and late region 3 (pHB F) RNA. After ligation of the plasmids, they were used to transform bacteria (LE392) and isolated from colonies which were ampicillin resistant but sensitive to tetracycline (as detailed in Maniatis et. al., 1982). Restriction digests of purified plasmid DNA and the Southern blotting (see b) confirmed the identity of these plasmids.

b) Southern blot to identify and confirm the fragments cloned above. Ad12 DNA was cut with restriction enzymes Bam HI (indicated on figure by B) or Hind III (H) and the fragments were separated on a 1% agarose gel in 1 x TBE. Following separation the fragments were blotted to nitrocellulose (Maniatis, et. al., 1982) and hybridized with radiolabeled probes made from one of the two new plasmids (lanes 1,2 and 5,6) or total Ad12 DNA (lanes 3 and 4). pHBBD hybridizes only to the Ad12 Bam HI D fragment and to overlapping Hind III fragments. Similarly, pHBFB only hybridizes to the Bam HI F fragment and related Hind III fragments.
pUC118 (Vieira and Messing, 1987) vectors (Fig.4). They are, respectively, pH118BC-r (59.6 to 73 m.u.), pH118HB-r (18.2 to 31.9 m.u.), pH118BB-1 (73 to 88.2 m.u.), and pUC118/E4r (90.2 to 100 m.u.) (S. Y. Zhang, Dept. of Biology, McMaster University, unpublished results). The viral sequences at 59.6 m.u., 18.2 m.u., 88.2 m.u. and 90.2 m.u. are adjacent to the vector sequences complimentary to the universal primer. An additional plasmid pH118HA-1 (66.5 to 81.9 m.u.) (S. Y. Zhang, unpublished) was used to generate a probe to detect late region 4 (L4) messages. Single stranded templates were generated as described by Vieira and Messing (1987).

6.b. Preparation of radiolabeled probes

DNA probes for hybridization to both DNA and RNA samples were labeled by nick-translation or primer extension.

6.b.i. Nick-translation

Plasmid DNA (usually 1 ug) was diluted to 40 ul in nick translation buffer (50mM Tris, pH 7.2, 10 mM MgSO₄, 0.1 mM dithiothreitol, and 50 ug/ml bovine serum albumen) together with 20uM each of dATP, dGTP, and dTTP. To this was added 100 uCi of [alpha-²³P]dCTP, 0.01 ug/ml DNase and 5 units of E.coli
DNA polymerase. This was incubated at 13°C for 80 minutes and stopped by putting on ice or addition of EDTA to 25mM final concentration (Maniatis et al., 1982).

6.b.ii. Primer extension.

Single stranded templates were utilized which had the sequence of interest inserted close to a vector site which complements a universal primer. Vectors used were either M13 or pUC118 derived. 8 ng of universal primer was mixed with each ug of template in 5 ul of primer extension buffer (20 mM Tris, pH 7.6, 120mM NaCl, and 13.2 mM MgCl₂). This was heated to 90°C for 5 minutes and allowed to cool to room temperature for 45 minutes to allow annealing of the primer to the template. Then 1 ul each of 10mM dATP, 10mM dGTP and 10mM dTTP was added together with 5 ul (50 uCi) of [alpha-³²P] dCTP and 1 ul of 40mM dithiothreitol (2.7 mM final). The mixture was then incubated at room temperature for 30 minutes in the presence of 5 units of E.coli DNA polymerase large fragment (Klenow). The reaction was then stopped by the addition of EDTA (20mM final) or left on ice until purification of the labeled probe (Meinkoth and Wahl, 1984).

The DNA probes made by the above two methods were separated from unincorporated isotope by centrifugation (1600g
X 4 minutes) through 1 ml sephadex G50 columns (Maniatis et. al., 1982). The spun-through DNA was denatured by boiling for 5 to 10 minutes and then added to the hybridization mixture.

7. Analysis of DNA

7.a. Alkaline sucrose gradients

7.a.i. Labeling procedure

Infected KB or CV1 cells (1 X 10^6 to 3 X 10^6 cells) were resuspended in 5 ml of prewarmed alpha medium supplemented with 10 % calf serum in small plastic tissue culture flasks (25 cm² growth area), after adsorption of virus. These were then incubated at 37°C in a humid atmosphere of 5% CO₂. At appropriate times after infection, cells were labeled with \(^{3}H\)-thymidine.

For continuous labeling, 7.5 uCi/ug of label was added at 2 ug/ml together with 2 ug/ml of nonradioactive thymidine to ensure the availability of the \(^{3}H\)-thymidine over long labeling periods. For the Ad2 experiments, label was added at approximately 8 to 10 hrs p.i. when cells were to be harvested at 24 hrs or added at 8 and 24 hrs when the harvest was at 40 hrs p.i. In the experiments involving Ad12 and CV1 cells, label was usually added at 12 to 14 hrs p.i. and cells were collected at 32 to 36 hrs p.i. The change is due to the
slightly slower lytic cycle of Ad12 (compared to Ad 2 or 5). The label was added at the time of maximum DNA synthesis.

In some cases the cells were labeled with a pulse of \(^3\text{H}\)-thymidine (7.5 uCi/ug at 2 ug/ml) for a period of 1 to 2 hrs at a later time in infection (18 to 28 h). The cells would then be collected by low speed centrifugation and washed with the prewarmed medium. Unlabeled thymidine was then added to these cells at 2 ug/ml to prevent further labeling and incubated for a further 5 hrs till harvested.

Labeled cells were then harvested by gently scraping cells from the surface with a rubber policeman and collected by low speed centrifugation (340 g X 10 minutes). The cells were then resuspended to 0.4 ml. with 0.01M Tris, pH 8.0 or TE (0.01M Tris, pH 8.0 and 1mM EDTA) buffer and frozen at \(-70^\circ\text{C}\) or lysed immediately for analysis on sucrose gradients.

7.a.ii. Sucrose gradient analysis

The size of single-stranded DNA molecules was assayed by alkaline sucrose gradient centrifugation. Approximately 2.5 \(\times\) 10^5 infected and labeled cells (about 2 \(\times\) 10^4 cpm), together with purified \(^{14}\text{C}\)-labeled DNA from Ad 2 virions (1 \(\times\) 10^4 cpm) were prepared for sedimentation by lysis in 0.4 ml. of lysing buffer (0.5M NaOH, 10mM EDTA, 0.1M NaCl, and 1% SDS) for 6
8 hrs.

The resulting lysate was layered on top of a 5 - 20% linear sucrose gradient containing 0.3M NaOH, 2mM EDTA, . 0.1M NaCl and 0.1% SDS. This was done carefully with a large mouth sterile pipette to avoid shearing the DNA. A cushion of 40 or 50% alkaline sucrose was added to the bottom of the centrifuge tubes to prevent complete sedimentation of DNA.

Centrifugation was done at 24,000 rpm for 13 hrs at 20°C in a Beckman SW40 or SW41Ti rotor. Approximately 30 fractions were collected by dripping from the bottom of the centrifuge tube. To each fraction 100 µg of salmon sperm DNA was added and the DNA was precipitated by adding 4 ml of cold 10% TCA (trichloroacetic acid). These were put on ice for about 20 minutes. The fractions were then collected onto nitrocellulose membrane filters, with suction, and rinsed with 5% TCA. The filters were baked for at least an hour at 80°C and the amount of radioactivity present on the filters was determined by liquid scintillation counting, using 5 ml of an Omnifluor-toluene solution (4 g Omnifluor per litre toluene).

7.b. DNA extraction and degradation assay

Cells were harvested and washed twice in 1x PBS (w/o)
and then resuspended in 100 (2 x 10⁶ cells) or 200 (5-10 x 10⁶ cells) ul of TE (10mM Tris-HCl, pH8.0, 1mM EDTA) buffer. An equal volume of 2x lysis buffer (0.02M Tris-HCl, pH7.9, 0.02M EDTA, 0.2M NaCl) was added and mixed gently prior to addition of Pronase to 1mg/ml and then SDS to a final concentration of 0.5%. Lysates were incubated overnight at 37°C. NaCl was added to 1M final concentration and the lysates were then mixed by tipping. They were then incubated at 4°C overnight (12-24 hrs) (Hirt, 1967). High molecular weight DNA and cellular debris was then removed by centrifugation in a microcentrifuge for 30 minutes at 4°C. The supernatant was removed to a clean, autoclaved microfuge tube. RNase was added at 50 ug/ml and samples were incubated for 1-2 hrs at 37°C prior to gel electrophoresis. Gel loading buffer (20% glycerol, 0.5% bromophenol blue) was then added and equal volumes were loaded on 0.8-1% agarose gels in 1x TBE (45mM Tris, 45mM boric acid, 2mM EDTA, pH 8.0) buffer. Generally 2-4x10⁵ cell equivalents were used. In cases where equivalent amounts of DNA were loaded, DNA samples were purified by ethanol precipitation after extracting with phenol, phenol/chloroform, and chloroform. DNA samples were quantitated by photospectrometry at 260nm (1 O.D. equivalent to 50 ug DNA/ml). Loading buffer was added to these samples prior to loading. Gels were run 12-18 hrs (overnight) at 25-30 volts and then stained with
ethidium bromide 0.5 ug/ml. The gels were then photographed with a polaroid camera with a red filter while being illuminated by UV light.

7.c. Slot blot analysis
7.c.i. Sample preparation

Purified DNA was prepared for slot blot analysis by lysing cells overnight, as for the above described degradation assay. DNA was then extracted by treating the lysates with phenol (2X) and chloroform (2X) and precipitating with ethanol at -70°C. The precipitate was resuspended in TE buffer and treated with RNase at 50 ug/ml. for 1 h at 37°C. The DNA was again extracted with phenol and chloroform and re-ethanol precipitated. The resuspended DNA was quantitated by photospectrometry (1 O.D. = 50 ug/ml).

These samples were then prepared for slot blotting as described by Schleicher and Schuell, Inc. (Transfer and Immobilization of Nucleic Acids to S & S Supports, Schleicher and Schuell, Inc., 1987). Appropriate dilutions of DNA were made equivalent (1 ug total) by the addition of carrier DNA (salmon sperm DNA). All samples were denatured by the addition of NaOH to 0.3M and heated to 65°C for 1 hr. After cooling, an equal volume of 2M ammonium acetate, pH 7.0, was added and
the samples were applied to nitrocellulose filters in a slot
blot apparatus (Schleicher and Schuell, Inc.) under light
vacuum. The nitrocellulose had been pretreated by soaking in
distilled H₂O and then 1M ammonium acetate, pH 7.0. After
sample application, the wells were washed once with 1M
ammonium acetate, pH 7.0 and the filter was baked at 80°C for
two hours.

7.c.ii. Hybridization

Baked filters were prepared for hybridization by
incubation in a prehybridization buffer (50% formamide, 5 X
Denhardt's ( 1 X = 200 ug/ml. each of Ficoll,
polyvinylpyrrolidone and BSA), 0.5% SDS, 5 X SSPE ( 1 X =
0.18M NaCl, 10mM NaPO₄, pH 7.7, and 1mM EDTA), and 100 ug/ml.
of sonicated, denatured, salmon sperm DNA)
for 4 to 16 hrs at 42°C. This solution was then replaced by
fresh buffer (as above) containing denatured, radiolabeled
probe and incubated for 16 to 24 hrs (overnight) at 42°C.
These conditions are generally as outlined by Scheicher and
Schuell, Inc. (1987), as are the following washing steps.
After hybridization, the filters were removed from the
hybridization solutions and washed twice in 1 X SSC and 0.1%
SDS for ten minutes each at room temperature. They were then
washed twice at 42 to 50°C for 15 minutes each in 0.1 X SSC and 0.1% SDS. The filters were then dried at room temperature and exposed to X-ray film (KODAK XAR-1) (Bonner and Laskey, 1974).

8. Analysis of RNA

8.a. Extraction and purification

Cells (1x10^6) were harvested at each time point and pelleted at 340 x g for 10 minutes, washed twice with 1x PBS (w/o), and pelleted. When extracting cytoplasmic RNA, pellet were lysed in Ricciardi's lysing solution (0.25M NaCl, 1.5 mM MgCl₂, 0.01M Tris pH 7.9, and 1.4% NP40) (Ricciardi et. al., 1979) for 10-15 minutes on ice. Lysis was monitored by microscopic observation. Nuclei were pelleted at 3000 rpm x 10 minutes at 4°C and the supernatant was added to 2x volume of 6M guanidium isothiocyanate (GIT) solution (6M guanidium isothiocyanate, 0.75% N-lauryl sarcosine, 37.5mM sodium citrate pH 7.0, and 0.15 M beta-mercaptoethanol) and vortexed for 2 minutes. In the preparation of total cell RNA, washed cells were resuspended directly in a 4M GIT solution (4M guanidium isothiocyanate, 0.5% N-lauryl sarcosine, 25mM sodium citrate pH 7.0, and 0.1M beta-mercaptoethanol and vortexed for 2 minutes (Chirgwin et. al. 1979). These lysates
were also sonicated for 60 seconds to fragment high molecular weight DNA. All lysates were layered on 5 ml. of 5.7M CsCl (pH 5.0) and the RNA was pelleted by centrifugation at 136 000 x g for 20-24 hrs (Grisaín et. al., 1974). The pellet was resuspended in either 0.3M sodium acetate pH 7.0 or a 0.5% solution of SDS which was made to 0.3M sodium acetate after the RNA was dissolved. The RNA was ethanol precipitated two times before resuspension in 200 ul. of H₂O. The concentration of RNA was determined by measuring the absorbance of a diluted aliquot of each sample at 260 nm (1 O.D. was equivalent to 47 µg/ml RNA; Mak, 1971).

All solutions and H₂O used were made RNase free by treating with diethylpyrocarbonate (DEPC), at a 0.1% concentration, overnight and then autoclaved. All glassware used was baked for at least 4 hrs before use at 250°C and where possible, sterile disposable plastic pipettes were used.

8.b. Slot blot analysis

8.b.i. Sample preparation.

RNA was analyzed by slot blotting using the procedure described by Schleicher and Schuell, Inc. (Transfer and Immobilization of Nucleic Acids to S. and S. Solid Supports, 1987). Enough RNA for serial dilutions (3 to 4 for each
sample) and probing by various probes was denatured in a solution of 6x SSC (1x = 0.15M NaCl, 15mM NaCitrate, pH 7.0), and 7.4% formaldehyde (final) for 15 minutes at 60°C. They were then diluted with the 10x SSC to the appropriate concentrations needed. Yeast tRNA were added to some samples, as needed to give all samples equivalent amounts of RNA, just prior to loading. This RNA was treated in a manner identical to cellular RNA. Some other samples were treated with 50 ug/ml RNase for 1 hr at 37°C prior to denaturation and these samples were made up to equivalent amounts of total RNA with tRNA just before loading. Samples were applied to nitrocellulose filter in a slot blot apparatus, in volumes of at least 400 ul, under light vacuum. Slots were then washed with 50% 1M sodium acetate pH 7.0. The filters were then baked for 90 minutes at 80°C to fix the RNA.

8.b.ii. Hybridization.

Baked filters were hybridized and washed using the conditions recommended by Schleicher and Schuell, Inc. (1987). The filters were prehybridized before labeled probe addition at 42°C in buffer containing 50% formamide, 5x Denhardt's solution, 0.25-0.5% SDS, 100 ug/ml sonicated and denatured salmon sperm DNA, and 5x SSPE. After pre-hybridization for at
least 2 hrs (to overnight) the buffer was replaced with fresh buffer solution containing denatured, radiolabeled probe. Incubation at 42°C was continued overnight. The hybridization buffer was then removed and the filters washed twice at room temperature in 2x SSC and 0.1% SDS for 10-20 minutes. They were then washed twice in 0.1x SSC and 0.1% SDS at 50-56°C for 30 minutes each. The filters were dried at room temperature and exposed to x-ray film (KODAK XAR-1) (Bonner and Laskey, 1974).

8.c. Northern blot analysis

RNA was also analyzed by northern blotting as described in the BRL publication of Focus: 8.2., 1982 (BRL Life Technologies Inc., Burlington, Ontario). Each RNA sample (the amount would depend on the number of gels used: generally 15 ug was loaded per lane) was first pelleted and dried under a vacuum (speed-vac) and then resuspended in HEPES/EDTA buffer containing formaldehyde and formamide. For every 15 ug of RNA, 2.4 ul of buffer A (0.14 M HEPES (pH7.8), 2.94 mM Na₂EDTA) and 4.6 ul of a formaldehyde - formamide solution (89 ul of 37% formaldehyde and 250 ul formamide) was added and samples were heated to 70°C for 10 minutes. They were then quenched on ice and 1.5 ul of loading buffer added (made up as 322 ul buffer
A, 5mg of bromophenol blue, 0.4 g sucrose, 178 ul formaldehyde and 500 ul formamide). Samples were loaded on a 1-1.4% agarose gel in 1x Hepes/EDTA (0.05M Hepes pH7.8, 1mM Na$_2$EDTA) containing 6% (final) formaldehyde. Gels were generally run for 3-4 hrs at 75-80 volts (till dye front had migrated at least 8 cm). The formaldehyde was removed from the gel after running by two washes in H$_2$O for 15 minutes each. The gel was blotted as described by Schleicher and Schuell, Inc., (1987). The gel was inverted and placed on 2 thicknesses of 3MM paper that was supported by a glass or plastic plate and placed in a tray containing 20x SSC blotting buffer. A piece of nitrocellulose, previously wet with H$_2$O and soaked in blotting buffer, was placed on the gel and covered with 3 thicknesses of 3MM paper and a stack of paper towels. A weight was added to the top and the RNA was allowed to blot onto the nitrocellulose, by capillary action of the buffer, for 18 to 24 hrs. The RNA was then fixed to the nitrocellulose by baking at 80°C for 2 hrs. The filters were hybridized, washed, and exposed to x-ray film (Kodak XAR-1) in a manner identical to that of the RNA slot blots.
9. Protein Analysis

9.a. Labeling and extraction.

At indicated times after infection culture media was removed from cell monolayers and replaced with just enough medium 199 (contains no methionine and referred to as met-medium) to cover the cells. The cells were incubated in this media for 1/2 to 1 hr prior to addition of labeled methionine to reduce intracellular pools of methionine. This was seen to increase the amount of TCA precipitable, labeled protein. If floating (detached) cells were detected prior to addition of met-medium, they were collected by low speed centrifugation (170g x 15 minutes) and added back to the plates in a minimal amount of met-medium. Cells were then labeled with \[^{35}\text{S}]\) methionine (Amersham) or a mixture of \[^{35}\text{S}]\)-methionine and \[^{35}\text{S}]\)-cysteine (\[^{35}\text{S}]\)-trans)(ICN Biomedicals Canada Ltd. Montreal, Quebec) for 1 to 4 hours at 37°C.

Cell plates were placed on ice after labeling and the cells detached by gentle scraping with a rubber policeman. The cells (0.5-1 x 10⁷ cells) were then pelleted, 340g x 15 minutes, washed 1-2x with cold 1x PBS, and then resuspended in 200 ul to 400 ul of Schweizer's buffer (10% glycerol, 0.1M tris pH 8.0, 0.1m NaCl, 5mM KCl,1mM CaCl₂, and 0.5mM MgCl₂) containing 1.4% NP-40 (BRL). Total cell lysates were prepared
by sonication of the resuspended cells for 90 seconds and centrifugation in a microcentrifuge for 20 minutes to remove cellular debris. Cytoplasmic extracts were prepared by incubation of resuspended cells on ice for 10-15 minutes and removal of nuclei by centrifugation at 1600g x 10 minutes (Mak and Mak, 1983). In both cases, lysis and destruction by sonication were confirmed by microscopic observation.

The amount of label in each lysate was determined by precipitating aliquots from each sample in 10% TCA. These were then collected on 0.45 μm (pore size) nitrocellulose filters by suction. Filters were rinsed in 5% TCA before drying in a 80 °C oven for at least 30 minutes. Radioactivity on the filters was determined by scintillation counting after immersion of the filter in 5 ml of scintillation cocktail (Omnifluor in toluene).

Non-immunoprecipitated lysates to be analysed on SDS PAGE were prepared by mixing aliquots of equivalent radioactivity to loading buffer (0.0625M Tris pH 6.8, 2% SDS, 10% glycerol, 5% beta-mercaptoethanol, and 0.001% bromophenol blue) (Laemli, 1970) and boiling for 3 minutes just prior to loading.
9.b. Immunoprecipitation

9.b.i. Sera

Anti-tumour serum AB6a-C3 was used to detect both Ela and Elb proteins, and anti-tumour serum A7R-C4 recognized only Ela proteins. Both anti-sera were extracted from rats bearing tumours induced by injection of adenovirus transformed rat cell lines (Mak et. al., 1984; I. Mak, unpublished).

19c is a monoclonal antisera, recognizing the carboxy-terminus of the Ad5 Elb 19 kDa protein, and was obtained from P. Branton (Dept. of Pathology, McMaster, University).

9.b.ii. Protein A Beads

0.3 grams of protein A sepharose beads (Pharmacia) were suspended in 8 mls of Schweizer's buffer containing 1% NP-40 and allowed to swell overnight at 4°C. They were then pelleted for 10 minutes at 240 x g and resuspended in 11 ml. of the same buffer.

9.b.iii. Immunoprecipitation.

Lysates of equivalent TCA precipitable counts were mixed with 20-40 ul. of anti-tumour sera and 200-400 ul. of
suspended protein A beads (Mak and Mak, 1983). The volume of all lysates were made equal by the addition of Schweizer's buffer and 1.4% NP-40. The samples were then rotated for 4 hrs to overnight at 4°C. Then, the beads were pelleted by centrifugation (180 x g for 10 minutes) at 4°C, washed 3x in LiCl buffer (0.1M Tris pH 8.2, 0.2M LiCl, and 0.14M betamercaptoethanol) and the final pellet resuspended in 35 ul. of Laemli loading buffer. The beads were then boiled for 3 minutes and pelleted by centrifugation for 1 minute in a microcentrifuge. The supernatant was then loaded onto a polyacrylamide gel.

9.2. Polyacrylamide gel electrophoresis

Two types of polyacrylamide gels were used. The system most often used was made with a 30% stock of acrylamide consisting of an acrylamide to bis-acrylamide ratio of 158:1 (Mak and Mak, 1983). These gels were made 15.2% polyacrylamide in 0.4 M Tris pH8.8 and 0.1% SDS with stacking gels of 7% polyacrylamide containing 0.13 M Tris pH 6.8 and 0.1% SDS. The other was the type originally described by Laemli (1970). This used a 30% stock of acrylamide with the acrylamide to bis ratio at 37.5:1. 12.5% gels were made in 0.375M Tris pH 8.8 and 0.1% SDS with 3% stacking gels in 0.125M Tris pH 6.8 and
0.1% SDS. The gels were usually run at 50-60 volts for about 18 hrs.

After electrophoresis, the running gels were fixed for 30-60 minutes in a 47% methanol: 1.14M acetic acid solution. The gels were then washed twice, for 30 minutes each, in dimethyl sulfoxide (DMSO), prior to soaking in a DMSO and 23% PPO (2',5'-diphenyloxazole) solution for at least 4 hours to overnight. The PPO was then precipitated by washing the gel in two changes of H2O for at least 30 minutes. The gels were then dried over a steaming water bath while under vacuum and exposed to x-ray film (Kodak XAR-1) (Bonner and Laskey, 1974).

For quantitation, gels were scanned using an LKB laser densitometer equipped with an internal digital integrator. Exposures were selected which would best enable direct comparison of bands of interest using identical scanning parameters.
RESULTS

A. ANALYSIS OF DNA DEGRADATION AND VIRAL YIELDS IN KB CELLS INFECTED WITH Ad2 MUTANTS: lp3, lp5 and d1250.

1. DNA degradation analysis of KB cells infected by Ad2 lp mutants at various multiplicities

Ad2 lp mutants lp5 and lp3 were identified and isolated on the basis of a 'large plaque'(lp) phenotype on human cell cultures (HeLa) (Chinnadurai, 1983). This phenotype was also the basis of selection of the Ad12 cytocidal (cyt) mutants (Takemori et. al., 1969). However, the Ad2 mutants do not have the distinctive cytopathic effect on infected cells characteristic of the Ad12 cytocidal (cyt) mutants (Subramanian et al., 1984). Studies were undertaken to determine if the lp mutants have a DNA degradation phenotype similar to the cyt mutants. Other studies indicate that these various phenotypes may be separable, although an altered E1B 19 kDa product is involved in each (White et. al. 1984; Subramanian et. al. 1984).

KB cells were infected with Ad2 lp3, lp5, wt or Ad12 cyt 61 at multiplicities ranging from 40 - 400 virions per
cell. The infected cells were either labeled continuously with 
$^3$H-thymidine from 8 to 24 hrs p.i., or pulse labeled for 1.5 
hrs at 18 hrs p.i. and chased with cold thymidine for 6 hrs 
until harvest. The total labeled DNA from these infections 
were analysed by alkaline sucrose gradient sedimentation. The 
resulting labeled DNA sedimentation profiles show that at 
m.o.i.s up to 400 virions/cell, lp3 or lp5 do not cause the 
degradation of intracellular DNA (Fig. 7), in contrast to the 
Ad12 cyt mutants and the mutant Ad5 d1313 (which lacks ElB). 
Most of the labeled DNA from Ad2 lp infected cultures 
cosedimented with the marker ([$^{14}$C]-labeled) virion DNA, 
indicating little or no DNA degradation. (The marker DNA 
profile is usually a small, distinct peak that spans about 4 
fractions: see Fig. 8). The profiles of the cyt mutant DNA 
show a broad peak of low molecular weight (LMW) DNA 
sedimenting much slower than the viral marker (Fig. 7: i,j). 
This would be expected for DNA molecules which have been 
degraded to smaller fragments and is similar to the results 
obtained by Ezoe et. al. (1981), with the Ad12 cyt mutants. 
The pulse label procedure was used to try to increase the 
sensitivity of the assay. The short labeling at later times, 
and short chase time, could reduce the labeling of DNA which 
is packaged and protected from degradation. As the results of 
the m.o.i. = 400 infections show however (Fig. 6:c,d,and g),
Figure 7. Sedimentation profiles of intracellular DNA from KB cells infected with one of Ad2 wt, lp3, lp5, Ad5 dl313, Ad12 cyt 68, or Ad12 cyt 61. Sedimentation was done in a 5 to 20% alkaline sucrose gradient and is depicted from right (top) to left (bottom). Multiplicity of infection (M.O.I.) ranged from 40 to 400 virions/cell. Arrows indicate the position of the marker viral DNA ([\(^{14}\)C]-thymidine labelled Ad2 DNA) peak cosedimenting with sample [\(^{3}\)H]-thymidine DNA.

Profiles from:

a. lp3 infected cells: m.o.i. = 40  
   b. lp3 infected cells: m.o.i. = 200  
   c. lp3 infected cells: m.o.i. = 400  
   d. Ad2 wt infected cells: m.o.i. = 400  
   e. lp5 infected cells: m.o.i. = 40  
   f. lp5 infected cells: m.o.i. = 200  
   g. lp5 infected cells: m.o.i. = 400  
   h. Ad2 wt infected cells: m.o.i. = 200  
   i. Ad12 cyt 68 infected cells: m.o.i. = 200  
   j. Ad12 cyt 61 infected cells: m.o.i. = 200  
   k. Ad5 dl313 infected cells: m.o.i. = 200

Profiles c, d, and g were obtained from pulse labeled samples (see text), the rest were from continuously labeled samples.
there is no evidence of degradation. The above results show that there was no obvious difference in lp - mutant DNA profiles on the basis of the m.o.i. used.

2. Ad2 lp mutant viral yield analysis

Degradation of viral DNA, as occurs in cyt-infected KB cells, leads to a decrease in the amount of genomic length viral DNA and therefore would suggest a reduction in the amount of progeny virus produced from the infected cell. Studies comparing the yields of cyt and wt Ad12 infections confirm this supposition (Ezoe et. al., 1981; Takemori et. al., 1969). In comparisons of KB cells infected with parental Ad12 or cyt 70 virus there was at least a 13 fold reduction in progeny virus for Ad12 cyt70 (Takemori et. al., 1969). Equivalent yields of the Ad2 lp mutants and wt virus in KB cells would support the lack of DNA degradation observed above.

The first two experiments utilized the labeled DNA from experiments in which DNA was assayed for degradation. After labeling with $[^3]H$ - thymidine from 8 to 24 hrs, infected cells were harvested and lysed with sodium deoxycholate (as described in Materials and Methods). The purified virions were combined with unlabeled Ad2 virus to be
used as a visible marker. Viral bands were collected from a preformed CsCl gradient after 5 hrs of centrifugation and an aliquot of this was used to determine the total radiolabel (cpm) present. From this a relative estimate of yield was determined for each mutant infected at a multiplicity of infection of 40 virions per cell (Table 1:a) and 200 virions per cell (Table 1:b).

A third experiment (Table 1:c), in which the viral yield was calculated by measuring the optical density of the viral band in a CsCl gradient, produced results similar to the previous experiments. In this experiment, the viruses were unlabeled and collected from a large enough cell population to provide a visible band in the gradient. The result of these experiments are recorded as an average yield ratio (ratio of mutant to wt yields). The yields of lp3 were 47% that of wt and the lp5 yields were 72% that of wt (Table 1). The lp yields were lower than that of the wild type virus but not by the approximate 10 fold difference observed between the cyt mutant and Ad12 wt virus (Takemori et. al., 1969; Ezoe et. al., 1981). Therefore the yield results here correlate well to the lack of DNA degradation in lp infected cell cultures. The differences observed could be due to intrinsic differences in the viral types or preparations or be due to effects of the mutations on some other aspect of viral reproduction in
Table 1.
Viral yields of Ad2 lp mutants and wild-type virus in KB cells.
Yields were determined by measuring the total counts (cpm) of virions extracted from infected cells which comigrated in a cesium chloride (CsCl) gradient with a visible band of Ad2 marker virions (a: a m.c.i. of 40 virions/cell was used, and b: the m.o.i. was 200 virions/cell). Alternatively (c) the viral yields were determined by measuring the O.D. 260 of the viral band, containing no marker virus, in a CsCl gradient. Cells for this experiment were infected at an m.o.i. of 200 virions/cell.

<table>
<thead>
<tr>
<th></th>
<th>lp3 yield / wt yield</th>
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<th>lp5 yield / wt yield</th>
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</thead>
<tbody>
<tr>
<td>a</td>
<td>0.29</td>
<td></td>
<td>0.59</td>
<td></td>
</tr>
<tr>
<td></td>
<td>0.54</td>
<td></td>
<td>1.13</td>
<td></td>
</tr>
<tr>
<td>b</td>
<td>0.30</td>
<td></td>
<td></td>
<td>0.51</td>
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<tr>
<td></td>
<td>0.47</td>
<td></td>
<td></td>
<td>0.50</td>
</tr>
<tr>
<td></td>
<td>0.77</td>
<td></td>
<td></td>
<td>--</td>
</tr>
<tr>
<td>c</td>
<td>0.43</td>
<td></td>
<td></td>
<td>0.86</td>
</tr>
</tbody>
</table>

Average yield ratio: 0.47 +/- 0.16 (S.D) 0.72 +/- 0.24 (S.D)

m.o.i. - multiplicity of infection
S.D. - standard deviation
the absence of DNA degradation.

3. DNA degradation and viral yield analysis from lp mutants grown at various temperatures

3.a. DNA degradation analysis

In 1982, Ho et al., isolated Ad5 host range mutants which possess a cold sensitive host range phenotype (Ho et al., 1982). In 293 cells (a human cell line which contains and expresses the Ad5 E1 region), these viruses grew well at both 32.5 °C and 38.5°C, but only grew well in HeLa cells at 38.5°C. Two mutants were found to map in E1A, and one was mapped to E1B. The E1B mutant is defective for transformation at all temperatures and the E1A mutants are cold-sensitive for transformation. Because both Ad12 cyt mutants and the Ad2 lp mutants are also defective for transformation (Mak and Mak, 1981; Chinnadurai, 1983), the Ad2 lp mutants were examined for possible temperature sensitivity of the DNA protection function and possible effects on viral yields.

KB cells were infected with one of Ad2 lp3, lp5 or wt and grown at temperatures of 33°C, 37°C, and 39°C. Half of the cells were labeled with ³H-thymidine at 9 hrs p.i. and harvested at 24 hrs p.i. The other half were labeled at both 8 and 24 hrs p.i. (to ensure continuous labeling) and
harvested at 40 hrs p.i. The longer labeling period was used as the cells grown at 33°C may have slower growth kinetics and full expression may be delayed. Alkaline sucrose gradient sedimentation was performed with labeled DNA and the sedimentation profiles can be seen in Figs. 8, 9, and 10. The first figure shows profiles from cells grown at 33°C for 24 and 40 hrs, (Fig. 8:a,b). The profiles of both sets are similar and show no evidence of DNA degradation. The major peaks of labeled DNA from lp3 and lp5 (Fig. 8) cosediment with the viral marker DNA. The same pattern is observed with Ad2 wt infected cells (Fig. 8) and indicate that little or no degradation has occurred. However the profiles from 40 hrs p.i. (Fig. 8:b) show a larger proportion of label in the LMW DNA fractions. This is most likely due to the larger amount of cell death and subsequent degradation of cellular DNA that occurs at later times post infection. The viral DNA peaks in the profiles of the 40 hr infections are greater than those from 24 hrs, although similar TCA precipitable counts (cpm) of lysate were loaded per gradient. This is most likely due to greater viral DNA synthesis (and incorporation of label) over the longer time course.
Figure 8. Sedimentation profiles in 5 - 20% alkaline sucrose gradients of intracellular DNA from infected KB cells grown at 33°C. Sedimentation is from right (top) to left (bottom). m.o.i. = 200 virions/cell. Cells were harvested at 24 hrs (a) and 40 hrs p.i. (b). The arrows or smaller peaks indicate the position of the marker viral DNA (Ad2) peaks. Profiles are identified in the figure by the virus used.
Figure 9. Sedimentation profiles in 5 - 20% alkaline sucrose gradients of intracellular DNA from infected KB cells grown at 37°C. Sedimentation is from right to left. M.O.I.= 200 virions/cell. Cells were harvested at 24 hrs (a) or 40 hrs p.i. (b). Arrows or small peaks indicate the position of marker viral DNA (Ad2) peaks. Profiles are identified by the virus used. m indicates DNA from mock infected cells.
Figure 10. Sedimentation profiles in 5 - 20% alkaline sucrose gradients of intracellular DNA from infected KB cells grown at 39°C. Sedimentation is from right to left. M.O.I.= 200 virions/cell. Cells were harvested at 24 hrs (a) or 40 hrs p.i. (b). Arrows or small peaks indicate the position of marker viral DNA (Ad2) peaks. Profiles are identified by the virus used.
The lp-infected cells incubated at 37°C (Fig. 9) show DNA sedimentation profiles similar to that of the Ad2 wt infected cells, lp infected cells at 33°C (Fig. 8), and those seen previously (Fig. 7). This confirms earlier observations of the lack of DNA degradation activity at this temperature. Again there was a broadening near the base of the viral peak (forming a shoulder) in the profiles from lp infected cells at 40 hrs. This was not as apparent in the Ad2 wt infected cells. There may be a low baseline activity of DNA degradation in the mutant infected cells, or a slightly higher rate of cell death, although this is by no means extensive.

DNA degradation was evident in lp3 infected cells incubated at 39°C, showing that the mutation does seem to render the 19K protein temperature sensitive in its ability to prevent DNA degradation (Fig. 10). At 24 hrs p.i. there is a large peak of viral DNA cosedimenting with the marker DNA and an approximately equal sized peak of low molecular weight DNA (Fig. 10:a). By 40 hrs p.i., however, the peak of degraded DNA is much greater and broader than the viral DNA peak (Fig. 10:b). The degradation observed is comparable to that seen in cyt infected cells (Fig. 7: i,j) and Ad5 dl313 (Fig. 7: k). In contrast, Ad2 lp5 DNA profiles from infected cells incubated at 39°C are similar to that of Ad2 wt at this temperature, except for a rather large 'shoulder' of slower
migrating species at 40 hrs p.i. (Fig. 10). Again there maybe a basal degradation activity that may be slightly enhanced by the higher temperature and/or longer time course.

In summary, DNA degradation was observed in Ad2 lp3 infected KB cells at high (39°C) temperatures. This suggests that the E1B 19K product is temperature sensitive for the DNA protection function. No degradation was apparent in the Ad2 lp5 infected cultures under any of the conditions tested.

3.b. Viral yield

Viral yields were also determined from KB cell infections by the different lp mutants at the three temperatures used above. It was expected that the yield of the lp 3 mutant may be reduced at 39°C, due to virus DNA degradation, compared to those at 33 or 37°C.

Three yield determinations were done in the manner described earlier, using [3H]-labeled virions from infected cells labeled till 40 hrs (Table 2:a) or 24 hrs p.i. (Table 2:b, c). The labeled virions were mixed with at least 3 X 10^{11} unlabeled virions and centrifuged in a preformed 1.32 g/cm^3 to 1.34 g/cm^3 CsCl gradient. The viral band was collected from the bottom of the gradient. An aliquot was TCA precipitated and counted (by scintillation) to obtain a relative estimate
of yield (in cpm).

The yields obtained are quite variable, as the yield ratios for the three experiments show, but some general observations can be made. The lowest yield obtained was from lp3 infected cells incubated at 39°C which was 10 fold reduced compared to Ad2 wt at this temperature (Table 2, expt. c). However, the lp5 yield in this experiment was also low: 25% of wt. The average yield of lp3 virions was lowest at 39°C, although lp3 yields were generally lower than wt at all temperatures. The lp5 virus is quite viable at all temperatures tested as the average lp5 yield was higher than wt. Therefore the yield data correlates well with the observed sucrose gradient results. The lp3 virus causes degradation of virion DNA at 39°C, which results in lower yields of virus. The reduction in yields observed here does not seem as extensive as that observed with the cyt mutants (Takimori et. al. 1969; Ezoe et. al. 1981) but this may be due to differences in the methods used to assay virus yield.

These experiments show that at 37°C, the mutations in the lp mutants do not affect the 19 kDa function of preventing DNA degradation. The degradation of DNA in lp3 infected cells at 39°C does suggest that this mutant may produce a temperature sensitive product which allows degradation at elevated temperatures. Since the E1B 19 kDa protein
Table 2.
Comparisons of Ad2 lp and wt viral yields from infected KB cells incubated at different temperatures. Yields were determined by measuring the radioactivity (cpm), of \(^{3}\text{H}\)-thymidine labeled virions comigrating with an Ad2 virion marker band (unlabeled) in a preformed cesium chloride gradient. Infected cells were grown at 33°, 37°, and 39°C and harvested at 40 h p.i. M.O.I. was 200 virions per cell.

<table>
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<th>Temperature °C</th>
<th>Ad2 wt (Yield cpm X 10^4)</th>
<th>Ad2 lp3 (Yield cpm X 10^4)</th>
<th>lp/wt ratio</th>
<th>Ad2 lp5 (Yield cpm X 10^4)</th>
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Average yield ratios (+/- S.D.)

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<th>37°C</th>
<th>39°C</th>
</tr>
</thead>
<tbody>
<tr>
<td>lp3</td>
<td>0.83 +/- 0.64</td>
<td>0.88 +/- 0.48</td>
<td>0.37 +/- 0.21</td>
</tr>
<tr>
<td>lp5</td>
<td>1.3 +/- 0.85</td>
<td>2.24 +/- 1.33</td>
<td>1.29 +/- 0.74</td>
</tr>
</tbody>
</table>
is the only gene product known to be altered in the lp3 mutant, it would be reasonable to suggest that this is the thermosensitive protein. Another observation from this study was that DNA degradation does not necessarily lead to extensively reduced viral yields. This is shown more clearly in the next section in which an Ad2 mutant, dl250, which has a deletion within the 19 kDa protein - encoding sequences, is assayed for its ability to degrade DNA and ability to produce virus.

4. DNA degradation and viral yield analysis of Ad2 dl250

4.a. Alkaline sucrose gradient sedimentation analysis

Ad2 dl250 is a deletion mutant constructed by the deletion of a 145 bp fragment from the Ad2 E1B 19 kDa protein coding region (Subramanian et. al., 1984). DNA from KB cells infected by this mutant was assayed by alkaline sucrose gradient sedimentation to determine what effect a lack of E1B 19 kDa product would have on DNA integrity.

KB cells were infected with Ad2 dl250 or Ad2 wt virus in a manner identical to that used for the above lp experiments. A typical gradient profile from continuously labeled (from 8 to 24 hrs) infected cells is shown in Figure
Figure 11. Alkaline sucrose gradient profiles of DNA from KB cells infected by Ad2 wt or d1250. Cells were labeled with \(^{3}H\)-thymidine from: A) 8 to 25 h p.i. or B) 18 to 19.5 h p.i. and chased a further 5.5 h with unlabeled thymidine. The top of each set of figures show DNA profiles from wt infected cells and the bottom show DNA profiles from d1250 infected cells. Arrows indicate the position of the viral marker DNA peak. m.o.i. was 200 virions/cell.
11:a. The top profile is from wt infected cells, the bottom from dl250 infected cells. Intracellular DNA from Ad2 dl250 infected cells exhibited a profile very similar to that of the cyt mutants (Fig. 7: i,j). Along with a peak of viral DNA there was a larger, broader peak of LMW DNA. A second pulse-labeled sample shows a sedimentation profile quite similar to the first (Fig. 11: b). In both cases the Ad2 wt virus displayed a single large viral DNA peak. Therefore, the dl250 mutant does degrade intracellular DNA in infected KB cells. In both profiles shown above there is also a prominent peak of viral genome sized DNA. This would suggest that yields of this virus may still be quite high despite the DNA degradation. This was examined next.

4.b. Viral yield

Viral yields were determined from two dl250 infections in a manner similar to that of the lp yield determinations. Extracted, labeled virions were banded together with unlabeled marker virions in a preformed CsCl gradient. The resulting visible band was collected in 5 drop fractions and the total peak fractions were taken as an estimate of the yield. The TCA precipitable counts (cpm) per fraction were plotted to get an idea of the distribution of the virions within the visible
Figure 12. Ad2 d1250 Viral Yield
Infected KB cells were labeled with $[^3H]^{-}$thymidine, lysed and then layered on top of a preformed CsCl gradient (of density 1.32 to 1.35). Radioactivity (cpm) was determined from 5 drop fractions of the gradient in the region of a visible Ad2 viral band. The value determined from the total area of the resulting peak was used as a relative estimate of viral yield. These are shown in Table 3. The peaks obtained for each virus in separate but identical gradients were superimposed in this figure.
Table 3.
Ad2 d1250 Viral Yield

<table>
<thead>
<tr>
<th>Virus</th>
<th>Area of peak*</th>
<th>Relative Yield</th>
</tr>
</thead>
<tbody>
<tr>
<td>Ad2 wt</td>
<td>0.31</td>
<td>1</td>
</tr>
<tr>
<td>Ad2 d1250</td>
<td>0.12</td>
<td>0.39</td>
</tr>
<tr>
<td>Ad2 wt</td>
<td>0.36</td>
<td>1</td>
</tr>
<tr>
<td>Ad2 d1250</td>
<td>0.195</td>
<td>0.54</td>
</tr>
</tbody>
</table>

Average relative yield of d1250: 0.47

* The areas were determined by cutting, from a piece of paper, plots obtained from each viral band and measuring their weight, in grams.
marker bands. In two experiments (Fig. 12 and Table 3), the yield of Ad2 d1250 was about 47% that of the wt viral yield, based on the total area under the peaks. The virions had a similar distribution in both wildtype and mutant profiles, suggesting that there is not an overabundance of defective or incomplete virions in the mutant samples. Although the d1250 viral yields were reduced compared to wt yields, there still remained a substantial (47% of wt) amount of intact virions. This correlates well with the observation made from the sucrose gradient data in that there was still a large amount of intact virion DNA present. The yields of the d1250 mutant were also very similar to those obtained from the Ad2 lp mutants (Table 1).
B. ANALYSIS OF CV1 CELLS INFECTED BY Ad12.

1. DNA degradation in Ad12 infected CV1 cells

Ad12 infection of the non-permissive cell type, CV1 (an established monkey cell line), results in a dramatic cytopathic effect (CPE), which resembles the cytocidal phenotype produced by the Ad12 "cyt" mutants on human cells (my unpublished observations). The CPE seen in CV1 cells after Ad12 infection is characterized by extensive cellular destruction by 24 to 36 hrs after infection, resulting in a large amount of cellular debris present in the infected CV1 cultures. In contrast, human KB cells infected by Ad12 round up and start lifting at this time but remain intact. Additionally, infected CV1 cells remain single, upon lifting, rather than clumping into the cell aggregates characteristic of infected human cells.

The enhanced CPE produced by Ad12 in CV1 cells suggested that perhaps E1B was not being fully expressed in these cells. Support for this idea comes from several sources. Analysis of hamster embryo fibroblasts, another non-permissive cell type, infected by wt Ad12 showed the extensive
DNA degradation typical of the adenovirus "cyt" mutants (Lai Fatt and Mak, 1982). It was suggested that the observed phenotype was due to incomplete expression of the Ad12 E1B region (Lai Fatt and Mak, 1982; and references within). Also, the Ad12 "cyt" mutants and the Ad2 mutant d1250 have defects in the E1B regions which are responsible for the observed cytopathic effect and DNA degradation in human cell cultures (Lai Fatt and Mak, 1982; Schaller, 1990; Chinnadurai, 1983; Subramanian, et.al., 1984, this study). It was therefore of considerable interest to the objectives of this thesis to determine if DNA degradation was also a factor in the CV1 infections.

1.a. Analysis of DNA degradation with agarose gels

One of the most frequently used assay systems to determine DNA degradation in infected cells is the analysis of DNA extracted by the modified "Hirt" procedure on agarose gels (as described in Materials and Methods). The size of the DNA in the running samples is analyzed on agarose gels and visualized by ethidium bromide staining. Figure 13a shows the results from CV1 and KB cells infected at a m.o.i. of 1200 viral particles per cell and harvested at 20, 28, and 44 hrs p.i. Mock infected cells were harvested at 24 hrs p.i. DNA
Figure 13. a. DNA degradation assay of low molecular weight DNA from modified 'Hirt' extracts obtained from Ad12 infected CV1 and KB cells. Equal volumes of lysate (from 4 x 10^5 cells) were loaded on a 1% agarose gel in 1X TBE and electrophoresed at 30 V for 18 hrs. The gel was then stained with 0.5 ug/ml ethidium bromide (EtBr) and photographed. The first 4 lanes are from CV1 cells: mock infected (lane C) or infected by Ad12 wt and harvested at 20, 28, and 44 hrs p.i., as indicated. The next 4 lanes are from similarly infected KB cells. Lane m is a Hind III digested Ad12 DNA (strain 3569) marker. The 6 lanes on the right are identical lysates from infected CV1 and KB cells, but digested with Hind III after one phenol extraction and ethanol precipitation.

b. Similar DNA degradation assay as in (a) but 5 ug of DNA (as determined by uv adsorbance at 260 nm) from each lysate was used rather than equivalent volumes. The first 5 lanes are from uninfected (lane C) or infected CV1 cells harvested at 9, 24, 34, and 45 hrs p.i., as indicated. The next five are from similarly infected KB cells. Marker (m) is from Eco RI digested Ad12 DNA.
degradation, as indicated by a broad smear of low molecular weight DNA throughout the lane, can be seen as early as 20 hrs p.i. but is evident at 28 and 44 hrs p.i. in the infected CV1 cells. The mock CV1 and infected KB controls showed little or no evidence of degradation (Fig. 13:a). The prominent band seen in all lanes is cellular or mitochondrial DNA as it is present in both infected and mock-infected CV1 and KB cells. The Hind III digested extracts, seen in the second half of the figure, show that although DNA was being degraded there was still intact viral DNA as late as 44 hrs p.i. In this experiment described above equal volumes or cell equivalents were used for analysis. Lysates containing equivalent amounts of DNA (as determined by spectrophotometry) were also loaded on similar gels and analysed. This was done to confirm that the amount of DNA in the cell-equivalent samples was approximately the same for each sample. Figure 13:b, an assay using 5 ug of purified DNA from infected CV1 and KB cells, shows that degradation occurred in infected CV1 cells at 34 and 45 h p.i. with no corresponding effect in the Ad12 infected KB cells.

Several other experiments were performed to determine whether multiplicity of infection (m.o.i.) had
an effect on DNA degradation (not shown). They indicated that DNA degradation occurs when the virus was added as low as 300 virions per cell, or as high as 4000 virions per cell. Subsequent infections were usually done at m.o.i.'s of 1000-1500 virions per cell, as more than 90% of the CV1 cells showed evidence of infection at these higher multiplicities (personal observation).

1.b. Analysis of degradation by alkaline sucrose density gradient centrifugation

The degradation of DNA in CV1 cells infected by Ad 12 was confirmed using 5%-20% alkaline sucrose gradients in the same manner as they were used to study the Ad2 lp mutants in the previous section (Part A, Results). Infected CV cells (at an m.o.i. of 1200 virions/cell) were labeled with [3H]-thymidine, either continuously from 21 to 40 hrs p.i., or as a pulse for 1 hr at 28 hrs p.i. followed by a chase with excess cold thymidine (2ug/ml) to 40 h p.i. The results shown in Figure 14 indicate a relatively small peak of intact viral length DNA and a large broad peak of low molecular weight (degraded) DNA, especially with the continuously labeled DNA. The cause of the relatively smaller viral peak in this profile (Fig. 14:a) compared to that in the pulse-labeled DNA profile
Figure 14. Sedimentation profiles of intracellular DNA from Ad12 infected CV1 cells, in 5-20% alkaline sucrose gradients. Sedimentation profile is from right (top) to left (bottom). m.o.i. = 1200 virions/cell. Arrows indicate the position of the marker viral DNA peak.

Panel A. Cells continuously labeled from 21 - 40 hrs p.i.

Panel B. Cells labeled from 28 to 29 hrs p.i. and then chased until 40 hrs p.i. with excess unlabeled thymidine.
(Fig. 14:b) is not known, but may reflect a loss of incorporation of \(^{3}H\)-thymidine late in the infection. The pulse label may indicate that complete viral genomes are first synthesized, and subsequently degraded, as with the Ad12 cyt mutants (Ezoe et. al., 1981).

To demonstrate this, intracellular DNA was labeled from 12 to 14 hrs p.i., and followed by a chase of fresh medium containing an excess (2 ug/ml) of unlabeled thymidine until 36 hrs p.i. The cells were then harvested at the end of the labeling period (Fig 15:a) or following the chase period (Fig 15:b). Complete viral genomes, generating the peak of viral sized DNA, were first synthesized (Fig. 15:a) and then are subsequently degraded (Fig. 15:b). In the experiment shown, the degradation does not seem to be as extensive as that shown previously, but does show some evidence of degradation. A typical profile was produced from KB cells infected by wt Ad12, which display little or no degradation (Fig. 15:d). An Ad12 infection of CV1 cells was also labeled continuously from 12 to 34 hrs p.i. This produced a profile which displayed both a distinct peak of intact viral DNA and a large amount of lower molecular weight DNA (Fig. 15: c). The results of both types of labeling conditions indicate that degradation does occur in CV1 cells infected by Ad12. The
Figure 15. Sedimentation profiles of intracellular DNA from infected CV1 and KB cells, in 5-20% alkaline sucrose gradients. Sedimentation is shown from right (top of gradient) to left (bottom). CV1 cells were labeled from 12 to 14 hrs p.i. and chased in the presence of unlabeled thymidine for 0 (a) and 22 (b) hrs, at which time the cells were harvested. Infected CV1 cells were also labeled continuously from 12 to 34 hrs p.i. (c), as were infected KB cells (d); m.o.i. = 1200 v/cell. Arrows indicate the position of the viral marker DNA (Ad2) peaks.
possible cause for this was explored by assaying Ad12 early gene expression in the CV1 cells.

2. Early gene expression in Ad12 infected CV1 cells

2.a. Early gene region E1 protein expression

Evidence cited in the Introduction suggests that region E1B may not be fully expressed in some non-permissive infections, thus leading to the degradation phenotype. To determine if this might be the case in the Ad12 infected CV1 cells, [³⁵S]-methionine labeled lysates were analysed by immunoprecipitation with E1 specific antisera.

CV1 and KB (control) cells were infected with Ad12 (1000 virions/cell) and labeled for 4 hours at 20 to 24 hrs post infection, following a 1/2 h preincubation in medium lacking methionine (met-). Lysates containing equal amounts of TCA-precipitable counts (cpm) were immunoprecipitated by AB6a sera, which recognizes Ad12 E1 tumour antigens (E1A and E1B proteins).

Figure 16 demonstrates that very little or no 19 kDa protein is evident in the infected CV1 cells at 24 hrs p.i. In this experiment, a large difference in the amount of immunoprecipitable 55 kDa products (a combination of E1A and E1B large 55 kDa proteins) is also seen between the two cell
Figure 16. SDS-PAGE analysis of Ad12 infected and mock infected CV1 and KB cells. Cells were labeled from 20 to 24 hrs p.i. after 0.5 h preincubation in media. Equivalent amounts (cpm) of lysate were immunoprecipitated with AB6a and analysed. Marker protein sizes are shown and the E1 55 kDa and E1B 19 kDa products are indicated by arrows. The CV1 and KB lysates were loaded onto the same gel and exposed to the same X-ray film for an identical time (3 days).

Cm: mock-infected CV1 cells
Cl2: Ad12 infected CV1 cells
M: protein size markers (indicated in kilodaltons (K))
Km: mock-infected KB cells
Kl2: Ad12 infected KB cells
E1B large 55 kDa proteins) is also seen between the two cell types at this time. However, this observation was variable between experiments, as demonstrated below, and may reflect alterations in the E1B 55 kDa protein level.

In permissive systems, E1A is expressed very early in infection and reaches a maximum at intermediate times after infection, 10-14 hrs, and then declines after DNA replication (Saito et. al., 1980). The E1B 55 kDa protein however does not accumulate as early, becoming detectable at intermediate times after infection and remaining relatively high even late in infection (Saito et. al., 1980). In CV1 cells it is possible that the E1B 55 kDa protein may not be expressed while the E1A 55 kDa protein is expressed. Since there was no available serum which detects only the Ad12 E1B products, the E1B-55 kDa protein levels were estimated by comparing the protein immunoprecipitated by a total E1 (E1A and E1B) serum (AB6a) and that immunoprecipitated by an E1A-only serum (A7Ra).

The immunoprecipitation described above was performed using labeled cells harvested at 11 and 26 hours post infection. Lysates containing equal amounts of TCA-precipitable counts (cpm) were immunoprecipitated with either AB6a (E1) or A7Ra (E1A) serum as seen in Figure 17. Approximately the same amount of 55 kDa product(s) were immunoprecipitated by both AB6a and A7Ra sera from the CV1
Figure 17. a. SDS - PAGE analysis of immunoprecipitates obtained from Ad12 infected and labeled CV1 and KB cells. Overexposure of the first four lanes of (b). This indicates that no 19 kDa product (which in this gel system runs at or slightly below the 18.4 kDa marker) is produced in Ad12 infected CV1 cells.

b. SDS - PAGE analysis of immunoprecipitates obtained from infected and labeled CV1 and KB cells. Cells were harvested at the times (in hours) indicated (above each lane) after a 4 hr labeling period, with C and K indicating CV1 or KB cells, respectively. Cm and Km refer to mock infected CV1 and KB cells, respectively. Equivalent amounts of labeled lysate (cpm) were immunoprecipitated with either AB6a (detects complete E1) or A7Ra (detects only E1A) antisera. The arrow indicates the E1A and E1B 55 kDa products. E1B 19 kDa can only be detected in the infected KB cells at 26 hrs (K26: immunoprecipitated by AB6a). The molecular weight of marker proteins (M) are indicated in kDa at the sides of panel B. The E1B 55 kDa product is indicated by the black triangle next to the K26 lysates immunoprecipitated by AB6a. The open triangle next to this lane indicates the migration position of the E1B 19 kDa protein.
infected cell lysates (Fig. 17).

The amount of 55 kDa product brought down by AB6a at 11 hrs p.i. was equivalent to that brought down by A7Ra at this time (as determined by densitometry). At 26 hrs, the amount of 55 kDa product immunoprecipitated by both antisera was less than at 11 hrs p.i., consistent with the kinetics of E1A expression. With the infected KB cell lysates, the situation was the same at 11 hrs: the amount of 55 kDa immunoprecipitated by A7Ra was equivalent to that detected by AB6a. However, at 26 hrs p.i., the amount of 55 kDa detected by AB6a was much greater than that detected by A7Ra, reflecting an increase in E1B 55 kDa at later times during infection. This was not seen in the CV1 cells infected by Ad12 at 26 hrs p.i.

Again the E1B 19 kDa protein was present in significant amounts only in KB cells and primarily at 26 hrs p.i. (Fig. 17:b). Even with an over-exposure of the CV1 lysates immunoprecipitated by AB6a, no 19 kDa product was evident (Fig. 17:a).

The level of E1A 55 kDa product, immunoprecipitated by A7Ra, seems greater in the infected CV1 cells at 11 hrs p.i. than in the corresponding KB cells (Figure 17). At 26 hrs p.i. there was little difference between levels of E1A 55
kDa in the two cell types. This may suggest that E1A was expressed at a higher level early in the CV1 infection.

During the course of this study, M. Schaller, a graduate student in our laboratory, (Dept. of Biology, McMaster University; Ph.D. Thesis, 1990) determined that the E1A 55 kDa protein band could be separated from the E1B 55 kDa product by changing the gel conditions from that used routinely in our laboratory (15.2% polyacrylamide, and a 157.9:1 acrylamide to bis ratio) to that described by Laemmli, 1970 (12.5% polyacrylamide in a 37.5 :1 acrylamide to bis ratio). This change altered the migration positions of the E1A (55 kDa) proteins to positions of approximately 41 kDa and 38 kDa, relative to the marker proteins, while the E1B 55 kDa product remains at the usual (55 kDa) position (M. Schaller, 1990). The labeling time was also reduced to 1 hr to minimize the effect of the short half life of the E1A proteins on levels of accumulation (35-55 minutes is the half life of Ad5 E1A according to Branton and Rowe, 1985). Again the lysates (at equivalent cpm) were immunoprecipitated with either AB6a or A7Ra. The results (Fig. 18) indicate that A7Ra immunoprecipitates (E1A) are are detected at levels slightly higher in CV1 cells at 7.5 hrs p.i. than that in KB cells at this time. The lower E1A band (38 kDa) was not distinct in both KB and CV1 infections at this time.
Figure 18. SDS-PAGE analysis of immunoprecipitates from Ad12 infected CV1 and KB cells. These cells were labeled for 1 hr prior to harvest, following a 0.5 hr preincubation in media.

Infected cells were harvested at 7.5 hrs or 24 hrs p.i. Mock-infected cells (lanes Cm or Km) were harvested at 24 hrs p.i. The polyacrylamide concentration was reduced compared to the earlier gels (12.5 as opposed to 15.2%) and the acrylamide to bis ratio was also reduced (see text). Equivalent lysates (cpm) were immunoprecipitated with either AB6a, A7Ra, or NR sera, as indicated. The E1B 55 kDa band is indicated by the circle next to the K24 lane immunoprecipitated by AB6a. The E1A specific products which now run at 38 to 40 kDa are indicated by the open triangles. The short labeling time drastically reduces the labeling of the 19 kDa in the KB cells.

The identity of the 79 kDa band seen prominently in the K24 lane has not been ascertained but it is most likely due to cross reaction of the antisera with the Ad12 hexon protein: this and other E1-antisera used in our laboratory precipitated other viral proteins especially at later times p.i. (S. Mak, personal communication).
The E1B 55 kDa was detectable only in infected KB cells at 24 hrs p.i., (at dot in Fig. 17) but not in infected CV1. The 19 kDa protein was not clearly visible in either cell type but this is may be a result of the shorter labeling time.

The preceding results show that Ad12 E1B is expressed only at very low levels in the non-permissive CV1 cells while the Ad12 55 kDa E1A protein is expressed at slightly higher levels in CV1 cells than in infected KB cells early in infection. These results, together with those from the previous autoradiographs suggest that DNA degradation in CV1 cells may result from a deficiency of E1B products. The levels of the 19 kDa product may be more important in this phenotype as the E1B 55 kDa product has never been implicated in the regulation of the DNA degradation phenotype (Babiss et.al., 1984b; Pilder et. al., 1986). The degradation phenotype seen in Ad12 infected CV1 cells is similar to that of 19 kDa protein-negative mutants of Ad2 and Ad12 in KB cells. These mutants express the E1B 55 kDa protein (Subramanian et.al., 1984; Pilder et.al., 1986; White et.al., 1986; Schaller, Ph.D. Thesis, McMaster University, 1990). Therefore it is possible that lack of the E1B 19 kDa protein may be the cause of the DNA degradation phenotype in the infected CV1 cells.

Protein expression of the other early regions: E2, E3, and E4 could not be determined due to the lack of specific
antisera. It is not known if any of these other regions are also involved in the DNA degradation phenotype. The next step taken was to analyze the levels of mRNA expression from the other adenoviral early regions. The relationship between mRNA levels and protein synthesis was estimated by comparing E1 mRNA expression with the protein results (Figs. 16 -18).

2.b. Expression of Ad12 early regions: RNA accumulation

2.b.i. Quantitation of RNA by slot blot analysis

To measure RNA accumulation, cytoplasmic or total cellular RNA was extracted from infected and mock infected cells by the guanidium isothiocyanate procedure described in Materials and Methods. Typically RNA was extracted from cells harvested at two or more time points throughout the infection. After the amount of RNA was measured by spectrophotometry, 3 to 4 dilutions of each sample were spotted on nitrocellulose filters and probed with $^{32}$P-labeled probes specific for the region of interest. The resulting bands on autoradiographs were measured by densitometry and the areas determined from the integrated optical densities of each peak (slot) were
plotted vs ug RNA. The slopes of each sample were used as a measure of RNA accumulation.

Expression from both cell types was estimated by measuring the optical density of bands formed on autoradiographs after identical exposure times of slots on X-ray film. This allowed comparison of the kinetics of accumulation between the two cell types. Because the specific activity and length of the probes varied, direct comparisons of relative accumulation between early regions could not be made. But comparisons between identical regions in the two different cell types could be made as they were probed at the same time with the same probe. E1A expression was used as an internal reference point to normalize the results in the case of cytoplasmic RNA. The E1A gene is the first region to be expressed upon infection and is independent of the expression of the other early regions (Grand, 1987). The previous protein data also suggested that E1A was expressed at levels equivalent to that in the permissive (KB) cells.

In all the experiments presented below, RNAsed treated samples were probed at the same time and showed that there was little or no detectable contaminating DNA. This correlated well with results obtained from test samples (centrifuged through guanidium-CsCl gradients) containing labeled DNA, which indicated that less than 1% of added DNA was recovered
in the collected RNA samples (S. Mak, Dept. of Biology, McMaster University, personal communication).

Immobilized RNA samples were probed with radiolabeled DNA specific for regions E1A (probe pHa5), E1B (pHB14R and pHB15R), E2A (pH118BC-r), E2B (pH118HB-r), E4 (M13mp18/E4r) and L4 (pHB118HA-l).

In the one cytoplasmic RNA experiment shown (Fig. 19), Ad12 infected CV1 and KB cells were harvested at 10, 24, and 34 hrs postinfection, and RNA was extracted to obtain a time course of RNA accumulation.

The E1A levels in both CV1 and KB infections were approximately the same at all times tested, the difference not being more than 1.6 fold (as determined by densitometry). E1A in the infected CV1 cells was 0.6 fold less than that in KB cells at 10 hrs p.i., and this ratio was reversed by 24 hrs p.i. (Fig. 19). The difference was less significant by 34 hrs p.i. This supports the assumption made earlier that E1A levels in both cell types are similar.

Based on the above data that shows E1A expression is equivalent in the two types of cells, the expression of the other early gene regions were determined in relation to this. This provides a value of expression "normalized expression" for each region in relation to E1A expression in the same sample. The relative values obtained from each region in KB
infected cells were then compared to the values obtained from each region in CV1 infected cells. Therefore the numbers shown in Table 4:B are relative values of gene expression rather than absolute. From these values we can determine general trends in gene expression, rather than an absolute or specific increase in expression. The more appropriate control, a cellular gene that was unaffected by adenovirus infection and expressed similarly in the two cell types, was not available.

The E1B RNA level in CV1 cells at 10 hrs p.i. was too low to accurately quantitate. At 23 hrs, just before DNA degradation is readily observable, two gene regions: E1B and L4 showed the greatest difference, with respect to E1A expression, in expression between the two cell types (Table 4). The other early regions tested at this time: E2A, E2B, and E4, were moderately underexpressed (relative to E1A) in infected CV1 cells compared to infected KB cells.

At 34 hrs p.i. the E1B region again showed the greatest difference in relative expression between the two cell types, suggesting a rather large decrease in amounts of detectable E1B RNA in the infected CV1 cells (Table 4). The differences seen in three other regions: E2A, E2B, and L4 also became quite substantial at this point. The relative amount of E4 expression was least affected in the CV1 infections, compared to expression in Ad12 infected KB cells.
Figure 19. a. Cytoplasmic RNA from infected CV1 and KB cells assayed by slot blot analysis. RNA was obtained from infected CV1 cells after 10, 23, 34, and 44 hrs p.i. and from infected KB cells 10, 23, and 34 hrs p.i. RNA from mock infected cells (m) harvested at 24 hrs p.i. was also assayed. Aliquots of 2, 4, and 6 ug RNA were spotted from each sample, as indicated. \[^{32}P\]-dCTP probes used were: phA5 for E1A, pHBl4R and pHBl5R for E1B, pHBl18 BCr for E2A, pH118 HBr, pMl3E4 for E4, and pH118 HAl to detect L4 transcripts. RNase treated controls were probed with all plasmids but no or very little hybridization was seen. The E2B probed controls are shown as an example. In this blot, row a contains the same three samples as the last row in the E2A filter: KB 24 hr RNA, untreated. The other slots in this panel are as follows: b1-CV1 mock, b2-CV1 10 h, b3-CV1 23 h, c1-KB mock, c2-KB 10 h, c3-KB 23 h, d1-CV1 34 h, d2-CV1 44 h, e1-KB 34 h, e2 and e3 are from COS cells and a second CV1 infection (24 h) respectively. Rows f and g contain 2, 4, and 6 ug of tRNA untreated (f) or treated (g) with RNase.
Table 4.
KB/CV1 RATIO OF RNA ACCUMULATION: RNA accumulation numbers (determined by densitometry) from each region have been normalized to E1A values obtained from identical samples of cytoplasmic RNA at the same time point (see text). The normalized results obtained from each region of the infected KB cells were then divided by the equivalent results from CV1 infected cells to determine relative expression levels in this experiment. The ratios have been rounded to the nearest whole number.

<table>
<thead>
<tr>
<th>Time p.i.</th>
<th>E1B</th>
<th>E2A</th>
<th>E2B</th>
<th>E4</th>
<th>L4</th>
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<tr>
<td>23 hrs</td>
<td>9</td>
<td>4</td>
<td>3</td>
<td>2</td>
<td>8</td>
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<tr>
<td>34 hrs</td>
<td>17</td>
<td>13</td>
<td>9</td>
<td>4</td>
<td>9</td>
</tr>
</tbody>
</table>

Relative expression of each gene region expressed as a KB to CV1 (KB/CV1) ratio of normalized (to E1A) expression.
Other experiments examining the accumulation of cytoplasmic RNA at 16 hrs and 24 hrs p.i. in this manner confirm that the ratios of expression of most Ad12 early regions (compared to E1A) are reduced to some extent in the infected CV1 cells compared to that determined for infected KB cells. The greatest difference between the two cell types at 24 hrs p.i. (at which time DNA degradation is evident) was in the E1B:E1A ratios. At later times, 24 hrs, it is still significantly reduced relative to expression in KB cells. Because the E1A levels at all times tested were approximately the same in the two cell types, the differences seen in expression of the other early regions would reflect a real decrease in cytoplasmic messages from most of these regions in infected CV1 cells.

This reduction in cytoplasmic mRNA levels may be enough to account, in part, for the reduction in E1B proteins in the CV1 system. The altered E1B to E1A mRNA ratios in the infected CV1 cells may eventually reflect an alteration in E1B protein levels compared to E1A products. Although the E1B/E1A protein ratios were not measured directly, the almost complete lack of 19 kDa product and low E1B 55 kDa protein levels in the infected CV1 cells (Figs. 16-18), would indicate that this ratio is less than the E1B/E1A mRNA ratio in these cells. This suggests that translational (or post-translational) events
regulating E1B expression may be more significantly different between the two cell types than earlier (transcription, mRNA transport, or mRNA stability) events. If the products of the E1B and E1A regions interact to regulate the prevention of DNA degradation (as suggested by White and Stillman, 1987), then it may be this alteration which leads to DNA degradation.

This difference on its own may lead to the DNA degradation phenotype, but the altered expression of the other regions may also be involved. As DNA degradation occurs in the absence of DNA replication (Pilder et al., 1984; White et al., 1984), and it occurs as early as 24 hrs p.i., involvement of an early gene region would be more likely than any of the late gene regions such as L4.

In a second experiment involving measurement of RNA expression, total cellular RNA rather than cytoplasmic RNA was analyzed (Fig. 20; Table 5). Expression of a cellular mRNA coding for human actin was also measured, as an additional internal control. Quantitation of actin expression by densitometric scanning showed that the levels of actin RNA were almost identical in both cell types at all times tested (9, 20, 32, and 48 hrs p.i.) (not shown). In both cell systems, actin levels were slightly enhanced by Ad12 infection at 9 hrs p.i., but were subsequently reduced to one quarter of the non-infected cell levels by 48 hrs p.i.
Figure 20. Total RNA from infected CV1 and KB cells assayed by slot blot analysis. RNA was purified from (m) mock infected cells (20 hrs p.i.) and infected cells harvested at 9, 20, 32, and 48 hrs p.i. These were probed with the same probes used earlier (as indicated) with the addition of pH18 Hbl for region L1 detection and a probe for the human alpha-actin message (obtained from R. Lee, Dept of Biology, McMaster University). 2, 4, and 8 ug of RNA were used from each sample for assay. As controls, Ad12 DNA was spotted on each filter, as well as DNase and RNased treated samples. Only those controls probed with actin and E1A are shown; no other RNased samples showed any response. Control samples are RNase treated: a1 - CV1 mock, a2 - CV1 9 h, a3 - CV1 23 h, b1 - CV1 32 h, b2 - CV1 48 h, b3 - KB mock, c1 - KB 9 h, c2 - KB 23 h, c3 - KB 32 h. or DNase treated: rows e, f, g, - same order as above Other samples on filters are as indicated.

The integrated optical densities were plotted as a function of RNA loaded. The slopes from the plots were used as a measure of RNA accumulation. Expression levels were then determined relative to actin or E1A levels.
**Table 5. Total Cell RNA Accumulation**

A. E1A RNA ACCUMULATION RELATIVE TO ACTIN EXPRESSION. The values of E1A expression determined by densitometry of Fig. 20 were compared to values obtained from identical samples probed with labeled actin DNA.

<table>
<thead>
<tr>
<th>time of harvest:</th>
<th>9</th>
<th>20</th>
<th>32</th>
<th>48</th>
</tr>
</thead>
<tbody>
<tr>
<td>E1A / Actin ratio</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>CV1 Ad12</td>
<td>0.1</td>
<td>0.21</td>
<td>1.55</td>
<td>2.78</td>
</tr>
<tr>
<td>KB Ad12</td>
<td>0.06</td>
<td>0.25</td>
<td>2.62</td>
<td>5.8</td>
</tr>
</tbody>
</table>

B. KB:CV1 RATIO OF RNA ACCUMULATION: RNA accumulation numbers from each region have been divided by actin values obtained from identical samples of cytoplasmic RNA at the same time point (see text). The normalized results obtained from each region of the infected KB cells were divided by the equivalent CV1 results to give relative expression levels.

<table>
<thead>
<tr>
<th>SAMPLE</th>
<th>REGION</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>E1A</td>
</tr>
<tr>
<td>20 hrs</td>
<td></td>
</tr>
<tr>
<td></td>
<td>1.2</td>
</tr>
<tr>
<td>32 hrs</td>
<td></td>
</tr>
<tr>
<td></td>
<td>1.7</td>
</tr>
<tr>
<td>48 hrs</td>
<td></td>
</tr>
<tr>
<td></td>
<td>2.1</td>
</tr>
</tbody>
</table>

C. KB/CV1 RATIO OF RNA ACCUMULATION NORMALIZED TO E1A: Expression values obtained by densitometry were determined in relation to E1A expression of the same samples. Comparisons between ratios obtained from infected KB cells and infected CV1 cells are shown. This data is thus similar to that in Table 4 (cytoplasmic RNA).

<table>
<thead>
<tr>
<th>SAMPLE</th>
<th>REGION</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>E1B</td>
</tr>
<tr>
<td>20 h</td>
<td></td>
</tr>
<tr>
<td></td>
<td>1</td>
</tr>
<tr>
<td>32 h</td>
<td></td>
</tr>
<tr>
<td></td>
<td>2</td>
</tr>
<tr>
<td>48 h</td>
<td></td>
</tr>
<tr>
<td></td>
<td>2</td>
</tr>
</tbody>
</table>
The E1A values determined as a fraction of actin levels (E1A/Actin) could be determined in this experiment (Table 5:A) and showed that the pattern of E1A expression (from total cell RNA) in the CV1 cells was similar to that detected in the cytoplasmic RNA analysis above (Fig 19). E1A expression is not more than 2 fold different between the two cell types infected by Ad12, at all times tested. In the infected CV1 cells E1A expression was higher, compared to that in KB cells, by a factor of 2 two fold at 9 hrs. By 48 hrs it was reduced to one-half that of E1A in infected KB cells: the KB to CV1 ratio was 2.1 (Table 5:B).

E1B expression in infected CV1 cells was barely detectable at 9 hrs p.i. It was equivalent to expression in infected KB cells at 20 hrs. At 32 hrs and 48 hrs p.i., E1B was expressed at a level 3 fold more in Ad12 infected KB cells than in infected KB cells. E2A and E2B expression was not significantly altered in the infected CV1 cells. E4 expression was most significantly altered in the infected CV1 cells. E4 was 7 fold greater at 20 hrs p.i. and about 4 fold greater between 32 hrs and 48 hrs p.i., in the infected KB cells compared to expression in infected CV1 cells.

The RNA values in this last experiment were also normalized with respect to E1A RNA, so the results of this experiment could be compared with the cytoplasmic RNA data
(Table 5:C), and the trend seen was similar to that obtained by normalization to actin. E4 was the only region which had a much higher expression ratio (6 fold) in the infected KB cells compared to equivalent CV1 infections, at 20 hrs p.i. (Table 5:C). At later times, E4 and E1B differed by only 2 fold between the two cell types, being greater in the infected KB cells. The other regions showed even less of a difference (between levels determined from the two cell types) when normalized to E1A.

The differences in the early region expression (relative to E1A expression) seen between the same regions in Table 4 and Table 5:C may be a consequence of the source of RNA examined. The fact that the RNA levels were determined from total cellular RNA in the latter experiment rather than cytoplasmic extracts, most likely made a significant difference in the relative accumulation detected. Indeed, unspliced and otherwise unprocessed RNA would be detected in assays of total cell RNA. The results obtained here suggest that there may be a defect in the transport of mRNA from the nucleus to the cytoplasm in the Ad12 infected CV1 cells. Alternatively, the stability of cytoplasmic mRNAs may be different in the two cell types. These defects may not be as evident in the assay of total cell RNA as it would be with cytoplasmic RNA.
2.b.ii. Qualitative analysis of Ad12 RNA species produced during infection of CV1 cells: Northern Blot analysis.

In addition to assaying RNA expression via slot-blotting, RNA was also analyzed by Northern blotting. While slot blots gave quantitative data on the amount of RNA being expressed from each specific region, they did not provide information on the number and size of mRNA species being produced. Many regions of the adenovirus genome code for a number of mRNAs differing in their start sites, splicing patterns and reading frames (see Review: Tooze, 1981). During infection there are also shifts from one type of message to another within the same regions, in response to various temporal regulatory factors. To determine if there is a difference in size or number of messages being expressed in infected CV1 cells, as compared to that in KB cells, (in addition to differences in total RNA synthesis) Northern blot analysis was utilized. Cytoplasmic RNA was collected from infected cells at various times after infection and purified as described previously. RNA from each sample was denatured, loaded on formaldehyde containing agarose gels and blotted to nitrocellulose after 3-4 hrs of electrophoresis at 80 V as described in Materials and Methods. Multiple gels were run simultaneously with
identical aliquots of the same RNA samples to allow for probing with radiolabeled \( ([^{32}P]-dCTP) \) DNA specific for the various Ad12 gene regions, as with the slot blots.

Equivalent amounts of RNA were loaded per lane (the concentration of RNA being determined by optical densitometry at a wavelength of 260nm), and this was confirmed by methylene blue staining of the blots after autoradiography. This staining detects mainly the position and amount of ribosomal RNA present.

In the blots shown, cytoplasmic RNA was obtained from CV1 and KB cells infected by Ad12 and harvested early (10 hrs) or late (26 hrs) after infection (Fig. 21). The E1A mRNAs (1.2 - 1.3 kb) levels were equivalent in both cell systems at both early (10 hrs) and later (26 hrs) times after infection (Fig. 21). This is in agreement with the earlier slot blot data. A larger, 2.5 to 3 kb, RNA product can be detected also. This product probably corresponds to a large 3 kb "readthrough" transcript, which originates at the 5' end of E1A and terminates at the 3' end of E1B (Saito et.al., 1981). This product was also seen in the E1B blots (Fig. 21: E1B).

The E1B blot demonstrates that there was no detectable E1B expression in the CV1 infections at 10 hrs p.i. At 26 hrs p.i., two RNA species, 0.9 kb and 2.2 kb, were expressed in the Ad12 infected CV1 cells, in addition to the larger
readthrough species, but a 1.3 kb message was not present at
the same level as the others. These three additional messages
correspond to the 22S, 13S, and 9S messages known to be
encoded from this region (see Fig. 3). The 22S message codes
for both the 55 kDa and 19 kDa proteins (in different reading
frames), the 13S for the 19 kDa product, and the 0.9S for a
structural protein (IX). In the Ad12 infected CV1 cells, all
four messages were not expressed to the degree that they were
in the KB cells at the same times (Fig. 21: E1B). Early in the
KB infections only the 2.2 kb and 0.9 kb bands are prominent,
but all the messages accumulate significantly by 26 hrs (Fig.
21). Therefore E1B is either underexpressed or expression of
this region was greatly delayed in the infected CV1 cells. The
ratio of 13S message to any of the other E1B products is also
lower in the infected CV1 cells than in infected KB cells.
Most of the differences detected between E1B expression in the
two cell types earlier (cytoplasmic RNA slot blot:Table 4) may
be a consequence of reduced cytoplasmic levels of the 1.3 kb
message.

The E2 (A and B) regions in these blots were not too
clear but showed similar patterns in both cell types, with
some exceptions (Fig. 21). The E2A blot showed a 1.8 - 2 kb
message, plus a faint, larger message (greater than 2.1
kb), in the infected CV1 and KB cells.
Figure 21. Northern blot analysis of cytoplasmic RNA from Ad12 infected CV1 and KB cells. Infected cells were harvested at 10 and 26 hrs p.i.; mock infected cells were harvested at 26 hrs p.i. 15 micrograms of RNA was loaded per lane per sample. Staining of the blots after autoradiography with methylene blue confirmed that identical amounts of RNA were present in each lane. Autoradiographs of blots probed for E1A and E1B are shown both in a short exposure, panel A, or in a long exposure of the labeled blot to film, panel B. Marker is Rsa I digested pBR322 DNA which gave fragments of approximately 2.1 kilobases (kb), 1.6 kb and 0.7 kb. The other 4 panels show autoradiographs obtained after hybridization of similar blots with labeled DNA specific for regions E2A, E2B, E3, and E4, as indicated. E4:b is a lighter exposure of the 26 hr infected KB RNA.

m - mock infected lanes
M - Marker DNA

The numbers along the side refer to the sizes of marker DNA fragments, as measured in kilobases (kb).
The 1.8 kb product is most likely the message for DNA binding protein (DBP), as S1 mapping has shown that it is encoded by a 1630 nucleotide message (Saito et. al. 1981). The larger product may be a E2B message which has overlapping promoter and leader sequences. The E2B messages have not been accurately mapped or identified for Ad12. The E2B blot showed this same message again plus a smaller 1 kb message (Fig. 21: E2B). This latter product was seen early in the infected KB cells, and possibly present in both cells at 26 hrs p.i. (Fig. 21). A smaller (less than 0.5 kb) message is also detectable late in both infections. The levels of expression of these and the other early gene regions were generally lower in the infected CV1 cells than in the corresponding KB infections at both time points (10 and 26 hrs p.i.). This is similar to the trends noted in the cytoplasmic RNA slot blots (Fig. 19; Table 4): reductions in the early region/E1A ratios for most early regions in infected CV1 cells. However, the expression detected in the Northern blots was not measured nor compared to an internal control to determine actual levels of expression. Indistinct bands and "smearing" of radiolabel in many lanes made measurements difficult, if not impossible.

Two major bands were detected by the E3 probe in the CV1 RNA. A 3 kb and 2.0 kb message predominate (Fig. 21: E3), most likely corresponding to the major messages of 3.2 kb and
2.0 kb noted by Saito et al. (1981). The pattern in KB cells is slightly different, with the appearance of two smaller products, 1.6 kb and 1.0 kb, at 26 hrs p.i. (Fig. 21).

In the E4 blot of Fig. 21, a 2.1 kb band and a 1.8 kb band were present in the CV1 cells at 26 hrs p.i. These two messages were seen as early as 10 hrs p.i. in the infected KB cells, along with a smaller (approx. 1.7 kb) message not seen in the CV1 infection. A lighter exposure of the KB 26 hr sample shows the presence of yet another smaller message (1 kb). The 2.1 kb mRNA species probably corresponds to the major species detected by Saito et al., 1981. The identity of the other messages is not known as the Ad12 E4 region has only recently been sequenced (Hogenkamp and Esche, 1990).

Expression of early region mRNA from these Northern blots and from others (not shown) were not too different in Ad12 infections of the two cell types, other than a general reduction of expression from most early regions. The major detectable changes in RNA patterns in the nonpermissive system include a reduction, or delayed expression, of the levels of E1B messages, with the most pronounced decrease involving the 1.3 kb message. Additionally, there was a reduction or disappearance of possibly two E4 products. Of course there may have been other changes in minor RNA species that were undetectable by this analysis. It is not known what effect the
reduction of the E2B message may have on expression. E2B in Ad5 and Ad2 is known to code for the proteins needed for DNA replication: the DNA polymerase and the terminal protein. E2B of Ad12 has not been fully sequenced nor characterized, but it is highly probable, due to the extreme similarity between Ad12 and Ad2 or Ad5 E1 regions, that it is organized in the same manner as in these latter adenoviruses. If DNA replication (synthesis) is relatively normal in the CV1 cells, this would indicate that E2B expression can not be severely altered. Similarly, expression of region E2A, which encodes a 72 kDa DNA binding protein, is also necessary for DNA replication. Alterations in its expression should be reflected in altered DNA replication. Therefore, this was examined next.

3. DNA replication

To ensure that apparently reduced levels of early mRNA in Ad12 infected CV1 cells was not due to lack of DNA replication (and thus lower viral genome copy numbers), DNA levels from various experiments were assayed by slot blot analysis and measured (in the same manner as with the RNA analysis). The slopes used to estimate relative DNA levels were obtained from the initial slope of each plot, before the points plateau. The 40 hr time point curves (for both cell
types) were not used for measurements due to the non-linear film response to the intense radioactive label. The viral DNA levels in the infected CV1 cells at 16 and 24 hrs were almost two fold greater than that in infected KB cells (Fig. 22; Table 6). Another DNA assay showed that at times ranging from 4 hrs p.i. to 34 hrs p.i., there was no greater than a two fold difference in viral DNA levels between infected KB and CV1 cells (Table 6).

On the basis of these and other slot blots which were not quantified but visually examined, there is less than a 2 fold difference between amounts of viral DNA in Ad12 infected CV1 and KB cells. The amount of viral DNA was slightly higher in the infected CV1 cells than in the infected KB cells. Reductions in levels or trends of early gene expression, with respect to E1A (Table 4), therefore did not result from lack of DNA genomes available for transcription. In fact, this is reflected in the near normal levels of RNA expression from most early regions when total RNA was measured (Table 5).

The approximately equivalent amounts of viral DNA present in both cell types at 4 hrs p.i. (Table 6:B) suggest that Ad12 virion adsorption was not significantly reduced in the CV1 cells, as equivalent amounts of viral specific DNA were present in both cell types. This would
Figure 22. Slot blot assay of Ad12 DNA replication in infected CV1 and KB cells. Total cellular DNA was extracted from infected cells at 0, 16, 24, and 40 hrs p.i. Aliquots of 0.2, 0.4, 0.6 and 0.8 ug DN\(^\text{a}\) from each sample were applied to a nitrocellulose filter and probed with \(^{32}\text{P}\)-labelled Ad12 DNA, as described in Materials and Methods.
Table 6. Relative DNA Replication in Ad12 infected CV1 and KB cells. Slopes were determined from plots generated for each sample time point from integrated optical densities (obtained by densitometry of DNA slot blot autoradiographs) vs micrograms of DNA loaded onto slots. These were used as a relative estimate of DNA replication as shown.

A. Values obtained from the DNA slot blot seen in Fig. 22. m.o.i. was 1200 virions per cell.

B. DNA was collected at 4, 24, and 34 hrs p.i. from Ad12 infected KB and CV1 cells. m.o.i. was 600 virions per cell.

<table>
<thead>
<tr>
<th>Time p.i.</th>
<th>CV1</th>
<th>KB</th>
<th>KB/CV</th>
</tr>
</thead>
<tbody>
<tr>
<td>A. 16</td>
<td>3.1</td>
<td>1.8</td>
<td>0.6</td>
</tr>
<tr>
<td>24</td>
<td>8.3</td>
<td>6.0</td>
<td>0.7</td>
</tr>
<tr>
<td>B. 4</td>
<td>0.02</td>
<td>0.02</td>
<td>1</td>
</tr>
<tr>
<td>24</td>
<td>0.15</td>
<td>0.11</td>
<td>0.7</td>
</tr>
<tr>
<td>34</td>
<td>0.28</td>
<td>0.18</td>
<td>0.6</td>
</tr>
</tbody>
</table>
correlate with data from studies with Ad2 which show that adsorption and internalization of adenovirus is normal in monkey cells (Feldman et. al., 1966; Malmgren et. al., 1966).

4. Late gene expression

To complete the survey of adenovirus type 12 expression in CV1 cells, the expression of the late (predominantly structural) proteins was also assayed, as this has never been determined previously. Published data from Ad5 or Ad2 infections have shown that most late proteins are expressed in CV1 cells, although at reduced levels, except for fibre protein (Klessig and Anderson, 1975; Farber et. al., 1978; Anderson and Klessig, 1983, 1984). This latter protein is present at very low or non-detectable levels. The reduction in levels of this protein has been determined to be mainly a result of aberrant splicing of the fibre message, which may affect its stability or ability to be translated (Klessig and Chow, 1980; Anderson and Klessig, 1984). Anderson and Klessig, 1985, also suggest that accumulation of the other late messages may also be slightly reduced.

The situation in Ad12 infected CV1 cells may be different. As shown above there were some differences in early gene expression between Ad12 infected CV1 and KB cells. E1B
protein levels were reduced in the infected CV1 cells and cytoplasmic EIB mRNA levels, relative to E1A (Table 4) seemed to be reduced in these cells. The northern blot data also seem to indicate a general reduction in levels of most early mRNAs. This was not the case when Ad2 early region RNA expression was compared in these two cell types (Anderson and Klessig, 1982). DNA degradation also has not been observed in Ad2 or Ad5 infections of CV1 cells: neither in this study (see Part C, Results) nor has it been reported in the literature. This degradation may also significantly reduce the amounts of late protein being produced.

[35S]-methionine labeled, total cell lysates from infected CV1 and KB cells were analyzed to determine if late viral proteins were being expressed. At the same time cellular protein expression was observed to determine if it was also being repressed, as occurs in permissive infections (Babiss and Ginsberg, 1984). The samples in the autoradiogram shown were labeled for four hours prior to harvest (Figure 23). Equivalent amounts of radiolabel from each lysate were loaded on 12.5 % polyacrylamide gels and processed in the usual manner. Figure 23:b shows the viral proteins present in a lysed sample of purified Ad12 virions (silver stained), to demonstrate the general pattern of virion structural proteins in a gel identical (although processed differently) to that
Figure 23. A. SDS-PAGE analysis of total lysates from infected CV1 and KB cells. Equal amounts (cpm) of lysate were separated on a 15.2% polyacrylamide gel. Cells were labeled for 4 hrs and harvested at the times (hrs p.i.) indicated above each lane, from 4 to 72 hrs p.i. The sizes of the marker proteins are indicated on the left, in kilodaltons (K).

B. SDS-PAGE of Ad12 virion proteins, separated in the same type of gel as above and silver stained. Two samples of Ad12 protein were run together with a marker lane. The Roman numerals refer to the identifiable Ad12 virion proteins in this gel and their counterparts in the previous autoradiograph.
from the radiolabelled experiment. The autoradiograph showed clearly that there was much less virus specific protein in the CV1 infected cells than in the permissive KB cells (Fig. 23). The only clearly discernible viral proteins in the CV1 system were the hexon protein (II) and protein IX (late in infection). The identity of these as virion products were confirmed using anti- Ad12 virion protein- sera against similar lysates (not shown).

The bulk of the reduction of late proteins could be due to insufficient accumulation of late messages. There may be delayed expression of these messages, but the cells are killed and lysed before significant late gene expression or accumulation can occur.

Analysis of late RNA by Northern blots supported this assumption. Equal amounts of cytoplasmic RNA from infected CV1 and KB cells were probed for the presence of L2, L3 or L4 (identical RNA samples to those used in Fig. 21 were used in this assay). The results show that at 26 hrs p.i., there was much less expression in the infected CV1 cells than in the parallel KB infection (Fig. 24). The pattern of RNA species in L4, although reduced, is similar to that in the L4 RNA from infected KB cells. Methylene blue staining of the ribosomal RNA on the blots after autoradiography confirmed that the transfer of RNA to the blots was equivalent for all samples.
Figure 24. Northern blot analysis of late mRNA from CV1 and KB cells infected by Ad12 and harvested 10 and 26 hrs p.i. Mock infected cells (lanes m) were harvested at 26 hrs p.i. The RNA in these blots are from the identical samples as analyzed in Figure 21. Blots for late region 2 (L2) and region 3 (L3) are shown, both as short exposures (Panel A) and longer exposures (Panel B). Late region 4 (L4) RNA was also probed (Panel C), using RNA from another similar experiment. The marker is Rsa I digested pBR322 DNA.
These results, while not measurable due to the lack of an internal control, do suggest that the late regions are underexpressed.

5. Viral yields in Ad12 infected CV1 cells

The absence of detectable virion proteins in Ad12 infected CV1 cells, as seen above, and the absence of viral production in Ad2 or Ad5 infected CV1 cells (see Introduction), suggest that very little virus would be produced in CV1 cells infected by Ad12. Several Ad12 yield assays were performed to confirm this suggestion and to confirm the results of the only other paper which demonstrated that CV1 cells were non-permissive for Ad12 infections (Rabson et al., 1964). Ad12 virions, labeled with $^{14}$C-thymidine, were harvested at 48 hrs p.i. from infected CV1 and KB cells and purified in a CsCl gradient together with enough ($4 \times 10^{11}$) unlabeled virus to form a visible band. The region of the gradient around the visible band was collected as described in Materials and Methods. The resulting radioactivity (cpm) was plotted (Fig. 25) and compared. There was very little, if any, labeled virus in the CV1 lysate (Fig. 25:b). A large amount of virus was present in the infected KB cell lysate (Fig. 25:a). Another control infection, using one of the Ad12
Figure 25. Comparison of viral yield from Ad12 infected CV1 and Ad12 wt or Ad12 cyt 61 infected KB cells. Infected cells were labeled with $[^3H]^{-}$-thymidine, harvested 48 hrs p.i., lysed, and layered on top of a preformed CsCl gradient (density of 1.32 to 1.36 gm/cc). Radioactivity was determined from 5 drop fractions of the gradient in the region of a visible viral band (made possible by the addition of 1 O.D. of unlabeled virus to the gradient). The resulting profiles are shown in this figure.
cytotoxic mutants (cyt 61) in KB cells, confirmed that the degradation phenotype can result in a lower viral yield (Fig. 25:c), as reported by others (Takemori et. al., 1968; Ezoe and Mak, 1974; Ezoe et. al., 1981).

Two plaque assays were also performed, with cell lysates collected from $1 \times 10^7$ CV1 or KB cells infected with Ad12 and harvested at 48 hrs p.i. These were assayed on MH12-C2 cells, a line of human embryonic kidney (HEK) cells transformed by the left 16% of the Ad12 genome which constitutively express Ad12 E1 proteins (I. Mak, unpublished data). The first assay showed that while a few plaques were detected from the CV1 lysates, the resulting titre $3 \times 10^5$ pfu/ml, was very much lower than that from KB cells, $2 \times 10^{10}$ pfu/ml (Table 7). In a second, similar assay, no plaques were detected from CV1 infected cells when either Ad12 or Ad5 was used but high titres were obtained from the infected KB cell controls (Table 7). The media from the infected CV1 cultures in this second assay was also tested for the presence of released virus and no plaques were obtained.
**Table 7.**

Ad12 growth: plaque assay of Ad12 grown on CV1 cells or KB cells and assayed on MH12-C2 cells (HEK cells which constitutively produce Ad12 E1 proteins. I.Mak, unpublished).

**Expt. 1**

<table>
<thead>
<tr>
<th>Cells from which virus was obtained</th>
<th>Titre (pfu/ml.)</th>
</tr>
</thead>
<tbody>
<tr>
<td>KB</td>
<td>$2.0 \times 10^{10} \pm 0.8 \times 10^{10}$</td>
</tr>
<tr>
<td>CV1</td>
<td>$3.0 \times 10^5 \pm 3.0 \times 10^3$</td>
</tr>
</tbody>
</table>

**Expt. 2**

| KB   | $7.2 \times 10^9 \pm 3.2 \times 10^9$ |
| CV1  | 0 |
| Ad5 in KB | $2.7 \times 10^{11} \pm 0$ |
| Ad5 in CV1 | 0 |

Infected cells for these assays were harvested at 48 h p.i. Media from infected CV1 cultures was also assayed for the presence of released virus but no plaques were observed. The above data was obtained from 5 to 7 dilutions of each stock, on 3 plates (60 mm² growth area) of MH12-C2 cells per dilution.
C. INFECTION OF CV1 CELLS BY THE Ad12/Ad5 RECOMBINANT VIRUSES T1227 AND T2743

The preceding results have shown that the Ad12 E1B 19 kDa protein levels are reduced in infected CV1 cells, compared to its expression in Ad12 infected KB cells. The DNA degradation phenotype in the Ad12 infected CV1 cells may be a direct result of this, as 19 kDa protein - negative mutants display the same phenotype (Subramanian et.al., 1984b; White et.al., 1986; Pilder et.al., 1986). It was noted previously that the Ad5 E1B mRNA is expressed in monkey (CV1) cells to the same extent as in human (KB) cells after Ad5 infection (Anderson and Klessig, 1982). As DNA degradation has never been reported in any studies of Ad2 or Ad5 infected CV1 cells, the 19 kDa protein would seem to be providing its postulated protective role against DNA nuclease action (White and Cipriani, 1989). White et.al., (1986) had also suggested that the E1A may act as a positive regulator of DNA degradation. The E1A of Ad12, which seems to be expressed normally in these cells, may be important in the induction of DNA degradation in CV1 cells. It would be of interest to determine if the serotype origin of the adenovirus E1B region, or any other
region, could lead to differences in DNA integrity in the non-permissive cell system. This could lead to further study of the mechanisms leading to DNA degradation. A good opportunity to begin to answer this question was made possible by the generation of two Ad12/Ad5 recombinant viruses, by T. Jelinek (Dept. of Biology, McMaster University). One virus, T1227, contains the first exon of Ad12 E1A joined to the second exon of Ad5 and the rest of the Ad5 genome. The other, T2743, contains the entire Ad12 E1A and the 5' half of Ad12 E1B: including the entire Ad12 19 kDa reading frame. The E1B 55 kDa becomes a hybrid Ad12/Ad5 protein. Both of these recombinant viruses can be grown on human (HeLa) cell cultures to wt Ad5 levels, which suggests that the hybrid E1 proteins are functional (T. Jelinek and F.L. Graham, unpublished results; Schramayr et al., 1990).

The first objective was to determine if the two recombinant viruses could produce wildtype levels of E1B in CV1 cells. If the serotype origin of the E1B promoter or coding region influences its expression in these cells, then one would expect normal E1B expression from T1227 but not from T2743. Alternatively, if the Ad12 E1A does not function properly in CV1 systems then neither mutant should produce wildtype levels of E1B protein.
1. E1B protein expression by the Ad12/Ad5 recombinant viruses T1227 and T2743

Expression of E1 proteins was analysed by the labeling of infected cells with \[^{35}S\]-methionine for 4 hrs and immunoprecipitation of the resulting lysates with antisera specific for Ad12 or Ad5 E1 proteins. Cells were labeled from 16 to 20 hr p.i. and equivalent amounts of lysates (based on cpm) were immunoprecipitated by specific antisera.

The Ad5 19 kDa-protein specific antisera, 19C, was used against lysates from CV1 and KB cells infected with one of the two recombinant viruses, wildtype Ad12, or wildtype Ad5. The results clearly showed that the 19 kDa product was made in all cells infected by viruses containing the Ad5 E1B region, including the T1227 infected CV1 cells (Fig. 26). The level of 19 kDa protein present not only equals that in Ad5 infected CV1 cells, but was also equivalent to that seen in Ad5 or T1227 infected KB cells. This monoclonal antisera only cross-reacted very slightly with the Ad12 19 kDa product, as seen in the infections of KB cells by Ad12 wt or T2743. Therefore, the levels of Ad12 19 kDa in the Ad12 or T2743 infected CV1 cells could not be determined by use of this sera.
Figure 26. SDS-PAGE analysis of immunoprecipitates from CV1 and KB cells infected by one of Ad12 wt (12), Ad5 wt (5), T1227 (T1), T2743 (T2), or coinfected by Ad12 and Ad5 or T1 and T2. The lysates were obtained from cell labeled for 4 hrs, from 20 to 24 hrs p.i. Lysates containing similar amounts of label (cpm) were immunoprecipitated by 19C antisera (from P. Branton, Dept. of Pathology, McMaster University) which is directed against the c-terminal end of the Ad5 19 kDa product. The 19 kDa products are indicated by the arrow (labeled 19K). Lanes are as indicated; m is from mock - infected CV1 and KB cells.

The protein marker sizes are also shown, measured in kilodaltons (K).
Preliminary results (not shown) with the AB6a scra, which detects Ad12 E1 proteins, suggested that the T2743 virus made very little of the E1B 19 kDa product. This was very similar to that seen in wildtype Ad12 infections of CV1 cells (as seen in Figs. 16 and 17). This observation however still needs to be verified.

The results obtained with the 19c antiserum (Fig. 26) demonstrate that Ad5 E1B 19 kDa, whether present in the Ad5 wt or T1227 viruses, was expressed in the CV1 cells. This contrasts with the low level of expression of Ad12 E1B products by Ad12 in the CV1 cells (Figs. 16 & 17). Together these results suggest that in the presence of Ad12 E1A, the Ad12 E1B is not expressed but the Ad5 derived E1B is expressed. The hybrid E1A product(s) produced by recombinant virus T1227 must be able to transactivate the Ad5 E1B region because E1B expression in infected cells requires transactivation by E1A for normal expression (Bos and ten Wolde-Kraamwinkel, 1983). Thus the Ad12 and Ad5 E1B regions are differentially regulated, although the nature of this regulation is not evident. The Ad12 E1A may interact with these two (Ad12 or Ad5) E1B regions differently to regulate their expression, or another Ad5 region (to the right of the E1 region) allows Ad5 E1B expression in infected CV1 cells whereas other Ad12 functions do not allow full Ad12 E1B
expression in these cells.

2. Effect of T1227 and T2743 infection of CV1 cells on DNA integrity

The E1B 19 kDa product is responsible for the prevention of DNA degradation in infected cells, as shown by the results with Ad2 d1250 infection of KB cells (see Results, Part A.) and by studies with other E1B 19 kDa protein mutants (see Introduction). The results presented above suggest that infection of CV1 cells with the virus T1227 should not cause DNA degradation, as the 19 kDa product is expressed at levels similar to that of wt Ad5 and T1227 in the infected KB cells (Fig. 26). Similarly, if prevention of DNA degradation requires simply the presence of an E1B 19 kDa protein, then there should be no degradation in the wt Ad5 infected CV1 cells. A difference in degradation patterns between Ad5 and T1227 would suggest that the serotype origin of the E1A region is somehow related to this. As well, if the degradation patterns of wt Ad12 and T2743 are different, this would suggest that another region on the Ad5 region (to the right of the Ad12/Ad5 joint) is also involved in the degradation phenotype.

To test this hypothesis, the DNA of recombinant virus
and wildtype (Ad12, Ad5) virus-infected CV1 cells was analysed by gel assay and alkaline sucrose gradient procedures. The agarose gel assays, used by other groups (Subramanian et al., 1984; White et al., 1986), produced very subjective results. Ad12 infection of CV1 cells always resulted in DNA degradation, as did cyt R. wtant or d1250 control infections (data not shown). T2743 infection of CV1 cells usually lead to DNA degradation, but CV1 infection by T1227 indicated DNA degradation about 50% of the time, as judged by this assay system. Due to the extreme subjectivity of this type of assay, and the variable results obtained from a number of similar experiments, degradation results were obtained from alkaline sucrose gradients.

Equal numbers of infected CV1 and KB cells were labeled with [³H]-thymidine (from 14 to 36 h p.i.) for analysis by alkaline sucrose gradient assay. The m.o.i. used was an equal 1000 particles per cell for each virus. All infected cells were harvested at 36 hrs after infection.

DNA degradation by recombinant virus infections of CV1 cells was clearly seen in the sucrose gradient profiles (Fig. 27). The patterns of degradation observed were different between the two CV1 infections. The degradation was very extensive in the T2743 infected cells, as the viral DNA peak was very small in relation to the peak of lower molecular
Figure 27. Alkaline sucrose gradient analysis of intracellular DNA from infected CV1 and KB cells. Sedimentation was in 5 to 20% alkaline sucrose gradients and is shown from right to left. The arrow indicates the location of the marker viral DNA peak. Cells were labeled from 12 to 36 hrs p.i. Profiles are labeled according to the cell type and virus used. m.o.i.= 1000 virions per cell.
weight DNA. This profile was very similar to that obtained from CV1 cells infected with the Ad12 wt virus (Fig. 27:f). The labeled DNA profile from the T1227 infected CV1 cells showed that the peak of degraded (low molecular weight) DNA was approximately the same size as the viral DNA peak (Fig. 27:a). This profile was similar to the DNA profile from the Ad12 cyt 61 infection (Fig. 27:g). CV1 cells infected by wt Ad5 did not produce a degradation profile (Fig. 27:h). With control KB infections only the Ad12 cyt mutant caused DNA degradation (Fig. 27:c,d,i,j,k). These results suggest that expression of the 19 kDa protein is not sufficient to prevent DNA degradation. In the infected CV1 cells DNA degradation may be dependent upon the type of E1A product(s) present. In the presence of an Ad12 E1A (in T1227), degradation occurs, unlike the situation in Ad5 infected CV1 cells: although both of these viruses contain an identical Ad5 DNA genome from the E1B region to the right end of the genome (from about 4 map units (m.u.) to 100 m.u.).

This assay procedure was repeated to confirm this result. Labeling conditions for gradient analysis was the same as above. It was continuous from 14 hr p.i. until cells were harvested at 36 hr p.i. The resulting sucrose gradient profiles showed results similar to the preceding sucrose gradient analysis (Fig. 28). The DNA profile from
Figure 28. Alkaline sucrose gradient analysis of intracellular DNA from infected CV1 and KB cells. Sedimentation was in a 5–20% sucrose gradient and the profiles are shown with sedimentation from right to left. Profiles are labeled according to the cell type and virus used. Arrows indicate the position of the viral DNA marker peak. Cells were labeled from 12 to 36 hrs p.i. m.o.i.=1000 virions/cell.
recombinant virus T2743 infected CV1 cells was very similar to that of Ad12 wt and Ad2 dl250 infected CV1 cells (Fig. 28: d,a,h). All showed a prominent peak of low molecular weight DNA and a very small, if any, peak of viral genome - sized DNA. CV1 infection by the recombinant T1227 yielded a profile which had a large peak of degraded DNA but also had a very significant viral DNA peak (Fig. 28:c). Unlike in the previous gradient analysis (Fig. 27:a) however, this viral DNA peak was not as great as the peak of degraded DNA. To take this analysis a step further, the counts making up the peak fractions and lower molecular weight DNA fractions were totaled and expressed as a fraction of total counts collected per gradient (Table 8). In both sucrose gradient experiments, the degradation pattern and ratio of viral to low molecular weight DNA of the T2743 infected CV1 cells parallel that of the wt Ad12 infected CV1 cells, much more so than the T1227 infected CV1 cells. These latter infections still gave definite evidence of DNA degradation. However the degree of degradation, when measured by the viral to L.M.W. DNA peak ratios (Table 8), was never as great as that induced by T2743 or wt Ad12 in CV1 cells or by Ad12 cyt 61 or Ad2 dl250 mutants in either cell type.
Table 8.

Relative proportions of viral and lower molecular weight (LMW) or degraded DNA in the alkaline sucrose gradients expressed as fractions of the total radioactivity (cpm) within each gradient.

<table>
<thead>
<tr>
<th>Sample (cell-virus)</th>
<th>Viral DNA Peak (A)</th>
<th>LMW DNA (B)</th>
<th>A/B</th>
</tr>
</thead>
<tbody>
<tr>
<td>Expt. 1: Fig. 27.</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>CV1-Ad12</td>
<td>0.09</td>
<td>0.84</td>
<td>0.11</td>
</tr>
<tr>
<td>KB-12</td>
<td>0.55</td>
<td>0.24</td>
<td>2.29</td>
</tr>
<tr>
<td>CV1-T1227</td>
<td>0.37</td>
<td>0.53</td>
<td>0.70</td>
</tr>
<tr>
<td>KB-T1227</td>
<td>0.77</td>
<td>0.14</td>
<td>5.50</td>
</tr>
<tr>
<td>CV1-T2743</td>
<td>0.12</td>
<td>0.72</td>
<td>0.17</td>
</tr>
<tr>
<td>KB-T2743</td>
<td>0.67</td>
<td>0.18</td>
<td>3.72</td>
</tr>
<tr>
<td>CV1-cyt 61</td>
<td>0.28</td>
<td>0.57</td>
<td>0.49</td>
</tr>
<tr>
<td>KB-cyt 61</td>
<td>0.05</td>
<td>0.90</td>
<td>0.06</td>
</tr>
<tr>
<td>CV1-Ad5</td>
<td>0.76</td>
<td>0.05</td>
<td>14.62</td>
</tr>
<tr>
<td>Expt. 2: Fig. 28.</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>CV1-T1227</td>
<td>0.23</td>
<td>0.76</td>
<td>0.30</td>
</tr>
<tr>
<td>KB-T1227</td>
<td>0.59</td>
<td>0.31</td>
<td>1.90</td>
</tr>
<tr>
<td>CV1-T2743</td>
<td>0.06</td>
<td>0.93</td>
<td>0.06</td>
</tr>
<tr>
<td>KB-T2743</td>
<td>0.63</td>
<td>0.24</td>
<td>2.63</td>
</tr>
<tr>
<td>CV1-Ad12</td>
<td>0.03</td>
<td>0.95</td>
<td>0.03</td>
</tr>
<tr>
<td>CV1-Ad5</td>
<td>0.82</td>
<td>0.15</td>
<td>5.47</td>
</tr>
<tr>
<td>CV1-d1250</td>
<td>0.03</td>
<td>0.95</td>
<td>0.03</td>
</tr>
</tbody>
</table>
In summary, both recombinant viruses T1227 and T2743 induced the DNA degradation phenotype in CV1 cells but it was more extensive in the mutant containing the Ad12 E1B promoter and 19 kDa coding region: T2743. This mutant shows essentially the same DNA degradation phenotype as the wt Ad12 virus does in CV1 cells. The observation of DNA degradation by the recombinant virus T1227 was not predicted from the protein results, since a 19 kDa product was made by this virus in CV1 cells. It is possible that the Ad12 E1A has an active role in inducing the DNA degradation which could not be completely suppressed by the Ad5 E1B. Unfortunately a reverse recombinant, in which an Ad5 E1A is joined to an Ad12 genome containing Ad12 E1B, was not available for extended analysis. Studies with this type of recombinant may have indicated if the presence of the Ad5 E1A could influence Ad12 E1B expression or lead to DNA degradation.
DISCUSSION

A. EFFECT OF E1B 19 kDa PROTEIN MUTATIONS IN Ad2 ON DNA INTEGRITY AND VIRAL YIELDS

1. DNA degradation

The Ad2 mutants, 1p3, 1p5, and d1250, were studied to examine one of the postulated functions of the E1B 19 kDa protein, namely the prevention of DNA degradation in infected cells.

The 1p mutants were isolated on the basis of their ability to form large clear plaques 6 to 8 days after infection of KB cell cultures, as opposed to the small fuzzy plaques generated by Ad2 wt virus (Chinnadurai, 1983). These mutants also have a reduced ability to transform rodent cell lines (Chinnadurai, 1983). A third mutant, d1250, which was generated by deleting a segment of the 19 kDa protein coding region of Ad2, also has an identical phenotype (Subramanian et.al., 1984a,b).

These phenotypic characteristics of these mutants are very similar to those of the Ad12 cytoidal mutants isolated by Takemori et. al., 1968. These Ad12 cyt mutants, which produce a more intense cytopathic effect (CPE) and cellular
destruction than Ad12 wt, also induce the degradation of both
viral and cellular DNA in infected human cell cultures (Ezoe
et al., 1981; Lai Fatt and Mak, 1982).

Various groups have accumulated considerable data
which demonstrate that an altered E1B 19 kDa product can lead
directly to the phenotypes described above (Takemori et al.,
1984; Pilder et al., 1984; White et al., 1984; Chinnadurai,
1983; Subramanian et al., 1984a, b; Schaller, 1990).

Although some of these effects may be the result of
the alteration of a single function, there is some evidence
which suggest that some of these characteristics may be
separable. (Subramanian et al., 1984b; White et al., 1986;
Zhang, 1991). For example, both lp3 and lp5 generate large
plaques on KB cell monolayers but neither generates the
cytocidal CPE (Subramanian et al., 1984b).

The results in this study support the idea of
separable functions in that neither lp mutant caused the
degradation of intracellular DNA at 37°C despite their ability
to form large clear plaques (Figures 7 and 9). Since these
data were collected, other studies with the mutant lp5 have
shown some evidence of DNA degradation (Subramanian et al.
1984a; White et al., 1984), but in the former study
(Subramanian et al., 1984a) the degradation caused by lp5
was not nearly as pronounced as with a 19 kDa protein negative
mutant (d1250). These data were also obtained using a more subjective agarose gel assay for degradation and are not as clearly demonstrated as with the sucrose gradient assay of the type used in the present study. Some problems with reproducibility was experienced in this study with the agarose gel assay.

DNA degradation was observed in KB cells which were infected by Ad2 lp3 and incubated at 39°C when the labeled, intracellular DNA was assayed by sucrose gradient analysis (Figure 10). This was contrary to the results obtained from lp3 infected cells incubated at 33°C or 37°C (Figures 7, 8, and 9). This suggests that the mutation in lp3, which results in the substitution of valine in place of alanine near the amino-terminus of the E1B 19 kDa protein, creates a thermosensitive product that is unable to prevent DNA degradation at the higher temperature. This may indicate a functional 'domain' for the prevention of DNA degradation at or near the amino terminus of the 19 kDa product. The lack of DNA degradation in KB cells infected by the lp5 mutant supports this suggestion. In this mutant there is a major change at the carboxy terminus (an additional 12-14 amino acids past the original terminus, as described in the Introduction) which has no apparent affect on DNA integrity. The d1250 mutant, which does contain the sequences for the
first 19 amino acids of the 19 kDa product, could not prevent 
DNA degradation but this may be the result of a very labile 
and non-functional product (no immunoprecipitable product 
could be detected in infected cells using an antibody against 
a 19 kDa-aminoterminal peptide (Subramanian et al., 1984a).

This domain hypothesis has been confirmed by the study 
of various Ad12 19 kDa protein mutants generated by site 
directed mutagenesis of Ad12 (S.Y. Zhang, Dept. of Biology, 
McMaster University; Ph.D. thesis in preparation). Mutations 
near the amino terminus were more likely to lead to DNA 
degradation in infected KB cells than were mutations near the 
carboxy terminus (Zhang, McMaster University, personal 
communication).

Although this area was not further pursued in my work, 
the existence of an Ad2 mutant which is thermosensitive for 
DNA degradation would be useful in the determination of the 
mechanisms involved in the degradation (or prevention thereof) 
of DNA. Cellular and viral products could be compared from lp3 
infected cells incubated at different temperatures or assayed 
after shift up to 39°C, to determine if any viral gene region 
(or cellular product) is altered in level of expression.
2. Viral yields

As expected from the lack of DNA degradation, the yields of the lp mutants were only slightly less than those of wt Ad2 at 37°C. The generally lower yields of the lp mutants could be due to slight effects the mutations have on DNA replication or viral production in the absence of DNA degradation.

The yields from lp3 infected cells incubated at 39°C, which was the temperature at which degradation was noted, were only reduced to about 1/3 that of wt virus (Table 2). Therefore, the DNA degradation observed from this virus at 39°C did not have a dramatic affect on the viral yield. Similarly, even though the 19 kDa-protein deletion mutant d1250 induced DNA degradation, its yield was still 40% that of the wt virus (Fig. 12; Table 3). It would seem that many intact genomes were still available for packaging even though DNA was being degraded in these infections. Some of this DNA may be protected from degradation by encapsidation, which begins soon after DNA replication and the onset of late viral protein synthesis.

Other groups have also shown that the 19 kDa product may not be essential for viral growth in HeLa cells (White et. al., 1984; Barker and Berk, 1987). In contrast, several
other groups have reported a substantial reduction in viral yields, as determined by plaque forming ability, of 19 kDa protein negative mutants when compared to wt yields (Pilder et. al. 1984; Subramanian et. al. 1984a; Bernards et. al.,1986). Possible reasons for the observed discrepancies may include the particular cell lines used for the viral assays or the assay methods utilized. The particular KB cell line used in this study may be more permissive for viral growth than those used some of the other studies. There is some precedence for this view from Takemori's study of the Ad12 cyt mutants (Takemori et. al. 1969), in which some mutants grew well in certain KB cell lines but not in other KB lines or human embryonic kidney cells. White et. al., 1986 have also reported that E1B 19 kDa protein mutant viruses have a growth advantage in growth-arrested human WI38 cells over the wild-type virus. This group has suggested that some cell lines may contain a product which can complement 19 kDa protein defects in viral growth (White et. al. 1986).

The observed discrepancies in 19 kDa protein mutant yields is not due to the way in which these yields were determined. The yield of virus in the present study was estimated by determining the relative amounts of radiolabelled viral DNA in purified viral particle preparations. These results correlate well with those measuring the lp mutant
viruses plaque-forming ability on human cell lines: both lp3 and lp5 had wt plaquing abilities on KB cells (Subramanian et al., 1984b). Other 19 kDa protein negative mutants however, in addition to the lp mutants, also have wt plaque-forming abilities (White et al. 1984; Barker and Berk, 1987).

Ad2 dl250 did display a much reduced (one hundred fold) plaque-forming ability compared to wt Ad2 in another study (Subramanian et al., 1984a,b). It is possible that this difference may result from an altered particle to pfu (plaque forming unit) ratio. A portion of the dl250 virion yields may have included defective or nonviable virions (which are included in the present virion particle assay), thus reducing its plaque forming ability, even though wild type amounts of virions are present. This has not been determined.

In conclusion, the 19 kDa product does not seem to be essential for viral propagation, in spite of its role in preventing DNA degradation. Based on this role, and others which include prevention of the cytocidal and large plaque phenotypes, the normal function of the 19 kDa protein protein may be to prevent early lysis. This would maximize the adenovirus' ability to package and produce large numbers of virions.
B. Ad12 INFECTION OF CV1 CELLS

The previous section suggests that variations in the type of host cell can have some effect on adenoviral infections. This has been clearly shown in adenoviral infections of monkey cells, which are non-permissive for the production of progeny virus. Ad2 and Ad5 infections of monkey cells have been intensively studied and the block is believed to be at a stage late in infection (see Introduction; Johnston et. al., 1985). Early region gene expression and DNA replication in CV1 cells are apparently normal (Anderson and Klessig, 1982; Friedman et. al., 1970; Hashimoto et. al., 1973). However there seem to be alterations in transcription from the major late promoter which result in lower levels of late gene transcription and increased premature termination (Klessig and Anderson, 1975; Johnston et. al., 1985). More significantly, the splicing of a RNA message encoding a major structural protein (fiber) is aberrant in monkey cells (Klessig and Chow, 1980; Anderson and Klessig, 1983; Anderson and Klessig, 1984). It is likely that a combination of these problems result in the non-permissiveness of the host monkey cells to Ad2/5 infection. In the present study it became apparent that there may be several differences between Ad12 and Ad2/Ad5 infections of monkey cells. Some of these factors
may also play a role in the non-permissiveness of monkey cells toward Ad12 infection.

1. DNA degradation

DNA degradation has never been reported to occur in Ad2 or Ad5 infected monkey cells. This lack of degradation correlates with the fact that Ad2 early gene expression is not altered in these cells (Anderson and Klessig, 1982). In contrast, DNA degradation does occur in Ad12 infected monkey (CV1) cells (Figs. 13-15). The degradation observed in this study parallels that seen in Ad12 cyt mutant infections of KB cells (Ezoe et al., 1981) and that seen in the Ad12 infection of hamster embryo fibroblasts, another non-permissive system (Lai Fatt and Mak, 1981). Lai Fatt and Mak (1981) suggested that this observed DNA degradation may be the result of incomplete E1B expression in these cells.

2. Early gene expression

The observations of degradation in Ad12 infected CV1 cells suggested an alteration in Ad12 E1B expression and this was supported by the present study. The E1B 19 kDa protein was produced at very low or undetectable levels in Ad12 infected
CV1 cells: levels much less than that observed in Ad12 infected KB cells (Figures 16 - 18). When equivalent cell lysates were immunoprecipitated by the anti-E1 antisera, the 19 kDa protein was always much lower in the infected CV1 cells. However the 55 kDa proteins (E1A and E1B) were variable in expression. Experiments in which both E1 specific and E1A specific antisera was used against these lysates indicate that most of this 55 kDa protein variation may be due to reduced E1B 55 kDa protein levels. Use of an antisera specific for the E1B 55 kDa protein would verify this.

The examination of total (nuclear and cytoplasmic) RNA levels in Ad12 infected CV1 and KB cells indicated that only region E4 expression was significantly different between the two cell types at 20 hrs p.i., just before DNA degradation occurs (Table 5). E4 was reduced 7 fold at this time, and about 4 fold at later times (32 to 48 hrs), in Ad12 infected CV1 cells compared to expression in infected KB cells. Interestingly, while E1B protein expression was much reduced in infected CV1 cells, total E1B RNA expression was equivalent in the two cell types at 20 hrs p.i., and only about 3 fold reduced in the CV1 cells at 32 and 48 hrs p.i. (Table 5:b).

The data obtained in this study indicated also that actin levels at all times tested were quite similar in both cell types after Ad12 infection. The E1A region was also used
as an internal control, as E1A levels in infected CV1 cells varied only slightly from that in infected KB cells: the difference was never greater than two fold (Table 5:A). This result was obtained from both the cytoplasmic and total RNA preparations.

The above total RNA results parallel those obtained when this early region RNA expression was measured relative to E1A levels (Fig. 5:C), showing the same general trends in gene expression from the early regions tested. These results could be compared with the cytoplasmic data shown in Table 4, in which an actin control was not used.

When early region expression was assayed at the cytoplasmic RNA level, the E1B levels (relative to E1A expression at the same time) were reduced to a greater extent than that of the other early regions in the infected CV1 cells (Figure 19; Table 4). This reduction of E1B levels was more pronounced than that of E2A, E2B, and E4, compared to the early region/E1A ratios determined from infected KB cells at 23 hrs p.i. Later in infection, 34 hrs p.i., the general trend showed a reduction in expression of all early region RNA in the cytoplasm of the infected CV1 cells, compared to that in the KB infections.

A striking difference between the total and cytoplasmic RNA early gene expression, when both were measured
relative to E1A expression, was the larger KB to CV1 ratios in the cytoplasmic RNA determinations. This may suggest that some sort of regulatory mechanism is taking place that reduces the relative amount of cytoplasmic mRNA originating from the Ad12 early regions in the infected CV1 cells. There may be an alteration in early viral mRNA transport between the nucleus and cytoplasm, or the stability of the viral messages may be reduced in CV1 cells. Experiments measuring nuclear vs cytoplasmic RNA levels and assessing mRNA stability in the two cell types upon infection by Ad12 are needed to determine if these are different in KB and CV1 cells.

The apparent reduction in relative cytoplasmic E1B RNA levels (Table 4) and in E4 expression (Table 5), in the presence of normal E1A expression, are the predominant changes in the Ad12 infected CV1 cells. These may be leading factors in the degradation of cellular and viral DNA, and in the non-permissiveness of CV1 cells for Ad12 infection. Certainly expression was not altered enough to adversely affect Ad12 DNA replication (Fig. 22, Table 6). This would imply that at least the E2A and E2B regions are expressed adequately in CV1: these regions encode the DNA binding protein (DBP), and the DNA polymerase and terminal proteins, respectively. These are the three major viral products required for DNA replication (reviewed by Chalberg and Kelly, 1989).
Northern blotting experiments suggest that none of the early regions other than E1B and E4 has an RNA pattern that is significantly different, in infections of these two cell types (Fig. 21). The major change in the E1B RNA pattern, in infected CV1 cells, is a reduction of the 13S message, which encodes the 19 kDa protein, compared to the other messages from this region. The 22S mRNA also encodes the 19 kDa protein but it also encodes the 55 kDa product. This message alone may not be sufficient to provide the 'normal' amounts of the 19 kDa protein seen in permissive systems, as the 55 kDa protein is also being translated from this message. In permissive Ad5 infections the 13S message accumulates to high levels at late times in infection, unlike the 22S message which levels off or declines (Glean and Ricciardi, 1988). If the 13S message is not following a similar pattern of expression in the infected CV1 cells, this may contribute to the observed reduction in 19 kDa protein expression. Because there are much reduced E1B 19 kDa and 55 kDa protein levels in the Ad12 infected CV1 cells in the presence of normal levels of total E1B RNA and relatively high E1B 22S message, reduction in protein expression must be regulated by some type of post-transcriptional control. This may include reduced translation initiation or protein stability, in addition to mRNA stability or transport. This
needs to be tested.

Region E4 also seems to be generally underexpressed in the infected monkey cells (Table 5, Fig 21). It appears that two transcripts from this region (as seen in KB cells and noted by Saito et al., (1981)) are not found in the infected CV1 cells (Fig. 21). However, it is not known what products these messages may encode. One or both of these may be analogous to an Ad5 message which encodes the ORF 3 or ORF 6 proteins (ORF refers to open reading frame). These products have been shown by several investigators to play an important role in the transport and/or stability of late viral mRNA, in conjunction with the E1B 55 kDa protein (Pilder et al. 1986; Sandler and Ketner, 1989; Bridge and Ketner, 1989; Bridge and Ketner, 1990). The product from ORF 6, a 34 kDa protein, has been demonstrated to be physically associated with the 55 kDa protein of E1B and it is this complex which appears to act together in regulating late viral mRNA transport and/or stability (Sarnow et al. 1984). At the same time, it functions to decrease the transport and/or stability of cellular messages (Sarnow et al., 1984; Bridge and Ketner, 1989). This E4 product may also stabilize the E1B 55K product (and perhaps the 19 kDa protein as well), increasing its half life in permissive systems. In the CV1 cells, if these product(s) are absent, the E1B products may not be able to
prevent DNA degradation or contribute to a permissive infection.

Studies of HIV mRNA regulation has shown that a viral product (Rev) is instrumental in expediting the transport of non-spliced viral RNA from the nuclei of infected cells (Chang and Sharp, 1989). It is possible that adenoviruses also use a means of regulating mRNA processing and transport of early messages and this process is not as efficient in monkey cells. Certainly there is evidence for the regulation of the transport of late adenoviral mRNA in nonpermissive cells (see Introduction) and this may extend to earlier messages as well.

3. Late gene expression and non-permissiveness

The above mentioned lower level expression of E1B and E4 may be a significant factor in the non-permissiveness of CV1 cells toward Ad12 production. As seen in Fig. 22, very little late viral products were observed in Ad12 infected CV1 cells. This phenotype is characteristic of that seen with E1B 55 kDa and E4 deletion mutants (Pilder et.al.,1986; Sandler and Ketner, 1989; Bridge and Ketner, 1989,1990). Also, Ad12 needs E1B expression to allow some late viral transcription in another non-permissive system: hamster (BHK) cells (Klimkat and Doerfler, 1987). Defects such as these may be
reflected in the relatively lower amounts of late (region L4) mRNA (relative to E1A expression) detected from the Ad12 infected CV1 cells (Table 4) and in the lower levels of the other late regions in Northern blots (Fig. 24). Other than a general reduction in expression, the pattern of detectable RNA species on Northern blots was not greatly different between the two types of infected cells. As would be expected from the decrease in late viral messages and very low structural protein production, little or no virions were produced (Fig. 25, Table 7). The fact that L1 expression seemed to be normal when nuclear RNA levels were examined may suggest that transcription from the major late promoter is normal (Table 5). Other steps in the processing or transport of these messages may be more important in the determination of the non-permissiveness of CV1 cells towards Ad12 infection.

The reduced E1B protein expression in Ad12 infected monkey cells seen in the present study is clearly different than the situation seen in Ad5 infected monkey cells (Anderson and Klessig, 1982; Results: Part C). A serotypic difference has been noted in 55 kDa involvement in DNA replication in infected human cells: the Ad12 E1B 55 kDa protein is involved in DNA replication, as shown by the lack of DNA replication of 55 kDa-protein mutants (Shiroki et.al., 1986; Breiding et.al.,1988). Ad2 or Ad5 E1B 55 kDa-protein mutants do not
have significantly altered DNA replication (Lassam et al., 1978; Babiss and Ginsberg, 1984). An apparent reduction of Ad12 E1B 55 kDa protein does not significantly affect DNA replication in CV1 cells, but may affect other functions such as late mRNA transport or transcription.

Comparison of the amounts and species of cytoplasmic and nuclear mRNA present in Ad12 infected CV1 and KB cells would help distinguish a problem in transcription as opposed to a mRNA transport defect. Additional studies would determine if rates of transcription initiation are altered or if stability of cytoplasmic mRNAs are affected in the monkey cell system.

C. CV1 INFECTION BY THE RECOMBINANT VIRUSES T1227 AND T2743

1. E1 expression

The availability of two Ad12/Ad5 recombinant viruses, T1227 and T2743, provided an opportunity to determine if serotype differences in the E1A or E1B regions of adenoviruses could influence DNA degradation. This may provide insights into the mechanism(s) by which the DNA degradation phenotype is regulated.

The mutant T1227 has an Ad5 E1B promoter region
driving Ad5 E1B expression. The E1A of this virus is part Ad12 (the complete first exons of both 12S and 13S messages) and part Ad5 (second exon of 12S and 13S mRNAs), as shown in Figure 4. Preliminary data indicates that this E1A is functional (T.Jelinek and F.L.Graham, Dept. of Biology, McMaster University, unpublished data; Schramayr et. al. 1990). Data obtained in the present study showed that this virus expressed E1B as efficiently as did wt Ad5, in both CV1 and KB cells (Fig. 26). This result also supports the assertion that the recombinant E1A was functioning normally in the CV1 cells, as the E1B region requires E1A transactivation for efficient expression (Berk et. al., 1979; Bos and ten Wolde-Kraamwinkel, 1983).

The other mutant, T2743, contains the complete Ad12 E1A and the 5' half of the Ad12 E1B region (Figure 4). The E1B promoter, the complete 19 kDa protein sequence, and approximately half of the 55 kDa protein sequence are derived from Ad12. The rest of the viral DNA, including the sequences encoding the 3' terminal portion of the 55 kDa protein, consists of Ad5 sequences. Preliminary data suggests that the 19 kDa product is much reduced in T2743 infected CV1 cells (not shown), equivalent to that seen in wt Ad12 infected CV1 cells. This observation needs to be verified but would be expected as both Ad12 wt and T12743 viruses contain the same
primary sequences coding for an identical 19 kDa product. The E1B 55 kDa products of these two viruses are different due to the recombination event within the 55 kDa protein encoding sequences, but it is not known what effect this would have on its expression, protein stability or function.

There have never been any reports of DNA degradation in Ad5 infected CV1 cells. Most Ad5 early gene regions are expressed efficiently (at the RNA level: Anderson and Klessig, 1982) in these cells. The availability of the two recombinant viruses provided an opportunity to determine if the serotype origin of the 19 kDa protein, or its E1A transactivator, could influence the DNA degradation phenotype.

2. DNA integrity in T1227 and T2743 infected CV1 cells

Infection of CV1 cells by each of the recombinant viruses led to DNA degradation despite the fact that one of these, T1227, produced an E1B 19 kDa protein. This indicates that at least one other viral (or virus-induced) function is involved in the regulation of the DNA degradation phenotype, and the presence of the 19 kDa protein is not sufficient to prevent DNA degradation. Because T1227 differed from wt Ad5 only in the sequence of its E1A region, and wt Ad5 did not
induce DNA degradation, this implicates E1A in this "effector" role. The degradation of DNA caused by T1227 infection of CV1 cells was not as extreme as that produced by T2743 or wt Ad12 (Table 8). This suggests that the Ad5 E1B had at least a partial effect on the inhibition of DNA degradation. The presence of the Ad12 E1A in both of these mutants correlates with the DNA degradation phenotype.

Results from studies with an Ad12 cyt mutant, H12 cyt 61, which has a dominant degradation phenotype (degradation cannot be inhibited by coinfection with wt Ad12) in KB cells, have led to the suggestion that there was an active viral DNase function, in addition to the protection function provided by E1B (Lai Fatt, 1981). In support of the activated DNase function, there may also be a direct enhancement of a cellular nuclease activity due to increased transactivation, as suggested by observations of an enhanced acidic nuclease activity in 19 kDa mutant (H2cyt141) infected HeLa cells (D, Halluin et al., 1985). This may be a result of a "suicidal" host cell defense mechanism which is normally blocked or delayed by the presence of 19 kDa.

White et al. (1988) and White and Stillman, (1987), have indicated that the expression of the E1A 12S or 13S mRNA was necessary to induce DNA degradation in human cells infected with viruses carrying an E1B 19 kDa protein defect.
E1A and the E2-DBP were also overexpressed in infected human cells, particularly after DNA synthesis, in the absence of 19 kDa (White et al., 1988). This is normally the time of maximal 19 kDa protein expression. Therefore it seems that Ad5 E1B 19 kDa protein may play a role in the inhibition of E1A-dependent early gene expression, including that of E1A. They conclude that DNA degradation may be a direct result of increased E1A transactivation, leading to an accelerated infection in the absence of an E1B 19 kDa product. In the absence of normal regulation, E1A may also transactivate a some type of nuclease activity, by virtue of its ability to transactivate most adenoviral and a number of host cell promoters. (see review by Flint and Shenk, 1989). However it may be modulation of E1A activity, rather than enhanced expression, that leads to DNA degradation (White et al., 1988). In the present study, although E1A RNA expression was not enhanced in Ad12 infected CV1 cells, there was a slight increase of the E1A 55 kDa product levels in infected CV1 cells at 7.5 hrs p.i., compared to levels in infected KB cells (Figs. 17, 18). Its stability may be enhanced in the CV1 cells, leading to increased activation of a nuclease activity.

In the CV1 system, perhaps the Ad12 E1A is a more potent nuclease activator than the Ad5 E1A, or conversely, the Ad5 E1B may not be able to inhibit Ad12 E1A effectively. In
the wt Ad12 and T2743 infections of CV1 cells, there may be too little E1B expression to regulate E1A expression and transactivation, leading to the degradation phenotype. The Ad5 E1B in T1227 also could not completely prevent DNA degradation. Perhaps it cannot interact efficiently with the Ad12 E1A in the CV1 cells, as it can in KB cells or with the Ad5 E1A. The differences in the primary sequences of E1A and E1B (between the two adenovirus serotypes) may be enough to lead to different interactions with mRNA transport, stability, splicing or a host of other regulatory factors in the CV1 cells.

A reverse recombinant, having an Ad5 E1A and the Ad12 E1B promoter and coding region in an Ad12 background, may be of further use in assessing the relative importance of these two regions in DNA degradation.

The actual role of the E1B 19 kDa product in preventing DNA degradation in a permissive infection is still not clear, but some type of regulation of E1A seems most likely. Determination of the mechanism by which the 19 kDa protein acts to regulate E1A expression needs further study. The results from the CV1 infections by wt Ad12 suggest that E1A protein may be overexpressed slightly, but post-transcriptional regulation or modulation of E1A function could also be a significant mechanism of control. Perhaps it is the
ratio of E1B products to E1A products that is important in this regulation. White et. al. (1984), in coinfection experiments with wt and 19 kDa protein mutants, had indicated that the 19 kDa protein mutation had a recessive phenotype in the presence of equal or greater amount of wt virus. If the amount of mutant virus was greater than the wt virus then the mutant phenotype (cyt, deg) would prevail. They have suggested that perhaps the ratio of E1B product to some other viral gene product was important in the prevention of DNA degradation (White et.al., 1984). The incomplete inhibition of degradation by T1227 in CV1 cells may be the result of an altered ratio of E1A to E1B products. Potential regulation based on the ratios of E1A to E1B could be a direct or indirect effect. There is no evidence of a physical association between the products of these two E1 regions, so a direct effect may be unlikely, although possible. E1B may be involved in a stage in the regulation of expression of a gene that has been transactivated by E1A. The exact mechanism will need to await further research to elucidate the interactions of the adenoviral E1A and E1B products.

The localization of the 19 kDa protein to nuclear and plasma-membrane surfaces (Granà and Galimore, 1984; White et.al.,1984) and association with cytoplasmic intermediate filaments and nuclear lamin proteins (White and Cipriani,
1989, 1990) suggests a mechanism for the prevention of DNA degradation. The 19 kDa product may help stabilize these structures during infection, delaying the cytocidal phenotype in normal wildtype infections of human cells. Perhaps in stabilizing the nuclear membrane it may prevent cellular or viral nucleases from entering the nucleus (White et.al., 1984). Lamins are important in determination of chromatin structure (reviewed by Gerace and Berke, 1988), and disruptions in the arrangement of these during infection, in the absence of the 19 kDa protein, may lead to degradation. In support of this view, the 19 kDa protein has been shown to stabilize plasmid DNA in transfection assays, leading to the apparent increase in gene expression from these plasmids due to their stability (Hermann and Mathews, 1989). Thus the 19 kDa protein may play a crucial role in maintaining host cell structure and integrity sufficiently long enough to enable progeny virions to be produced.
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