RNA ACCUMULATIONS IN
ADENOVIRUS E1B MUTANTS
THE EFFECTS OF ADENOVIRUS REGION E1B MUTATIONS ON THE ACCUMULATIONS OF VIRAL SPECIFIC RNAs

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The effects of adenovirus region ElB mutations on the accumulations of viral specific RNAs.

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ABSTRACT

The expression of the adenovirus (Ad) genome is co-ordinately regulated and the various early regions of the genome are known to exert feedback controls on each other. The region E1B is known to encode functions which are required for oncogenic transformation by adenovirus yet its potential regulatory role is poorly understood. In this study the regulatory role(s) of the region E1B was investigated at the level of RNA accumulation. The Northern blot technique using various cloned early regions as probes and RNAs from E1B mutants Ad 12, Ad 2 and Ad 5 were used.

The levels of region E1A specific RNAs were found to be aberrant. Thus for Ad 12 cyt 68 and Ad 2 dl 250 the levels were higher than wild type ones at late times whereas for Ad 5 dl 313 and hr-6 the levels were consistently lower than Ad 5. This implies that the region E1B normally encodes functions which are involved either directly or indirectly in the efficient accumulation of region E1A specific RNA.

Other regions also seemed to be affected in these mutants. In cyt 68, region E1B RNA was higher than wild ones at late time, indicating an auto-regulatory mode of control for this region. The
expression of region E3, E4 and L5 RNA were all perturbed by the E1B mutation as some of the mutants either accumulated higher or lower than wild type levels of RNA.
I wish to express my sincere thanks to the following persons who helped me with this thesis. First, to my supervisor, Dr. S. Mak, for his help, criticisms and guidance throughout this project; Mrs. I. Mak, for supply of viruses; Dr. F.L. Graham for supply of hr-6 and Ad 5 DNA and his willingness to help; Dr. R. Morton for letting me use his word processor; my fellow graduate students Dody Bautista for excellent photographic help and John Gysbers and Mike Schaller for useful and interesting discussions; last but not least, to my family: My wife, Devianee, for displaying undaunting spirit, support, encouragement and above all for patiently converting my hieroglyphs into typescript at every stage of this thesis; and my daughter, Pamela, and son, Roy, for sacrificing me the time that rightly belongs to them.
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I. INTRODUCTION

I.1. Viruses as Probes for Gene Expression

Gene expression and its regulation are the molecular basis of many diverse biological processes such as differentiation (Maltzman et al., 1981), membrane biogenesis (Ghosh, 1980) and cellular transformation and oncogenesis (Bishop, 1983). Biological systems in which a set of genes is co-ordinately expressed, such as the operons of prokaryotes, lend themselves easily to genetic studies (Rosenberg and Court, 1979). Infection of a eukaryotic cell by an appropriate virus represents one eukaryotic system that exhibits co-ordinately regulated genes (Maltzman et al., 1981). Adenovirus (Ad) is a eukaryotic virus that has been intensively studied because it has a well defined genome that is temporally expressed. Viruses, being obligate intracellular parasites, depend on cellular functions for the expression of their genome; by doing so they may unmask events that occur in uninfected cells: Adenovirus has proved to be an excellent model for the biogenesis of cellular mRNA as the virus is dependent on cellular mechanisms for the synthesis of its mRNA (Nevins, 1982). Furthermore, these viruses may also serve as useful models for identifying and characterizing genes implicated in cellular proliferation and oncogenesis as all adenoviruses are capable of
transforming cells in vitro and some serotypes can induce tumours in hamsters (Heubner, 1967; Petterson, 1982). The present study focusses on some aspects of the adenovirus gene expression.

I.2.0. Basic Biology of Adenoviruses

I.2.1. Taxonomy of the Human Adenoviruses

The human adenoviruses comprise 39 serotypes (Wadell, 1980). Based on their abilities to induce tumours in Syrian hamsters, they were originally classified into three subgroups; A, B and C (Huebner, 1967). Group A (serotypes 12, 18 and 31) is highly oncogenic, inducing tumours within two months and with high frequency. Group B (serotypes 3, 7, 11, 14, 16 and 26) is weakly oncogenic and induces tumours after 4 to 8 months. Group C, comprising of the remaining serotypes, is non-oncogenic. Based on DNA homology, viral polypeptides and growth requirements, some members of the group C has been further subdivided into subgroups D, E, F and G (Wadell et al., 1980).

Within a given group, the DNA homology ranges from 85 - 95 % (Green et al., 1979). The functional organization of all human Ad viruses seems to be similar; however, the biological functions may not necessarily be identical.
I.2.2. Virion Morphology and Composition

Adenoviruses are non-enveloped viruses measuring 70 - 80 nm in diameter and the adenovirion capsid is icosahedral in morphology (Horne, 1959). The capsid consists of 252 capsomeres; 240 hexons form the facets of the icosahedron and 12 pentons are located at the corners of the capsid. The pentons consists of the penton base and the fiber, a non-covalently attached protein (Ginsberg et al., 1966). An endonuclease activity has been found to be associated with the pentons of most human adenoviruses (Marusyk and Wadell, 1975). Analysis of the viral structural proteins by denaturing gel electrophoresis have yielded at least eleven proteins designated, in Roman Numerals, II to XII (Ginsberg et al., 1966).

Within the capsid is the genome which is a linear double-stranded DNA molecule of 35 - 36 kilobase (Kb) consisting of an 'r' and an 'l' strands (Green et al., 1967). The 'r' strand is transcribed rightwards while the 'l' strand is transcribed leftwards (Flint, 1981). Covalently attached on the 5' end of each DNA strand is a terminal protein of 55,000 dalton (Stillman and Bellet, 1977). The DNA sequence at the end of the molecule are inverted terminal repeats (ITR) of about 102 - 136 base pairs (Steenbergh et al., 1977).
1.2.3. **Types of Virus-Cell Interactions**

The interaction of the adenovirus genome with a cell is a complex process and the outcome depends on both the viral serotypes and the cell types. Human KB cell lines are permissive to adenoviruses resulting in a lytic infection with high virion yield (Flint, 1981).

African Green Monkey Kidney Cell line is a semi-permissive host in which the viral yield is very little, even though DNA synthesis takes place (Klessig and Anderson, 1975). This block to replication has been assumed to be a late event in the replication cycle since the synthesis of several late viral proteins, especially fiber, is severely reduced (Baum et al., 1972).

Infection of primary rodent cells often results in an abortive cycle depending on the serotype used: For Ad 2 or 5 these cell-lines are permissive but, if replication of the virus is blocked, a small fraction of the cells containing the integrated genome survive as transformed cells (Rapp and Westmoreland, 1976). However, the hamster cell is totally non-permissive for Ad 12; viral DNA synthesis is completely blocked (Doerfler, 1970) but early tumour antigens and a subset of the early mRNAs are expressed (Ortin et al., 1976). A small fraction of the cells may infrequently survive as transformed cells by the interaction of Ad 12 with such cells.
1.3.0. Permissive Infection by Adenoviruses

The expression of the adenovirus genome is co-ordinately regulated in a lytic infection, showing an early and a late phase separated by an intermediate phase of DNA replication (Flint, 1981). The reproductive cycles of different groups of adenoviruses differ in their time course (Mak, 1969). For example, for group C viruses namely Ad 2 or 5, viral DNA synthesis begins at 7 hours post-infection whereas for group A, e.g. Ad 12, the cycle is delayed by a few hours (Mak and Green, 1968).

During the early phase, only a fraction of the genome is expressed as mRNA (Fujinaga et al., 1968; Green et al., 1971), which directs the synthesis of non-structural viral proteins. At this time, viral mRNA constitutes a minor proportion of the total RNA of the cell; however, at the late time, virtually all the mRNAs bound to the cells polyribosomes are viral in origin (Thomas and Green, 1966). Most of the late mRNAs direct the synthesis of viral structural proteins.

1.3.1. Transcription of the Adenovirus Genome in the Early Phase

By the techniques of liquid hybridization (Ortin et al., 1976; Green et al., 1971), hybridization to separated strands of restriction fragments (Tibbetts and Petterson, 1974; Smiley and Mak,
1979); Northern blot analysis and S1 mapping (Sharp et al., 1974) it has been found that early RNA is transcribed from five widely separated regions of the genome designated E1, E2A, E2B, E3 and E4 (Flint, 1977; Galos et al., 1979). The region E1 has been further subdivided into two separated transcriptional units; E1A and E1B (Wilson and Darnell, 1979). See Figures 1-1. Early region E1 RNA is transcribed from the 'r' strand between 1.5 and 11.2 map unit (mu); early region E2A, from the 'l' strand between 61.6 and 74.9 mu; early region E2B from the 'l' strand between 11.2 and 23.5 mu; early region E3, from the 'r' strand between 76.8 and 86.0 mu; and early region E4 from the 'l' strand between 91.4 and 99.1 mu (Berk and Sharp, 1978; Chow et al., 1979; Stillman et al., 1981; Gingeras et al., 1982). Small amounts of RNA from the 'r' strand hybridizing to region 16.5 to 40.0, representing the late L1 mRNAs, have also been detected at early times (Shaw and Ziff, 1980).

Studies of the promotores by nascent chain analysis (Evans et al., 1977), ultraviolet inactivation (Berk, 1978) and by 5,6-dichloro-1-β-D-ribofuranosyl-benzimidazole (Sehgal et al., 1979) have shown that all early adenovirus transcription units are preceded by an initiation site for RNA polymerase II and each unit, except E2B, has its own promotors. However, the rate of transcription from each promotor differs and for some regions, like E2, different promotors are used as infection progresses (Chow et al., 1979).

From kinetics studies it is known that the E1A promotor is the first to be expressed. For Ad 2 and 5, E1A transcripts can be
Figure 1-1. A schematic drawing, showing the principal organization of the adenovirus type 2 genome (modified from Flint, 1980). Arrowheads show the location of 3'-ends of the mRNAs and the promoter sites are indicated with brackets. Selected polypeptides which have been assigned to different regions are indicated. Thick lines represent mRNAs which are expressed early after infection, in the absence of viral DNA replication and unfilled arrows indicate sequences present in late mRNA. Thin lines indicate mRNAs which are expressed at intermediate and late times after infection. Five separate co-termination families of late mRNA L1 to L5 are present. The three segments which are spliced together to form the tripartite leader (1, 2, 3) are also shown as well as the location of the i-leader. Two small RNAs, VA RNAI and RNAII, map around position 30.
detected 15 minutes after infection and the maximum rate of transcription is maintained for at least 6 hours (Wilson and Darnell, 1981). The region E1A encodes three species of mRNA of 9S, 12S and 13S. The 13S and 12S mRNA are processed from alternative 5' splice sites to a common 3' splice site of the same initial transcript and are translated into polypeptides of 243 amino acids and 289 amino acids. These proteins share common amino and carboxy peptides but differ in the length of the internal amino acid sequences (Perricaudet et al., 1979; Halbert et al., 1979; Esche et al., 1980; Smart et al., 1981). The 9S mRNA splices into an alternative 3' reading frame and thus only has amino peptides in common with 12S and 13S RNA (Svenson et al., 1983).

The transcription rate from the E1B promotor appears to be constant throughout the infection by Adenovirus type 2 (Wilson and Darnell, 1981). The region E1B encodes 13S and 22S mRNA spliced from alternative 5' splice site to a common 3' splice site and an independently promoted unspliced 9S mRNA. Both the 13S and 22S mRNAs direct the synthesis of a major 19K tumour antigen. However, the 22S is unique in that it also directs the synthesis of 58K tumour antigen by utilizing an internal translation initiation site for a different reading frame (Bos et al., 1981). The unspliced 9S mRNA contains a single open reading frame encoding the virion structural polypeptide IX (Alestrom et al., 1980).

The early regions E2A and E2B of the DNA use the same promotor but the E2A mRNAs are expressed before the E2B RNA (Stillman et al.,
1981). Expression of region E2B is the last early transcription unit to be activated, RNA synthesis begins around 3 hours post-infection and reaches a maximum at intermediate time (Nevins et al., 1979). Although three differently spliced mRNAs are seen for region E2A, only a single 72K, DNA binding (DBP) has been detected (Berk and Sharp, 1978). By electronmicroscopy, four differently spliced mRNAs have been identified for region E2B (Stillman et al., 1981). These mRNAs direct the synthesis of three proteins; a 87K protein which is the precursor of the 55K terminal protein (pTP); a 140K protein which is the virally encoded polymerase and an uncharacterized polypeptide of 75K.

Transcription from region E3 begins around 1.5 hours post-infection and reaches a maximum at 3 hours. Region E3 has been shown to encode 6 - 8 major species of mRNAs (Berk and Sharp, 1978). All have a common 5' segment that is spliced to sequences beginning at co-ordinate 78.6. At least three different proteins have been identified, the well characterized one is the 19 K membrane-bound glycoprotein.

Raskas et al. (1984) have found the Ad 2 E4 transcripts to be constant throughout infection. Region E4 is complex and at least six transcripts of varying sizes have been identified by electron microscopy (Chow et al., 1979). All of these mRNAs are derived from a nuclear precursor by the removal of introns and are co-terminal with a poly (A) addition site at co-ordinate 91.3 (Berk and Sharp, 1977). Six polypeptides have been identified by in vitro translation of the
five messages but the structural relationship between the polypeptides and the mRNAs are still being actively studied. Thus for Ad 2 region E4, Raskas et al. (1984) have identified multiple splice sites in one of the 3.0 Kb precursor RNA that can generate as many as 18 to 24 RNAs which could in turn direct the synthesis of at least 14 different polypeptides. It is also known that a polypeptide of 44K which had previously been assigned to region E1 is a product of the region E4 (Downey et al., 1983).

1.3.2. **Viral DNA Replication**

At the intermediate time of a lytic infection, the Ad virus DNA is replicated (Flint, 1981). This process has been intensively studied as a model for eukaryotic DNA replication (Kornberg, 1982). From various studies it is known that the 'r' and the 'l' strands can independently initiate replication in their respective ITRs containing the origins of replication (Chalberg and Kelly, 1979 & 1982). Replication proceeds by strand displacement of non-template strand. In *vitro* studies have shown that at least three virally encoded proteins; the DBP of region E2A, and the DNA polymerase and the pTP of region E2B, are involved in the process. The pTP forms an initiation complex with dCMP and the 3' hydroxyl end of the bound dCMP residue acts as a primer for DNA synthesis. Elongation occurs by attachment of the virally encoded DNA polymerase to the initiation complex. A
number of unidentified cellular proteins such as factors I and II are also postulated to be involved in the replication process but the exact mechanism has still not been completely elucidated (Stillman, 1983).

1.3.3. **Transcription of the Adenovirus Genome in the Late Phase**

After DNA replication, the transition from early to late transcription occurs and the major late promotor at co-ordinate 16.5 mu controls most of the transcriptional activity. A primary transcript, consisting of approximately 28,000 nucleotides, that is then spliced in different fashions to generate the mRNA for all proteins in the viral capsid (with the exception of protein IX) is produced (Chow et al., 1977). Three short segments, derived from map co-ordinates 16.5, 19.6 and 26.6 are joined to form a common tripartite leader sequence that is then attached to different mRNA bodies to form a common tripartite leader. Within the major late transcription unit, five major poly(A) addition sites exist and this has been used as a basis to classify the late mRNAs into five 3' co-terminal families designated L1 to L5 (Shaw and Ziff, 1980). See Figure 1-1.

The L1 mRNA directs the synthesis of polypeptides of 52K and 55K in addition to the synthesis of polypeptide II.

The major proteins directed by L2 messages are polypeptide III
which forms the penton base and polypeptides VII and V constituting the core proteins.

The L3 messages direct the synthesis of polypeptide pVI; the hexon polypeptide II and a virally encoded protease required in the maturation of the virions (Weber and Bhatti, 1980).

The L4 messages direct the synthesis of the polypeptide pVIII which forms the hexon associated protein.

L5 messages direct the synthesis of polypeptide IV constituting the adenovirus fiber.

1.4.0. Regulation of Adenovirus Gene Expression

Because of their co-ordinately regulated genomes and the availability of various kinds of viral mutants, adenoviruses have become a favourite model for the study of gene expression by molecular biologists. The production of viral mRNAs and their polypeptide products follow a complex programme which is subjected to a variety of inter-locking regulatory mechanisms. Regulatory events appear to occur at the level of initiation and termination of transcription, processing, mRNA stability and translation (Logan and Shenk, 1982; Nevins, 1982). When these regulatory mechanisms are interfered with, they may in turn manifest themselves by affecting the biological properties and phenotypes of the respective viruses. This phenomenon for each region, as far as is known, is reviewed below.
1.4.1. Region E1A

By the use of region E1A specific mutants or the technique of microinjection, it has been established that region E1A encoded product(s) can act as regulators for the expression of other early regions. The two mutants which have been particularly useful in this respect have been the Ad 5 deletion mutant, dl 312 which has its E1A region DNA deleted (Jones and Shenk, 1978) and the host-range group I (hr-I) mutants which map in region E1A (Harrison et al., 1977). Both these mutants were originally selected on the Ad 5 transformed cells, 293 cell lines which contain sequences from the left end of the Ad 5 genome (Graham et al., 1977).

Thus Ad 5 E1A deletion mutant dl 312 (Jones et al., 1979) or group I host range mutants (Berk et al., 1979) have been used to establish that one or more products encoded by region E1A are necessary for the efficient accumulation of cytoplasmic RNA from region E1B, E2, E3 and E4. By site directed mutagenesis (Montell et al., 1982) or in vitro translation (Riccardi, 1980), it has been shown that the product of the 13S message is required for this enhancement effect. The exact mechanism of E1A regulatory effect is still not known. However, it has been postulated that the E1A gene product may function by inhibiting the function of a cellular repressor that is presumed to suppress the promotors of the other
Evidence for E1A products directly enhancing the activity of specific promoters has also been demonstrated. For instance, using the technique of microinjection of cloned segment of viral DNA in cell cultures, Rossini (1983) has shown that products encoded by region E1A differentially control the expression of the E2A DNA binding protein. The DBP produced from the early promotor is stimulated by E1A but the DBP produced from the late promotor is inhibited by products from region E1A. The effect of E1A region on the expression of the E3 promotor has been similarly studied by Ferguson et al. (1984) who injected Xenopus oocytes with E1A protein produced from E. Coli and found region E1A product(s) to enhance the expression of the E3 promotor.

1.4.2. Region E1B

Studies from a variety of E1B mutants implicate the region E1B as encoding multifunctional regulatory roles. It encodes functions that can act both as positive and negative regulators for the expression of other early regions; functions that are involved in the switch from early to late phase and functions that are needed in the interactions of the virus with the host-cell.

For Ad 5, Ross et al. (1980), using host-range group II (hr-II) mutant mapping in region E1B (Harrison et al., 1977), have
demonstrated that this region can act as a negative regulator for the accumulation of proteins from regions E2 and E3. They found the levels of these proteins to be elevated in the hr-II mutant when compared to the Ad 5. Using an hr-II mutant, hr-6 that synthetizes no immunoprecipitable level of the E1B 58K polypeptide (Lassam et al., 1979), Rowe et al. (1984) have postulated that this region can act as a positive regulator for the synthesis of E1A proteins: They observed that the level of E1A proteins was lower in the mutant infected cells than in the wild type infected ones at early time.

However, similar evidence at the level of RNA expression is lacking and inconclusive. Most workers have found the level of E1A RNA not to be significantly different in wild type viruses and their E1B mutants. For example, using the hr-II mutant, hr-7, Berk et al. (1979) found the levels of early RNA to be of the wild type one. Jones and Shenk (1979) using dl 313, an Ad 5 E1B mutant which has all its E1B genome including the E1A polyadenylation site deleted, found the RNA hybridizing to region E1 to be fainter than that from the wild type. However, they did not consider this observation as significant. Similarly Lai Fatt (1983) using the low oncogenicity Ad 12 E1B mutants, namely the cyt mutants which were originally isolated by Takemori et al. (1968), did not report any significant difference between the levels of wild type and mutant E1A RNAs. If the protein data can be taken to reflect the RNA level, one expects to see a difference in the level of region E1A RNA in the absence of a functional region E1B. The only group to provide circumstantial
evidence to that effect was that of Van der Elsen et al. (1983) who observed the level of E1A transcripts to be significantly higher in transformants obtained from E1A plus E1B when compared to those obtained by E1A alone.

Studies with Ad 5 dl 313 or Ad 12 cyt mutants (Lai Fatt and Mak, 1982; Ezoe et al., 1981) or Ad 2 dl 250 mutant which lacks 145 bp that specifically affect the 19K protein (Chinnadurai, 1983; Gysbers, 1984) suggest that one E1B product may function as an antinuclease factor. This conclusion is based on the observation that all these E1B mutants are characterized by DNA degradation phenotypes. Based on the dominant nature of (cyt 61), one of the cyt mutants, it has been postulated that the Ad virus may encode a nuclease gene that is located distal to the E1B region (Lai Fatt and Mak, 1982). From the complementation study of Ad 5 and cyt mutants, the E1B 19K polypeptide is thought to be involved in this anti-nuclease activity. The evidence is further supported by the lack of complementation between dl 250 and cyt 68, a cyt mutant that does not synthetize any detectable level of 19K polypeptide (Mak et al., 1984).

Characterization of in 206 B, a host range mutant of Ad 12 defective in region E1B, shows that both the E1B-encoded 58K and 19K polypeptides may be involved in functions affecting viral DNA replication as well as the efficient accumulations of L1 and L5 mRNAs (Fukui et al., 1984). Babiss and Ginsberg (1984) have also shown, from studies with Ad 5 E1B-deletion mutants (dl 110 and dl 163), that the E1B products may function by interacting with additional
viral or host product to shut off host protein synthesis as well as in the efficient accumulations of L1, L3 and L5 messages.

1.4.3. Region E2A and E2B

From studies using Ad 5 temperature sensitive mutant, ts 125, mapping in region E2A, Carter and Blanton (1978b) have shown that this region is involved in its autoregulation as the E2A RNA is over produced at the non-permissive temperature. Nevins et al. (1980), using the same mutant have shown that region E2A is involved in the shut off of region E4 transcription since they found the level of E4 transcript to be repressed at the permissive but not at the restrictive temperatures. Handa et al. (1983) have directly demonstrated, by in vitro DNA binding studies, that the E2A-72K protein inhibits E4 transcription by binding to the E4 region. Babich and Nevins (1981) have also provided evidence that a functional E2A, 72K DBP is required for the rapid turnover of E1 early mRNA, as they found the level of E1 RNA to be overproduced at the non-permissive temperature in Ad 5 ts 125. The possible regulatory role(s) of region E2B has not been well defined yet.

1.5.0. Aims of the Study
Based on the evidence presented above, the E1B mutants, in particular those characterized by DNA degradation phenotypes, seem to encode multi-regulatory functions. However, most of the studies using region E1B mutants have focussed on limited pulse-labelling analysis of protein synthesis. Similar studies for the RNA phenotypes are lacking and inconclusive. In view of the potential regulatory roles of this region it was decided to undertake a systematic study of the RNA phenotypes of some E1B mutants. The central question in this project was what is the effect of such E1B mutations on the transcriptional expression of the other regions of the adenovirus genome. In addition, by studying the transcriptional expression of the other regions it was anticipated to localize the expression of a putative nuclease gene. Based on the observation that the DNA degradation phenotypes of cyt 61 is dominant Lai Fatt and Mak (1983) have postulated that the Adenovirus may encode a nuclease gene. In this study, it was assumed that if this nuclease gene exists it could be over-expressed at the transcriptional level, as the nuclease activities are over-expressed in the cyt mutants.

The study was essentially divided into two parts. Part one consisted of constructing or characterizing specific probes for the different early regions of the Ad 12 genome. In the second part, the transcriptional expression of the adenoviruses' genomes, as monitored by the Northern blot technique, was investigated.
II MATERIALS AND METHODS

II.1.0. Recombinant DNA Techniques

Probes for Ad 12 region E1 to "E4" and Ad 5 region E1A and "E4" were used for the detection of virus specific RNA in this study. The Ad 12 clones were pHAS, 0 to 4 mu, for region E1A; pHBl4R, 4.5 to 6.7 mu, for region E1B; pBH1C, 63.3 to 68 mu, for region "E2A"; pH40, 14.2 to 24.0 mu, for region "E2B"; pBH1B, 73 to 88 mu, for region "E3" and pBH1E, 88 to 100 mu, for region "E4". The Ad 5 clones pHE1, 0 to 4.5 mu, for region E1A and pH14, 89 to 97 mu, for region "E4" were obtained from Dr. F.L. Graham, McMaster University. Extensive homology exists between Ad 5 and Ad 2 (Green et al., 1979), this enables one to use the Ad 5 probes for the detection of Ad 2 RNA. Except for region E1A and E1B recombinant clones the remaining ones contain sequences which are also complementary to late transcripts. Because results obtained from these probes at late times are less definitive than at early times these probes have been accordingly qualified. See Figure 3-1 for a summary and origins of the Ad 12 recombinant clones.

II.1.1. Cloning of Specific Early Regions
All the probes, with the exception of pBH1C ("E2A") and pBH1B ("E3"), are already available as they have been cloned into pBR322 by various investigators in Dr. Mak's laboratory. The cloning strategy used for constructing pBH1C and pBH1B is schematically illustrated in Figure 2-1.

Twenty five micrograms of Ad 12 DNA (strain 1131) was restricted with 25 units of Bam H1 in TA buffer (Tris Acetate buffer composed of 33 mM Tris Acetate pH7.9, 66 mM Potassium acetate, 10 mM magnesium acetate, 100 ug/ml of Bovine Serum Albumin and 0.05 mM of dithriothreitol) for 3 hours at 37°C and extracted with phenol (equilibriated with isotonic buffer). The phenol was removed with ether and the DNA precipitated with 95 % ethanol. One ug of the cloning vector pBR322 was similarly restricted with Bam H1 and, the 5' ends were dephosphorylated with bacterial alkaline phosphatase in phosphatase buffer (10 mM MgCl₂, 1mM ZnCl₂, 10 mM spermidine, 0.5 M Tris-Cl, pH 9.0) to prevent re-circularization. The DNA was then re-extracted with phenol as before. Ligation of the viral plasmid DNAs was carried out at 14°C overnight using 100 Weiss Unit of T4 ligase in ligation buffer (50 mM Tris, pH 7.4, 10 mM MgCl₂, 10 mM dithriothreitol, 1 mM spermidine, 10 mM ATP and 1mg/ml of Bovine Serum albumin. E. Coli (strain LE 392, Ampicillin and, Tetracycline Sensitive) was then transformed by the Calcium Chloride procedure as follows: A culture of E. Coli in the logarithmic phase (0.4 to 0.60D at 260 nm, approximately equal to 6 x
Figure 2-1. Cloning strategy used for obtaining pBH1B and pBH1C. Ad 12 DNA was restricted with Bam HI and shot-gun cloned in pBR322. Details are outlined in the text. Abbreviations used are map units, (mu), Ap and Tc show the locations of the respective Ampicillin and Tetracycline genes in pBR322.
10^7 cells/ml) grown in Luria broth (10 g/L Bactotryptone, 5 g/L Bacto-yeast extract and 10 g/L NaCl) was chilled on ice for 10 minutes then pelleted at 4,000 g for 5 minutes. The pellet was re-dissolved in 1/2 the original volume in transformation buffer (50 mM CaCl_2 and 10 mM Tris-Cl, pH 8.0), held at 4°C for 15 minutes then the cells were pelleted and re-suspended in 1/15 the original volume in transformation buffer and held at 4°C overnight. The ligated DNA was added to the CaCl_2 treated cultures which were heat-shocked at 42°C for 2 minutes to maximize the efficiency of DNA uptake. The cultures containing the appropriate antibiotics were then heated for 45 minutes at 37°C to allow the bacteria to recover and tenfold dilutions of the bacterial culture were plated on Luria agar containing ampicillin (15 ug/ml).

Insertion of viral DNA into the Bam H1 site of the pBR322 inactivates the Tetracycline resistance gene. Seventy-two randomly picked colonies growing on the Ampicillin agar were replica plated on separate agar plates containing Tetracycline and Ampicillin respectively. Only those colonies showing Ampicillin resistance and Tetracycline sensitivity were selected for further analysis. After the DNA was extracted by the modified Birnboim and Doly procedure (1979), they were analysed by restriction mapping.

II.1.2.0. Analysis and Characterization of Recombinant DNA Clones
11.1.2.1. Small Scale Analytical Purification of Plasmid DNA from *E. Coli*

The method of Birnboim and Doly (1979) was used for rapid isolation of plasmid DNA from *E. Coli*. Two ml of an overnight culture, grown from a single colony in Luria broth containing 25 ug/ml of chloramphenicol, was centrifuged for one minute in an Eppendorf tube and the pellet re-suspended and digested for 10 minutes with 5 ug/ml of lysozyme in 100 ul of an ice-cold lysozyme buffer (50 mM Glucose, 10 mM EDTA, 25 mM Tris-Cl, pH 8.0). The bacterial spheroplasts so formed were lysed and the DNA concomittantly denatured with a 200 ul solution of 0.2 N NaOH and 1 % SDS. The bacterial DNA was then selectively precipitated, with 150 ul of 3 M potassium acetate of pH 4.8 and was removed by centrifugation for 5 minutes. The plasmid DNA was precipitated from the supernatant with 2 volumes of ethanol at room temperature, and pelleted by centrifugation as before. The pellet was washed once with 70 % of alcohol and dissolved in 50 ul of TE buffer (10 mM Tris-Cl, pH 8.0, 1 mM EDTA, pH 8.0).

II.1.2.2. Large Scale Isolation of Plasmid DNA for Labelling

For the purpose of unambiguous restriction analysis and labelling with (32P) DNA was purified by the cesium chloride banding technique (Maniatis *et al.*, 1982). A scaled-up version of
the Birnboim and Doly procedure was used and the DNA was extracted from the plasmids as in the previous Section with the exception that 500 ml of bacterial culture was initially used. After dissolution in TE buffer the DNA was further purified by cesium chloride-ethidium bromide density gradient. One gram of cesium chloride per ml of DNA solution was used and 0.8 ml of Ethidium bromide (10 ug/ml) was added per 10 ml of DNA solution. This solution was centrifuged in a Beckman Type-50 rotor at 45,000 rpm for 36 hours at 20°C. The band containing plasmid DNA was collected and the Ethidium bromide removed by X5 extraction with a solution of equal volume of 1-butanol saturated with iso-amyl alcohol (Maniatis et al., 1982). The aqueous phase was extensively dialysed against several changes of TE buffer and the amount of DNA estimated by absorbance value at 260 nm (1.0 D unit 260=50 ug/ml DNA, Clark and Switzer, 1977).

II.1.2.3. Restriction Enzymes Analysis of Ad 12 Clones

All Ad 12 clones were characterized by appropriate restriction enzyme digest according to standard procedures (Maniatis et al., 1982). The digested DNA was then analyzed by two kinds of gel electrophoresis; the minigel and vertical gel (Maniatis et al., 1982). In both types 1 -1.2 % agarose gel was used; in the minigel, electrophoresis was carried at 60V for 2 hours while in the vertical gel electrophoresis was carried for 10 hours at 25V. In both cases
the electrophoresis buffer was TBE buffer (0.089 M Tris-borate, 0.089 M boric acid and 0.01 M EDTA). The DNA was stained with Ethidium bromide (0.5 ug/ml) and photographed using UV illumination.

II.1.2.4. Nick Translation of Cloned Viral DNA

These DNA probes were labelled with $^{32}$P-dCTP using a modification of the Nick Translation procedure of Rigby (1977). One ug of DNA was incubated for one hour at 13°C in a 50 ul reaction mixture containing Nick translation buffer (50 mM Tris-Cl, pH 7.2, 10 mM MgSO$_4$, 0.1 mM dithiothreitol, 50 ug/ml Bovine serum albumin, 0.25 ng of DNase I (Sigma) in 0.15 M NaCl, 10 mM of each of the following nucleotides dATP, dTTP and dGTP, 6 units of E. Coli DNA polymerase I and 100 uCi of $^{32}$P dCTP (New England Nuclear, Specific activity 300 - 600 Ci/mM). The reaction was stopped with 25 ul of 250 mM EDTA pH 7.9 and the final volume brought to 100 ul with TE buffer. The labelled DNA was separated from the unincorporated nucleotides by spinning the mixture (1600 g, 4 minutes at 4°C) in a Sephadex G-50 column (bed volume 0.9 ml) that has previously been equilibrated in TE buffer (Maniatis et al., 1982) and used in the Northern blot analysis.

II.1.2.5. Construction of r-Strand Probe Specific for Region L5
The strategy used for converting pBH1E into single strand, L5 specific probe is shown in Figure 2-2. A restriction enzyme (Sal I) which cuts once in the pBR322 sequence at a site near the viral insert was used. The exposed 3' ends were digested with 5 units of Exonuclease III in Exonuclease buffer (50 mM Tris-Cl, pH 8.0, 5 mM MgCl₂ and 10 mM 2-mercaptoethanol). In order to ascertain that at least part of the 'r' strand had been removed, an aliquot of Exonuclease III treated DNA was treated by nuclease S1 for 30 minutes at 37°C with 100 units of enzyme in S1 buffer (0.28 M NaCl, 0.05 M sodium acetate, pH 4.6, 4.5 mM ZnSO₄ and 20 ug/ml single stranded salmon sperm DNA), the size of the remaining double stranded fragment was then determined by electrophoresis as in section II. 1.2.1.

II.1.2.5.1. Labelling with Klenow Fragment of DNA Polymerase I

Region L5, 'r' strand DNA from pBH1E was labelled with Klenow fragment of DNA polymerase I. The DNA used was the six minute Exonuclease treated one from the previous section. The reaction conditions were identical to those used for Nick translation with the following exceptions. No DNase was used and instead of the holo enzyme, the Klenow fragment of E. Coli DNA polymerase was used.
Figure 2-2. **Strategy used for constructing L5 Specific Probe**.

Details are discussed in the text. Solid line represents viral DNA and dotted line pBR322 DNA. Clone pBH1E was restricted with Sal I and treated with Exonuclease III for various lengths of time. The size of the remaining double stranded fragment was determined after removing the single strands with S1 nuclease. The restriction enzyme results at the bottom right show the size of the treated DNA fragments. Notations used are: pBH1E restricted with Sal I; 2, Hind III digest of Ad 12 as markers; The numbers 3, 4 and 5 represent treatment of the Sal I restricted pBH1E with Exonuclease III then S1 for 2, 4 and 6 minutes respectively. The location of the 3460 bp, which is not visible in this reproduced photograph, is shown by an arrowhead.
CONSTRUCTION OF L5 PROBE

PROBE

CONSTRUCTION

OF

L5

P

BamH1

pBH1E

Sall

BamH1

3710 bp

4152 bp

5500 bp

100 mu

Exo III

Nuclease SI

3'-5' exonuclease

Klenow(2'-p')dNTP

BamH1

1

r

3460 bp
II.2.0. Biological and Biochemical Techniques

II.2.1. Tissue Cultures

Human KB cells were used for viral growth. Monolayers were grown and maintained on F11 medium containing 10% calf serum, 50 units/ml of penicillin and 50 ug/ml of streptomycin (Gibco Laboratory, Canada). The cultures were incubated at 37°C in a 5% CO₂ incubator. Suspension cultures were maintained at a density of 2-4 x 10⁵ cells/ml in Joklik's medium containing 5% horse serum (Gibco Laboratory, Canada).

II.2.2. Types and Sources of Viruses

The following viruses were used in this study: Ad 12 strain 1131; H12 cyt 61; H12 cyt 68; Ad 2 dl 250; Ad 5; H5 dl 313 and H5 hr-6. Ad 2 dl 250 was obtained from Dr. Chinnadurai laboratory (St. Louis, Missouri); Ad 5 and Ad 5 hr-6 was obtained from Dr. Graham (McMaster University). The authenticity of the hr-6 mutant had already been checked by plaque assay on 293 and Hela cells.

The cyt mutants and Ad 12 strain 1131 were originally
obtained as crude stock form Dr. Takemori (St. Louis, Missouri) and have been passaged many times in Dr. Mak's laboratory. The remaining viruses were obtained from Dr. Mak's laboratory.

II.2.3. Growth and Purification

II.2.3.1. Ad 12 Strain 1131

KB cells were infected at a multiplicity of infection (moi) of 200 virions per cell and adsorbed for 90 minutes at 37°C in Joklik's medium containing 1 % calf serum then maintained in suspension for 72 hours.

The method of Green and Pina (1963) was used for extraction and purification. Infected cells were pelleted and re-suspended in 10 mM Tris-HCl, pH 8.0; sonicated for 30 minutes and homogenized twice with Freon 113 at 4°C. The aqueous phase was concentrated on a cesium chloride cushion, at a buoyant density of 1.44 g/ml and the virus purified by isopycnic banding in cesium chloride at a buoyant density of 1.34 g/ml at 33,000 rpm for 20 hours at 5°C in a Beckman ultra-centrifuge. The concentration of the virions was estimated by measuring the absorbance at 260 nm; one absorbance unit corresponding to $4 \times 10^{11}$ virion/ml (Mak, 1971).
II.2.3.2. Mutants H12 Cyt 68

These were grown like the parental strain except that, due to the fragility of the infected cells, they were maintained as monolayers of infected cells and harvested at 48 - 50 hour post-infection. The method of Lawrence and Ginsberg (1967) was used for purification. Infected cells (3 - 4 x 10^7 cells/ml) were lysed by 0.5 % w/v sodium deoxycholate for 30 minutes at room temperature and treated with DNase I (25 ug/ml) in 20 mM MgCl₂ for one hour at 37°C then purified as in the previous section (starting on CsCl cushion).

II.2.4.0. Isolation of Cytoplasmic RNA from Virus Infected Cells

KB cells were infected at an moi of 200 virions per cell for all viruses except in experiments where the moi was varied. For the Ad 5 and hr-6 the multiplicity of infection was 5 pfu/cell. The degree of infection of each virus was monitored by observing the time of appearance and extent of cytopathic effect (CPE). After 48 hours aliquots of all infected cells were stained with Orcein and examined for the presence of intra-nuclear inclusion bodies which are characterized by crystals formed upon infection of the Adenovirus.

Infected cells were harvested, washed once with phosphate
buffered saline (pH 7.4) and re-suspended in isotonic buffer (0.15 M NaCl, 10 mM EDTA, 10 mM Tris-Cl, pH 7.8) at a concentration of 3 - 5 x 10^7 cells/ml then lysed for 5 minutes with an equal volume of isotonic buffer containing 1.4 % NP40 (Shell Chemical Corp.) and 10 mM vanadyl-ribonucleoside complex (VRC) as an RNase inhibitor (Maniatis et al., 1982). The nuclei were pelleted at 3,000 g, for 10 minutes, the cytoplasmic fraction was adjusted to 1 % SDS and 25 mM EDTA to solubilize the proteins then repeatedly extracted with a 1:1 chloroform-phenol mixture equilibrated with isotonic buffer (10 mM sodium acetate, pH 6.0, 100 mM NaCl and 1 mM EDTA) containing 0.1 % hydroxyquinoline until all the VRC was removed. This was achieved when the organic phase no longer turned black (Maniatis et al., 1982). The resultant aqueous phase was re-extracted twice with a mixture of chloroform : iso-amyl alcohol (24:1), adjusted to 0.15 M NaCl and precipitated with 2.5 volume of 95 % ethanol at -70°C overnight. The RNA was pelleted at 24,000 g, 30 minutes at 4°C and redissolved in 1/20th the original volume in sterile distilled water. The concentration of RNA was estimated by absorbance reading at 260 nm; one absorbance unit corresponding to 47 ug/ml of RNA (Reed et al., 1977).

II.2.4.1. Fractionation of RNA by Electrophoresis in Agarose Gel

The RNA samples were denatured by heating in 2.2 M
formaldehyde and 50% formamide at 60°C for 15 minutes then analyzed by two kinds of electrophoresis under denaturing conditions in 1.9% horizontal agarose gel (Lehrach et al., 1977). In both types, the electrophoresis buffer contained 2.2 M formaldehyde, 5 mM sodium acetate, 1 mM EDTA and 100 mM MOPS (Morpholinopropanesulfonic acid), pH 7.0. For the purpose of Northern blot analysis, electrophoresis was carried out at 40 V for 16 hours (2 V/cm) whereas for the purpose of quantitating the RNA electrophoresis was carried at 100 V for half an hour.

II.2.4.2. Transfer of RNA to Nitrocellulose Filter

The RNA containing gel was soaked in an excess of 50 mM NaOH for 40 minutes then neutralized with a solution containing 3 M NaCl, 0.5 M Tris-Cl, pH 7.4 for one hour and transferred to nitrocellulose filter as follows. The gel was then placed on 2 sheets of Whatmann 3 MM chromatography paper saturated with 20 x SSC (Standard Sodium Citrate) and the borders sealed with plastic saran wrap. Nitrocellulose filter paper (Satorius) was placed on the gel which was overlaid with Whatmann 3 MM paper, followed by a stack of paper towels and a light weight. Transfer was done overnight, the filter was fixed by baking for 3 hours at 80°C and stored in sealed plastic bags until used for hybridization.
II.2.4.3. Hybridization of RNA to DNA by Northern Blots

This was done by a two step procedure using the method of Thomas (1980). To reduce non-specific binding of the probe, the nitrocellulose filter containing RNA was pre-incubated for 4 hours at 42°C in a sealed polyethylene bag containing 10 ml of pre-incubated buffer composed of 50 % Formamide, 5 x SSC, 5 x Denhardt's reagent (0.02 % w/v bovine serum albumin, 0.02 % w/v polyvinyl pyrrolidone 0.02 % Ficoll), 50 mM sodium phosphate pH 6.5 and 250 ug/ml of denatured single strand salmon sperm DNA. The pre-incubated buffer was removed and the probe which has been denatured for 5 minutes at 100°C in 10 ml of hybridization buffer (50 % formamide, 5 x SSC, x 1 Denhardt's reagent, 20 mM sodium phosphate, pH 6.5, 100 ug/ml denatured salmon sperm DNA and 10 % sodium dextran sulphate) was added to the bag containing the pre-incubation RNA. Hybridization was carried for 12 - 16 hours at 43°C. The filters were then washed three times (5 minutes each) with 2 x SSC and 0.1 % SDS at room temperature followed by three fifteen minute washes in 0.1 x SSC and 0.1 % SDS at 50°C. The filters were oven dried for 20 minutes at 80°C and the radioactivity detected by autoradiography by exposing the filters to a Kodak XRP-5 X-ray film for 1 to 3 days then processed by an automatic film developer.
II.2.4.4. Quantitation of Radioactivity Hybridized to RNA

RNA was quantitated by microdensitometry. The autoradiographs were scanned by using a MK III CS double beam recording microdensitometer (Joyce Loebl Co., England). The area under the densitometer scans was used as a measure of the relative amount of radioactive probe hybridized, and hence the relative amount of RNA present. The relative area from each resultant peak was integrated using an automatic area analyzer (MOP-3, Carl Zeiss) then plotted versus the corresponding RNA concentration. The level of RNA accumulated was calculated from the relevant slopes. In keeping with the order of accuracy of the technique used, the slopes were rounded to the nearest whole number.
IIII. RESULTS

III.1. Restriction Analysis of Ad 12 Recombinant Clones used as Probes

Ad 12 recombinant clones which had been purified by banding in Cesium chloride-Ethidium bromide gradients were analyzed by restriction mapping with the appropriate enzymes. For some clones a combination of enzymes was used to unambiguously identify a diagnostic fragment. The results of such analyses are given in Figure 3-1. Lanes A, B and C show the molecular weight markers which are Ad 12 DNA restricted with Hind III, EcoRI and Bam HI enzymes respectively. The lanes numbered 1 to 8 represent the appropriate clones as explained in the Figure legend.

Clone pHA5 contains 0 to 4 μm (1 μm = 350 bp) of Ad 12 DNA inserted into the plasmid pBR322. Since the viral insert contains a Kpn I site at 1.4 μm and the plasmid contains a unique Hind III site double digestion of pHA5 with Hind III and Kpn I produces a diagnostic 836 bp fragment (lane 1). This fragment is composed of 490 bp (1.4 μm) of Ad 12 DNA and 346 bp of plasmid DNA spanning the Hind III and Bam HI sites.

Clone pHBl4R contains 2.1 μm of DNA (735 bp) spanning the Acc I site at 4.6 μm and the Hind III site at 6.7 μm. On double digestion
Figure 3-1. **Origin and Analysis of Adenovirus 12 Clones**. An idealized Adenovirus 12 genome is shown from 0 to 100 map units (μu). The Bam H1 fragments C, B and E refer to strain 1131 and the Hind III B fragment (18 to 32 μu) refers to Huie strain. See text for details of all clones. Two micrograms of Ad 12 DNA was restricted, electrophoresed on 1% Agar for 13 hours at 30V and photographed after staining with ethidium bromide. The restriction digest results at the bottom refers to each clone. Notations used for Ad 12 markers are: A, Hind III digest; B, EcoRI digest; C, Bam HI digest. The corresponding fragments are alphabetically labelled. Abbreviations used for the clones are: 1, pHA5 restricted with Hind III and Kpn I; 2, pHB14R restricted with Hind III and Acc I; 3, pH40 restricted with Hind III; 4 and 5 , pBH1C restricted with Bam HI and EcoRI respectively; 6 and 7, pBH1B restricted with Bam HI and Hind III and 8 and 9, pBH1E restricted with Hind III and Bam HI respectively. The diagnostic fragments as discussed in the text are also indicated by an arrowhead.
with Acc I and Hind III enzymes a diagnostic 735 bp fragment (lane 2) was obtained.

Since clone pH40 contains the Hind III B fragment of Ad 12 genome (Huie strain), a Hind III B fragment was obtained by restriction with the Hind III enzyme (lane 3).

Clone pBH1C contains Bam H1 C fragment (lane 4) which yielded a diagnostic EcoR1 F fragment (lane 5) on digestion with EcoR1 enzyme.

Clone pBH1B contains a Bam H1 B fragment (lane 6) enclosing a diagnostic Hind III H fragment. On digestion with Hind III, the identity of this clone was confirmed (lane 7).

Clone pBH1E contains the Bam H1 E fragment as was confirmed by the Bam H1 digestion of the clone (lane 9).

III.2.0 Analysis of Viral Specific RNA

III.2.1. Adenovirus Group A and Mutants

III.2.1.1. Adenovirus 12 and Cyt 68 Mutants

Total cytoplasmic RNA was isolated at 71/2, 12 - 13 and 23 hours post-infection to represent RNA produced at "early", "intermediate" and "late" time of the Ad 12 replicative cycle. Sixty
38 micrograms of cytoplasmic RNA was used for Northern blots.

Figure 3-2 shows the results of probing the RNA extracted from KB cells infected with Ad 12 or with cyt 68 at an moi of 200 virions per cell. The identity of each probe is shown at the top of each panel. Negative control (uninfected cellular RNA) is shown in lane 7 of each panel. Size markers used throughout these experiments were denatured Hind III restriction fragments of Ad 12 DNA (lane Mr). The Hind III digest was used to construct a standard curve (Figure 3-3) for molecular weight estimation. The locations of standard cellular RNA species were determined by similarly fractionating $^3$H-Uridine labelled total cellular RNAs.

It is seen that for region E1A at early time both Ad 12 (lane 1) and cyt 68 (lane 2) infected cells accumulated one major species of RNA that co-migrated as a band of 1.3 to 1.4 Kb in size. The cyt 68 specific RNAs were visibly less than the Ad 12 ones. At intermediate time Ad 12 (lane 3) and cyt 68 (lane 4) maintained the same pattern. However, at late time the pattern was reversed. The amount of hybridizing Ad 12 specific RNA (lane 5) was visibly less than those of cyt 68 (lane 6). The region E1A of Ad 12 virus is known to encode three major species of mRNAs; 13S (1.1 Kb); 12S (0.98 Kb) and 9S (0.6 Kb). Sawada and Fujinaga (1980) have reported that the 12S and 13S RNA co-migrate as one major band whereas the detection of the 9S species was not always seen. These observations are consistent with the present one. However, Lai Fatt (1983) has detected five major species of RNA ranging from 3.1 to 0.8 Kb in size.
Figure 3-2. Northern Blot Analysis of Adenovirus 12 and Cyt 68 RNA Detected by Hybridization to cloned \(^{32}\text{P}\) Labelled Viral DNA.

Sixty micrograms of total cellular RNA was fractionated in 1.9 % denaturing agarose gel for 10 hours at 40V, transferred to nitro-cellulose and hybridized to viral DNA. The region E1A RNA was detected with clone pHA5; region E1B with pHAB14R; region "E2A" with pBH1C; region "E2B" with pH40; region "E3" with pHBH1B; and region "E4" with pBH1E. Molecular weight markers were: Mr, mixture of Hind III digested Ad 12 hybridized to pHA5. The units for the sizes are in Kb. The approximate location of total cellular RNA was determined by fractionating \(^{3}\text{H}\)-Uridine labelled cellular RNA under the same experimented conditions of electrophoresis. Notations used are:

channel 7, mock-infected KB cells RNA. Channels 1 and 2 respectively represent Ad 12 and cyt 68 RNAs at 7 hours post-infection; channels 3 and 4 respectively represent Ad 12 and cyt 68 RNAs at 12 hours post-infection and channels 5 and 6 respectively represent Ad 12 and cyt 68 RNAs at 23 hours post-infection.
Figure 3-3. Calibration Curve used for the Estimation of the Sizes of Viral Specific RNA. Hind III digested Ad 12 DNA was fractionated under identical conditions to the RNA and hybridized to nick-translated total viral DNA. Experimental details are as given in the text.
Distance travelled (m)
This difference can be partly accounted for by the fact that in this case total cytoplasmic RNA was used whereas Lai Fatt selected only poly(A)+ RNA. RNAs containing 3' poly(A) tails are variable in length—a property which may limit the resolution of molecules with small differences in mobility (Browerman, 1976). It is also known that the conditions of fractionation may affect the resolution of RNA species (Lehrach et al., 1982). As the conditions in the present study and those of Lai Fatt were not identical, the apparent difference may partly be accounted by this.

No RNA species hybridizing to probe for region E1B could be detected at early times in Ad 12 (lane 1) or cyt 68 (lane 2) infected KB cells. Barely visible species of RNA of 1.2 to 2.4 Kb were seen in Ad 12 (lane 3) and cyt 68 (lane 4) at intermediate times. These RNA species increased in intensity at late times. For Ad 12 (lane 5) it was clearly less than that of cyt 68 (lane 6). The present observations are in agreement with those of Sawada and Fujinaga (1980) who did not observe any hybridizing RNA at early times. Similarly, Perricaudet et al. (1980) found no cDNA sequences complementary to region E1B of Ad 12 among 10,000 cDNA clones of early mRNA from Ad 12 infected KB cells and no hybridization was detected between single stranded (32P)-DNA copies of this mRNA and the Hind III-I fragment (6.7 to 10.8 mu). However, Lai Fatt (1983) found four major bands of 2 - 3 to 0.5 Kb in size at early times using Hind III-I fragment of Ad 12 or the Accl-J fragment (4.6 - 6.7 mu) as probes. The E1B probe, pH14R, used here also contains 4.6 to 6.7 mu of the
adenovirus 12 genome yet no early transcripts could be detected. Since the studies of Lai Fatt were performed using uncloned restriction fragments, the possibility of contamination with other incompletely digested restriction fragments cannot be ruled out.

By means of probe "E2A" one major species of RNA of 1.8 Kb was seen at early times for both Ad 12 (lane 1) and cyt 68 (lane 2). This RNA was visibly of equal intensity in levels for both viruses and is indicative of the fact that the degree of infection for both viruses had proceeded to the same extent. At intermediate times the pattern changed for both Ad 12 (lane 3) and cyt 68 (lane 4) as the amount of RNA accumulated increased. This pattern was maintained for Ad 12 (lane 5) and cyt 68 (lane 6) at late time also. However due to poor resolution conditions it was not possible to determine whether or not the increased amount of RNA detected was due to the presence of novel species. Berk and Sharp (1977) using S1 mapping reported three species of E2A specific RNA ranging from 2.2, 1.96 and 1.8 Kb for Ad 5. The E2A RNA observed at intermediate and late times for Ad 12 seem to fall within these ranges, thus confirming the Northern blot profiles of group A and C Adenoviruses.

For region "E2B" at early times no hybridizing RNA species were detected for Ad 12 (lane 1) or cyt 68 (lane 2). This observation is in agreement with those of Stillman (1981) who found the Ad 5 E2B transcript to be the rarest of early transcripts. At intermediate time one major species of RNA of about 2 Kb in size was seen for both Ad 12 (lane 3) and cyt 68 (lane 4). The amount of RNA
accumulated by Ad 12 was visibly higher than that of \textit{cyt 68}. At late times both Ad 12 (lane 5) and \textit{cyt 68} (lane 6) were accumulating visibly similar amount of RNA. As for region E2A, it was not possible to determine if the increased amount of RNA was due to novel species of RNA.

For region "E3" at early time three major species of RNA ranging from 2.9 Kb to 1.5 Kb was observed for both Ad 12 (lane 1) and cyt 68 (lane 2). Essentially the same pattern was maintained at intermediate (lanes 3 and 4) and late (lanes 5 and 6) times. However, at late times the level of \textit{cyt 68} RNA (lane 6) was visibly higher than that of Ad 12 (lane 5) and possibly novel species of RNA were being detected. The finding of four major species of RNA is consistent with that of Anderson and Klessig (1982) who observed four species of 2.7 Kb, 2.6 Kb, 1.8 Kb and 1.0 Kb mRNA by Northern blot analysis of Ad 2 infected cells. The region E3 Northern blot profile of Ad 12 and Ad 2 thus seemed to exhibit the same pattern.

For region "E4" three major species of RNA of 2.9 to 1.5 Kb in size were observed at early times in both Ad 12 (lane 1) and \textit{cyt 68} (lane 2) infected cells. This pattern was essentially the same as that observed for region E3 at both intermediate (lanes 3 and 4) and late (lanes 5 and 6) times. Again at late time the levels of RNA accumulated in \textit{cyt 68} infected cells (lane 6) were visibly higher than those of Ad 12 ones (lane 5). The observation of three major species of RNA is in agreement with those Ad 2 reported by Anderson and Klessig (1982) who also observed three major species of RNA of
sizes falling within the range reported here. This further confirms the similarity of structural organization of Ad 12 and Ad 2 region E4 as probed by Northern blots.

III.2.1.2. Adenovirus 12 and Cyt 61 mutants

Ad 12 cyt 61 is an Ad 12 mutant that is dominant in contrast to cyt 68. Since it has been suggested that mutation maps elsewhere (Lai Fatt and Mak, 1982), it was of interest to study its Northern blot profile in comparison to the E1B mutant cyt 68. The results of probing RNA extracted from Ad 12 and cyt 61 infected cells at early and late times are shown in Figure 3-4. The probes used are indicated at the top of each panel and the molecular weight markers are as described for Figure 3-2. Qualitatively the viral transcripts were similar to those seen for Ad 12 and cyt 68 but quantitatively there appeared to be a difference. Thus for region E1A, one major species of 1.4 Kb RNA was present at both early (lane 2) and late times (lane 4) as had been observed for cyt 68. However, the levels of RNA accumulated by Ad 12 infected cells were consistently higher (lanes 1 and 3) than those of cyt 61 (lanes 2 and 4) infected ones. Similarly for region E1B no detectable level of E1B transcript were detected at early times in both Ad 12 (lane 1) and cyt 61 (lane 2) infected cells. However, at late time two major species of 2.4 Kb and 1.2 Kb were detected in Ad 12 (lane 3) and cyt 61 (lane 4). These
Figure 3-4. Northern Blot Analysis of Adenovirus Type 12 and Cyt 61 RNA Detected by Hybridization to Cloned $^{32}$P Labelled Viral DNA. See Figure 3-4 for details. Sixty microgram of total cellular RNA was used and electrophoresis was carried for 18 hours at 40V. Notations used are: Mr for Hind III digest of Ad 12 as marker, Ms for total cellular RNA as marker. The units for sizes are Kb. Channels 1 and 2 respectively represent Ad 12 and cyt 61 RNAs at 7 hours post-infection and 3 and 4 respectively represent Ad 12 and cyt 61 RNAs at 23 hours post-infection.
The image contains a series of gel electrophoresis patterns labeled as E1A, E1B, "E2A," "E2B," E3, and E4. Each gel strip is numbered from 1 to 4. The gels show bands at different molecular weights, indicated by the Mr and Ms markers on the left and right sides of the figure, respectively. The bands are numbered 4-7, 1-4, and additional markers like 5-3, 3-3, 2-3, and 1-0 are also present.
ElB specific transcripts were apparently of a higher concentration in Ad 12 than in cyt 61 infected cells.

For region "E2A" the Northern blot pattern deviated from that of cyt 68. In the present case four major species of RNA ranging from 2.2 Kb, 1.9 Kb, 1.8 Kb and 1.7 Kb were seen at a higher level in cyt 61 infected (lane 2) than in Ad 12 infected cells (lane 1) at early times. The pattern however seemed to alter as the infection progressed; at late times Ad 12 (lane 3) accumulated more RNA than did cyt 61 (lane 4).

The accumulation of RNA hybridizing to regions "E2B", "E3" and "E4" specific probes followed essentially the same qualitative and quantitative pattern as those for cyt 68. Thus for region E2B no major transcripts were seen at early times (lane 2) but two major species were seen at late times (lane 3). Similarly three major species of RNA were seen for region "E3" (lanes 2 and 4) as for region "E4" (lanes 2 and 4). At late times the accumulation of RNAs in cyt 61 infected cells were of higher intensity for region "E3" and "E4" (the respective lane 4) than for Ad 12 (the respective lane 3).

III.2.2. Adenovirus Group C and Mutants

Although the cyt mutation has been mapped by complementation studies, the exact nature of the mutation is unknown. In an effort to better understand the nature of the mutation(s), it was decided to
turn to more defined mutants of Ad 2 and Ad 5. Ad 2 dl 250 and Ad 5 dl 313 are both characterized by a DNA degradation phenotype, similar to the cyt mutants. Since the region E1A and E4 RNA seemed to be affected most by the E1B mutation in cyt 68, it was decided to examine the pattern of RNA accumulation of dl 250 and dl 313 mutants. To compensate for the differences in the time course of the groups A and C replicative cycles, "early" RNA was extracted at 5 1/2 hours post-infection and "late" RNA, at 15 hours post-infection.

III.2.2.1. Ad 2 and dl 250

The results of probing RNA extracted from Ad 2 and dl 250 infected cells are shown in Figure 3-5. Molecular weights markers are the same as described for Ad 12 in Figure 3-2. The probes used are shown on the top of each panel. For region E1A at early times one major species of RNA of approximately 1.3 Kb in size was seen for both Ad 2 (lane 1) and dl 250 (lane 2). This pattern was also maintained at late times although the RNA accumulated in the dl 250 infected cells appeared to be degraded.

For Ad 2 (lanes 1 and 3) and dl 250 (lanes 2 and 4) infected cells the accumulations of RNAs hybridizing to region "E4" were of three types exhibiting a range of 2.0 to 1.7 Kb in size. The RNA produced by dl 250 infected cells at late times (lane 4) was visibly higher than those of Ad 2 infected ones (lane 3). The results of the
Figure 3-5. Northern Blot Analysis of Adenovirus Type 2 and dl 250 RNA Detected by Hybridization to $^{32}$P Labelled Viral DNA.

Details are as given in Figure 3-5. E1A RNA was detected by pHE1 and the "E4" RNA by pH14. M represents Hind III digest of Ad 12 detected by hybridization to 16% of Ad 12 DNA (clone RC6). Channels 1 and 2 represent Ad 2 and dl 250 respective RNA at 5 hours post-infection and channels 3 and 4 represent Ad 2 and dl 250 RNA at 15 hours post-infection.
region E1A and E4 RNA for Ad 2 reported here are consistent with the published results of Ad 2 by Anderson and Klessig (1982).

III.2.2.2  Ad 5 and Mutants

The results of probing RNA extracted from Ad 5 and dl 313 infected cells are shown in Figure 3-6. The ($^{32}$P)-labelled probes are indicated at the top of each panel and the molecular weight markers are as described previously. For region E1A at early time one major species of RNA of 1.3 kb was seen for Ad 12 (lane 1) but a slightly higher (approximately 1.5 kb) species of RNA was seen for dl 313 (lane 2) infected cells. However, the level in dl 313 was visibly lower than that in Ad 5 infected cells. At late time only one major species of RNA was present in Ad 5 (lane 3) and dl 313 (lane 4) infected cells. At that time the levels of RNA accumulated by both viruses were barely visible, an observation that indicates that the accumulation of E1A specific RNA is reduced at late time.

For region E4 three major species of RNA of about 2.3 to 1.0 kb were seen in Ad 5 (lane 1) and dl 313 (lane 2) infected cells at early times. This pattern was maintained at late times, but the Ad 5 infected cells (lane 3) accumulated visibly higher levels of "E4" specific RNA than did the dl 313 infected ones (lane 4).

These observations for Ad 5 region E1A and E4 specific RNAs are in agreement with those previously reported by Berk and Sharp.
Figure 3-6. Northern Blot Analysis of Adenovirus Type 5 and dl 313 RNA Detected by Hybridization to $^{32}$P Labelled Viral DNA. See figure 3-6 for probes and markers used. Abbreviations used: channels 1 and 2 represent Ad 5 and dl 313 respective RNA at 5 hours post-infection; channels 3, 4 represent Ad 5 and dl 313 respective RNA at 15 hours post-infection. Top: Northern blot. Bottom: modification of Northern blot used for the quantitation of RNA (shown in Figures 3-17 and 3-18). a, b, c represent concentration of 5, 10 and 15 ug of RNA respectively.
III.3.0. Quantitation of Levels of Accumulated RNA

In order to gain a quantitative appreciation of the differences in the levels of RNAs observed by the Northern blot analysis microdensitometry was used. For this purpose, measured amounts of RNAs were electrophoresed for a short length of time at high voltage to circumvent the problem of resolving the total RNA into its component species. The amount of RNA present was then estimated by densitometric scanning of the autoradiograms. The amount of radioactive probe was used as a relative measure of hybridized RNA present. For all the quantitation data, a linear response was observed for the varying concentrations of RNA used, indicating that the amount of radioactive probe hybridized was a true measure of the amount of viral specific RNA that were present.

III.3.1. Adenovirus 12 and Mutants

The results of the accumulation of the levels of E1A-specific RNA in Ad 12 and cyt 68 infected cells at early and late times are shown in Figure 3-7. The horizontal axis represents the concentrations of RNA used and the vertical axis represents the
Figure 3-7. **Quantitation of Adenovirus 12 and cyt 68 E1A RNA at early and late times**. Varying concentrations of RNA were electrophoresed under denaturing condition for half hour at 100V and detected by hybridization to $^{32}$P labelled cloned E1A DNA. The radio activity hybridized to each RNA was quantitated by densitometric scanning and the area of the peak integrated by an automatic area analyzer. These areas are plotted versus their respective RNA concentrations. Notations used are: C.Lt, _ cyt 68_ late RNA; W Er, Ad 12 Early RNA; W Lt, Ad Late RNA; C Er, _ cyt 68_ Early RNA and CO, RNA from mock-infected cells. a, b, c, d, e, and f represent concentrations of 10, 20, 30, 40 and 50 ug of RNA per lane.
relative peak areas obtained from densitometric scanning. The inset at the top left hand corner shows the actual autoradiogram from which the relative areas were calculated. It is seen that no hybridisation was detected in the mock-infected, negative control (inset CO). From the various slopes it was found that the levels of RNA accumulated in the Ad 12 infected cells at early times, were about three times greater than the corresponding level at late times. But for the cyt 68 mutant, this trend was reversed; the level of late E1A RNA was about 5 times greater than the corresponding level at early time. The ratio of the level of Ad 12 to cyt 68 RNA at early time was approximately 1 : 5. Whereas the same ratio at late time was almost reversed being 1 : 0.42. These levels were consistent with those visually estimated by the Northern blot technique.

Since the dl 313 mutant is known to show a multiplicity dependent leakiness, it was decided to investigate the effect of moi on the appearance of cyt 68 E1A early messages. The results are shown in Figure 3-8. Over the range tested, 50 to 1600 moi per cell, the difference in the levels of wild type and cyt 68 RNA was maintained. At the lower moi of 50, 100, 200 the level was about three times less in the cyt 68 infected cells than in Ad 12 ones. At an moi of 400 the difference was reduced to only a twofold factor but thereafter the difference widened until at an moi of 1600 the wild type was accumulating 8 times as much RNA as the cyt.

Figure 3-9 shows the results of accumulation E1B-specific transcripts at intermediate and late times from Ad 12 and cyt 68
Figure 3-8. **Effect of moi on the Accumulation of ElA Messages in Ad 12 and cyt 68.** Relative accumulation of early RNA in Ad (o) and cyt 68 (o) infected KB cells infected at moi's of 50, 100, 200, 400, 800 and 1600 virions per cell. Ten micrograms of RNA from each moi was used for quantitation of the RNA. The relative areas under the densitometer scans were used as a measure of radioactivity of (32p) of the RNA for ten micrograms of RNA.
Figure 3-9. Quantitation of Ad 12 and cyt 68 region E1B RNA.
Details are essentially as given in figure 3-8. The probe used was pHBl4R. Symbols used are: (●) Ad 12 intermediate RNA; (o) Ad 12 late RNA; (■) cyt 68 intermediate RNA; (□) cyt 68 late RNA.
M 1crograms of RNA

PEAK AREAS

Micrograms of RNA

10 20 30 40 50
infected cells. Details are essentially as described earlier. From the various slopes it is seen that at intermediate time Ad 12 accumulated twice as much RNA as did cyt 68. However, at late times this pattern changed; the levels of E1B-specific transcript in cyt 68 infected cells were approximately four times those in Ad 12 infected ones. These results are also consistent with the visual estimates of the Northern blot analysis.

The accumulations of transcripts hybridizing to region "E2A" probe from Ad 12 and cyt 68 infected cells at early and late times are shown in Figure 3-10. From the various slopes it is seen that at early times both Ad 12 and cyt 68 accumulated approximately equal amounts of RNA, the respective ratio being 1.5 : 1. Within the experimental range of the technique, this difference is not considered to be significant. But at late times Ad 12 accumulated twice as much RNA as did cyt 68.

Figure 3-11 shows the accumulation of Ad 12 and cyt 68 transcripts hybridizing to probe "E2B" at intermediate and late times. From the relevant slopes it was calculated that at intermediate time Ad 12 accumulated approximately three times as much RNA as did cyt 68. However, at late times cyt 68 accumulated twice as much RNA as did Ad 12.

The accumulations of transcripts hybridizing to probe from region "E3" at late times are shown in Figure 3-12. From the slopes it is seen that at late times cyt 68 accumulated approximately three times RNA as did Ad 12. This pattern is again consistent with the
Figure 3-10. Quantitation of Ad 12 and cyt 68 E2A RNA at early and late times. Details are same as in Figure 3-8. Symbols used are:

- (●) Ad 12 early RNA,
- (o) Ad 12 late RNA,
- (■) cyt 68 early RNA,
- (□) cyt 68 late RNA.
Micrograms of RNA
Figure 3-11. Quantitation of Ad 12 and cyt 68 "E2B" intermediate and late RNA. Details are given in Figure 3-8. Symbols used are:

- (●) Ad 12 intermediate RNA,
- (o) Ad 12 late RNA,
- (■) cyt 68 intermediate,
- (□) cyt 68 late RNA.
Figure 3-12. Quantitation of Ad 12 and cyt 68 "E3" RNA at late times. Details are as is given in Figure 3-8. Symbols used are: (o) Ad 12 late RNA and (□) cyt 68 late RNA.
visual estimate of the Northern blots.

The levels of "E4" specific RNA extracted from Ad 12 and cyt 68 infected cells at late time are shown in Figure 3-13. It is seen that the ratio of cyt 68 : Ad 12 late "E4" RNA was approximately 5 : 1, confirming the visual impression seen in the Northern blot.

III.3.2. Accumulation of RNA Hybridizing to L5 Probe

The accumulation of region "E4" RNA at late times seems to be affected by the E1B mutants. This region also contains sequences complementary to the late, L5 RNA which has been shown to be affected by E1B mutation (Fukui et al., 1984). It was therefore decided to investigate the level of L5 specific RNA using specifically constructed 'r'-strand specific probe.

III.3.2.1. Construction of L5 Specific 'r' Strand Probe

Figure 2-1 illustrates the strategy used for constructing 'r' strand probe. Clone pBH1E contains both the 'l' (E4) and 'r' (L5) strands of the Ad 12 DNA. To convert this into a single "r" strand specific DNA, the plasmid was opened once and the '3' ends of the DNA strands were asymmetrically digested with Exonuclease III enzyme. To predict the products of this approach it was first necessary to
Figure 3-13. Quantitation of Adenovirus 12 and cyt 68 E4 RNA at early and late times. Details and notations are the same as in Figure 3-8.
ADENOVIRUS 12 & CYT 68 E4 RNA

MICROGRAMS OF RNA

AREA (X 10^9)

A B C D E

C L

W L
determine the orientation of the viral insert. There are two possible orientation for the insertion of the viral fragment but these can be distinguished by the location of the asymmetrically placed Hind III site in the plasmid. In the orientation shown in Figure 3-1, the predicted sizes of the fragments are 4708 and 3806 base pairs respectively. These sizes were confirmed in a Hind III digest of pBH1E (Figure 3-1,lane 8).

Digestion of the pBH1E clone with Sal I opens the plasmid once and exposes the viral DNA flanked by two 3710 bp and 650 bp of asymmetric pBR322 DNA, as shown in Figure 2-1. On digestion with Exonuclease III for 6 minutes and estimation of the double stranded fragment it was found that the size of this fragment was approximately 3460 bp. Since the original double stranded molecule was 8512 bp, 5052 bp must have been removed. Assuming that the rate of digestion of the Exonuclease enzyme to be the same on both sides of the DNA, this means that 2526 bp (that is 5052/2) had been digested and that approximately 1876 bp of viral DNA (2526 - 650 of flanking pBR322 DNA) of the 'r' strand were available for labelling by Klenow polymerase.

III.3.2.2. Quantitation of L5 Specific RNA

The accumulation of L5 specific RNA at late times and cyt 68 infected cells are shown in Figure 3-14. Using the L5 specific probe it is seen that cyt 68 (line 68 SS) accumulated three times as much
Figure 3-14. Quantitation of Ad 12 and cyt 68 RNA Hybridizing to E4 and L5 Probes at Late Times. See Figure 3-8 for experimental details. Symbols used: 68 ds and ss, cyt 68 RNA detected by pBHIE and L5 probes respectively; 12 ds and ss, Ad 12 RNA detected by pBHIE and L5 probes respectively.
ADENOVIRUS 12 & 68 E4 RNA

- 68ds
- 12ds
- 68ss
- 12ss

MICROGRAMS OF RNA
RNA as did Ad 12 (line 12 SS). As seen in the same Figure this ratio was consistent with that observed with the double stranded probe.

III.3.3. Adenovirus Group C and Mutants

III.3.3.1. Adenovirus 2 and dl 250

The results for Ad 2 and dl 250 E1A RNA are shown in Figure 3-15. From the respective slopes it is calculated that the level of E1A RNA in Ad 2 infected cells at early time was found to be approximately equal to twice that produced at late time, confirming the visual impression that had been observed by the Northern blot analysis. For the dl 250 mutant this trend was reversed at late time; the level of E1A RNA was about 3 times greater than that at early time. However, the ratio of dl 250 to Ad 2 RNA at early times was 2 : 1 and at late times, 3 : 1.

The levels for region "E4" RNA are shown in Figure 3-16. From the respective slopes it is seen that, at late time, the level of "E4" RNA for Ad 2, was twice that at early time. For the dl 250 a similar trend was seen. The level of dl 250 RNA at late time was 4 times greater than that at early time. The ratio of dl 250 to Ad 2 RNA at late time was approximately 5 : 1. These ratios were again consistent with the trend observed in the Northern blot analysis.
Figure 3-15. Quantitation of Ad 2 and \textbf{dl 250} E1A RNA at early and late times. Experimental details are as given in Figure 3-8. Probe used was pHE1. Symbols used are: (■) \textbf{dl 250}; (□) \textbf{dl 250} late RNA; (●) Ad 2 E1 RNA and (○) Ad 2 late RNA.
Figure 3-16. Quantitation of Ad 2 and \( \text{dl 250} \) E4 RNA accumulated at early and late times. Experimental details were as in Figure 3-8. Probe used was pH14. Symbols used are: (●) Ad 2 early RNA; (○) Ad 2 late RNA; (■) \( \text{dl 250} \) early RNA; (□) \( \text{dl 250} \) late RNA.
III.3.3.2. Adenovirus 5 and Mutants

The levels of dl 313 RNA was quantitated from the autoradiograms shown in Figure 3-6. The results for region E1A specific RNA are shown in Figure 3-17, it is observed that at both the early and late times dl 313 accumulated lower than wild type levels of RNA. At early time, the ratio of dl 313 to Ad 5 was 1 : 3 and at late time it was 1 : 4.

The levels of Ad 5 and dl 313 RNA for region "E4" are shown in Figure 3-18. The pattern was similar to that of region E1A but the ratios were higher. Thus at early time the ratios of Ad 5 to dl 313 E4 RNA was 5 : 1 and at late time the same ratio was 6 : 1. These levels confirm the visual estimate seen in the respective Northern blots.

Since hr-6 is an E1B mutant that does not degrade DNA, it was of interest to examine its pattern of RNA accumulation.

Figure 3-19 shows the results for region E1A RNA. At early times Ad 5 and hr-6 accumulated approximately six times as much RNA as they did at late time. However, the level of Ad 5 RNA at early times was thrice as much as that of hr-6 and at late time Ad 5 accumulated twice as much RNA as hr-6. Thus the hr-6 displayed a pattern of E1A RNA accumulation that broadly resembled that of cyt 61.
Figure 3-17. Levels of E1A RNA accumulated by Ad 5 and dl 313 were detected by using pHE1 as explained in Figure 3-8. Symbols used are:

- (●) Ad 5 early RNA;
- (○) Ad 5 late RNA;
- (■) dl 313 early RNA;
- (□) dl 313 late RNA.
Figure 3-18. Levels of "E4" RNA accumulated by Ad 5 and dl 313 were detected by using probe pH14 as explained in Figure 3-8. Abbreviations used are: (●) Ad 5 early RNA; (o) Ad 5 late RNA; (■) dl 313 early RNA and □ dl 313 late RNA.
Figure 3-19. Quantitation of Ad 5 and hr-6 E1A RNA accumulated at early and late times. Experimental details are given in Figure 3-8. The probe used was pHE1. Abbreviations used are: (●) Ad 5 early RNA; (o) Ad 5 late RNA; (■) hr-6 early RNA and ( ) hr-6 late RNA.
Micrograms of RNA

Area of peaks
The results of "E4" specific RNA for Ad 5 and hr-6 are shown in Figure 3-20. The pattern mirrored the ElA expression. At early times, both Ad 5 and hr-6 accumulated twice as much RNA as they did at late times. However, the levels of Ad 5 RNA were consistently 3 times higher than those of hr-6 at all times.

The results of the quantitation data for Ad 12 and cyt 68 mutants are also summarized in Table 3-1, and those for Adenovirus group C and their corresponding mutants are summarized in Table 3-2.
Figure 3-20. Quantitation of Ad 5 and hr-6 E4 RNA Accumulated at Early and Late Times. Experimental details are as given in Figure 3-8 and the probe used was pH14. Symbols used are: 5 E and 5 L, Ad 5 RNA accumulated at early and late times respectively; hr E and hr L, hr-6 RNA accumulated at early and late times respectively.
Table 3-1. Summary of Relative Levels of RNA Accumulation in Adenovirus 12 and cyt 68

<table>
<thead>
<tr>
<th>Regions</th>
<th>Time of Accumulation</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Early: WT: Mutant</td>
</tr>
<tr>
<td></td>
<td>E1A</td>
</tr>
<tr>
<td></td>
<td>E1B</td>
</tr>
<tr>
<td></td>
<td>E2A</td>
</tr>
<tr>
<td></td>
<td>E2B</td>
</tr>
<tr>
<td></td>
<td>E3</td>
</tr>
<tr>
<td></td>
<td>E4</td>
</tr>
</tbody>
</table>

Abbreviations: WT: Wild Type, ND: Not Done
Table 3-2. Summary of Relative Levels of RNA Accumulation in Adenovirus Group C and Mutants.

<table>
<thead>
<tr>
<th>Virus Pair</th>
<th>Region E1A RNA Ratios</th>
<th>Region &quot;E4&quot; RNA Ratios</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Early</td>
<td>Late</td>
</tr>
<tr>
<td></td>
<td>WT : Mutant</td>
<td>WT : Mutant</td>
</tr>
<tr>
<td>Ad 2 &amp; dl 250</td>
<td>1 : 2</td>
<td>1 : 3</td>
</tr>
<tr>
<td>Ad 5 &amp; hr-6</td>
<td>3 : 1</td>
<td>6 : 1</td>
</tr>
<tr>
<td>Ad 5 &amp; dl 312</td>
<td>3 : 1</td>
<td>4 : 1</td>
</tr>
</tbody>
</table>

Abbreviations: WT : Wild Type  
Ad : Adenovirus
IV. DISCUSSION

The co-ordinately regulated expression of the adenoviral genome has made it a favourite model for the study of gene expression. The production of viral mRNAs and their polypeptide products follow a complex programme which is subjected to a variety of inter-locking regulatory mechanisms. Regulatory events appear to occur at the level of initiation and termination of transcription, processing, mRNA stability and translation (Logan and Shenk, 1982; Nevins, 1982). However, one of the most intensively studied aspect of the adenovirus gene expression is the regulation of one region of the genome by another. The demonstration of such regulation has been facilitated by the availability of viral mutants. Thus it has been clearly demonstrated that one or more products encoded by region E1A are required for the efficient accumulation of cytoplasmic RNA from regions E1B, E2, E3 and E4. For instance Ad 5 E1A mutants such as deletion mutants _dl 312_ (Jones _et al._, 1979) or group I host range mutants (Berk _et al._, 1979) are found to accumulate reduced amount of regions E1B, E2, E3 and E4 mRNA. Similarly, early region E2A has been found to be involved in the efficient shut off of region E4 transcription as Nevins _et al._ (1980) have found that the E4 transcripts in the E2A mutant _ts 125_ to be repressed at the permissive but not at the non-permissive temperature.
The region E1B seems to encode multifunctional regulatory roles. First of all, based on the reduced levels of E2 and E3 proteins synthetized in the Ad 5 E1B mutant hr-7 infected cells, Ross et al. (1980) have postulated that the region E1B can act as a negative regulator for the expression of regions E2 and E3 proteins. On the other hand Rowe et al. (1984) have postulated that the region E1B can equally act as a positive regulator for the expression of regions E1A proteins. Studies with Ad 5 dl313 and Ad 12 cyt mutants (Lai Fatt, 1982) or Ad 2 dl250 mutant (Gysbers, 1984) have shown yet another regulatory role for region E1B. All these mutants are characterized by a DNA degradation phenotype, implying a loss of function that determines an antinuclease activity. In this study it was asked, besides the loss of the antinuclease function, what other potential regulatory roles do the region E1B encode? Towards this end, the accumulations of steady state levels of RNAs from the various early regions of the adenovirus were detected by the use of specifically cloned early region probes and the technique of Northern blot, DNA-RNA hybridization. Principally the early regions were studied because these regions are known to be involved in the interaction of the Ad 12 virus with hamster cells. In these cells Ad 12 induces DNA degradation yet only the early regions of the virus are expressed (Ortin, 1976), as mRNAs. This observation implies that the early regions are in some ways involved with the expression of the nuclease activity.

This study was divided into two parts; cloning and analysis
of viral RNA. As the cloning aspect and the qualitative aspects of the Northern blot analysis have been adequately elaborated in the result sections they will not be considered here; rather the quantitative results of the modified Northern blots and their implications will be discussed in detail. However, before considering any possible regulatory effects, it is pertinent at the outset to review the merits of the probes used; the limitations of the techniques employed and the rationale for the choice of the mutants.

The approach taken in choosing representative Ad 12 restriction fragments to be cloned as probes for the various early regions was to assume that the transcriptional maps of group A (Ad 12) and group C (Ad 2 and 5) are structurally alike. This assumption is founded on the following evidence: First of all, based on biological properties it has been demonstrated that Ad 12 can complement mutants of Ad 5 with defects in the transforming region (Rowe and Graham, 1981) or in DNA degradation functions (Lai Fatt and Mak, 1982). Finally, based on results obtained by liquid hybridization technique it has been shown that the organization of Ad 12 transcription map is similar to that of Ad 2 and 5 (Smiley and Mak, 1978; Ortin et al., 1976). The use of these probes in terms of determining RNA that are expressed only at early or late times or throughout infection merit further qualifications. Except for probes for regions E1A and E1B, those for regions "E2A", "E2B", "E3" and "E4" may contain sequences which are complementary to the major late transcripts. Therefore, the results obtained from these probes, particularly after the onset of
DNA replication at late times may be less definitive. Hence the qualification of these probes. It is for this reason that, when it was necessary to determine the exact origin of a given transcript, single stranded probe for region L5 of Ad 12 was constructed.

The Northern blot technique, although quite sensitive, only detects steady-state levels of RNA accumulation. This level may not be directly proportional to the levels of mRNA available for translation, since the mRNA concentration will be influenced by the stability of each species. Also, the possibility of rapid turn-over rates for individual RNA species does not make it possible to correlate the accumulation rate with the synthetic rate. Finally, control of transcription may be influenced by such post-transcriptional events as splicing or polyadenylation each of which may in turn affect the presence or absence of a given species (Nevins and Wilson, 1981; Darnell, 1982). However, this is not to be construed that control at the level of accumulation does not exist. Indeed the 72K DBP has been shown to be involved in controlling the rapid turnover and stability of E1 mRNA for Ad 5 (Babich and Nevins, 1981).

One of the principal ways of identifying the genes or gene products involved in a particular event is through the use of conditional mutants that exhibit the phenotype of interest. DNA degradation was a phenotype shown by most of the mutants used here. The DNA degradation mutants fell into two groups; those mapping in region E1B and those mapping outside region E1B. The mutants mapping
in the region E1B were H12 cyt 68; Ad 5 dl 313; Ad 5 hr-6 and Ad 2 dl 250. H12 cyt 61 was also used as a negative control as the mutation in this virus has been mapped to be outside the E1B region (Lai Fatt and Mak, 1982). Choice of these mutants was made in an effort to identify or implicate any possible regulatory E1B polypeptides. For instance, it is known that dl 313 synthetizes no detectable levels of either 58K or 19K E1B polypeptides (Flint, 1981) while the hr-6 mutant synthetizes no detectable level of E1B 58K polypeptides (Lassam et al., 1978). Both H12 cyt 68 and H2 dl 250, on the other hand, are defective in the 19K polypeptides; cyt 68 synthetizes no detectable levels of 19K polypeptides (Mak et al., 1984) while dl 250 mutant has the bulk of the N terminus of the 19K polypeptide deleted and also synthetizes no detectable level of 19K (Subramanian et al., 1984). Thus efficient use of these mutants may elucidate the potential regulatory polypeptides involved.

IV.2. Pattern of RNA Accumulation and Regulation

The implications of the quantitative results of the pattern of RNA accumulations will be considered separately for each region. For the sake of coherency, the E1B and non-E1B mutants will also be discussed separately.
IV.2.1. Pattern of RNA Accumulation of E1B Mutants

IV.2.1.1. Early Region E1A

With respect to the E1A-specific RNA, the mutants studied displayed three main phenotypes, henceforth called A, B and C for the purpose of discussion. Phenotype A, displayed by H5 hr-6 and H5 dl 313, was characterized by a pattern of expression that mirrored the wild type in all aspects except the levels of RNA. Phenotype B was displayed by H12 cyt 68 and was marked by delayed expression at early times but higher than wild type levels at late time. Finally, phenotype C, characterized by consistently higher than wild type levels of RNA, was displayed by Ad 2 dl 250 mutant.

In the class A RNA phenotype, the levels of E1A specific transcripts at both early and late times were consistently lower than the corresponding wild type ones as visually estimated in the Northern blots and subsequently confirmed by quantitation. For example, the level of RNA accumulated by Ad 5 was 2 - 3 times more than that of hr-6 and five times more than that of dl 313. The relative levels of wild type and mutant RNA reported here are at variance with those previously reported by other workers. For example, Lai Fatt (1983) did not report any significant difference in the levels of E1A specific RNA in Ad 12 and cyt mutants. Similarly, Jones and Shenk (1979) did not report any significant difference between the levels of
Ad 5 and dl 313 early region E1A RNA. Berk et al. (1979) using hr-7, an Ad 5 hr-II mutant that is phenotypically similar to hr-6, reported wild type level of early RNA in hr-7 infected cells. However, in all these studies, the RNAs were not quantitated and the disagreement may, in part, be due to this approach. In fact a re-examination of the published results shows that some differences between mutants and wild types do exist but might have been missed earlier. Jones and Shenk actually reported the intensity of the RNA bands in dl 313 to be fainter than those in Ad 5 although they did not consider this as a significant observation. Furthermore, although phenotypically alike, hr-6 and hr-7 may be genotypically different, hence it may not be strictly valid to compare the relative levels of RNA in these two mutants. Due to various steps involved in the selection of Poly(A+) RNA, differential recovery can easily occur. To circumvent this problem, the viral RNA were quantitated with respect to a stable cellular RNA. Hence the results of quantitation obtained in this study may be a realistic one. Semantically, it could still be argued that the decreased levels of E1A transcripts seen in the mutants here are due to poor or differential infections. However, this is ruled out by the facts that the extent of CPE and distribution of inclusion bodies were the same in the wild type viruses and their corresponding mutants, indicating that infections had progressed to the same extent in mutant and wild type viruses. The present observation that the levels of E1A RNA are lower in the absence of functional E1B region is circumstantially supported by the work of Van
den Elsen et al. (1983). Using S1 mapping and micro-densitometry, they found the concentration of E1A-specific RNA to be lower in cells transformed by region E1A alone than in those transformed by region E1A plus E1B.

Ad 12 cyt 68 typifies phenotype B and showed a pattern of E1A RNA accumulation that was different from that of the corresponding wild type one. The cyt 68 showed a delayed E1A RNA accumulation, at both the early and intermediate times, the levels of RNA were consistently lower that those of Ad 12 by at least five times. The low level of E1A transcripts in the early phase of cyt 68 infection was found not to be related to an effect of moi. As this difference was essentially maintained from an moi of 50 to 1600 virions per cell. At late time the pattern almost reversed, with cyt 68 accumulating more RNA than Ad 12. Since in the early time both cyt 68 and Ad 12 accumulated visibly equimolar amounts of E2A RNA this difference in pattern cannot be attributed to differences in the degree of infection.

Mutant dl 250 typifies phenotype C. It showed an E1A RNA phenotype that was consistently higher than that of Ad 2 at both early and late times. At late times the levels in the mutant infected cells were twice as high as those in the wild type ones.

A common denominator in the mutants showing aberrant patterns or levels of E1A specific RNA is the absence of a functional E1B region. It follows a priori that a functional E1B region or its products may be either directly or indirectly involved in the
efficient accumulation and stability of region E1A RNA. This is a concept that has been postulated by other workers. As already mentioned Van den Elsen (1983) was the first to propose that such a regulatory mechanism may exist. Rowe et al. (1984), using hr-6 mutant, observed that at early times the level of E1A 44K protein synthesis was lower in the mutant than in Ad 5 infected cells, thus implicating region E1B in the expression of E1A proteins. That region E1B can indeed have a regulatory role on other regions is further supported by studies examining the effect of E1B mutations on the transcriptional expression of the adenovirus genome. Thus both Fukui et al. (1984) and Babiss et al. (1984) have demonstrated that a functional E1B region is required for the efficient accumulation of L1, L3 and L5 messages. Taken together, these observations strongly argue for a regulatory role of E1B encoded products in the efficient accumulation of Ad RNAs and the possibility of region E1B controlling the expression of region E1A becomes a viable plausibility. A critical examination of the mutants used may enable one to speculate on the possible regulatory E1B products involved in the accumulation of E1A specific RNA. The low levels of E1A specific RNA in dl 313 at early and late times provide circumstantial evidence for the involvement of both the E1B 19K and 58K polypeptides. The low level of E1A specific RNA in hr-6 would further implicate the E1B 58K protein in functions affecting the efficient accumulation of E1A RNA. However, the delayed expression of E1A early RNA in cyt 68 would seem to implicate the 19K polypeptide as also being involved in this
process. Alternatively, since the exact nature of the cyt 68 mutation is not known, it is conceivable that cyt 68 may be genenotopically mixed mutant with mutations in both the 19K and the 58K polypeptides. Furthermore, in d1 250 and cyt 68 infected cells the levels of E1A specific RNA were higher at late times thus implying that the 19K polypeptide may have a dual role to play in the expression of region E1A specific RNA. At early times, either alone or together with 58K, it could increase the level of E1A specific RNA and at late times it may be involved in the shut off of the E1A transcript or in processes affecting the stability of the messages. The dual and opposite effects of 19K polypeptides can be reconciled by envisaging that each function may map within a particular domain of the protein. This analogy is not without precedence; the E1A 13S mRNA encoded products that are known to be multi-functional in nature, playing a role in early gene regulation (Berk et al., 1979), cellular transformation (Van den Elsen, 1983) and in the modulation of the host-immune response (Bernards et al., 1983). Presumably these functions are carried out by different domains of the E1A proteins. Suggestive as the role for the 19K polypeptide maybe, it must be remembered that it is still not possible to rule out a hitherto uncharacterized E1B protein as being involved in this regulatory mechanism. Ad 12 E1B region is also known to encode a 17K E1B polypeptide (Esche et al., 1980; Mak and Mak, 1984) and Ad 2 is known to encode a 20K E1B polypeptide (Lucher et al., 1984); neither of these polypeptides have been fully characterized and hence their
functions remain essentially undefined and speculative.

IV.2.1.2. Early Region E1B

Due to lack of readily available probes only region E1B specific RNA of Ad 12 and cyt 68 mutants were examined.

Since no region E1B-specific transcripts were detected at early times only the levels of intermediate and late RNAs were quantitated. The more dramatic effect was seen at the late time when cyt 68 accumulated significantly higher than wild type level of RNA. There are various possible interpretations of these observations. The simplest is that the high level of E1B transcripts is due to the enhancing effect of the elevated E1A RNA, a finding which is not unexpected based on the work of Berk et al. (1979). On the other hand, the failure of the early region E1B RNA to return to wild type levels may be equally intrinsic to the E1B mutation. From the DNA degradation studies (Lai Fatt and Mak, 1983) the region E1B of the adenovirus genome has been postulated to encode an anti-nuclease factor which suppresses the activity of a putative nuclease gene. Hence the region E1B may encode, among other products, a repressor type of molecule. From the prokaryotic models, repressor molecules example the lac-I repressor of the E. Coli lac operon (Jacob and Monod, 1961), are generally autoregulated and when mutated they are over-produced. Though not conclusive, it is tempting to speculate
based on the persisting high levels of E1B transcripts, that the region E1B of the adenovirus genome may encode suppressor types of molecules that can behave as classical repressors.

IV.2.1.3. Early Region E2A and E2B

At the early time transcripts from region "E2A" of Ad 12 and cyt 68 infected cells were not significantly different but an increase was noted in the cyt 68 RNA at late time. The significance of this difference remains to be determined. The region E2A is known to encode the 72K DNA binding protein which play a role in DNA replication. Ross et al. (1980) using hr-7, an E1B mutant of Ad 5, observed a 3 - 4 fold increase in region E2A proteins. The results of the RNA data obtained here would seem to support this general trend i.e. E1B region can act as a negative regulator for the expression of region E2A. On the other hand it is equally possible that the increase in E2A transcripts is due to the enhancing effect of the elevated level of E1A transcript. However, this possibility is rendered unlikely from the results of Rossini's work (1983). By microinjection of cloned segments of viral DNA into cultured cells, Rossini (1983) observed that E1A differentially controlled the expression of the region E2A DBP; at early times it had an enhancing effect but at late times it had an inhibitory effect on the synthesis of the DBP. If the expression of region E2A RNA reflects the
expression of DBP, one expects to see a reduced level of E2A RNA in cyt 68 infected cells at late time. However, this was not observed here, hence the results are more consistent with region E1B acting as a negative regulator of region E2A as postulated by Ross et al. (1983).

The level of "E2B" specific transcripts was consistently higher in cyt 68 infected cells than they were in Ad 12 infected ones. This again implies that the region E1B may normally act as a negative regulator of E2B region also.

The region E2B is known to encode two major proteins involved in DNA replication, the precursor of the terminal protein of 80K and the 140K adenovirus DNA polymerase. Both the E2B and E2A regions have the same promoter but different polyadenylation sites therefore the relative level of E2A : E2B may be regulated by the choice of polyadenylation sites. For example, Stillman et al. (1981) have shown that the E2B is the rarest of Ad 5 transcripts and the ratio of E2B : E2A is heavily biased (1 : 100). The results obtained in this study support Stillman's observation since no E2B transcripts were detected at early times when the E2A specific RNA was abundant.

IV.2.1.4. Early Region E3

For region "E3" the cyt 68 infected cells accumulated higher levels of RNA than did the Ad 12 infected ones. By injecting Xenopus
oocyte with E1A protein Ferguson \textit{et al.} (1984) have demonstrated that the E1A products enhances the expression of the E3 promotor. The present observation, namely increased level of E3 RNA in the presence of high level of E1A RNA, would be consistent with Ferguson's observation. These results are equally consistent with those of Ross \textit{et al.} (1980) who have observed increased level of E3 proteins when the region E1B is mutated.

IV.2.1.5. Early Region E4

The mutants fell into two classes with respect to their E4 RNA phenotypes. Those with higher levels of late RNA and those with reduced levels of late RNA. Higher levels of late RNA were exhibited by cyt 68 and \textit{dl 250} whereas reduced levels were shown by \textit{dl 313} and hr-6. A critical examination of the corresponding E1A RNA phenotypes of the mutants shows that the efficient accumulation of E4 transcripts shows a dependence on the E1A specific transcripts. This is a corollary that is consistent with published results of at least three groups. First, Nevins and Winkler (1981) have shown that the E1A 13S products are required for the efficient accumulation of E4 RNA. Similarly, Berk \textit{et al.} (1979) observed the level of E4 RNA to be reduced in the Ad 5 E1A hr-I mutant. Finally, Jones \textit{et al.} (1979) using the E1A \textit{dl 312} mutant found the level of E4 RNA to be decreased. The mechanism of action of E1A products on the E4 promotor
is not known. However, Nevins (1981) has proposed that the E1A regulatory product functions by inactivating a cellular repressor product that normally inhibits the other viral promoters. Quantitative analysis of the effects of proteins synthesis inhibitors on activation of early transcription units have shown that the most dramatic increase in the rate of transcription occurs for region E4 (Katze et al., 1981; Persson et al., 1981). One possible explanation could be that the E4 promotor is the most sensitive to the E1A products. Hence the increased levels of E4 transcript observed in the presence of increased levels of E1A RNA and the reduced levels of E4 transcripts seen in the presence of low levels of E1A RNA can be rationalized by this argument.

IV.2.1.6. Accumulation of L5 Specific RNA

The region E1B has been recently shown to be involved in the efficient accumulation of L5 specific mRNA. For instance Babiss et al. (1984) and Fukui et al. (1984) have both shown that the accumulation of L5 messages is diminished in E1B mutants defective in 19K and 58K polypeptides. In this study the results of probing with the "E4" probe which contains sequences complementary to the L5 specific transcripts had shown that the expression of "E4" RNA were aberrant in the E1B mutants. It was therefore decided to investigate the accumulation of L5 RNA using the L5 'r' strand specific probe
constructed by Exonuclease III digestion of the '1' strand of the "E4" clone. The authenticity of the L5, 'r' specific probe could not be confirmed by the use of the complementary E4, '1' specific probe because the exonuclease digestion approach was unsuccessful in this case. However, since this technique has been found to be successful in the construction of strand specific probes for use in hybrid select (Mak et al., 1984), the L5 probe so constructed is assumed to be the right one. The expression of L5 messages was found to be over-produced in cyt 68 infected cells. This observation is in contrast to those of Babiss and Fukui who had found the expression of L5 RNA to be reduced. A number of explanations is possible: One explanation is that the mutation in the responsible E1B products of cyt 68 is not in the same domain as those observed by Fukui et al. (1984). Alternatively, this effect could be due to such other factors as effect of moi or differences in the replicative cycles of the viruses and the relative stabilities of the messages. The region L5 is known to encode the fiber antigen the significance of this observation remains to be determined and is further discussed below.

IV.2.2. Ad 12 and cyt 61

These results of probing the RNA produced in cyt 61 infected cells will only briefly be considered as they have already been discussed under the Northern blot result sections.
Cyt 61 is a mutant that possibly maps outside the E1B region and exhibits many atypical properties. For instance it is near wild type in its transformation properties (Mak and Mak, 1982) yet it exhibits a dominant DNA degradation phenotype (Lai Fatt and Mak, 1982). The exact location and nature of the cyt 61 mutation is not known. However, its pattern of RNA accumulation is interesting because it serves to demonstrate that not just region E1B can perturb the expression of the Adenovirus genome. Its main features were that early region E2A RNA and regions E3 and E4 late RNAs were over-expressed. The significance of these observations remain open to interpretation and are considered in the speculative remarks.

IV.3.0. Speculative Remarks and Significance

IV.3.1. Possible Location of the Nuclease Associated Gene

Since the cyt mutants are associated with an antinuclease factor, it has been postulated that the adenoviruses may encode a putative nuclease gene that is located distal to the E1B region. This gene may be expressed either at early or late times (Lai Fatt and Mak, 1982). If these genes are overproduced in the cyt mutants it was assumed that they would be over-expressed at the transcriptional level. The only region that was found to be over-expressed at early
time was the E2A region of the cyt 61 mutant. This region encodes the 72K DBP protein which has a multifunctional role. Amongst other functions, the DBP has been shown to inhibit the hydrolysis of single-stranded DNA by a DNase isolated from KB cells (Nass and Frenkel, 1978). Thus it is conceivable that the nuclease activity associated with cyt mutants is cellular in origin and that it is triggered by the virus infection. In keeping with this argument one would assume that the region E2A may normally act as anti-nuclease except when it is mutated. However, the region E2A is apparently normal in cyt 68 and the nuclease activity of the cyt mutants degrade both single stranded and double stranded molecules (Ezoe and Mak, 1980; Lai Fatt, 1983). This may limit the region E2A as a strong anti-nuclease candidate. The only other regions that are elevated at late times in the absence of boosting E1A effect, are regions E3 and E4 in cyt 61. The elevated level in cyt 68 was found to be for L5 RNA directing the synthesis of fiber antigen. Marusyk and Wadell (1975) have found an endonuclease activity to be associated with the penton antigen which is directed by L1 RNA. If it is assumed that the nuclease activity is associated with the fiber, then the possibility that their penton preparation was contaminated with fiber antigen cannot be ruled out. It must be remembered that this is highly speculative and no solid evidence exists for this claim.

IV.3.2. Regulation of Early Regions
The evidence for a regulatory role of region E1B on region E1A is compelling from this study. However, due to the various feedback control system existing within the adenovirus genome, it is not possible to conclude whether this is a direct or indirect effect. The most direct way to prove this would be by microinjection of cloned DNA containing either regions E1A plus E1B and E1A alone in KB cells then assaying for E1A promotor activity. It is predicted that the level of E1A transcript would be higher in cells injected with the E1A plus E1B DNA than those injected with E1A alone.
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