CHARACTERIZATION AND MAPPING OF A DNA DEGRADATION FUNCTION (CYT) IN ADENOVIRUS

By

© RICHARD BERNARDO LAI FATT, B.Sc.

A Thesis

Submitted to the School of Graduate Studies

in Partial Fulfilment of the Requirements

for the Degree

Doctor of Philosophy

McMaster University

January 1983

DNA DEGRADATION FUNCTION IN ADENOVIRUS

DOCTOR OF PHILOSOPHY (1983)
(Biology)

McMASTER UNIVERSITY
Hamilton, Ontario

TITLE: Characterization and Mapping of a DNA Degradation Function (Cyt) in Adenovirus

AUTHOR: Richard Bernardo Lai Fatt, B.Sc. (University of Miami, Fla.)

SUPERVISOR: Professor S. Mak

NUMBER OF PAGES: xv, 154

ABSTRACT

The cyt mutants of the highly oncogenic adenovirus type 12 (Ad12) produce low yields of virus and are generally weakly tumorigenic. In this study, four cyt mutants examined were shown to induce extensive degradation of DNA in infected cells. Complementation tests using two cyt mutants, host range and host range-deletion mutants of Ad5, and wild type Ad12 and Ad5 in various cell types, suggested that two viral functions are involved in the control of DNA degradation. One of these functions, a DNase-inhibitory function, mapped by genetic complementation in early region E1b of the Ad5 genome. A transcriptional study of early region E1 of the Ad12 genome identified mRNA species complementary to the left 10.5 percent of the genome by the method of RNA-blot hybridization. Ad12 appeared to possess a region E1b physically corresponding to that of Ad5, suggesting that the DNase-inhibitory function also maps in this region in Ad12. Results implicated the association of this function with an E1b-19,000 dalton polypeptide of both serotypes. The second viral function appeared to influence positively the induction of DNA degradation. This DNase-effecter function appeared to be an early viral function. Results of time course-superinfection experiments in which cyt mutant-infected cells were superinfected with wild type strains of Ad12 and Ad5 and analyzed for the properties of DNA degradation and virus yields, support the hypothesis that the degradation of DNA is determined by the relative abundance of the DNase-inhibitory factor and the DNase-effecter, and that the two factors might functionally

interact with each other.

A supplementary study on the interference with the DNA replication of Ad12-strain 1131 by an Ad12-strain Huie variant, Ad12-Huie (M8), suggested that the interference capacity lies in a nucleotide sequence repeat structure at the right-terminus of the Ad12-Huie (M8) genome.

ACKNOWLEDGEMENTS

I wish to thank my supervisor Dr. S. Mak for his considerable interest, guidance, and helpful discussions throughout the course of this work. I am also grateful to Mrs. I. Mak for her technical advice and interesting discussions, Dody Bautista for his assistance with the preparation of photographs, and to many others, particularly Ms. Rosemary Redfield, who worked in the laboratory over the years for their help and valuable discussions.

I am thankful to Dr. F. Graham (McMaster University, Hamilton) for his helpful discussions and for providing the Ad5 host range mutants hr 1 and hr 6 and the 293 human cell line, and to Dr. T. Shenk (New York Univ., Stoneybrook, N.Y.) for the Ad5 deletion mutants dl 312 and dl 313.

I am especially grateful to Miss Janie Katona for her continued encouragement and for the typing of this thesis.

TABLE OF CONTENTS

LIST OF FIGURES

LIST OF TABLES

INTRODUCTION

Adenoviruses

The adenoviruses are comprised of about 80 identified viruses classified into six groups based on their natural host species: human, simian, bovine, canine, murine and avian (Pereira et al., 1963). The most widely studied and characterized group is the human adenoviruses of which there are at least 35 members (Beladi, 1972; Keller et al., 1977; Stadler, Heirholzer and Oxman, 1977). The human adenoviruses were isolated largely from lymphadenoid tissue of patients with respiratory disease and have been classified into six major classes based on several independent criteria: hemeagglutination and antigenic cross-reactivity, epidemiology, DNA sequence homology, nucleotide content of DNA, and oncogenicity in rodents (Table 1-1). Although they are important etiological agents of human respiratory disease (Parrott, 1960; Hope-Simpson and Higgins, 1969; Hilleman, 1957), interest in these viruses greatly increased after it was demonstrated by Trentin et al. (1962) that human adenovirus type 12 (Ad12) induces malignant tumors following innoculation into newborn hamsters. This landmark observation initiated tremendous research into the molecular biology of adenoviruses, particularly with respect to cellular transformation and tumorigenesis. It has since been shown that many adenoviruses of different classes have oncogenic potential.

Morphology and Composition of the Virion

The virus particle is nonenveloped with icosahedral symmetry,

Table 1 -]

HUMAN ADENOVIRUS HOMOLOGY CLASSES

Class	Representative Serotypes	Hemag-glutination Group *	% DNA Homology +	% G + C	Oncogenicity in Rodents	Target Tissue	Epidemiology
A	12, 18, 31	IV	a 48-69% b 8-20% c 50-80%	48%	high	gastrointestinal tract	cryptic gastrointestinal infection
B	3, 7, 11, 21	I	a 89-94% b 9-20% c 50-80%	51%	weak	pharynx lungs (upper & lower respiratory tract) hemorrhagic cystitis (lower urinary tract) conjunctivits (eye)	acute epidemic infection
C	1, 2, 5, 6	III	a 99-100% b 10-16% c 50-80%	58%	nil	pharynx (upper respiratory tract)	latent throat infection; cryptic gastrointestinal infection
D	8, 9, 19	II	a 94-99% b 4 -23%	58%	nil	keratoconjunctivitis (eye)	acute epidemic infection
E	4	III		58%	nil	upper respiratory tract	
F	EA				nil	gastrointestinal tract	enteritis-associated enteric infection

* I. Complete agglutination of monkey erythrocytes; II. Complete agglutination of rat ethryocytes; III. Partial agglutination of rat erthrocytes; IV. Minimal agglutination response.

\+ a. Homology of members of same group; b. Homology of members of different groups; c. DNA sequence homology of members of different groups (0-4.5 map units and 15-17 map units).

Reprinted from Sambrook et al. (1981)

containing a linear, double-stranded DNA molecule of molecular weight approximately 23 x 10^6 (van der Eb and van Kesteren, 1966; Green et al., 1967). The outer protein shell consists of 252 capsomers, 240 of which are termed hexons forming the planes of the triangular facets and 12 of which are termed pentons located at the vertices (Ginsberg et al., 1966). Projecting from each penton base is a spike-like structure, the fibre (Valentine and Pereira, 1965; Norrby, 1966; Geldeblum et al., 1967; Pettersson, Philipson and Höglund, 1968). Three smaller polypeptides (VI, IIIa and IX) are also associated with the capsid.

Within the capsid structure is the core, consisting of the double-stranded DNA genome in tight association with two core proteins, polypeptides V and VII (Laver et al., 1968; Prage, Pettersson and Philipson, 1968; Prage et al., 1970; Laver, 1970; Prage and Pettersson, 1971; Russell, McIntosh and Skehel, 1971; Everitt et al., 1973). Covalently attached to the 5'-end of each DNA strand is a protein of m.w. 55,000 (Stillman and Bellet, 1979; Stillman, 1981; Rekosh et al., 1977). The DNA sequence at the ends of the molecule are inverted terminal repeats of about 102-136 base pairs (Steenbergh et al., 1977; Tolun, Alestrom and Pettersson, 1979; Arrand and Roberts, 1979) and about 180 nucleotides from the terminus of each strand is an internal self-complementary sequence of about 100 nucleotides in length, capable of forming an approximately 50 base pair hairpin structure (Padmanabhan and Green, 1976). The significance of these sequences and structures are not clear, but they are probably involved in viral DNA replication.

Virus-Host Interactions

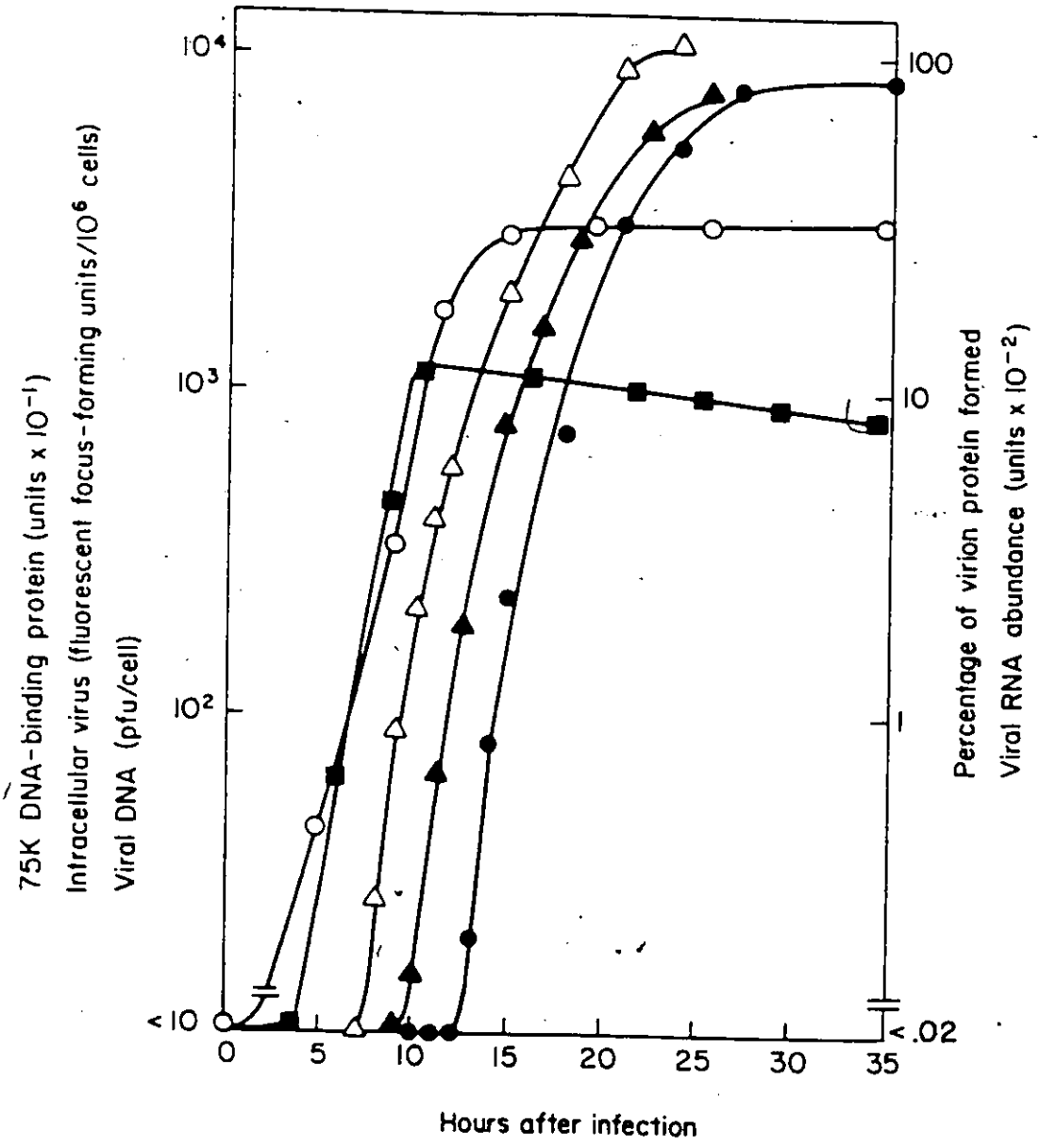
Infection of cells in culture by human adenoviruses can take one of three courses: permissive, semi-permissive, or abortive infections, the outcome depending on both the serotype of the virus and the type of cells. Infection of human cells such as HeLa and KB cell lines is permissive, and the yield of virus is highest compared to infection of cell lines of other host origin. Infection of simian cells such as African Green Monkey Kidney (AGMK) cell line is an example of semi-permissive infection, with a block in virus production late in the replication cycle, and very little virus is produced. Infection of primary rodent cells often results in an abortive infection in which the virus is blocked in its replication, but from which a very small fraction of the cells may survive as transformed cells. Adenovirus transformed cells often have oncogenic potential when injected into the appropriate host.

Permissive Infection by Adenovirus

The reproductive cycle of human adenoviruses as studied in KB and HeLa cell lines can be divided temporally into two main phases, early and late, delineated by the onset of viral DNA synthesis.

The virus particle is sequentially uncoated as it translocates from receptors on the cell surface to the nucleus of the cell, where only the core enters (Lonberg-Holm and Philipson, 1969; Philipson, Lonberg-Holm and Pettersson, 1968; Morgan, Rosenkranz and Medris, 1969; Chardonnet and Dales, 1972). This sequence of uncoating takes about 2 hours (Philipson, Lonberg-Holm and Pettersson, 1968). At this stage of infection viral gene expression begins. Figure 1-1 shows the

Figure 1-1 Time course of adenovirus-2 productive infection in suspension culture of KB cells. (●) intracellular virus measured as fluorescent focus-forming units/10^6 cells; (○) total virus-specific RNA measured by hybridization of labelled RNA to adenovirus-2 DNA; (Δ) synthesis of viral DNA (data from Green et al., 1971); (▲) virion protein (hexon antigen) measured by complement fixation; (■) 75K DNA binding protein. (Reprinted from Tooze, 1980).


75K DNA-binding protein (units x 10^{-1})
Intracellular virus (fluorescent focus-forming units/10^6 cells)
Viral DNA (pfu/cell)
10^4
10^3
10^2
< 10
100
10
1
< .02
Percentage of virion protein formed
Viral RNA abundance (units x 10^{-2})
0
5
10
15
20
25
30
35
Hours after infection

kinetics of production of viral RNA, viral proteins, viral DNA and total intracellular virus for Ad2.

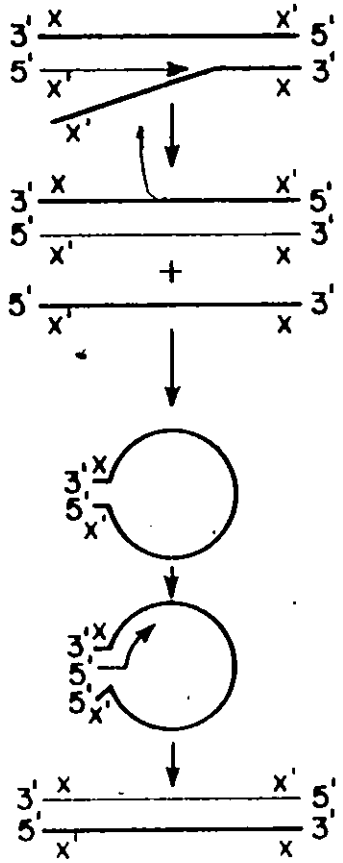
Host DNA synthesis begins to be inhibited at about the onset of viral DNA synthesis and is completely shut down within 4-7 hours thereafter (Ginsberg, Bello and Levine, 1967; Piña and Green, 1969). Synthesis of host proteins is also inhibited with similar kinetics (Ginsberg, Bello and Levine, 1967) and the basic cellular synthetic machinery is apparently redirected towards the synthesis of viral macromolecules. Mature infectious virions within the cell appear as early as 15 hr, but virus accumulates in the nuclei until released much later due to the eventual death of the infected cell. The timing of these processes are delayed by a few hours in the group A adenoviruses.

Viral DNA Replication

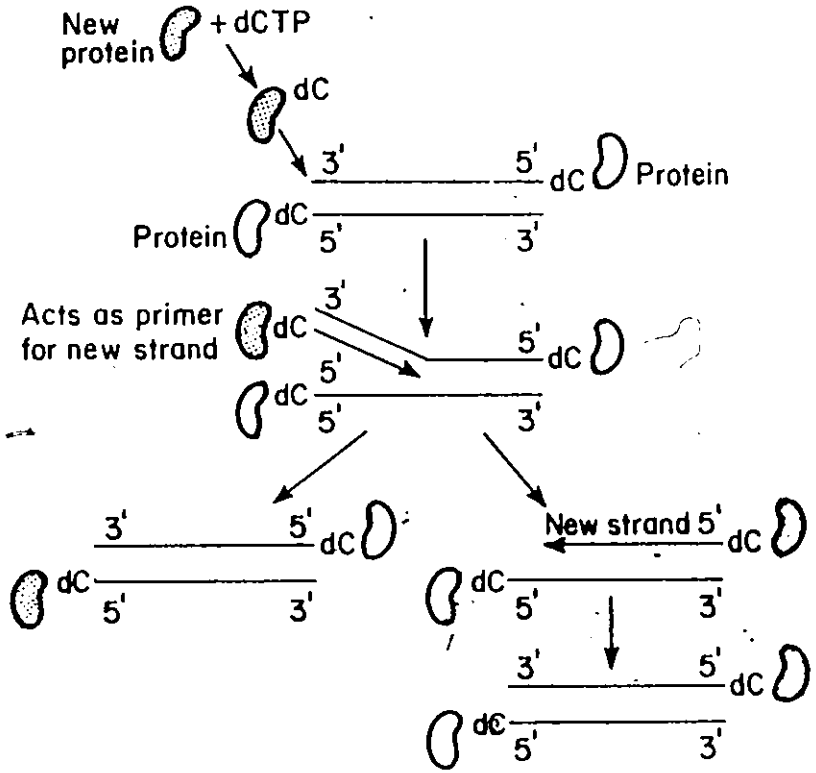
Adenovirus DNA appears to replicate by a unique process among eukaryotic systems. Extensive research into the replication of this apparently simple, linear double-stranded DNA molecule has generated contradictory data and different models of mechanisms involved. In recent years, however, with more direct analyses of replicating molecules with the use of restriction endonucleases, electron-microscopy, and *in vitro* assay systems, a generally accepted model of the overall process has evolved.

This strand displacement-continuous synthesis model as originally proposed by J. Sambrook (See Daniell, 1976) is diagrammed in Figure 1-2a. DNA synthesis is initiated off one strand only as template at, or close to, one end of the double-stranded molecule. Synthesis of this new

Figure 1-2 (a) A model of synthesis of adenoviral DNA (Daniell, 1976) in which synthesis of a new strand displaces the parental strand. If the sequences of the inverted terminal repetition base-pair to form the panhandle intermediate shown, then the double-stranded panhandle has the same terminal structure as parental viral DNA and will presumably be recognized by the enzyme complex responsible for initiation of viral DNA synthesis. Although not shown here, r strands displaced during the first step in synthesis could obviously form an analogous panhandle intermediate, with the same terminal, double-stranded sequence.
(b) A similar displacement mechanism in which the possible role of the 5'-terminal protein in initiation of adenoviral DNA synthesis is illustrated , (redrawn from Rekosh et al. 1977). (Reprinted from Tooze, 1980).


3' x x' 5'
5' x' x 3'
x'
3' x x' 5'
5' x' x 3'
+
5' x' x 3'
3' x
5' x'
3' x
5' x'
3' x x' 5'
5' x' x 3'

a


New protein
+ dCTP
dC
3' 5' dC Protein
Protein dC
5' 3'
Acts as primer for new strand
3'
dC 5' dC
5'
dC 5' 3'
3' 5' dC
dC 5' 3'
New strand 5' dC
dC 5' 3'
3' 5' dC
dC 5' 3'

b

strand is continuous until complete displacement of the other parental strand, generating a double-stranded molecule identical to the original and a single-stranded full length molecule. The inverted terminal repeat sequences of this single-stranded molecule can base pair to now generate a double-stranded structure identical to an end of the parental genome. Initiation of strand synthesis can occur at this duplex, and copying of the entire strand generates a double-stranded molecule to complete the cycle. A recent extension to this model has provided a role for the protein covalently attached to the 5'-terminus of each strand (Figure 1-2b). It has been suggested that the protein functions as a primer by the covalent binding of a deoxycytidine residue from which DNA synthesis can proceed (Rekosh et al., 1977; Stillman and Bellet, 1979; Stillman, 1981; Challberg, Desiderio and Kelly, 1980; Pincus, Robertson and Rekosh, 1981). This cytidine residue remains as the 5'-terminal nucleotide of each strand.

Based on genetic data, viral products apparently involved in viral DNA replication are the terminal protein, a single-strand DNA binding protein, and a third product (see "Genetics of Adenovirus", p. 16), possibly a DNA polymerase. Recent studies which more closely examined the DNA polymerase activity involved in adenovirus replication suggest that at least a viral encoded product which is resistant to the drug aphidocoline is involved in viral DNA replication (Pincus, Robertson, and Rekosh, 1981; Lichy, Horwitz, and Hurwitz, 1981). All other functions involved appear to be of host origin.

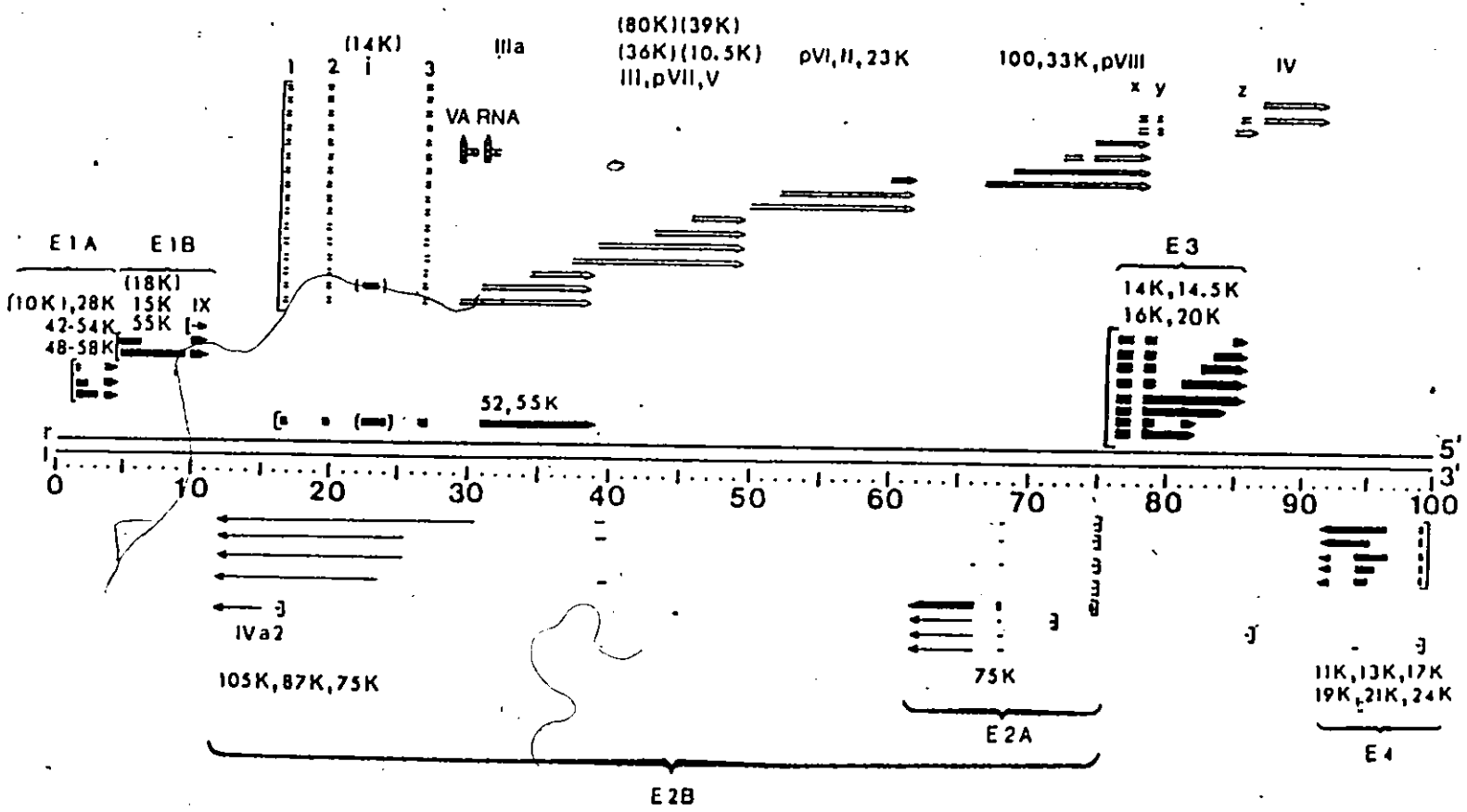
Genome Organization and Expression

The most detailed transcriptional and protein mapping studies have been done with Ad2. However, from studies done on other serotypes, including Ad12 of subgroup A (Ortin et al., 1976; Smiley and Mak, 1978) it appears that all human adenoviruses are genetically organized in a similar fashion.

Early and late genes are defined as those which begin to be expressed as mRNA before and after, respectively, the onset of viral DNA synthesis. Four non-overlapping regions of the genome encode the early functions and early mRNA is transcribed from both strands of the DNA (Fig. 1-3). The late functions are encoded in one large transcriptional block from 16.7 to 100 map units of the r-strand only, the rightward transcribed strand (Fraser et al., 1979). The locations in the genome of genes encoding viral proteins are also indicated in Fig. 1-3. All the mRNAs except that for polypeptide IX are spliced from larger, co-linear transcripts (Chow and Broker, 1980).

The control of gene expression in adenovirus is very complex yet orderly, and not well understood (reviewed by Ziff, 1980). There are many potential stages of regulation post-transcriptionally, and these are probably influenced to a large extent by host factors and mechanisms which are utilized by the virus. Several instances of apparent viral controlled gene expression have been demonstrated. The different transcriptional blocks in the viral genome, each with their own promoter(s), are transcribed at varying rates and at different time periods throughout infection (Nevins et al., 1979). Even different genes under the control of the same promoter and overlapping in DNA

Figure 1-3 A schematic drawing, showing the principle organization of the adenovirus type 2 genome (reprinted from Pettersson and Akusjarui, private communication). 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 cotermination families of late mRNA 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 VA RNAII, map around position 30.


(14K)
IIIa
(80K)(39K)
(36K)(10.5K)
III,pVII,V
pVI,II,23K
100,33K,pVIII
IV
1
2
3
x
y
z
VA RNA
E1A
E1B
(18K)
(10K),28K
42-54K
48-58K
15K
55K
IX
E3
14K,14.5K
16K,20K
52,55K
5'
3'
0
10
20
30
40
50
60
70
80
90
100
IVa2
105K,87K,75K
75K
E2A
E2B
11K,13K,17K
19K,21K,24K
E4

sequence can be expressed at different levels of mRNA and protein, for example, the 58,000 dalton protein and the 19,000 dalton protein and their mRNAs of region E1b (Chow, Broker and Lewis, 1979). There is some indication that the 72,000 dalton single-stranded DNA binding protein encoded in region E2a regulates the level of expression of the early genes (Carter and Blanton, 1978a; 1978b; Blanton and Carter, 1979; Nevins and Winkler, 1980; Richardson and Westphal, 1981). There is also evidence to suggest that gene products of early region E1a and possibly two immediate early gene products under control of the major late promoter (Lewis and Matthews, 1980) are required for the expression of all other regions (Berk et al., 1979; Jones and Shenk, 1979b; Persson, et al., 1981; Nevins and Wilson, 1981). In order for the late genes to be transcribed, it also appears that prior replication of the viral DNA template is necessary (Thomas and Mathews, 1980). The complexities of gene expression in adenovirus are only now being realized.

Enzymatic Activities in Adenovirus Infected Cells

Virus infection of host cells often induce novel enzymatic activities due to the expression of viral encoded enzymes. However, with adenovirus, few, if any, enzymatic activities are encoded by the virus. This is, perhaps, somewhat peculiar since the molecular biosynthesis occurring late in cells productively infected with adenovirus is almost totally of viral products. Activities of enzymes, particularly those involved in nucleic acid metabolism, have been examined in adenovirus infected cells. DNA polymerase activity remains constant in exponentially growing KB cells when infected with Ad12 or Ad31, and

thymidine kinase activity increases only slightly (1.2-3.0 fold) under these conditions (Green, Piña and Chagoya, 1964; Piña and Green, 1969). RNA polymerase II which is responsible for viral mRNA synthesis (Wallace and Kates, 1972; Price and Penman, 1972a) and RNA polymerase III which is responsible for the synthesis of the small virus-associated RNA (VA-RNA) species (Price and Penman, 1972b; Weinmann, Raskas and Roeder, 1974; Weinmann et al., 1976; Söderlund et al., 1976) appear not to be virus encoded. The adenovirus encoded single-stranded DNA binding protein and the 5'-terminal protein, both involved in viral DNA synthesis have not been shown to function enzymatically.

Three kinds of enzymatic activities however, have been reported to be closely associated with, but not necessarily intrinsic to, viral gene products. The first is a protein kinase activity immunoprecipitable from Ad5 and Ad12 infected cells using antisera from animals bearing tumors induced by the virus, but not with sera from normal animals (Branton et al., 1981). The second is a deoxyribonuclease activity associated with the pentons of the virion capsid. Endonuclease activity copurifies with the penton fraction from viruses belonging to subgroups A, B, and C (Burlingham and Doerfler, 1972; Marusyk, Morgan and Wadell, 1975; Cajean-Feroldi, Chardonnet and Chantepie-Auray, 1977; Tsang and Marusyk, 1980). Similar endonuclease activities are also present in Ad2 and Ad5 infected and uninfected human cell lines (Burlingham and Doerfler, 1972; Reif, Winterhoff and Doerfler, 1977; Tsuro et al., 1978; Padmanabhan, et al., 1979). There are disagreements, however, between the various laboratories as to the substrate specificity, optimal

conditions, and end products of digestion of the different endonuclease activities. The reasons for the discrepancies are unclear, and it has not been demonstrated that any of the nucleases are virus encoded. A third enzymatic activity is a DNA polymerase activity which copurifies with adenoviral proteins required for viral DNA replication (Enomoto et al., 1981).

Transformation by Adenovirus

Adenovirus infection of permissive cells results in virus replication and cell death as described earlier. Infection of semi-permissive and nonpermissive cells, however, can result in the morphological transformation of a minority of the cells. The transformed cells are often tumorigenic depending on both the serotype of the virus and the host animals in which they are innoculated.

Consistent transformation is obtained under semipermissive conditions such as rat embryo cells infected with Ads 2 and 5. In these cultures, a proportion of the cells are productively infected and eventually die, but a few of the nonpermissive cells survive to form dense foci of transformants. The efficiency of transformation varies with the tissue origin of the cells, probably due to differences in permissivity (Casto , 1973; Gallimore, Sharp and Sambrook, 1974). The tumorigenic potential of the transformed rat cells appear to parallel that of the virus with which they were transformed; rat cells transformed by Ad12 are generally highly tumorigenic, while those transformed by Ad2 and Ad5 are generally non-tumorigenic. Some rat cells transformed by Ad2, however, are tumorigenic in immunosuppressed, syngeneic hosts (Gallimore, 1973).

The only totally nonpermissive system studied to any extent is Ad12 infection of hamster cells. Ad12 is completely blocked in viral DNA synthesis in hamster cells (Shimojo and Yamashita, 1968; Doerfler, 1969, 1970), but expresses early tumor antigens (Strohl, 1969a,b; Raska and Strohl, 1972) and much if not all of the early mRNA expressed in permissive infection (Ortin et al., 1976). Transformation of hamster cells by Ad12 occurs infrequently, however. This is perhaps due to the severe chromosomal aberrations induced by Ad12, which could lead to cell death (Stich and Yohn, 1970; Stich, 1973; zur Hausen, 1968, 1973). Hamster cells, which are permissive for Ad2 and Ad5, can only be transformed by these serotypes when the virus is inactivated by UV irradiation (Lewis, Rabson and Levine, 1974) or by temperature-sensitive mutation and assayed at the nonpermissive temperature (Williams, 1973).

The viral genes necessary and sufficient for transformation appear to be all located in early region I (0-12%) of the viral genome. All adenovirus transformed cells so far examined contain and express at least these sequences (Gallimore, Sharp and Sambrook, 1974; Flint et al., 1976). Using purified viral DNA restriction fragments, Graham et al., (1975) demonstrated that only fragments containing the left-hand 8% or more of the viral genome could transform rat embryo cells. Most, if not all, of the mRNA and polypeptide products encoded in this region of the genome have been identified. However, the functional roles of these gene products and the mechanism of transformation and tumorigenesis by adenovirus have only begun to be elucidated.

Genetics of Adenovirus

The study of mutants are invaluable in identifying gene products and functions for the understanding of biological processes. Adenovirus mutants can generally be divided into two classes, early and late, determined by their ability to replicate their DNA. The late mutants, all of which are temperature-sensitive, isolated so far from several serotypes have identified 15 complementation groups affecting the synthesis, transport, and assembly of viral structural proteins. Of greater interest, perhaps, are the early mutants which might be expected to be defective in functions involved in gene regulation, DNA replication, transformation, tumorigenesis, and other virus-host interactions. The varieties of adenovirus early mutants isolated include temperature-sensitive, host range and deletion-substitution mutants, most of which were derived from Ad5 and Ad2.

(i) Temperature Sensitive Mutants. Only two complementation groups of Ad5 ts mutants affect early functions. One group, represented by H5 ts 36, has recently been mapped in early region E2b between map units 18.5 and 22.0 (Galos et al., 1979). This region overlaps with the region encoding the protein which is covalently linked to the 5'-end of each viral DNA strand (Stillman et al., 1981), and the mutant does not synthesize viral DNA at the non-permissive temperature (Ensinger and Ginsberg, 1972; Wilkie, Ustacelebi and Williams, 1973). H5 ts 36 and H5 ts 37, both of the same complementation group, are the only ts mutants that are defective in transformation at the non-permissive temperature (Williams and Young,

and Austin, 1975). Results of temperature-shift experiments suggest, however, that the function affected is only required transiently for the establishment but not maintenance of transformation (Williams, Young and Austin, 1975). In support of this view, the region of the viral genome encoding the H5 ts 36 function is not required for transformation by viral DNA fragments (Graham et al., 1975) and is not consistently present in virus transformed cells (Gallimore, Sharp and Sambrook, 1974; Flint et al., 1976).

The second early complementation group, of which H5 ts 125 is the best characterized representative, maps in the region 59.0 - 71.0 map units, within region E2a, encoding the 72,000 dalton single-strand DNA binding protein (van der Vliet and Levine, 1973; Levin et al., 1975; Lewis et al., 1976; Grodzicker et al., 1977). The protein is more labile at the non-permissive temperature (van der Vliet et al., 1975) and the virus is defective in the initiation (van der Vliet and Sussenbach, 1975) and elongation (Flint, Berget and Sharp, 1976a; van der Vliet, Landberg and Jansz, 1977) of viral DNA synthesis. This mutant is not defective in transformation (Ginsberg and Young, 1975).

Similar ts mutants have been isolated from Ad2. With Ad2, however, three complementation groups affect viral DNA synthesis (Kathman et al., 1976). H2ts 206 will complement both H5ts 36 and H5ts 125, yet does not synthesize viral DNA at the nonpermissive temperature. H2ts 206 is presumably defective in a third function other than the SS DNA binding protein and the 5'-terminal protein, that is required for DNA replication.

In Ad12, three complementation groups of early mutants have been identified (Shiroki and Shimojo, 1974). All three groups affect the initiation of viral DNA synthesis, and only one group has been assigned to a protein product, the 60,000 dalton single-stranded DNA binding protein of Ad12. It is interesting to note that conditional lethal mutants of adenovirus have not been isolated which affect functions in early regions 1, 3 and 4.

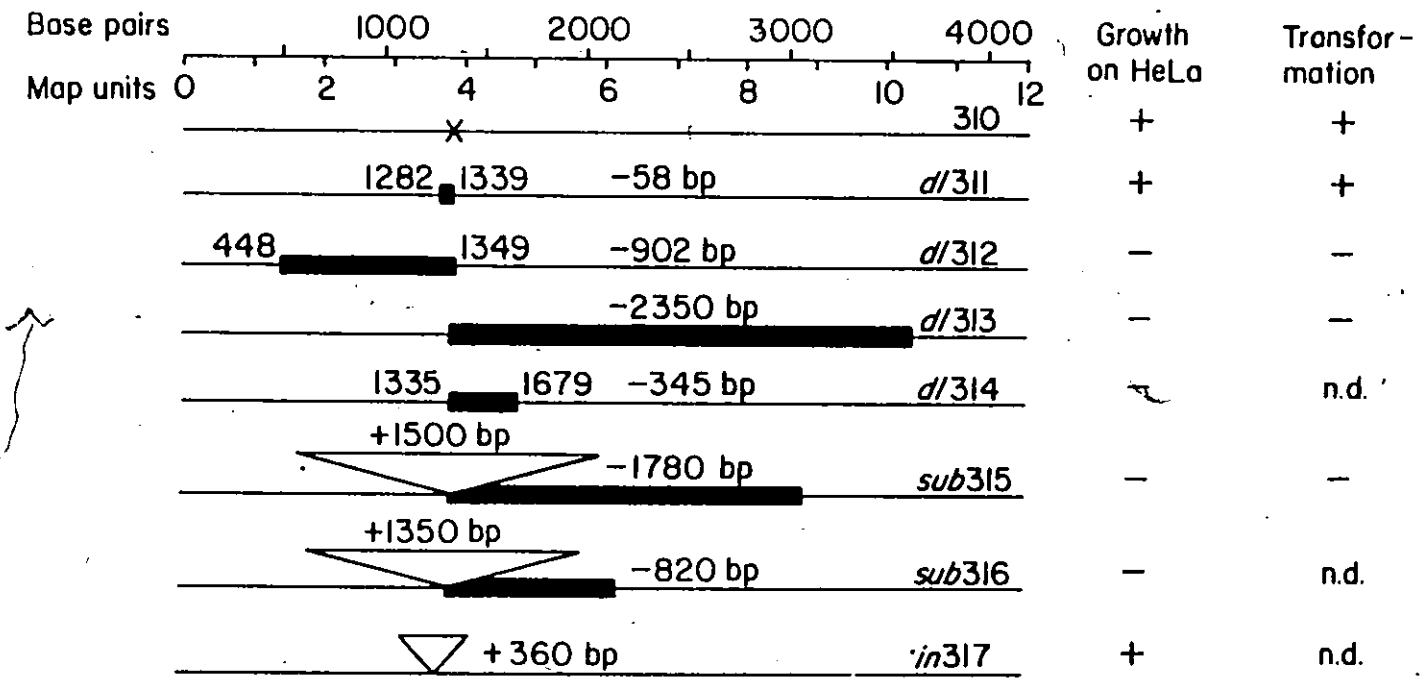
(ii) Host Range Mutants. Host range (hr) mutants of several types have been isolated from Ad5. One group of hur mutants does not replicate in hamster cells (Takahshi, 1972; Minekawa et al., 1976; Harrison and Williams, 1978). These mutants are not well characterized although one mutant, H5 hr 65, has been shown to complement all complementation groups of ts mutants of Ad5 (Harrison and Williams, 1978).

The most important and well studied group of mutants are those isolated by Harrison, Graham and Williams, (1977). These mutants were selected for growth in 293 cells, human embryonic kidney (HEK) cells transformed with sheared Ad5 DNA (Graham et al., 1977), but not in HeLa cells. The nine mutants segregate into two complementation groups. The Group I mutants are defective in both transformation (Graham et al., 1977) and viral DNA synthesis (Lassam, Bayley and Graham, 1978; Ross et al., 1980) and appear to map in the left-hand 4.4% of the Ad5 genome, or early region Ela (Frost and Williams, 1978; Galos et al., 1980). The Group II mutants are also defective in transformation, but do synthesize viral DNA (Lassam, Bayley and Graham, 1978) and map between 6.1 and 9.4 map units, or early region Elb (Frost and Williams, 1978; Galos et al., 1980). The Group II mutants have the ability to grow on primary HEK cells.

(iii) Deletion-Substitution Mutants. The first mutants in which the precise physical locations of the mutations are known are the deletion-substitution mutants of Ad5 isolated by Jones and Shenk (1978). The important mutations originate at the restriction endonuclease XbaI site at 4.0 map unit, and the structures of eight mutants are given in Figure 1-4. The mutants were selected for resistance to the enzyme XbaI and for efficient growth in 293 cells. dl310 and dl311 will grow in HeLa cells, but relatively inefficiently. dl312, dl313 and sub-315 are all defective in growth on HeLa cells. dl312 and sub-315 are defective in transformation, while dl313 infection of rat cells at high multiplicities can give rise to partially transformed cells (Shiroki et al., 1981; Mak and Mak, in press). dl312 and dl313 have been the subjects of extensive study because of their deletions affecting transformation. The Group I and Group II mutants and the dl mutants have contributed immensely towards identifying the viral products and their functions involved in transformation.

(iv) Weakly Tumorigenic Mutants of Ad12. The first mutants of adenovirus to be isolated were a group of cytocidal (cyt) mutants of Ad12 by Takemori, Riggs and Aldrich (1968). The mutants were originally selected for altered plaque morphology on human amnion FL cells and HEK cells, but not for any particular functional alterations. Fortunately, perhaps, the first plaque purified mutant was found to be weakly tumorigenic compared to the highly tumorigenic parental virus, a necessary property for the study of viral oncogenesis. A few hundred

Figure 1-4 Structure of deletion and substitution mutants of adenovirus 5 (Jones and Shenk, 1978). (———) Adenovirus-5 genome from 0 to 12 map units; (▬▬) regions of deleted viral DNA. Mutant 310 apparently carries a point mutation, X, because no rearrangement could be detected (Reprinted from Tooze, 1980).


Base pairs
1000
2000
3000
4000
Map units
0
2
4
6
8
10
12
Growth on HeLa
Transfor-mation
310
+
+
1282
1339
−58 bp
dl311
+
+
448
1349
−902 bp
dl312
−
−
−2350 bp
dl313
−
−
1335
1679
−345 bp
dl314
n.d.
+1500 bp
−1780 bp
sub315
−
−
+1350 bp
−820 bp
sub316
−
n.d.
+360 bp
in317
+
n.d.

mutants were isolated from two parental stocks of Ad12: the prototype Huie strain (Ad12-Huie) and the 1131 strain (Ad12-1131) of Pereira and Macallum (1964). Cyt mutants arise spontaneously at a frequency of about 10^{-5}, and ultraviolet irradiation of parental stocks to 10^{-1} to 10^{-2} survival increases this frequency by about 5-fold.

An initial study (Takemori, Riggs and Aldrich, 1968) of many cyt mutants established several characteristic properties of the mutants. They produce large clear plaques as opposed to small fuzzy-edged plaques produced by the parental viruses on HEK cells. In contrast to the adenovirus type cytopathic effect (CPE) of marked rounding and aggregation of infected cells (cyt^+), the mutants produce a CPE of extensive cellular destruction (cyt). In mixed infections with parental virus, the cyt^+ CPE is dominant.

The cyt mutants are much less tumorigenic than the parental virus when injected into newborn hamsters (only 1 of 43 tested was as tumorigenic as the parental virus). However, in mixed innoculation with low tumorigenic field strains of Ad12 and with weakly oncogenic Ad3 and Ad7, tumor induction is higher than the sum of that of each virus type alone. This cooperation in tumorigenesis is not obtained with the non-oncogenic Ad2, Ad5, or Ad4.

Complementation and recombination analysis of the CPE and tumorigenicity markers between pairs of cyt mutants suggest that a single structural gene sequence is affected in the mutants (Takemori, Riggs and Aldrich, 1968; Takemori, 1972). Many of the cyt mutations were separable by recombination, and the resulting intragenic recombinants selected for cyt^+ CPE were again highly tumorigenic like

wild type virus (Takemori, 1972).

Comparative studies (Ezoe and Mak, 1974; Ezoe, 1976) on physical and biological properties of a representative spontaneous cyt mutant, H12 cyt 70, to its parental Ad12-1131 found no difference in buoyant density of the virions. The kinetics of synthesis of early and late viral antigens and the synthesis of viral DNA were also found to be similar. The yield of H12 cyt 70 virus from three different human cell lines (HEP-2, KB and HEK), however, were consistently about 4-5 fold lower than that of the parental virus.

Analysis of intracellular viral DNA in infected KB cells revealed some interesting differences between H12 cyt 70 and the parental virus (Ezoe, Lai Fatt and Mak, 1981). DNA hybridization experiments showed that cells infected with H12 cyt 70 contain approximately 37% of the viral DNA found in cells infected with the parental virus. This is greater than that expected from the relatively low virus yields. Alkaline and neutral sucrose gradient sedimentation analyses of infected-cell lysates demonstrated, however, that much of the intracellular DNA, both viral and cellular, is fragmented in H12 cyt 70 infected cells compared to in Ad12-1131 infected cells. Pulse-chase labelling experiments demonstrated that the small DNA molecules (the majority being 4-22% of the length of the viral genome) are the result of degradation of larger molecules and not due to premature termination of DNA synthesis nor incomplete ligation of replication intermediates. This degradation of DNA is absent in cells coinfected with H12 cyt 70 and the parental virus. It has been suggested that

the degradation of intracellular DNA may be responsible for the low virus yield and cyt$^+$ CPE. A similar function of inducing degradation of DNA has also been described with a mutant of Ad2; H2 ts 111 produces DNA degradation in infected KB cells when incubated at the non-permissive temperature, and both viral and cellular DNA are degraded also (D'Halluin et al., 1980). Studies to date, however, have not identified any enzyme responsible for degrading the DNA, nor whether it is of viral or host origin.

Purpose of Study

The induction of DNA degradation by cyt mutants of Ad12 in KB cells is of great interest for several reasons. First, a strongly induced enzymatic activity appears to be involved, quite uncharacteristic of adenovirus infection of exponentially growing KB cells. Secondly, the cyt mutants are defective in several properties including, most importantly, transformation and tumorigenicity. The viral gene(s) involved in the induction of DNA degradation might also be involved in transformation and tumor induction. The importance of this cyt property of induction of DNA degradation warrants further studies on its functional characterization and on the genome location of the gene(s) involved.

The studies in this thesis, therefore, further investigate the process of induction of DNA degradation in cells infected with adenoviruses. The approach of genetic complementation analyses using several cyt mutants of Ad12 and region E1 mutants of Ad5 in both permissive and non-permissive cell systems was employed in elucidating two viral gene

functions involved in DNA degradation. These results in addition to a transcriptional study of region E1 of Ad12, mapped one function in region E1b of the adenovirus genome. Superinfection experiments with a *gyt* mutant and wild type viruses suggested a working hypothesis of the mechanism of the induction of DNA degradation by adenoviruses.

Regarding the format of presentation of these studies, in the interest of clarity each study is organized into a chapter with a brief introduction, the results, and discussion. The "materials and methods" of all the studies, however, are compiled into a single section preceding these studies. A general discussion of the studies is presented as the final chapter.

CHAPTER 1

MATERIALS AND METHODS

Cells

(i) Human KB Cells. This cell line, which originated from a human epithelial cheek carcinoma, was passaged either as monolayer cultures in glass prescription bottles (Brockway, 32 oz) or as suspension cultures. Monolayer cultures were maintained in F11 medium (Gibco) supplemented with 10% calf serum (Gibco), penicillin-streptomycin (50 units - μgs/ml) (Gibco), and fungizone (1.25 μg/ml) (Gibco) in an atmosphere of 5% CO_2 in air at 37°C. Cells were routinely subcultured when they reached confluency by scraping them from the glass surface with a rubber policeman and aliquots of the cell suspension seeded into fresh bottles with prewarmed medium. Suspension cultures were maintained at a cell concentration of 2-4 x 10^5/ml in Joklik modified MEM (spinner medium, Gibco) supplemented with 5% horse serum (Gibco), at 37°C. Cells were kept in suspension by constant stirring with a teflon coated magnetic stir bar. Cell concentration was maintained by dilution with prewarmed medium.

(ii) Primary Hamster Embryo Cells. All manipulations were done asceptically. Embryos were removed from pregnant hamsters on the 13th to 14th day of gestation and were decapitated, minced and treated with 0.125% Bacto-trypsin (Difco) in citrate-saline (1% KCl, 0.44% Na acetate) with stirring for 15 min at 37°C. A crude cell suspension was obtained by filtering through cheesecloth. Cells were pelleted and resuspended in Alpha-MEM (Gibco) supplemented with 10% fetal calf

serum (Gibco) and penicillin-streptomycin (100 units - µgs/ml) and plated at a cell density of about 4 x 10^4 cells/cm^2 in plastic petri dishes (Falcon). Medium was changed after 1 day, and on the second day cells were subcultured by trypsinization and re-seeded onto plastic dishes. Cells were used in experiments between the fourth and sixth subculturing.

(iii) Human 293 Cells. This cell line, transformed by sheared Ad5 DNA (Graham et al., 1977; Aiello et al., 1979) was grown as monolayer cultures in plastic flasks or petri dishes. The methods for maintenance and subculturing are as described for primary hamster embryo cells.

Viruses

Virus stocks of Ad12 strain 1131 (Ad12-1131) and Ad12-Huie were obtained from N. Takemori and M. Green, respectively. Ad12-Huie (M8) is a stock derived from a single plaque of Ad12-Huie, isolated by I. Mak. Crude stocks of cyt mutants H12 cyt 70, H12 cyt 52, H12 cyt 61, and H12 cyt 62 were obtained from N. Takemori (St. Louis Univ. Medical School, MO). Virus stocks of wild type Ad5 and host range mutants Ad5 hr1 and Ad5 hr6 were obtained from F.L. Graham (McMaster University, Hamilton, Ont). Stocks of deletion-host range mutants Ad5 d1 312 and Ad5 d1 313 were obtained from T. Shenk (New York Univ., Stoneybrook, N.Y.).

Virus Growth and Purification

(i) Ad12-1131, Ad12-Huie (M8), and Ad5. KB cells grown in suspension cultures were concentrated to 1 x 10^7 cells/ml in Joklik medium containing 1% calf serum (adsorption medium) and mixed with virus at a multiplicity of about 200 virions/cell. Adsorption was for 90 min. at 37°C with the cells kept in suspension by gentle stirring with a small teflon-coated magnetic stir bar or by moderate rotation of the vessel in a "roller wheel" apparatus at approximately 48 r.p.m. The cells were then diluted to a concentration of 3 x 10^5 cells/ml in spinner medium and maintained as a suspension culture. After 72 hr for Ad12 or 50 h for Ad5, the cells were harvested by centrifugation at 200 g (about 1,000 r.p.m. in a clinical centrifuge) for 10 min. and the cell pellet washed with phosphate buffered saline without Ca^{++} and Mg^{++} (PBS/w/o).

Extraction and purification of the virus from greater than 10^8 cells were carried out essentially according to the procedure of Green and Piña (1963). Cells were resuspended in 10 mM Tris-HCl, pH 8.1, disrupted by sonication to release virions, homogenized with freon 113 at 4°C and the virus concentrated from the aqueous phase by sedimentation onto a cesium chloride (CsCl) cushion of density 1.44 gm/ml. The concentrated virus was purified by two rounds of isopycnic banding in CsCl of density 1.34 gm/ml by centrifugation in a Beckman 50 Ti rotor at 33,000 rp.m. for 20 h, 0°C. The concentration of virions was determined by U.V. absorption at 260 nm wavelength (A260)

with one absorbance unit corresponding to 4×10^{11} virions/ml. Virus was stored at a concentration of 1-2 absorbance units in Tris-buffered saline (Winocour, 1963), containing 30% glycerol at -50 to -70°C.

Extraction of virus from less than 10^8 cells was done essentially according to the procedure of Lawrence and Ginsberg (1967). Cell pellets were resuspended to a concentration of $3\text{-}4 \times 10^7$ cells/ml in 10 mM Tris-HCl, pH 7.4. The cell suspension was made 0.5% w/v sodium deoxycholate and lysed at room temperature for 30 min to release virus. After adjusting the suspension to 20 mM $MgCl_2$, DNase I was added to a concentration of 25 µg/ml and incubated at 37°C for 1 h, the suspension was then centrifuged at 1400 g for 25 min. at 5°C. The supernatant containing the virus was extracted with an equal volume of freon 113 twice. Virus in the aqueous phase was further purified by isopycnic centrifugations as described above.

(ii) Cyt Mutants of Ad12. Virus growth and purification procedures were essentially the same as for the wild type viruses with the exception that after virus adsorption, the cells were kept as monolayer cultures in 32 oz. prescription bottles until harvested at about 50 h. This procedure was necessary due to the extreme fragility of the infected cells which prevented the use of suspension cultures and long periods of incubation.

(iii) Radioisotopically-labelled Virions. Virus labelled with ^3H or ^{14}C to be used as DNA or virion markers were prepared by adding (^3H)-thymidine (20 Ci/mM, 1.35 µci/ml) or (^{14}C)-thymidine (40-60 mCi/mM, 1.3×10^{-2} µCi/ml) (New England Nuclear) to the infected culture at 14 h post-infection until harvested.

Infection of Cells for DNA Degradation Assay

(i) KB Cells.

a) Primary infection: KB cells, grown in suspension culture to a density not greater than 3.5×10^5 cells/ml, were concentrated to 1×10^7 cells/ml in adsorption medium. Virus was adsorbed for 60 min to cells kept in suspension by means of a "roller wheel" apparatus as described earlier. After adsorption, cells were separated from un-adsorbed virus by low speed centrifugation. The infected cells were seeded in plastic flasks (3×10^6 cells/Falcon 25 cm^2 flask).

b) Superinfection: At appropriate times after primary infection, the infected cells were gently scraped into suspension and sedimented by low speed centrifugation. The cell pellet was resuspended to a concentration of 1×10^7 cells/ml in adsorption medium and adsorbed with the second virus. The same procedure for adsorption and seeding of cells were followed as for primary infection.

(ii) 293 Cells. Monolayers of cells at close to confluency in Falcon 25 cm^2 flasks were overlayed with 0.5 ml of adsorption medium containing virus and incubated for 2 h at 37°C. The adsorption medium was then removed and fresh growth medium was added.

(iii) Primary Hamster Embryo Cells. Cells in their 4th to 6th passage were trypsinized and resuspended in adsorption medium. 5×10^6 cells in 0.7 ml were mixed with virus (2000 - 5000 virions/cell) in a Falcon 25 cm^2 flask, and the cells were kept in suspension during adsorption for 90 min at 37°C by placing the flask horizontally in a shaking water-bath. The cells were then diluted in growth medium and seeded at 2.5×10^6 cells per 25 cm^2 flask.

Adsorption Efficiency Assay

KB cells were infected with H12 cyt 70 at a multiplicity of 300 virions/cell as described for primary infection. The cells were plated at 5 x 10^6 cells/flask (Falcon 75 cm^2) in growth medium. At the appropriate time points, cell were gently scraped from the surface, pelleted at 200 g for 5 min, then resuspended in 0.5 ml of fresh adsorption medium. (^{3}H)-labelled Ad12-1131, labelled with (^{3}H)-thymidine, was added at 2000 virions/cell and adsorbed at 37°C in the "roller wheel" apparatus for 90 min. Cells were then centrifuged at 200 g for 10 min, and duplicate aliquots of 0.2 ml of the supernatant were made 0.1% SDS, treated with cold 5% trichloroacetic acid (TCA), filtered onto nitrocellulose membrane filters which were washed with cold 5% TCA then dried. The amount of radioactivity on each filter was determined by liquid scintillation counting. The cell pellet was washed with 1 x PBS w/o, lysed with 0.1% SDS, and the radioactivity determined as with the supernatant.

Radioisotopic-Labelling of Cells for DNA Degradation Assay

(i) Continuous labelling: Infected cells were labelled with (^{3}H)-thymidine at a final concentration of 15 µCi/ml, 7.5 µCi/µg, at 14 h post-primary infection. For cultures which were superinfected at times later than 14 h after primary infection, the (^{3}H)-thymidine was added immediately after superinfection. Cells were harvested at 36-40 h after primary infection for all cases.

(ii) Pulse-chase Labelling: Infected cells in monolayer cultures were labelled with (^{3}H)-thymidine (20 Ci/mM) at 10 µCi/ml for 60 min. The medium containing unincorporated (^{3}H)-thymidine was decanted and centrifuged to collect any cells which were concommitantly decanted. These cells were resuspended in fresh growth medium and added back to the monolayer cells, and unlabelled thymidine was added to a final concentration of 5 µg/ml during the chase period of 6 h.

Alkaline Sucrose Gradient Sedimentation Assay for DNA Degradation

1 x 10^6 cells in 0.1 ml of 10 mM Tris-HCl, pH 8.1 together with purified Ad5 virions containing (^{14}C)-labelled DNA (3.5 x 10^3 CPM) as a marker, were lysed by the addition of 0.2 ml of lysing reagent (0.5 N NaOH, 10 mM EDTA, 0.1 M NaCl, and 1% SDS) for 6 h at room temperature. Alkaline sucrose gradients were of 5% to 20% sucrose solution containing 0.3 N NaOH, 2.0 mM EDTA, 0.1 M NaCl, and 0.1% SDS. Gradients were made in Beckman nitrocellulose tubes (5/8" x 4"). Cell lysates were carefully layered onto the gradients by means of a wide-mouthed plastic pipette (Falcon disposable). Centrifugation was at 24,000 rpm for 13 h at 20^o in a Beckman SW27.1 rotor. About forty fractions were collection from each tube by dripping from a hole pierced through the bottom of the tube. The radioactivity in each fraction was determined by liquid scintillation counting after cold TCA precipitation onto nitrocellulose filters.

Cesium Chloride Density Gradient Sedimentation Assay

A purified virus preparation from infected cells which had been labelled with (^{3}H)-thymidine was mixed with approximately 2 x 10^{11} Ad5 virions containing (^{14}C)-labelled DNA as both visible and radioactive

markers in 0.5 ml of 10 mM Tris-HCl, pH 8.1, and carefully layered on top of a preformed CsCl density gradient with a linear density range of 1.32 - 1.36 gm/ml in a Beckman nitrocellulose tube (9/16" x 3 3/4"). Centrifugation was carried out at 20,000 r.p.m. for 5 h at 5°C in a Beckman SW40 rotor. Fractions of 5 drops each were collected in the region of the visible virus band. Radioactivity was determined as with the fractions from the sucrose gradients.

DNA Extraction and Purification

(i) Total intracellular DNA: Total intracellular DNA was extracted according to the procedure of Mak (1969). The cell pellet was washed with PBS, w/o, resuspended in extraction buffer [1 x SSC (0.15 NaCl, 0.015 M sodium citrate), 10 mM Tris-HCl, pH 8.0, 0.3 M sodium trichloroacetate, and 5 mM EDTA] and lysed with 0.5% SDS for 10 min, at room temperature. Pronase (Calbiochem Inc., B grade) (predigested for 2 h at 37°C) was added to a concentration of 800 µg/ml at 37°C for at least 3 h. The mixture was then extracted three times with phenol saturated with 50 mM sodium phosphate pH 6.8, at 0°C. The aqueous phase was removed, adjusted to 0.15 M NaCl, and nucleic acids precipitated by the addition of 2 volumes of 95% ethanol and stored at -20°C overnight. The precipitate was collected by centrifugation at 8,000 g for 15 min. at 5°C. The pellet was resuspended in 1 x SSC with pancreatic RNase (Worthington; preheated to 90°C for 10 min.) added to a concentration of 50 µg/ml and incubated for 30 min at 37°C. The mixture was again phenol extracted three times as above, then three times with equal volumes of ether, and the DNA solution dialyzed extensively against 0.1 x SSC at 4°C.

(ii) Viral DNA: The virus collected from CsCl density gradients was dialyzed against 10 mM Tris-HCl, pH 8.1, at 4°C, to remove Cs Cl. The suspension was then adjusted to 0.15 M sodium phosphate pH 6.0, 7.5 mM EDTA, 1% SDS and 800 µg/ml pronase (predigested at 37° for 2 h). After incubation at 37°C for 1 h, the mixture was extracted three times with phenol as described earlier. The aqueous phase was then dialyzed against 0.1 x SSC or 10 mM Tris, pH 7.5.

All DNA concentrations were determined by A_{260} with one absorbance unit corresponding to 50 µg/ml.

Isolation of Replicating Viral DNA Molecules (partially single-stranded DNA)

Viral DNA was preferentially extracted from infected cells essentially according to the procedure of Hirt (1967). Cells were washed and resuspended in buffer containing 0.13 M NaCl, 50 mM Tris-HCl, pH 7.4. SDS was added to a final concentration of 0.6%, and cells were allowed to lyse at room temperature for 20 min. 5M NaCl was added to a final concentration of 1.0 M, the viscous suspension mixed gently by inversion of the vessel, and stored at 4°C for at least 10 h. The mixture was centrifuged at 17,000 g for 15 min at 4°C to pellet cellular DNA and the supernatant containing mostly viral DNA collected and further purified by phenol extraction.

Single-stranded (s.s.) DNA molecules were selectively removed from the viral DNA preparation by hydroxylapatite column chromatography. Hydroxylapatite, kindly provided by I. Mak, was prepared according to the procedure of Muench, 1971. DNA was dissolved in a buffer containing 10 mM Sodium phosphate, pH 6.8, and applied to the hydroxylapatite

column, 1.5 ml packed volume, prepared in the same buffer. Both s.s. and d.s. DNA binds to the hydroxylapatite in this buffer. Finally s.s. DNA molecules were eluted from the column with buffer containing 0.14 M sodium phosphate, pH 6.8. Molecules containing d.s. DNA were then eluted with buffer containing 0.4 M sodium phosphate, pH 6.8. This final DNA preparation was dialyzed extensively against 0.1 x SSC and precipitated with ethanol.

Fully d.s. DNA molecules were then selectively removed from the DNA preparation by benzoylated, napthoylated, DEAE cellulose (BND cellulose) column chromatography. A column of BND-cellulose (Sigma Chem. Co.), 1.5 ml packed volume was prepared in glass-distilled H_2O, then washed extensively in sample application buffer (0.3 M NaCl, 10 mM Tris-HCl, pH 7.2, 1 mM EDTA). The DNA was resuspended in application buffer and applied to the column. The effluent was reapplied twice to obtain maximum binding of the DNA, then the column washed extensively with application buffer. Totally d.s. DNA molecules were eluted with buffer containing 1.0 M NaCl, 10 mM Tris-HCl, pH 7.2, and 1 mM EDTA to remove any contaminating materials.

Determination of Single-Stranded DNA Content by Nuclease S1 Digestion

The percentage of (^{3}H)-labelled DNA in the form of s.s. DNA was determined by the percentage of radioactivity susceptible to digestion by nuclease S1. A quantity of (^{3}H)-labelled DNA to be analyzed for content of (^{3}H)-labelled s.s. DNA was resuspended to 1.0 ml in nuclease S1 digestion buffer containing 30 mM sodium acetate pH 4.5, 1.8 mM $ZnCl_2$, 0.3 M NaCl and 40 µg of denatured calf thymus DNA (Sigma Chem.

Co.). 3 x 10^3 units of nuclease S1 (Miles Research Products) was added and the mixture incubated for 1 h at 37°. The mixture was cooled on ice, 100 µg of carrier DNA was added and the nuclease S1 resistant DNA was precipitated by addition of cold 50% TCA to a final concentration of 100% and incubation on ice for 20 min. The precipitated d.s. DNA was collected onto nitrocellulose filters (0.2 micron pore size) and the radioactivity was determined by liquid scintillation counting.

DNA-DNA Hybridization

The hybridization of labelled DNA to unlabelled DNA immobilized on nitrocellulose filters was done essentially according to the procedure described by Warnaar and Cohen (1966). Viral DNA was denaturated at 100°C for 10 min in 0.1 x SSC, then immediately cooled in an ice-bath. The DNA solution was then adjusted to 2 x SSC and filtered slowly onto nitrocellulose filters which were presoaked in 2 x SSC, at a concentration of 2 µg of DNA per filter (25 mm in diameter). The filters were baked at 80°C for 4 h.

The labelled DNA to be hybridized to the DNA-filters was adjusted to 0.1 x SSC and fragmented by sonication (Biosonic, 1.5 min at power setting of 30% output). The fragmented DNA was denatured at 100°C for 10 min and quickly cooled. The solution was then adjusted to 2 x SSC, 0.1% SDS and a 1.0 ml aliquot was added to each vial containing a DNA-filter. The vials were incubated at 65°C for 20 h. The filters were then washed exhaustively by suction with buffer containing 0.1 x SSC, 3 mM Tris-HCl, pH 9.4 and dried at 80°C. The radioactivity bound

to each filter was determined by liquid scintillation counting.

Restriction Endonuclease Digestion of DNA and Gel Electrophoresis

DNA was digested with the restriction endonucleases Bam Hl and EcoRl (Bethesda Research Laboratories or Borhingher Mannheim) according to the instructions of the suppliers. DNA fragments were electrophoresed in 1% agarose slab gels (16.5 cm x 16.5 cm) cast between vertical glass plates (3 mm apart). Samples of restricted DNA were made 0.5% SDS, 0.005% bromophenol blue, and 5% glycerol and loaded under electrophoresis buffer (40 mM Tris-HCl, pH 7.8, 5 mM sodium acetate, 1 mM EDTA) into the wells of the gel. Electrophoresis was at room temperature at 30 volts for approximately 10 h.

Fluorography

Fluorography procedure was as described by Bonner and Laskey (1974). After electrophoresis, gels were dehydrated in 20 volumes of methanol with intermittent agitation for 30 min. This step was repeated once. The gel was then immersed in approximately 5 volumes of 10% w/v Omnifluor in methanol for 3 h with intermittent agitation. This solution was removed and replaced with distilled water to precipitate the Omnifluor within the gel. The gel was then vacuum dried at 100°C onto Whatman 3 MM paper. The dried gel was then placed on Kodak XRP-5 film, pre-sensitized by a brief flash of white light as described by Laskey and Mills (1975). Exposure was at -70°C for 2 days in most instances. The film was developed in an automatic Kodak X-ray film processor. Microdensitometry was performed on a Joyce Loebl Mk. III CS instrument, and the areas under the peaks determined with a planimeter.

Preparation of Cytoplasmic RNA

All operations were done at 0°C in plastic vessels which had been washed in 95% ethanol and autoclaved. Cells were washed once with and resuspended in isotonic buffer (0.15 M NaCl, 10 mM EDTA, 10 mM Tris-HCl, pH 7.8) at a concentration of (3-5) x 10^7 cells/ml. An equal volume of isotonic buffer containing 1.4% NP40 (Shell Chemical Corp.) was added and gently mixed. Cell lysis was monitored by phase contrast microscopy and was generally completed within 10 min. One-half volume of isotonic buffer was added and gently mixed. The nuclei were then pelleted at 850 g for 10 min, 0°C, and the supernatant was collected. The nuclei were washed once with isotonic buffer, pelleted and the supernatant pooled with the first. The cytoplasmic fraction was made 1% SDS and extracted by vigorous shaking at room temperature for 10 min with an equal volume of extraction reagent consisting of approximately 75% phenol, 24 % chloroform, 1% isoamyl alcohol and 0.1% 8-hydroxyquinoline, saturated with isotonic buffer. After extraction, the mixture was cooled in a methanol-ice bath until phenol crystals appeared on the surface of the extraction vessel, then centrifuged at 24,000 g, 5 min, 0°C. The aqueous phase was removed and extracted twice more. The final aqueous phase was made 2% sodium acetate and the nucleic acids precipitated by the addition of 2 volumes of 95% ethanol at -20°C overnight or at -70°C for several hours. The precipitate was pelleted at 24,000 g, 30 min, 0°C. The pellet was washed once with 70% ethanol, 30% 0.15 M NaCl. The pellet was drained dry and then resuspended in DNase digestion buffer (10 mM Tris-HCl, pH 8.0, 10 mM NaCl, 10 mM $MgCl_2$) and digested with DNase I (Worthington) at 50 µg/ml for

1 h at 37°C. The solution was then re-extracted three times as above; the RNA was precipitated with ethanol and collected by centrifugation in a Beckman SW40 rotor at 30,000 r.p.m. for 90 min. The RNA pellet was resuspended in a small volume of 10 mM Tris-HCl, pH 7.9, and ethanol precipitated twice more. RNA concentrations were estimated by A_{260}, assuming 1 absorbance unit corresponds to 42 µg/ml.

Preparation of Poly(A)$^+$ RNA

Poly(A)$^+$ RNA was selected by oligo (dT)-cellulose (Collaborative Research Inc., Type 3) affinity chromatography. A column of 0.4 ml packed volume of oligo (dT)-cellulose was made in a 1 ml plastic syringe in application buffer (10 mM Tris-HCl, pH 7.9, 0.5 M NaCl), and washed extensively with the buffer. The RNA preparation was made 10 mM Tris-HCl, pH 7.9, 0.5 M NaCl, and applied to the column. The effluent was reapplied three times to assure maximum binding of Poly(A)$^+$ RNA to the oligo (dT). The column was then washed with approximately 5 volumes of application buffer. Poly (A)$^+$ RNA was eluted with 10 mM Tris pH 7.9 buffer. Poly(A)$^+$ RNA was precipitated with ethanol and pelleted in a Beckman SW 50.1 rotor at 35,000 r.p.m. for 90 min. The RNA was re-suspended in 10 mM Tris-HCl, pH 7.9 and stored at -70°C. The oligo (dT)-cellulose column was washed between use with 0.5 N NaOH to remove any non-specifically bound RNA.

Isolation of Endonuclease Restriction Fragments from Agarose Gels

DNA fragments were isolated from agarose gels according to the procedure of Girvitz et al. (1980). Viral DNA fragments generated by various restriction endonucleases were separated by horizontal agarose

gel (1.0 - 1.5% agarose, 5-7 mm thick) electrophoresis in buffer containing 40 mM Tris-HCl, pH 7.8, 5 mM sodium acetate, 1 mM EDTA and 0.5 µg/ml ethidium bromide, at 2V/cm until DNA fragments to be isolated, viewed under UV illumination, were clearly separated. A slit was made in the gel in front of and parallel to the DNA band to be isolated. A strip of Whatman 3 MM chromatography paper backed with dialysis membrane was inserted into the slit. The DNA band was then electrophoresed at 4-5 V/cm until it migrated completely into the paper. The paper together with the dialysis membrane backing was removed and the paper placed in a 0.5 ml Eppendorf microcentrifuge tube with a small hole pierced in the bottom with a 26 gauge needle. The 0.5 ml tube was placed inside a 1.5 ml Eppendorf tube and centrifuged for 1 min. in a Brinkman 6412 microcentrifuge. The paper was removed and wetted with a wash buffer (10 mM Tris-HCl, pH 7.6, 10 mM NaCl, 1 mM EDTA, .2% SDS), then used to "sponge" the dialysis membrane for any residual DNA. The paper was centrifuged and washed once with buffer or until paper was free of DNA as determined by UV illumination. The pooled solution of DNA was made 0.15 M NaCl and the DNA was precipitated with ethanol twice.

In Vitro Labelling of DNA with (^{32}P)

DNA was labelled by a modified procedure of Weinstock et al. (1978) as described by Redfield (1980). Approximately 0.2 µg of DNA was incubated for 10 min at 37°C with 0.25 ng of DNase I (Worthington) in 50 µl of 50 mM Tris-HCl, pH 7.9, 5 mM $MgCl_2$, 10 mM β-mercaptoethanol, 100 µg bovine serum albumin, and 10 µM each of dATP, dTTP and dGTP. The mixture was then combined with 100 µCi of (^{32}P)α-dGTP (New England Nuclear, specific activity 300-600 Ci/mM, air dried to remove ethanol)

and 6 units of E. coli. DNA polymerase I (Borhingher Mannheim), and incubated for 1 h at 12°C. The reaction was stopped by addition of EDTA to a final concentration of 25 mM. Protein was removed by phenol extraction, and the DNA was separated from unincorporated (^{32}P)α-dCTP by gel filtration through a 7 ml Sephadex G-50 column using a buffer containing 10 mM Tris-HCl, pH 8.0, 10 mM NaCl, 2 mM EDTA and 0.01% SDS. Fractions containing (^{32}P)-radioactivity eluting in the void volume were pooled and combined with 100 µg of salmon sperm DNA precipitated with ethanol. (^{32}P)-labelled DNA was redissolved in 0.1 x SSC.

Electrophoresis of RNA in Agarose Gel

RNA samples were denatured and electrophoresed in horizontal agarose gels (16.5 cm x 17.0 cm x 0.6 cm) by a modified procedure of Lehrach et al. (1977). RNA electrophoresis buffer (E-buffer) contained 2.0 M formaldehyde, 5 mM sodium acetate, 1 mM EDTA, and 100 mM MOPS, pH 7.0. Agarose (1.0 - 1.4% w/v) was melted in E-buffer minus formaldehyde and cooled to 75°C; formaldehyde was then added, mixed and poured into the gel apparatus. The RNA samples were suspended with 50% formamide (BDH Analar, deionized on Bio-Rad AG 501-X8 mixed bed resin) in 1 x E-buffer and incubated at 60°C for 5 min to denature the RNA. The mixture was adjusted to 1% ficoll, 0.005% bromophenol blue and loaded into the wells of the gel. Electrophoresis was for 10 h at 2V/cm.

Blotting of RNA to Nitrocellulose and Hybridization

The procedure of Wahl, Stern and Stark (1979) for the transfer of DNA to nitrocellulose and hybridization were modified to accomodate

RNA transfer and hybridization. The agarose gel containing RNA was immersed in 50 mM NaOH for 2 x 20 min incubations with intermittent shaking. The NaOH was decanted, the gel washed briefly with distilled water, then immersed in a buffer containing 3 M NaCl, 0.5 M Tris-HCl, pH 7.4, for 2 x 30 min incubations with intermittent shaking. The gel was placed on 2 sheets of Whatman 3 MM chromatography paper saturated with 20 x SSC and the borders of the gel and paper was covered with sheets of plastic Saran wrap. The nitrocellulose paper (Sartorius, 0.45 μ pore size) soaked in 20 x SSC was placed on the gel, then overlayed with 2 sheets of Whatman 3 MM paper dampened with 20 x SSC and overlayed with a stack (about 3 inches) of paper towels. A light weight was placed on top. After 4 hours, the nitrocellulose filter was removed and then baked at 80°C for 3 h.

Hybridization was carried out as follows:
The filter containing RNA was carefully placed in a polyethylene "freezer" bag containing approximately 10 ml of pre-incubation buffer containing 50% formamide, 5 x SSC, 5 X Denhardts reagent (0.02 w/v bovine serum albumin, 0.02% w/v polyvinyl pyrollidone, 0.02% ficoll), 50 mM sodium phosphate, pH 6.5, and 250 μg/ml of sonicated, denatured salmon sperm DNA then sealed and incubated for 1 h at 42°C. This preincubation buffer was removed and replaced with a hybridization solution (approx. 10 ml) containing 50% formamide, 5 x SSC, 1 x Denhardt's reagent, 20 mM sodium phosphate, pH 6.5, 100 μg/ml sonicated, denatured salmon sperm

DNA, 10% sodium dextran sulphate and (^{32}P)-labelled DNA probe which was denatured at 100°C for 5 min in 0.1 x SSC. The bag was sealed and incubated at 42°C for 16-20 h. The filter was removed from the bag and washed 3 times, ~~each with~~ 250 ml of 2 x SSC, 0.1% SDS for 5 min at room temperature. The filter was then washed 3 times each with 250 ml of 0.1 x SSC, 0.1% SDS at 50°C for 15 min. The filter was then dried at 80°C for 20 min, then autoradiography done with Kodak XRP-5 film.

Chapter 2

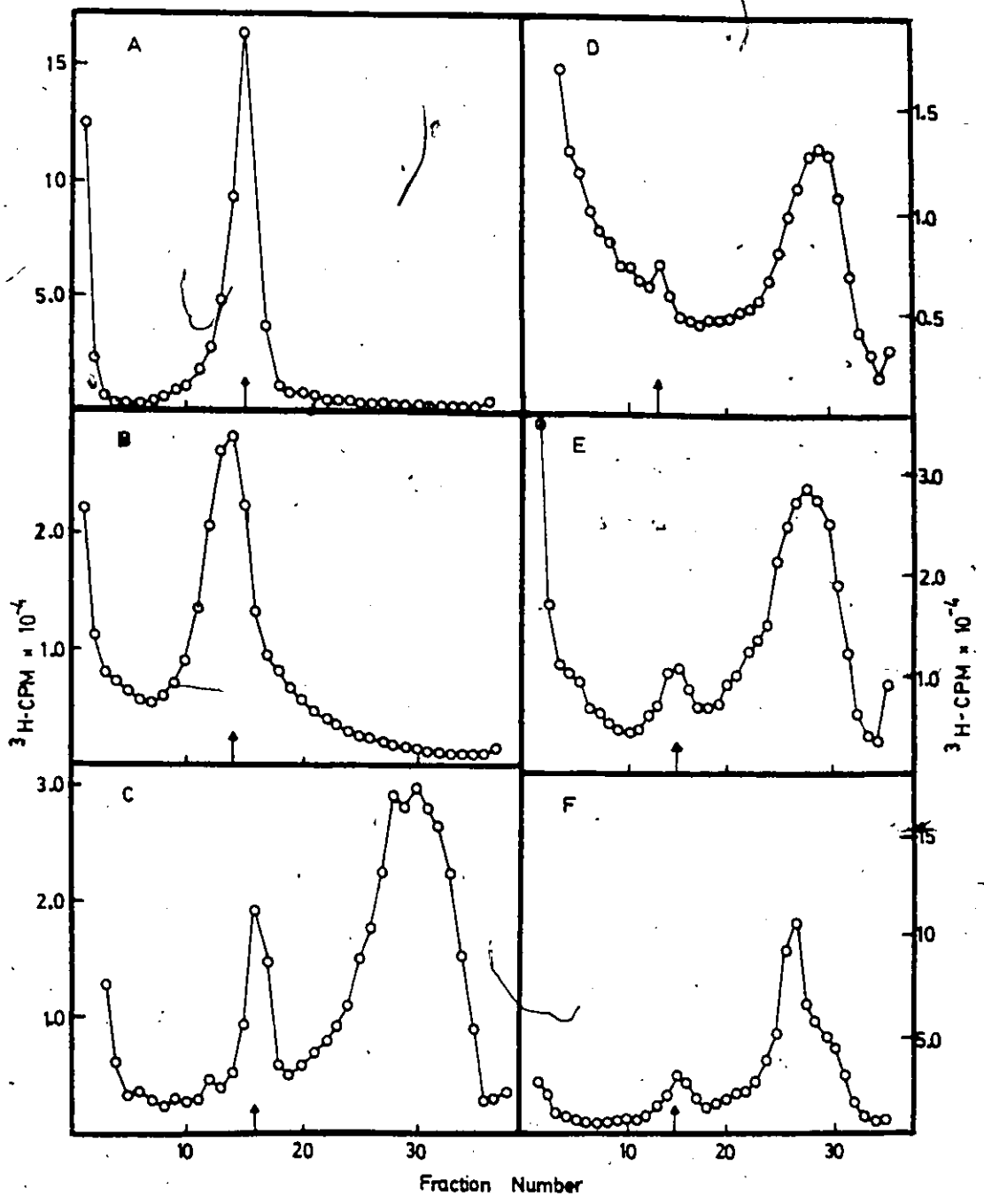
Induction of DNA Degradation in KB Cells by Cyt Mutants of Ad12

It was found by Ezoe (1976), that the cyt mutant H12 cyt 70, a spontaneously derived mutant of Ad12-1131, induces extensive degradation of intracellular DNA in KB cells, unlike the parental virus. It has been suggested from results of complementation and recombination analyses of the CPE marker between pairs of cyt mutants that a single cistron is affected in these mutants (Takemori, Riggs and Aldrich, 1968; Takemori, 1972). To determine if the function of inducing DNA degradation is a general property of cyt mutants, a plaque morphology revertant and three additional independent isolates of cyt mutants were tested for the ability to induce DNA degradation in KB Cells. Of the additional cyt mutants, one isolate, H12 cyt 52, was also spontaneously derived from Ad12-1131 and two isolates, H12 cyt 61 and H12 cyt 62, were derived from UV irradiated stocks of Ad12-Huie and Ad12-1131, respectively.

KB cells were infected with the wild-type Ad12-1131, the revertant and the four cyt mutants at various multiplicities of infection and labelled with (^{3}H)-thymidine (15 μCi/ml, 7.5 μCi/μg) from 14-36 h post-infection (p.i.). The cells were lysed and the size distribution of the labelled DNA was analyzed by alkaline sucrose gradient sedimentation.

Cells infected with the wild type Ad12-1131 and the revertant at a multiplicity of 150 virions/cell (Fig. 2-1, A,B) produced a narrow

Figure 2-1 Sedimentation profiles in alkaline sucrose gradients of intracellular DNA in KB cells infected with Ad12 parental virus (strain 1131), various cyt mutants and a revertant. Sedimentation is from right to left. Cells infected with: (A) parental Ad12-1131 virus [m.o.i. 150 virions/cell], (B) a revertant [m.o.i. 150/virions/cell], (C) H12 cyt 70 [m.o.i. 150 virions/cell], (D) H12 cyt 52 [m.o.i. 1,000 virions/cell], (E) H12 cyt 62 [m.o.i. 500 virions/cell], and (F) H12 cyt 61 [m.o.i. 150 virions/cell]. Arrows indicate the position of [^{14}C]-labelled Ad5 marker DNA.


A
B
C
D
E
F
15
10
5.0
2.0
1.0
3.0
2.0
1.0
1.5
1.0
0.5
3.0
2.0
1.0
15
10
5.0
10
20
30
10
20
30
Fraction Number
^{3}H-CPM x 10^{-4}
^{3}H-CPM x 10^{-4}

peak of radioactivity cosedimenting with the (^{14}C)-labelled viral DNA size marker, with a very small amount of radioactivity sedimenting slower. Cells infected with the cyt mutants H12 cyt 70 and H12 cyt 61 at 150 virions/cell, H12 cyt 62 at 500 virions/cell and H12 cyt 52 at 1000 virions/cell (Fig. 2-1, C,F,E and D, respectively), on the other hand, showed relatively little radioactivity cosedimenting with the viral DNA marker and a broad peak of radioactivity sedimenting as smaller DNA fragments ranging in size from approximately 0.02 to 0.05 the length of the viral genome. Mutants H12 cyt 52 and H12 cyt 62 required higher multiplicities of infection of 500-1000 virions/cell to show significant CPE and DNA degradation, but the wild-type Ad12 at these high multiplicities did not induce DNA degradation (data not shown). These results strongly suggest that the function of inducing DNA degradation in KB cells is a general property of cyt mutants.

Chapter 3

Mapping of an Adenovirus Function Involved in the Inhibition of DNA Degradation

INTRODUCTION

Revertants have been isolated from some cyt isolates and have been shown to regain the wild type characteristics of high tumorigenicity, plaque morphology and the lack of induction of DNA degradation during lytic infection (Takemori, Riggs and Aldrich, 1968; Takemori, Riggs and Aldrich, 1969; Ch. 2). The functions for cellular transformation and tumorigenicity are encoded in region E1 of adenoviruses (Flint et al., 1976; Gallimore, Sharp and Sambrook, 1974; Graham, Harrison and Williams, 1978; Sharp, Pettersson and Sambrook, 1974); it is possible that the function required for the prevention of DNA degradation is also encoded in this region.

Studies on the transcriptional map of Ad12 (Ortin et al., 1976; Smiley and Mak, 1978) and that of Ad2 and Ad5 suggest that the overall organization of the genomes of these serotypes are structurally similar. Furthermore, Ad12 can complement the functional defects encoded in region E1 of Ad5 and Ad2 (Brusca and Chinnadurai, 1981; Rowe and Graham, 1981; Williams and Galos, 1981). In this study Ad5 early mutants which have mutations in region E1 were used to map by complementation the cyt mutation of Ad12. These are the host range mutants (groups I and II) isolated by Harrison, Graham and Williams (1977) and the host range deletion mutants of Jones and Shenk (1978).

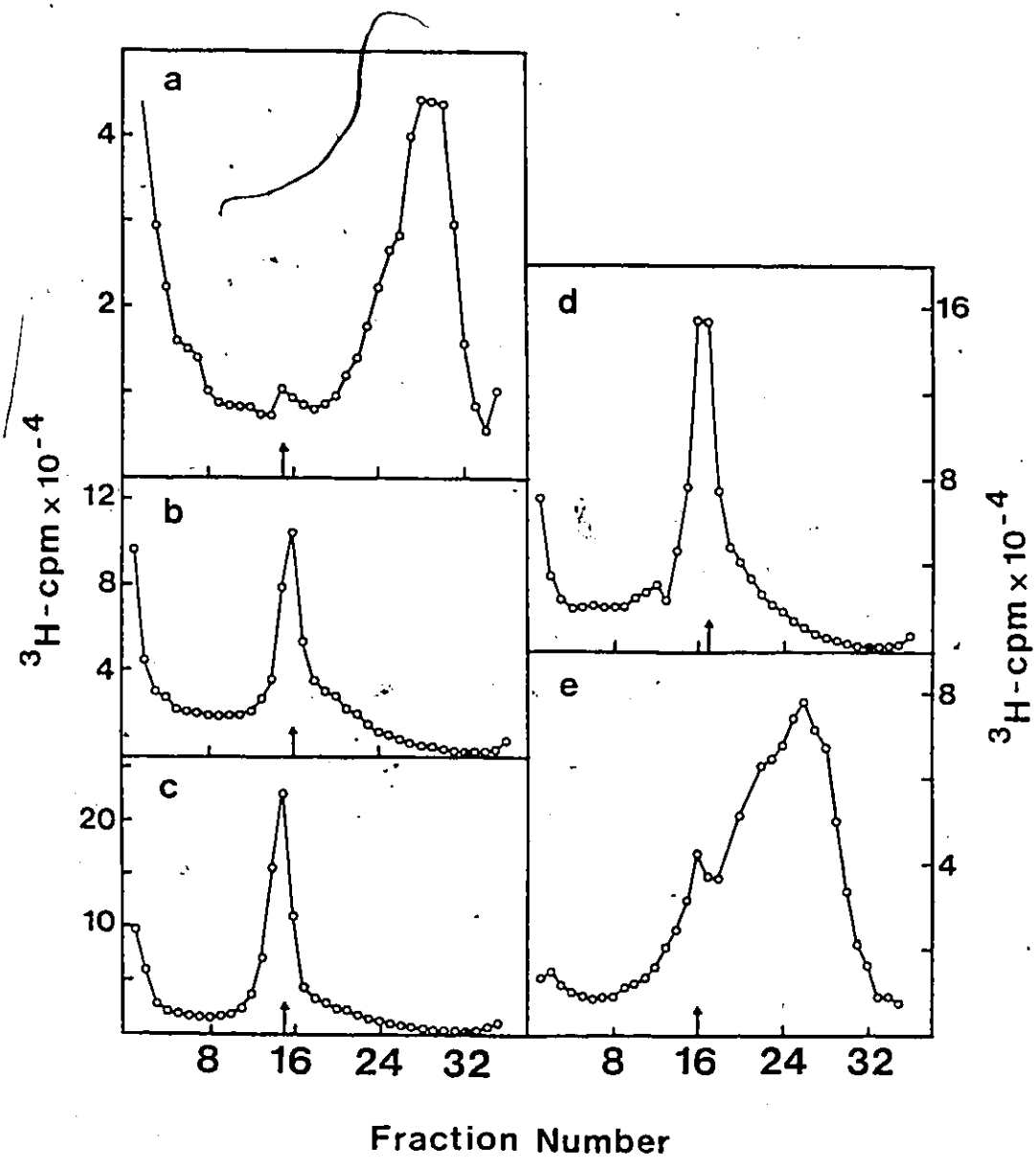
The group I mutants, represented by hr 1, are defective in viral DNA synthesis (Lassam, Bayley and Graham, 1978; Ross et al., 1980) and are mapped within 1.3-3.7 map units (Frost and Williams, 1978; Galos et al., 1980). The group II mutants, represented by hr 6 are DNA positive (Lassam, Bayley and Graham, 1978) and are mapped within 6.1 - 8.5 map units (Frost and Williams, 1978; Galos et al., 1980). The deletion host range mutants dl 312 and dl 313, with deletions between nucleotides 448-1349 and between 1334-3639 respectively (Colby and Shenk, 1981; Shenk et al., 1980) are both DNA negative (Jones and Shenk, 1979a).

RESULTS

DNA Degradation in KB Cells Coinfected with Ad12 Cyt Mutant and Host Range Mutants

KB Cells were infected with the cyt mutant H12 cyt 70 at a multiplicity of 150 virions/cell, then immediately superinfected with either hr 1, hr 6, dl 312 or dl 313 of Ad5 at an equal multiplicity. Infected cells were labelled (^{3}H)-thymidine (15 µCi/µl, 7.5 µCi/µg) from 14 h to 36 h, and the labelled DNA from cell lysates were analyzed in alkaline sucrose gradients (Fig. 3-1). Infection with H12 cyt 70 alone produced a small peak of DNA cosedimenting with the marker viral DNA ([^{14}C] labelled Ad5) and a broad peak of degraded DNA sedimenting slower (Fig. 3-1a) as described earlier. Coinfection with hr 1 and dl 312 both resulted in a relatively large peak of DNA cosedimenting with the marker and no degraded DNA (Fig. 3-1, b and c). Similar results were obtained in the coinfection with hr 6 (Fig. 3-1d). Coinfection of KB cells with dl 313, however, resulted in even more extensive DNA degradation than in cells infected with H12 cyt 70 alone (Fig. 3-1e). Therefore, hr 1, dl 312 and hr 6 complemented the DNA degradation function in H12 cyt 70 while dl 313 did not. This suggests that a function altered by the deletion in dl 313, but not by the mutation in hr 6 which maps within this deletion, appears to fall in the same complementation group as H12 cyt 70.

Figure 3-1 Sedimentation profiles in alkaline sucrose gradients of intracellular DNA in KB cells singly infected with cyt mutant H12 cyt 70 and doubly infected with H12 cyt 70 and various Ad5 mutants. The multiplicity of infection of each virus was 150 virions per cell. Sedimentation is from right to left. Cells infected with: a) H12 cyt 70, b) H12 cyt 70 and hr1, c) H12 cyt 70 and d1312, d) H12 cyt 70 and hr6 and e) H12 cyt 70 and d1313. Arrows indicate the positions of [^{14}C]-labelled Ad5 marker DNA.


a
b
c
d
e
^{3}H-cpm x 10^{-4}
^{3}H-cpm x 10^{-4}
4
2
12
8
4
20
10
16
8
8
4
8
16
24
32
8
16
24
32
Fraction Number

DNA Degradation in KB Cells Infected with Region E1 Mutants of Ad5

KB cells were infected with each Ad5 mutant at a multiplicity of 150 virions/cell and assayed for DNA degradation. The region E1a mutants, hr 1 and dl 312, produced no significant peak of DNA co-sedimenting with the marker and no indication of DNA degradation (Fig. 3-2, a and b), consistent with these mutants being DNA replication deficient (Jones and Shenk, 1979a). Hr 6 produced a large peak of DNA cosedimenting with the marker and no degradation of DNA (Fig. 3-2a), consistent with this mutant being normal in viral DNA synthesis (Lassam, Bayley and Graham, 1978). Multiplicities of infection ranging from 25 to 500 virions/cell with hr 6 still did not produce any DNA degradation (data not shown). dl 313, however, consistently produced extensive DNA degradation with no significant amount of DNA cosedimenting with the marker (Fig. 3-2b), suggesting that DNA degradation is an early function. However, it has been reported that the dl mutants display a multiplicity dependent leakiness (Shenk et al., 1980). It is, therefore, possible that viral DNA synthesis had occurred in the infected cells. To test this possibility, DNA from KB cells infected with dl 313 at a multiplicity of 200 virions/cell and labelled with (^{3}H)-thymidine at various 1 hr intervals between 14 and 36 h was hybridized to excess unlabelled Ad5 DNA. No significant amount of radioactivity was found to hybridize (Tables 3-1). dl 313, therefore, induced extensive degradation of host DNA in the absence of viral DNA synthesis, suggesting that a function deleted in dl 313 is required for the inhibition of DNA degradation.

Figure 3-2 Sedimentation profiles in alkaline sucrose gradients of intracellular DNA in KB cells infected with various Ad5 mutants at a multiplicity of infection of 200 virions per cell. Sedimentation is from right to left. Cells infected with: a) hr 1 (O) and hr 6 (●) and b) dl312 (O) and dl313 (●). Arrows indicate the positions of [^{14}C]-labelled Ad5 marker DNA.

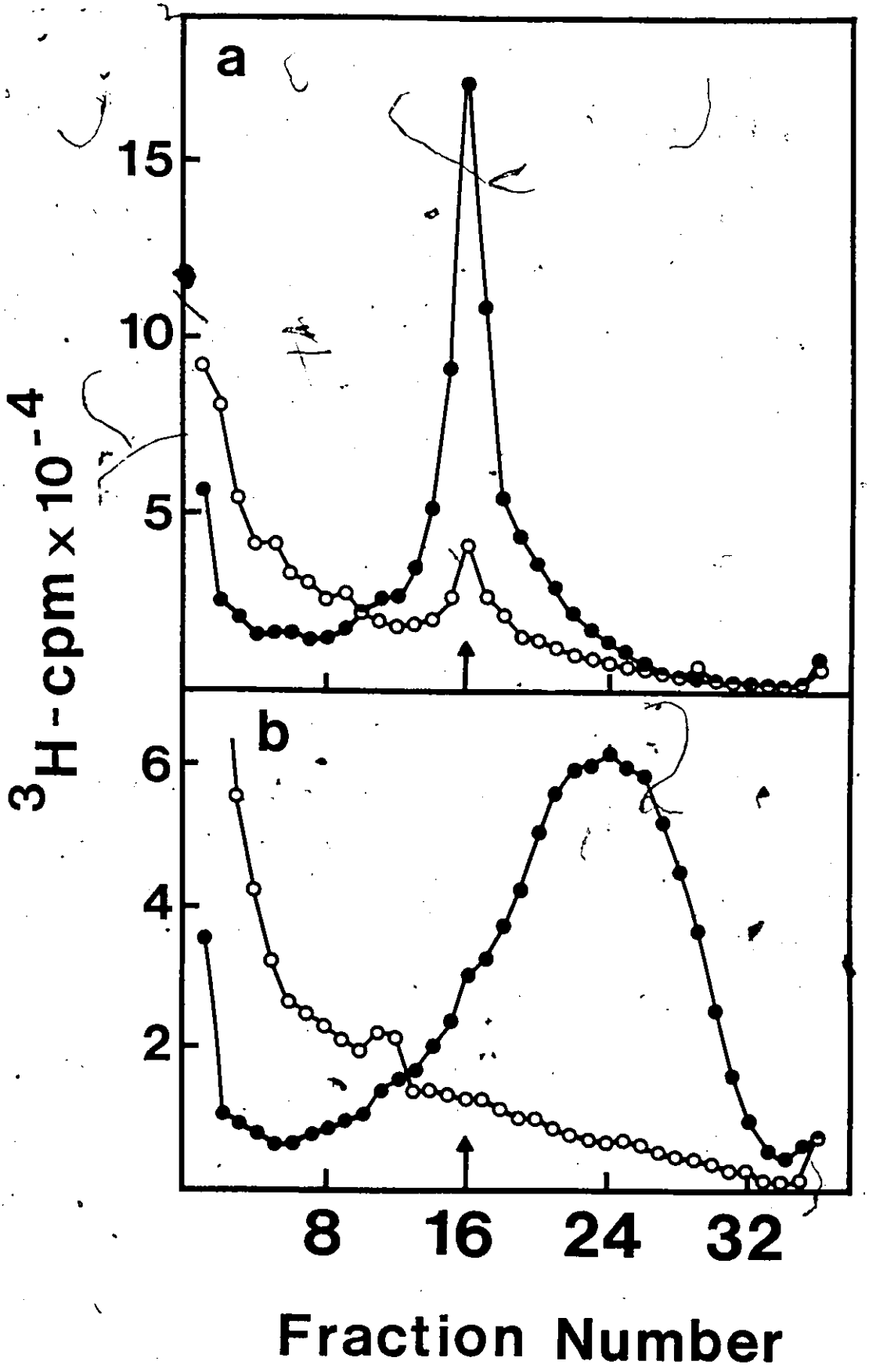

a
b
15
10
5
6
4
2
^{3}H-cpm x 10^{-4}
8
16
24
32
Fraction Number

Table 3-1

Synthesis of Ad5 dl 313 DNA as Determined by DNA-DNA Hybridization

Time Period of Labelling[a] (h p.i.)	Input CPM	CPM Hybridized to Ad5 DNA[b] (A)	CPM Hybridized to salmon sperm DNA[c] (B)	A-B	% of Input CPM complementary to Ad5
0-1	8016	39	55	-16	0
	8611	27		-28	0
5.5 - 6.5	8278	30	49	-19	0
	8380	35		-14	
11.5 - 12.5	13,315	88	55	33	0
	13,873	86		31	0
23 - 24	2517	56	18	38	1
	2202	54		36	1
36 - 37	1632	90	18	72	4
	1607	91		73	4
[^{3}H] Ad5 DNA	2656	2743	22	2721	~100
	2774	2843		2821	~100

a infected cells were labelled with [^{3}H]-thymidine (20 Ci/mM) at 10 µCi/ml

b 2 µg of Ad5 DNA per filter

c 2 µg of salmon sperm DNA per filter

Polypeptides Not Involved in DNA Degradation

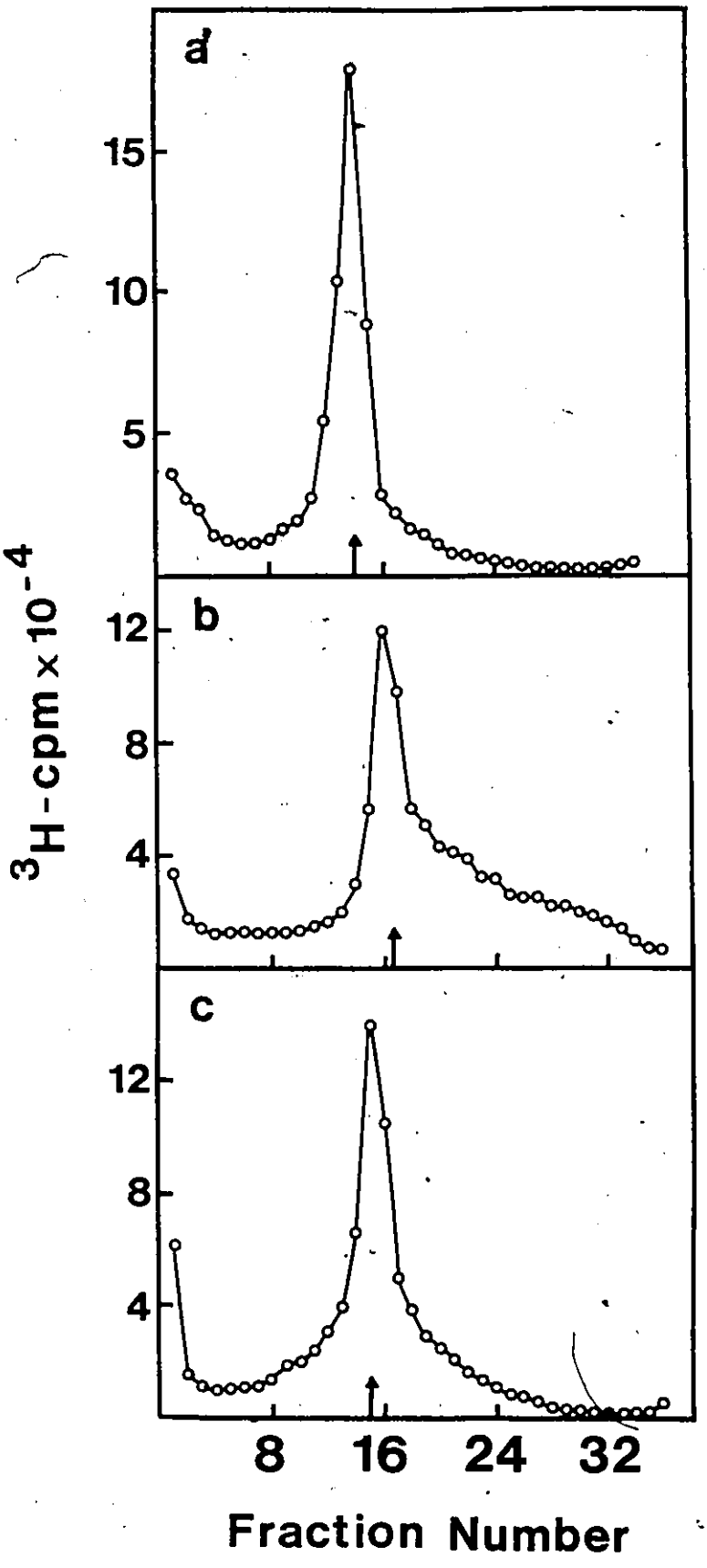
Region E1b of Ad5 codes for several polypeptides (Esche, Mathews and Lewis, 1980; Halbert, Spector and Raskas, 1979; Lassam, Bayley and Graham, 1979; Ross, Flint and Levine, 1980; Ross et al., 1980). Experiments were carried out to identify the polypeptide(s) involved in DNA degradation by complementing Ad5 dl 313 with cells or viruses that express only some of the polypeptides in region E1b. To interpret the complementation results, it is necessary to demonstrate at first that the function of dl 313 is recessive as it is in H12 cyt 70. Fig. 3-3a shows the result of coinfection of KB cells with dl 312 and dl 313. Clearly these two mutants complemented for viral DNA synthesis and prevented DNA degradation.

It has been shown that 293 cells do not express polypeptide IX, a structural protein encoded in region E1b, even upon productive infection with dl 313 (Colby and Shenk, 1981; Spector, Halbert and Raskas, 1980). Figure 3-3b shows the result of infection of 293 cells with dl 313. A large peak of DNA cosedimented with the marker and relatively little DNA sedimented slower. This suggests that polypeptide IX is not involved in the inhibition of DNA degradation.

Group II host range mutant hr 6 fails to synthesize the 58,000 dalton (58K) polypeptide encoded in region E1b (Lassam, Bayley and Graham, 1979; Ross et al., 1980), but synthesizes viral DNA (Lassam, Bayley and Graham, 1978). KB cells were coinfected with hr 6 and dl 313 at a multiplicity of 150 virions/cell each and assayed for DNA degradation. DNA degradation was not observed (Fig. 3-3c).

53

Figure 3-3 Sedimentation profiles in alkaline sucrose gradients of intracellular DNA in a) KB cells coinfected with mutants dl313 and dl312, b) 293 cells infected with dl313 and c) KB cells coinfected with dl313 and hr6. The multiplicity of infection of each virus was 200 virions per cell. Sedimentation is from right to left. Arrows indicate the positions of [^{14}C]-labelled Ad5 marker DNA.


a
b
c
15
10
5
12
8
4
12
8
4
^{3}H-cpm x 10^{-4}
8
16
24
32
Fraction Number

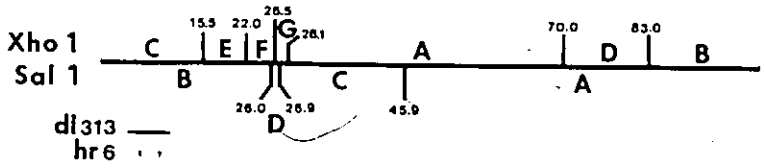
This result could have been due to an inhibition or exclusion of dl 313 by hr 6. To determine if complementation had occurred, intracellular viral DNA labelled with (^{3}H)-thymidine from 24 h to 36 h after infection was analyzed with restriction endonucleases. The restriction maps for the enzymes, Xho 1 and Sal I are shown in Fig. 3-4a. Neither enzyme cuts within the left hand 12 percent of the prototype Ad5 DNA where the mutations are located. These terminal fragments should differ between the mutants by the size of the deletion in dl 313, 7 percent of the viral genome equivalent. Figure 3-4b lanes 1 and 4 represent wild type Ad5 DNA which should be indistinguishable from hr 6 DNA. Xho 1 cleavage of the DNA from the coinfected cells shows both fragment C of hr 6 and fragment C* of dl 313 (lane 2). Sal I cleavage shows the presence of fragment B of wild type Ad5, and an apparent overabundance of fragment C which indicates the presence of fragment B* of dl 313 which comigrated. These results demonstrate that hr 6 and dl 313 complemented for viral DNA synthesis and that the 58,000 dalton polypeptide is not required for the prevention of DNA degradation.

DNA Degradation in Infected Hamster Embryo Cells

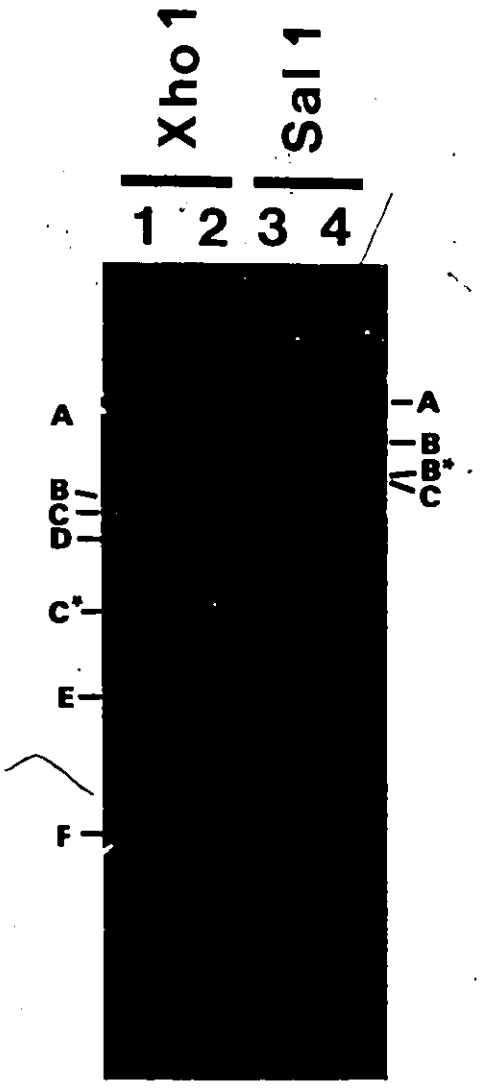
Ad5 dl 313 induced DNA degradation in the absence of viral DNA synthesis (Fig. 3-2d). It was of interest to determine if the cyt mutants could also induce DNA degradation in the absence of viral DNA synthesis. Hamster cells are non-permissive for Ad12, and viral DNA is not synthesized in the infected cells (Doerfler, 1969, 1970; Shimojo and Yamashita, 1968). These cells are, however, permissive for Ad5 (Takahashi, 1972; Williams, 1973).

Figure 3-4 a) restriction map of Ad5 DNA for endonucleases XhoI and Sal-I, showing locations of deleted sequences in dl313 and the mutation in hr6 in region E1 of the genome. b) fluorogram of restricted DNA from Ad5 virions (lanes 1 and 4) and from KB cells coinfected with mutants hr6 and dl313 each at a multiplicity of infection of 200 virions per cell (lanes 2 and 3). Infected cells were labelled with [^{3}H]-thymidine (15 µCi/ml, 7.5 µCi/µg) from 24 to 36 h post-infection. Asterisks denote fragments of dl313 DNA reduced in size by the deletion.

a


Xho 1
Sal 1
C
B
E
F
G
A
D
B
C
A
15.5
22.0
26.5
26.1
70.0
83.0
26.0
26.9
45.9
D
dl313
hr6

b


Xho 1
Sal 1
1
2
3
4
A
B
C
D
C*
E
F
A
B
B*
C

Primary hamster embryo cells were infected with the cyt mutants H12 cyt 70, H12 cyt 61 and Ad12 at a multiplicity of 5,000 virions/cell and with Ad5 and dl 313 at a multiplicity of 2,000 virions/cell and assayed for DNA degradation.

AD5 produced a sharp peak of DNA cosedimenting with the marker and no apparent DNA degradation (Fig. 3-5a) whereas dl 313 produced a broad peak of degraded DNA and no intact viral DNA (Fig. 3-5b). These results are similar to that produced in KB cells.

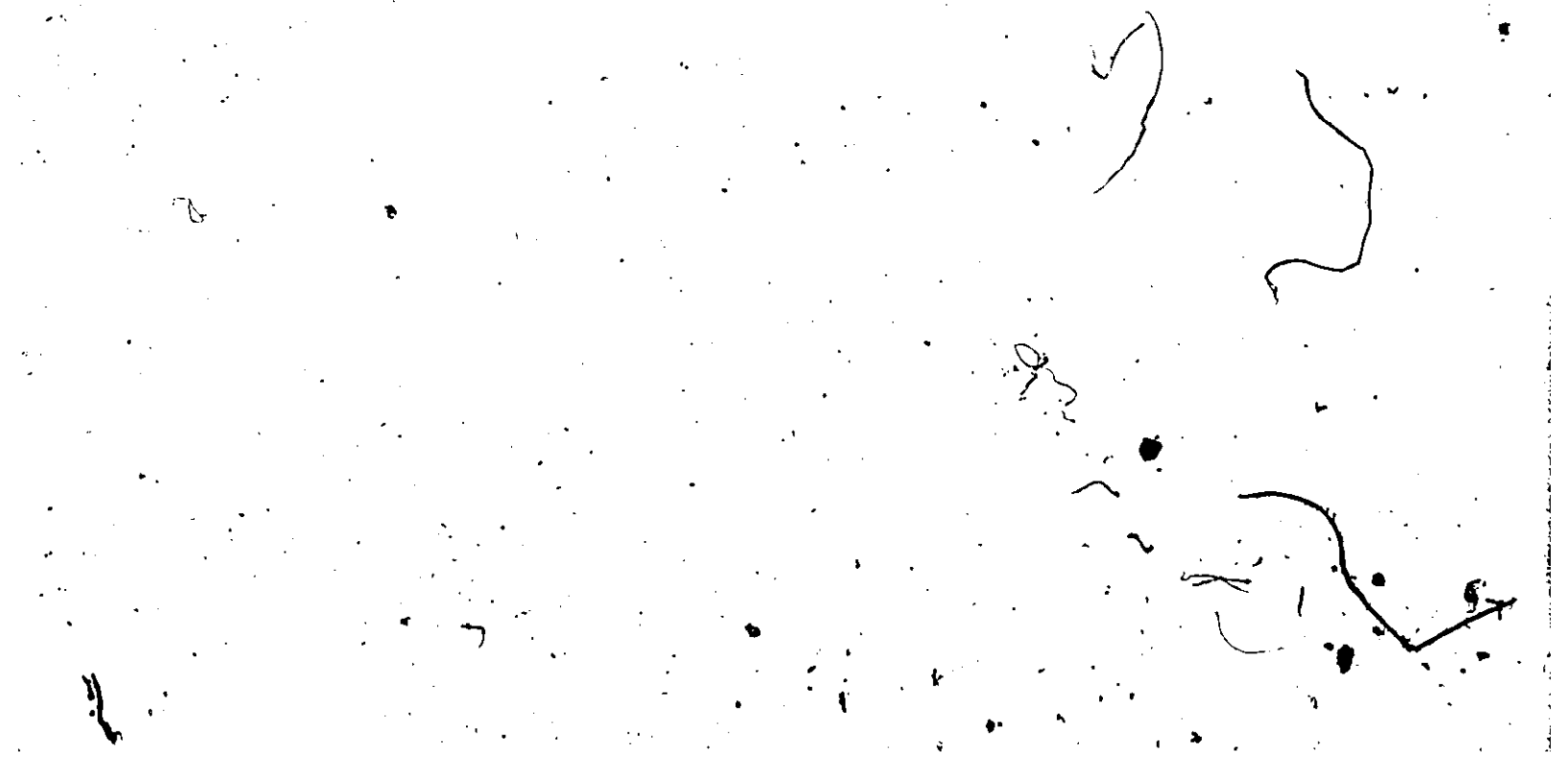
H12 cyt 70, H12 cyt 61, and wild type Ad12 produced no significant amount of DNA cosedimenting with the marker, but all produced a large peak of smaller DNA fragments (Fig. 3-5c, d and e), similar to those produced in dl 313-infected hamster cells (Fig. 3-5b). This indicates that both the cyt mutants and the wild type Ad12 induced DNA degradation in hamster cells.

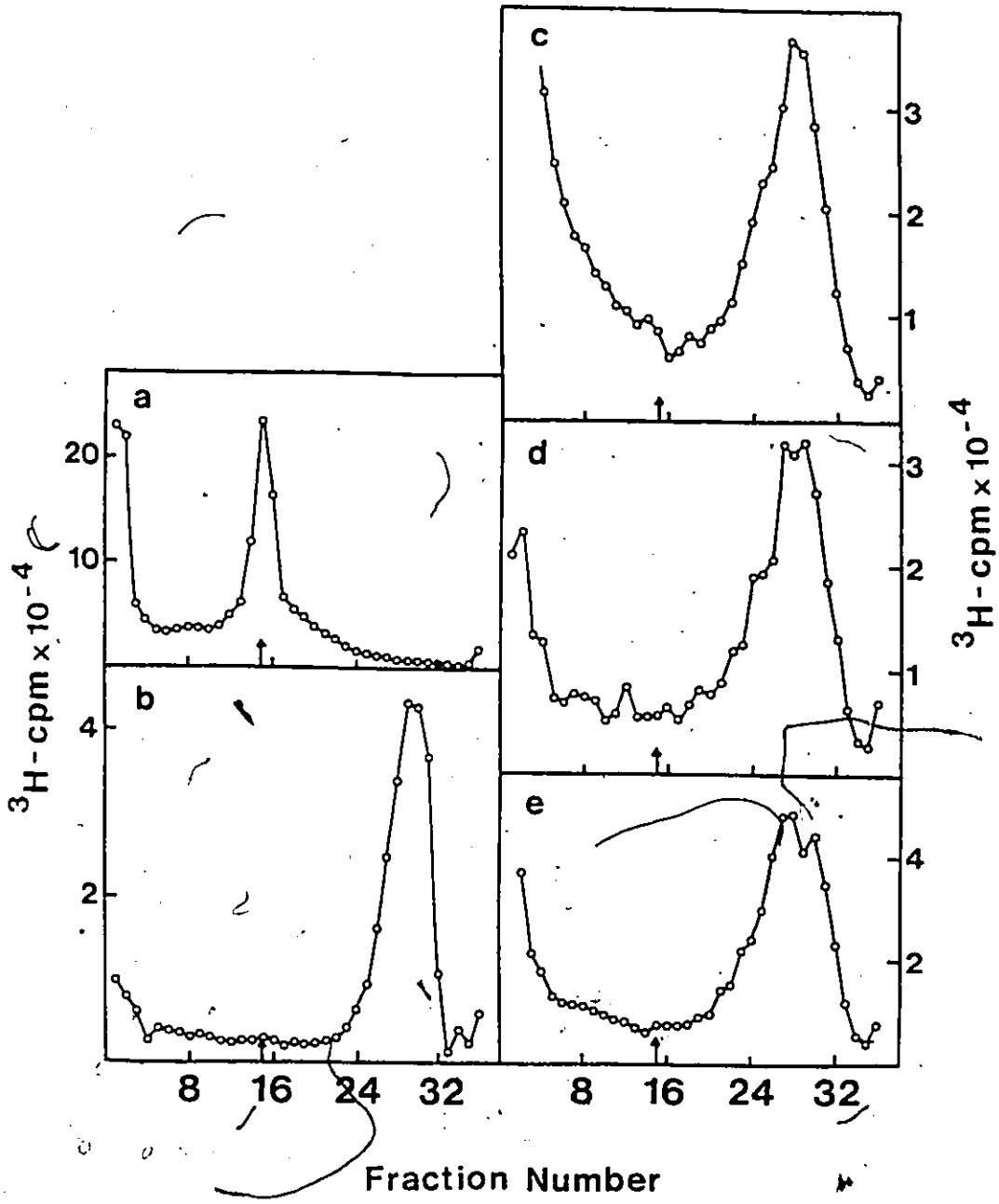
DNA Degradation in KB Cells Induced by H12 Cyt 61

The mutant H12 cyt 61 is an atypical cyt mutant in that it transforms rat embryo cells in vitro as efficiently as the parental Ad12 virus (Mak and Mak, in press). This unique mutant was therefore further studied in complementation experiments similar to those done with H12 cyt 70 to see if there were any differences concerning the function of virus-induced DNA degradation.

KB cells were first infected with H12 cyt 61 at a multiplicity of 150 virions/cell then immediately superinfected with the parental virus Ad12-1131 or with wild type Ad12-Huie at an equal multiplicity.

Figure 3-5 Sedimentation profiles in alkaline sucrose gradients of intracellular DNA in hamster embryo cells infected with a) Ad5 and b) d1313 at multiplicities of infection of 2,000 virions per cell and with c) H12 cyt 70, d) H12 cyt 61 and e) Ad12 at multiplicities of infection of 5,000 virions per cell. Sedimentation is from right to left. Arrows indicate the positions of [^{14}C]-labelled Ad5 marker DNA.

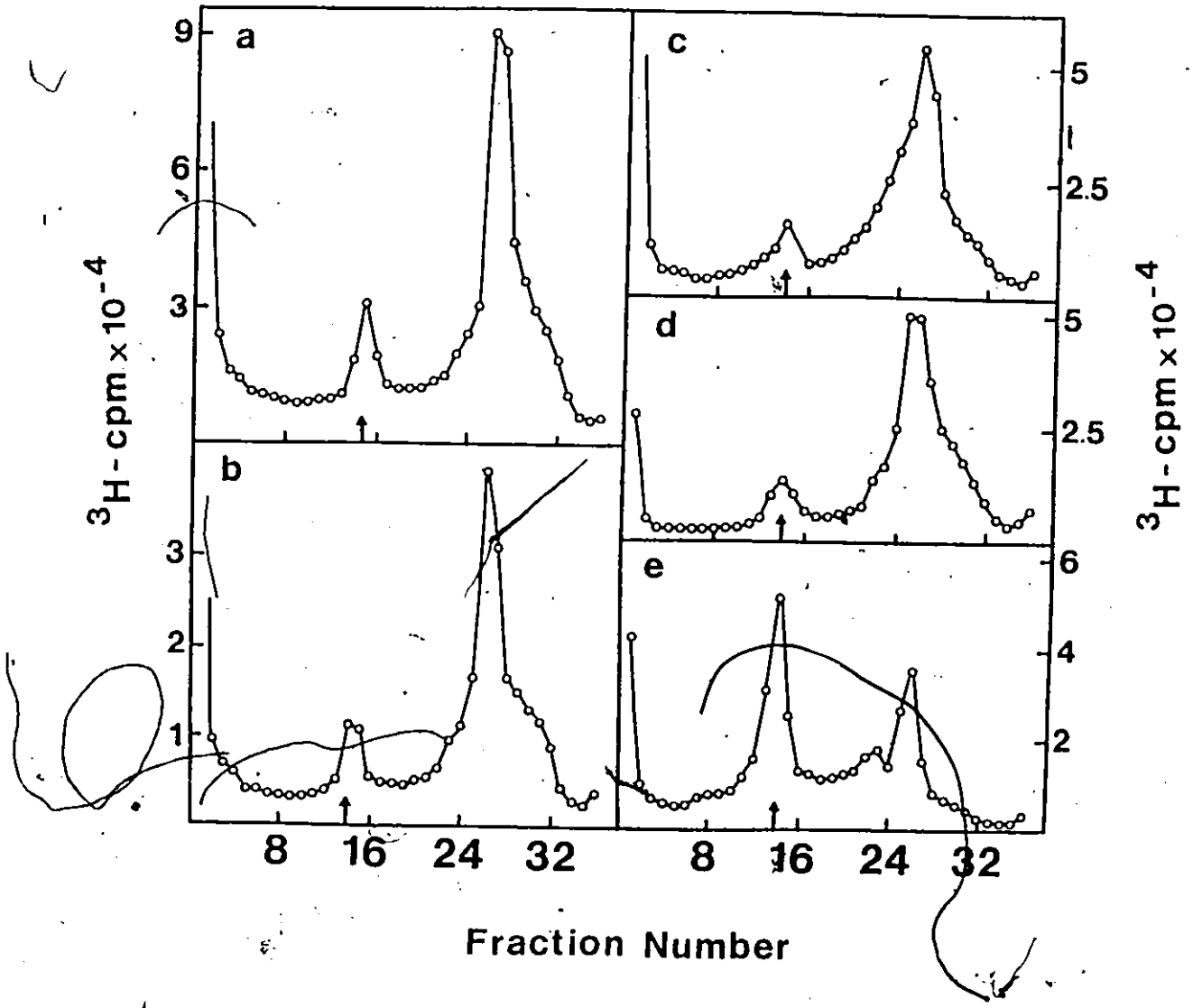



a
b
c
d
e
20
10
4
2
3
2
1
3
2
1
4
2
8
16
24
32
8
16
24
32
^{3}H-cpm x 10^{-4}
^{3}H-cpm x 10^{-4}
Fraction Number

Superinfection with Ad12-1131 did not inhibit DNA degradation (Fig. 3-6b). Similar results were obtained with Ad12-Huie (data not shown). To investigate the possibility that superinfection by the wild type viruses was being interfered with by the mutant, the reverse order of infections was also done. Similar results (data not shown) were obtained. This suggests that the mutation in H12 cyt 61 is dominant.

Coinfection of KB cells with H12 cyt 61 and mutants of Ad5 was also examined. Fig. 3-6c, d and e shows the results of coinfection with hr 1, hr 6, and dl 312, respectively. None of the Ad5 mutants in the coinfected cells strongly inhibited DNA degradation, including Ad5 hr 6 which is normal in viral DNA synthesis.

Figure 3-6 Sedimentation profiles in alkaline sucrose gradients of intracellular DNA in KB cells singly infected with cyt mutant H12 cyt 61 and doubly infected with H12 cyt 61 and various wild-type strains of Ad12 and mutants of Ad5. The multiplicity of infection of each virus was 150 virions per cell. Sedimentation is from right to left. Cells infected with: a) H12 cyt 61, b) H12 cyt 61 and Ad12 strain 1131, c) H12 cyt 61 and hr 1, d) H12 cyt 61 and d1312, and e) H12 cyt 61 and hr 6. Arrows indicate the positions of [^{14}C]-labelled Ad5 marker DNA.


a
b
c
d
e
9
6
3
3
2
1
5
2.5
5
2.5
6
4
2
8
16
24
32
8
16
24
32
^{3}H - cpm x 10^{-4}
^{3}H - cpm x 10^{-4}
Fraction Number

DISCUSSION

Results presented in Chapter 2 showed that infection of KB cells by cyt mutants of Ad12 lead to extensive DNA degradation. The results of the cyt mutation may be a) the over production of an endonuclease, b) the failure to produce a DNase-inhibitor, or c) the failure to produce a factor that protects the DNA from degradation. Coinfection of cells by a cyt mutant and wild type Ad12 can prevent the degradation of DNA (Ezoe, LaiFatt and Mak, 1981), suggesting that a diffusible substance can control the production of or inhibit the action of the DNase. The Ad2 temperature-sensitive early mutant H2ts 111 also induces degradation of viral and cellular DNA at the non-permissive temperature, and is suggested to be defective in a DNase-inhibitory factor (D'Halluin et al., 1979).

In this study, complementation tests between cyt mutants of Ad12 and region E1 mutants of Ad5 were employed in mapping a viral function involved in the prevention of degradation of intracellular DNA. Region E1a mutants of Ad5 did not induce DNA degradation in single infection of KB cells, and were able to complement this function in H12 cyt 70. Ad5 dl 313 on the other hand, failed to complement H12 cyt 70 and in single infection of KB cells, induced extensive degradation of host DNA. These results strongly suggest that both Ad12 and Ad5 encode functionally related DNase-inhibitory factors, and that in Ad5 this factor maps in region E1b.

It is unlikely that the deficiency in the DNase-inhibitory factor in dl 313 is a secondary effect of the deletion in region Elb since dl 313 appears to express normal or increased amounts of proteins encoded by all early regions except region El of the viral genome (Lassam et al., 1978; Ross et al., 1980). The failure of H12 cyt 70 and Ad5 dl 313 to complement each other in the prevention of DNA degradation is not due to incompatibility of the region El products, since wild type Ad12 has been shown to complement the replication of Ad5 dl 313 and group II host range mutants (Rowe and Graham, 1981; Williams, Ho and Galos, 1981). Thus the defect in the cyt mutant appears to lie in the functionally equivalent region Elb of Ad12.

At least three polypeptides have been identified to be encoded in region Elb of Ad5; polypeptide IX, an 18-19K polypeptide, and a 53-58K polypeptide (Alestron et al., 1980; Esche, Matthews and Lewis, 1980; Halbert, Spector and Raskas, 1979; Lassam, Bayley and Graham, 1979; Ross, Flint and Levine, 1980; Ross et al., 1980). Ad5 dl 313 did not induce DNA degradation in 293 cells which do not express polypeptide IX from the integrated viral sequences, even during productive infection with dl 313 (Colby and Shenk, 1981; Spector, Halbert and Raskas, 1980). hr 6 did not induce DNA degradation and was able to complement dl 313 in this function in KB cells. hr 6, a group II mutant, is clearly defective in the synthesis of at least the 58K polypeptide (Lassam, Bayley and Graham, 1979; Ross et al., 1980). Thus, these two polypeptides appear not to be required for the inhibition of DNA degradation.

This leaves the 19K polypeptide and any other polypeptides encoded in region E1b as candidates for the DNase-inhibitory factor. It is of interest to note that a preliminary analysis by immunoprecipitation with anti-tumor sera of polypeptides induced in KB cells infected with either H12 cyt 62 or H12 cyt 68 indicates that these two cyt mutants fail to induce a 19K polypeptide found with infection of KB cells by wild type Ad12 (H. Galet, personal communication).

The mutant H12 cyt 61 was dominant in inducing DNA degradation. This mutant is also different from other cyt mutants in having wild type transforming capability (Mak and Mak, personal communication). It appears, therefore, that the mutation in H12 cyt 61 affects a different viral product involved in the induction of DNA degradation from that affected in H12 cyt 70 and d1313, and probably does not map in region E1. Also region E1a mutants of Ad5 did not induce DNA degradation, and these mutants do not express region E1b products. These results suggest, therefore, that the expression of a second viral gene product is involved in inducing DNA degradation and that it acts positively. It appears that the mutation in H12 cyt 61 prevents the normal E1b factor from negatively controlling the expression or activity of this second factor.

The observation that wild type Ad12 induced degradation of DNA in hamster embryo cells is in keeping with earlier reports that Ad12 induces chromosomal aberrations in hamster cells (Stich, 1973; Stich and Yohn, 1970; zur Hausen, 1968; zur Hausen, 1973). In a study comparing the ability to induce chromosomal aberrations between wild type Ad12 and cyt mutants, no significant differences were found (Stich,

1973). Inactivation of virus by UV irradiation suggests also that the adenovirus induced chromosomal aberrations are probably due to the action of an enzyme controlled by viral genes (zur Hausen, 1967). The most direct explanation for these observations, based on the results of this study, is that the DNase-inhibitory factor in region E1b of Ad12-is not functionally expressed in non-permissive hamster cells. In support of this view, it has been reported that Ad12 complements the growth of the group II host range mutants of Ad5 in HeLa cells but not in the hamster cell line BHK 21 (Rowe and Graham, 1981). In addition, hybridization of cytoplasmic RNA from Ad12 infected cells to the Eco R1-fragment C (left 16 percent of the genome) showed that approximately 30 percent of the sequences expressed in productively infected KB cells are not expressed in abortively infected BHK 21 cells (Ortin, et al., 1976).

The induction of DNA degradation by d1 313 in KB cells and by Ad12 in hamster embryo cell demonstrate that viral DNA synthesis is not required for its occurence. This implies that the viral product which effects the nuclease activity is an early viral function. The possibility remains, however, that this product, under the control of the region E1b inhibitory factor, is normally expressed late. A defective inhibitory factor could result in its early expression.

Chapter 4

Messenger RNA Species Complementary to Region E1 of Ad12 Expressed in Lytic Infection of KB Cells

INTRODUCTION

The mutation in the Ad12 cyt mutant H12 cyt 70 has been mapped by complementation in the region of the genome functionally equivalent to region E1b of Ad5 (4.5 to 11.5 map units). It was of interest to examine possible effects of the cyt mutation on the transcription of this region of Ad12. The transcriptional map and gene organization of region E1 of Ad12, however, was not clearly established at that time. In fact, the few reported studies on Ad12 transcription suggested that region E1 of Ad12 perhaps differs in size and organization compared to that of Ads 2 and 5. Ortin et al. (1976) by hybridization of labelled DNA restriction fragments to excess mRNA from infected cells determined that the left 7.5 - 8.8 percent of the genome is expressed as mRNA early in productive infection of KB cells. Smiley and Mak (1978), by hybridization of labelled early mRNA from productively infected KB cells to restriction fragments of Ad12 DNA, determined that only the left 6.7 percent of the genome is expressed early. However, since the methods of detecting RNA sequences used in these studies of Ad12 are of relatively low sensitivity compared to those used in the more detailed studies of Ads 2 and 5, it was not conclusive that Ad12 differs significantly from Ad5 in region E1.

To more clearly establish the physical map location of the DNase inhibitory function in Ad12, it was necessary to define the location of

region E1b of Ad12. In this study, therefore, mRNA species complementary to region E1 of Ad12 and two cyt mutants were identified and mapped. These mRNA species were also compared to those reported to be transcribed from region E1 of Ads 2 and 5.

RESULTS

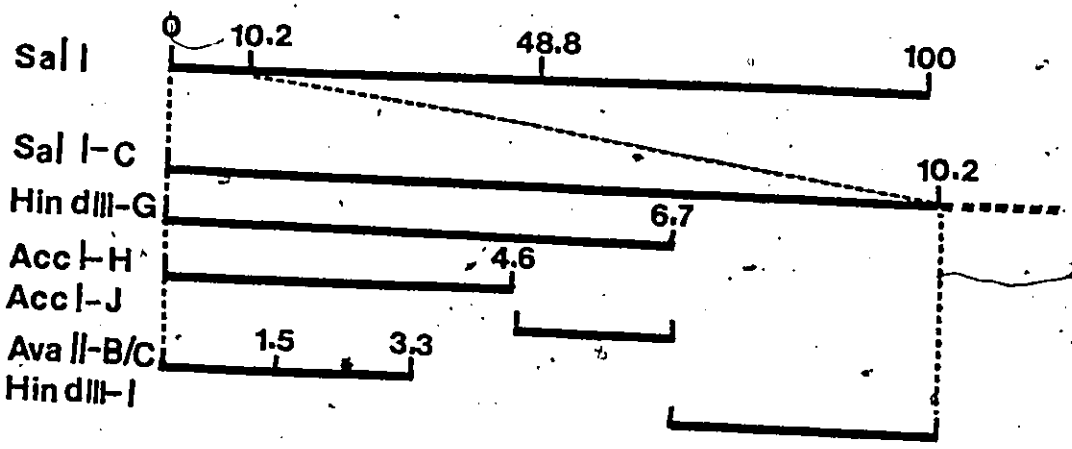
Experimental Approach

The method used for detecting specific mRNA species was a northern-blot hybridization procedure as described in Materials and Methods. Briefly, RNA under denaturing conditions was fractionated by agarose gel electrophoresis, transferred to nitrocellulose filters and hybridized with (^{32}P)-labelled restriction fragments of Ad12 DNA.

The approach taken in choosing the restriction fragments as hybridization probes was to assume that the transcriptional maps of region E1 of Ad12 and Ad5 are structurally similar. Figure 4-1 shows the restriction fragments of Ad12 used as probes for region E1 mRNAs. These fragments were prepared by preparative agarose gel electrophoresis. The Sal 1-C fragment (0-10.2 map units) should encompass almost the entire region E1 without overlapping with any other coding regions. Sal 1-C fragment was the source used for the preparation of all other probes representing subregions of E1. Hind III digestion of Sal 1-C generated fragments Hind III-G (0-6.7 map units) and Hind III-I (6.7-10.2 map units). Acc I digestion of Hind III-G generated fragments Acc 1-H (0-4.6 map units) and Acc 1-J (4.6-6.7 map units) fragments. Ava II digestion of Hind III-G generated fragments Ava II-A, B and C with B and C comigrating as a single band representing 0-3.3 map units. This selection of probes should allow the assignment of different mRNA species to potential E1a and E1b regions of Ad12.

mRNA was isolated at two time points during infection to repre-

Figure 4-1 Endonuclease restriction maps of Ad12 DNA showing fragments used as probes representing subregions of region E1.


0
10.2
48.8
100
Sal I
Sal I-C
10.2
Hin dIII-G
6.7
Acc I-H
4.6
Acc I-J
Ava II-B/C
1.5
3.3
Hin dIII-I

sent both early and delayed early species.

Identification of mRNA Species Complementary to Region E1 in Productively Infected KB Cells

Cytoplasmic poly(A)$^+$ RNA was isolated at 7½ h and 23 h after infection from KB cells infected at a multiplicity of 200 virions/cell with Ad12-1131, H12 cyt 70 and H12 cyt 61 and from cells infected at a multiplicity of 1000 virions/cell with H12 cyt 70.

Fig. 4-2 shows the results of probing the mRNA preparations with (^{32}P)-labelled Sal 1-C fragment. With wild type Ad12, there were at least three size classes of mRNAs present early with a few additional minor, higher molecular weight bands and a broad, lower molecular weight band present late. Size markers used throughout these experiments were denatured Hind III restriction fragments of Ad12-DNA. The three major bands detected early after infection correspond to sizes of 3.15 kilobases (kb), 2.35 kb, and a broad smear of 0.65 - 1.4 kb. There appeared to be no significant qualitative differences between wild type Ad12 and the cyt mutants even upon extended exposure of the autoradiogram.

To further resolve the different species of mRNA and map their location within region E1, the mRNA preparations were also hybridized with the subfragments Acc1-H, Ava II-B/C, Acc1-J and Hind III-I on separate blots (Fig. 4-3, -4, -5, -6, respectively). The data from these figures are summarized in Table 4-1, indicating rough estimates of the relative intensities of bands detected by a given probe at a given time after infection. The band migrating at 3.15 kb was clearly detected by all probes. The 2.35 kb band was detected much more strongly relative

Figure 4-2 . Hybridization of [^{32}P]-labelled Sal 1-C DNA to cytoplasmic poly(A)$^{+}$ RNA from KB cells infected at various multiplicities of infection with different viruses. RNA was isolated at 7.5 h and 23 h post-infection (p.i.) from KB cells infected with: 1,5) H12 cyt 70 at 200 virions/cell; 2,6) H12 cyt 70 at 1,000 virions/cell; 3,7) Ad12-1131 at 200 virions/cell; and 4,8) H12 cyt 61 at 200 virions/cell. Markers (m) represent denatured Hind III digest of Ad12 DNA.

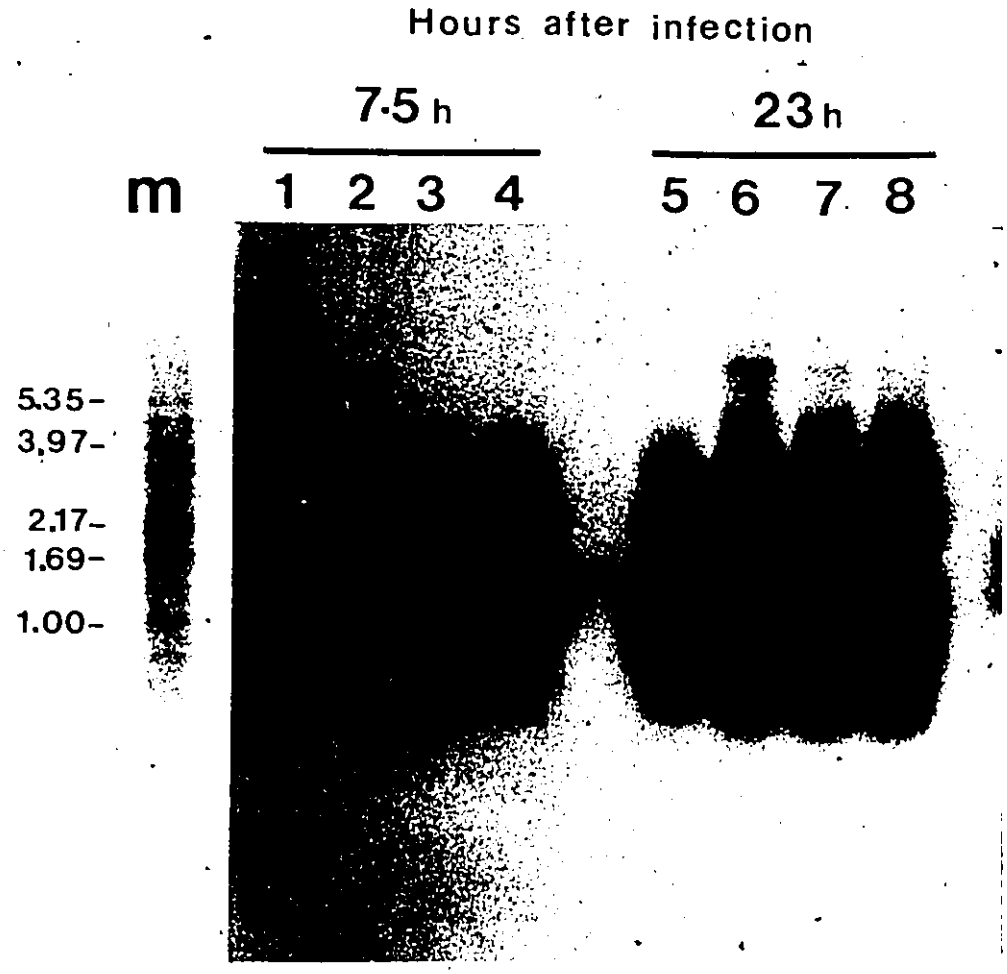

Hours after infection
7·5 h
23 h
m
1 2 3 4
5 6 7 8
5.35-
3,97-
2.17-
1.69-
1.00-

Figure 4-3 Hybridization of [^{32}P]-labelled AccI-H DNA to cytoplasmic poly(A)$^+$ RNA from KB cells infected at various multiplicities of infection with different viruses. RNA was isolated at 7.5 h and 23 h post-infection (p.i.) from KB cells infected with: 1,5) H12 cyt 70 at 200 virions/cell; 2,6) H12 cyt 70 at 1,000 virions/cell; 3,7) Ad12-1131 at 200 virions/cell; and 4,8) H12 cyt 61 at 200 virions/cell. Markers (m) represent denatured Hind III digest of Ad12 DNA.

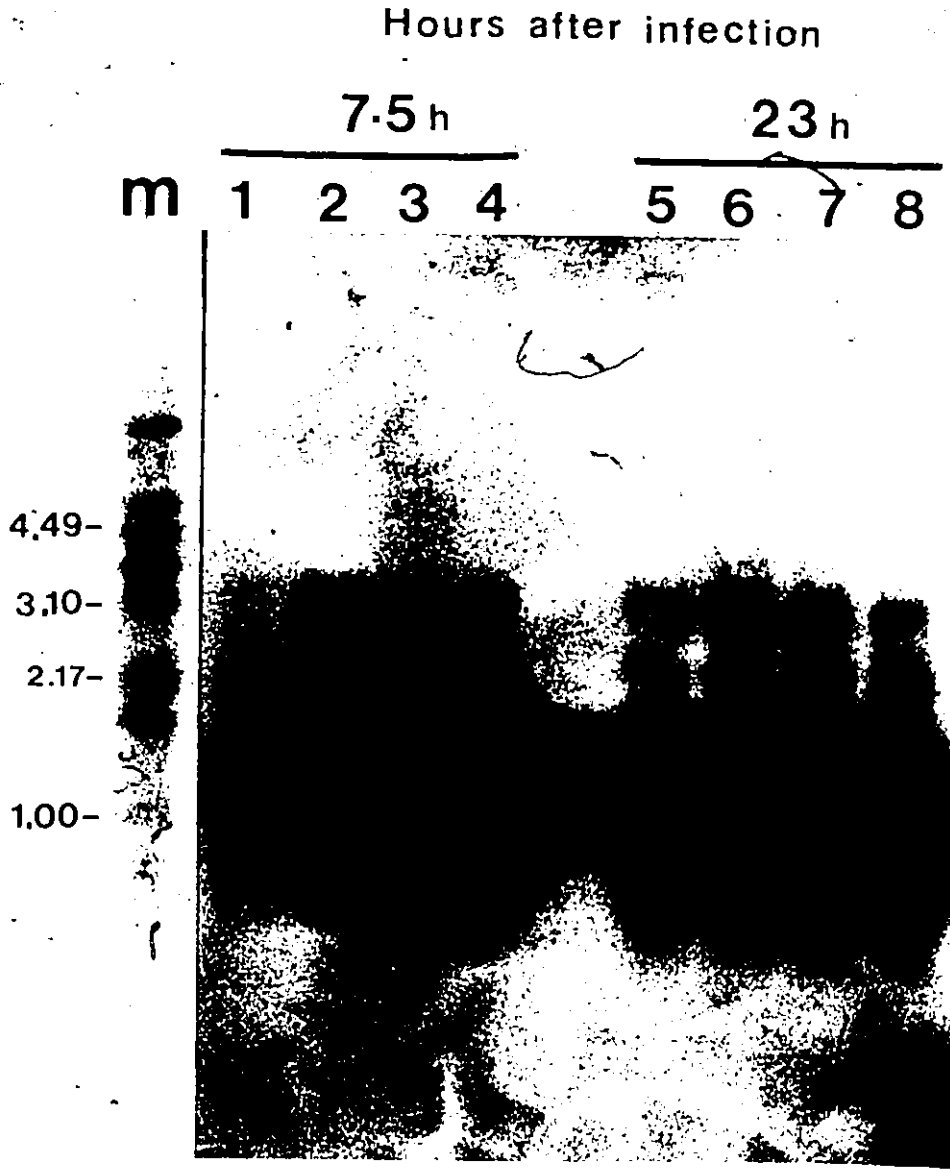

Hours after infection
7·5 h
23 h
m
1
2
3
4
5
6
7
8
4.49–
3.10–
2.17–
1.00–

Figure 4-4 Hybridization of [^{32}P]-labelled Ava II-B/C DNA to cytoplasmic poly(A)$^+$ RNA from KB cells infected at various multiplicities of infection with various viruses. RNA was isolated at 7.5 h and 23 h post-infection (p.i.) from KB cells infected with: 1,5) H12 cyt 70 at 200 virions/cell; 2,6) H12 cyt 70 at 1,000 virions/cell; 3,7) Ad12-1131 at 200 virions/cell; and 4,8) H12 cyt 61 at 200 virions/cell.

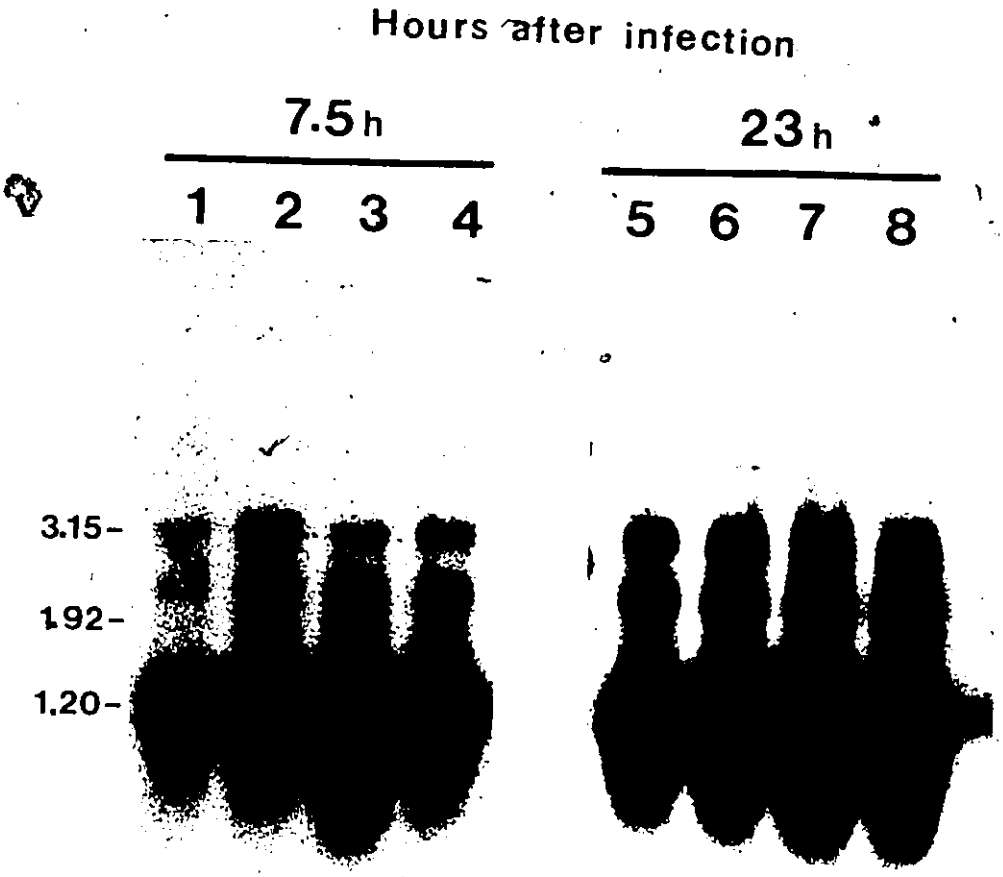

Hours after infection
7.5 h
23 h
1 2 3 4
5 6 7 8
3.15-
1.92-
1.20-

Figure 4-5 Hybridization of [^{32}P]-labelled Accl-J DNA to cytoplasmic poly(A)$^+$ RNA from KB cells infected at various multiplicities of infection with various viruses. RNA was isolated at 7.5 h and 23 h post-infection (p.i.) from KB cells infected with: 1,5) Hl2 cyt 70 at 200 virions/cell; 2,6) Hl2 cyt 70 at 1,000 virions/cell; 3,7) Adl2-1131 at 200 virions/cell; and 4,8) Hl2 cyt 61 at 200 virions/cell.

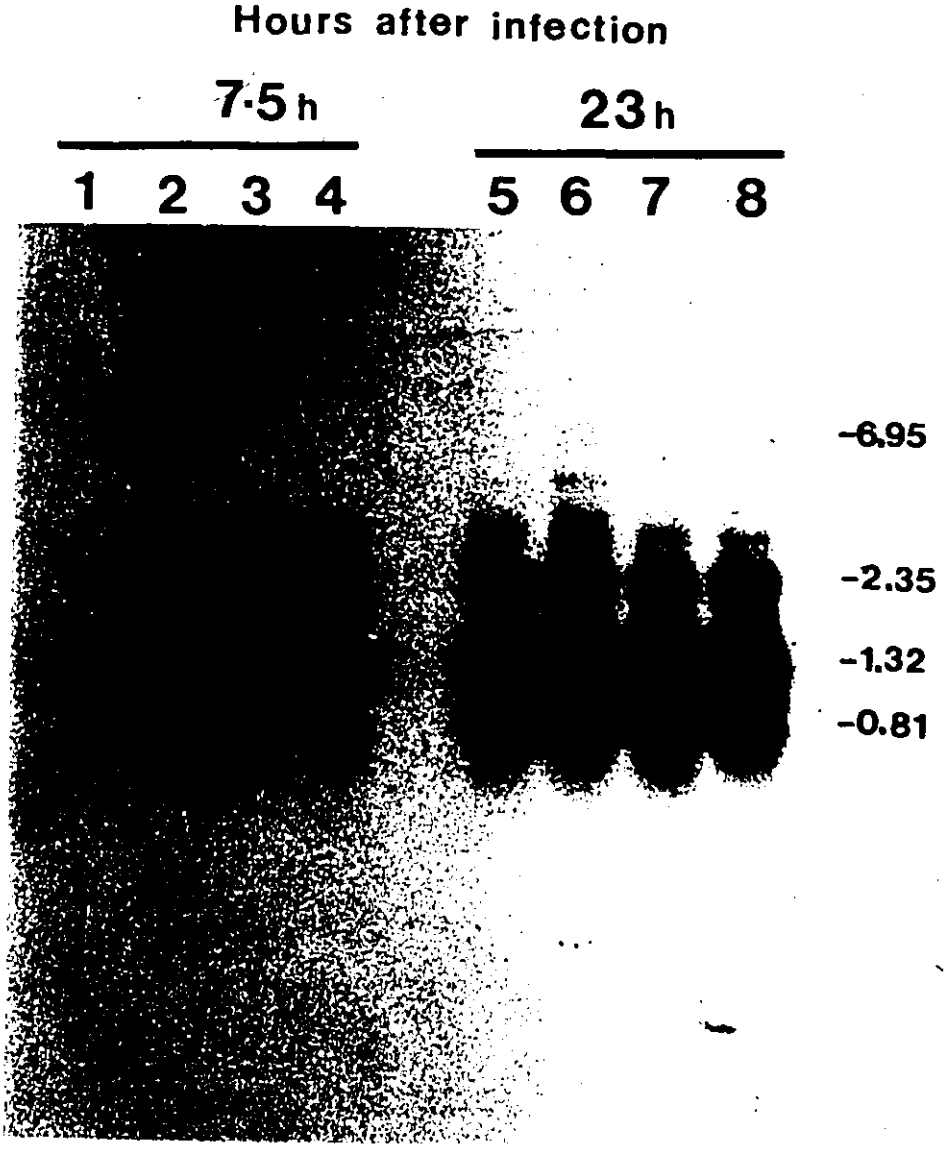

Hours after infection
7·5 h
23 h
1 2 3 4
5 6 7 8
-6.95
-2.35
-1.32
-0.81

Figure 4-6 Hybridization of [^{32}P]-labelled Hind III-I DNA to cytoplasmic poly(A)$^{+}$ RNA isolated at 7.5 h and 23 h p.i. from KB cells infected with Ad12-1131 at a multiplicity of 200 virions/cell.

Hours after infection

7.5h 23h

-2.35

-1.32

-0.81

-0.50

Table 4-1. Summary of mRNA species complementary to restriction fragments of region E1 of Ad12[a]

mRNA species	AvaII-B/C (0-3.3%)		AccI-H (0-4.6%)		AccI-J (4.6-6.7%)		HindIII-I (6.7-10.2%)	
	7.5 h	23 h	7.5 h	23 h	7.5 h	23 h	7.5 h	23 h
8600		(+)				+		
6950		(+)				+		
6150		(+)				+		+
4670		(+)				+		+
3150	+	+	+	+	+	+	+	+
2350	+	+	+	+	+++	+++	+++	+++
1920	+		+					
1700								
1320					>++++	++++	>++++	++++
1200	>++++	>++++	>++++	>++++				
1050						+		
830	+	+	(+)	+				
810						++		>++++
670	(+)[b]		(+)					
500								+

a Data summarized from Fig. 4-3 to Fig. 4-6. Indicated within each column are the relative intensities of species detected by a given [^{32}P]-labelled restriction fragment probe at a given time after infection.

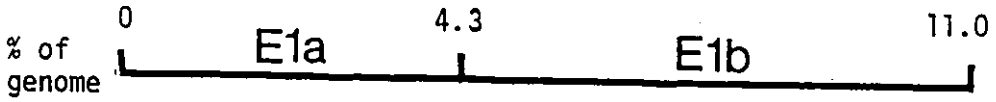
b (+) indicates a very weak signal detectable in long exposures of film.

to the 3.15 kb band with probes Acc-J and Hind III-I compared to those with probes Ava II-B/C and Accl-H. A band at 1.92 kb was detected by the latter two probes and was present at both early and late times. A band at 0.81 kb was detected at the late time by the Accl-J probe and is much more intense when probed by the Hind III-I probe in contrast to the 2.35 kb and 1.32 kb bands which are roughly equally detected by both probes. The band at 0.83 kb was detected by Accl-H and Ava II-B/C probes at the late time and by the Ava II-B/C probe weakly at the early time. A diffuse band at about 0.5 kb was detected only by the Hind III-I probe at the late time only. Minor bands include one at 1.7 kb detected by only the Accl-J probe at the late time. In the region of about 0.65 - 0.7 kb, faint smears are detected at the early time with the probes Accl-H and Ava II-B/C. These mRNA species were assigned to regions E1a and E1b (Fig. 4-7). Species larger than 3.0 kb were assumed to span the entire region E1.

Effects of Cyclohexamide, Cytosine Arabinoside and High Multiplicity of Infection on Accumulation of mRNA

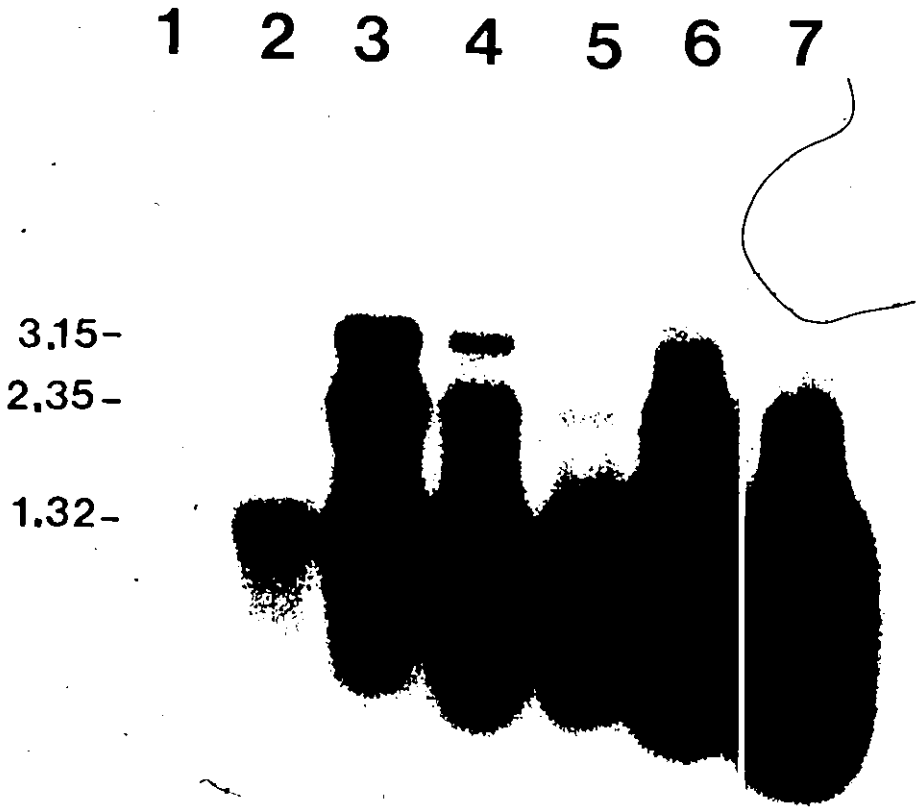
The poly(A)$^+$ RNA preparations used in these experiments were obtained from cells infected at a relatively low multiplicity and without the use of drugs that affect either protein or DNA synthesis. It was of interest to examine the possible effects of the drugs cyclohexamide, cytosine arabinoside and a high multiplicity of infection, all frequently used factors in transcriptional studies, on the accumulation of viral E1 specific poly(A)$^+$ RNA in KB cells. As shown in Fig. 4-8, cyclohexamide (Sigma Chem. Co.) added at 25 µg/ml from 1 h after infection or a

Figure 4-7 Allocation of mRNA species described in Table 4-1 to regions E1a and E1b of the Ad12 genome. Species larger than 3.0 Kb were not assigned since they are assumed to span the entire region E1.



1,200	>	++++	1,320	>	++++
2,350		+	810		++++
3,150		+	2,350		+++
1,920		+	1,050		+
830		+	500		+

Figure 4-8 - Effects of cyclohexamide, cytosine arabinoside and high multiplicity of infection on the accumulation of cytoplasmic poly(A)$^+$ RNA complementary to [^{32}P]-labelled Accl-J DNA in KB cells infected with Adl2-1131. 1) uninfected KB cells; 2) multiplicity of 200 virions/cell, 4 h p.i.; 3) multiplicity of 200 virions/cell, 8 h p.i.; 4) multiplicity of 200 virions/cell, cyclohexamide added at 25 µg/ml from 1-8 h p.i.; 5) multiplicity of 2000 virions/cell, 8 h p.i.; 6) multiplicity of 200 virions/cell, 23 h p.i.; and 7) multiplicity of 2000 virions/cell, cytosine arabinoside added at 20 µg/ml from 4-23 h p.i.


1 2 3 4 5 6 7
3.15-
2.35-
1.32-

multiplicity of 2000 virions/cell both reduced the abundance at 8 h of the 2.35 kb and the 3.15 kb mRNAs relative to the smaller species, as compared to cells infected at 200 virions/cell without the addition of drugs. Cytosine arabinoside (Sigma Chem. Co.) added at 20 µg/ml from 4 h to 23 h to cells infected at 2000 virions/cell also reduced the relative abundance of the high molecular weight mRNAs at 23 h compared to untreated cells infected at 200 virions/cell.

DISCUSSION

Not many details about the organization of coding sequences of region E1 and Ad12 and their transcription had been reported at the time of initiation of these studies. It was not clear as to the mapping of the cyt mutant's defective DNase-inhibitory function in Ad12 since there was no clearly established physical correlation of coding sequences of region E1 between Ad12 and Ad5. This study attempted to define the regions E1a and E1b of Ad12, and to determine any effects of cyt mutations on the production of mRNA from these sequences.

The method of northern-blot hybridization used in this study is quite sensitive as it detects steady state levels of mRNA with a complementary DNA probe of high specific radioactivity. However, there are several limitations to this method. mRNA species with short half-lives might not be readily detected amongst more stable species. The technique only determines that at least part of the nucleotide sequence of the mRNA detected by the probe is transcribed from sequences represented in the probe and does not allow fine mapping or structure determination of the mRNA. It should also be noted that the mRNA species contain 3'-poly(A) sequences which are variable in length and averaging greater than 200 bases (Browerman, 1976; Sawicki et al., 1973, 1978) thus limiting the resolution of similar sized molecules and diminishing the accuracy of size determination of progressively smaller molecules.

During the course of this work, several important transcriptional studies on region E1 of Ad12 were reported. Perricaudet, le Moullec and Tiollais, (1980) have cloned in Escherichia coli double-stranded complementary DNA (cDNA) copies of early mRNAs from Ad12 infected KB cells. Only two mRNA species, both encoded to the left of 4.5 map units, were identified in region E1. Bos et al. (1981) by comparing the nucleotide sequence homology of Ad12 with that of region E1b of Ad5, and by nuclease-S1 analysis from mRNA from an Ad12 transformed hamster cell line (HT3), have recently identified in Ad12 three mRNAs from region E1 encoded to the right of 4.5 map units. The structure of these mRNAs along with the structures of the Ads2 and 5 region E1 mRNAs are diagrammed in Fig. 4-9. The results of a third study by Sawada and Fujinaga (1980) on the mapping by nuclease S1 and northern-blot analyses of Ad12 E1 mRNAs in transformed rat cell line CY-1 [transformed with Eco R1-C (0-16.5%)], and in infected KB cells, are summarized in Table 4-2. The results of this investigation will perhaps be best discussed and interpreted in relation to these reported results with Ad12 and Ad5.

It appears from the reported studies that regions E1a and E1b of Ad12 are delineated at approximately 4.4 map units, similar to in Ad5. Therefore, probes Ava II-B/C and Accl-H correspond to region E1a and probes Accl-J and Hind III-I correspond to region E1b.

The RNA band at 3.15 kb, which hybridized to all the probes, is analogous to the 3.2 kb northern-blot band reported by Sawada and

Figure 4-9 The size and structure of DNA sequences represented in mRNA species encoded in region E1a of Ad12 and Ad2 as determined by sequence analysis of cloned cDNA copies of mRNA (Perricaudet et al., 1979, Perricaudet, le Moullec and Tiollais, 1980); and in region E1b of Ad12 and Ad5 determined by DNA sequence analysis and nuclease S1-mapping (Bos et al., 1981). Relevant restriction enzyme sites in Ad12 DNA shown are Av = Ava II, Ac = AccI, H = Hind III and S = Sal I.

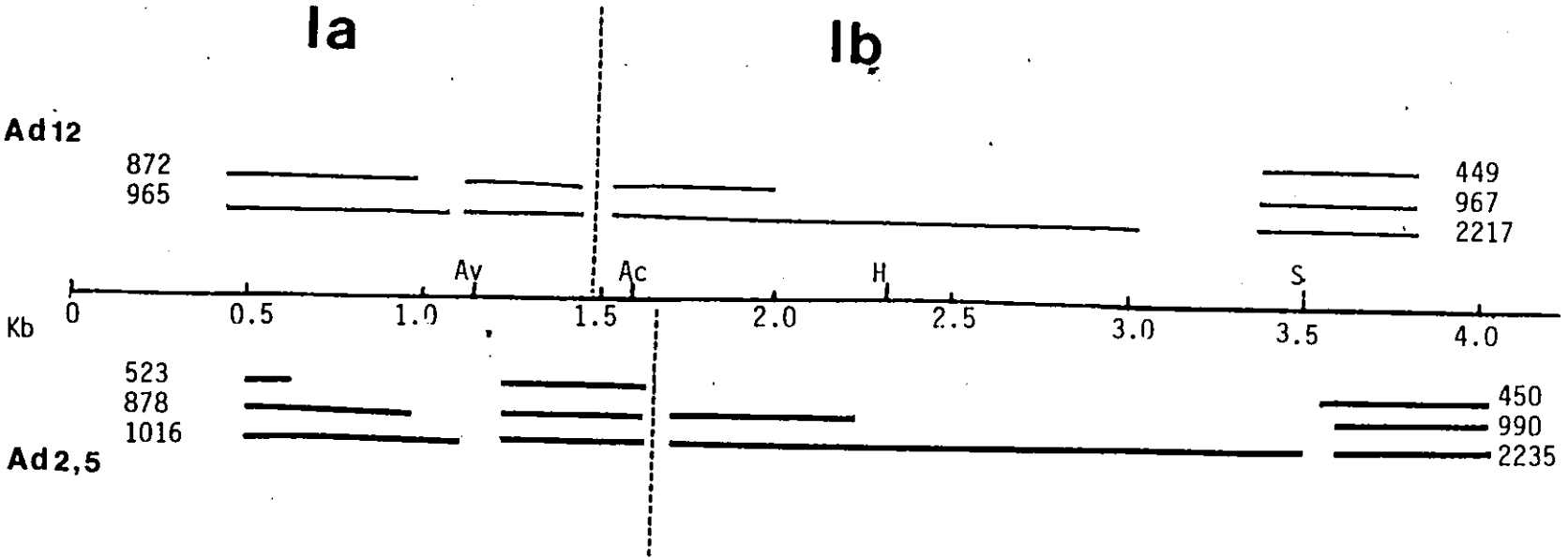

Ia
Ib
Ad12
872
965
449
967
2217
Ay
Ac
H
S
Kb
0
0.5
1.0
1.5
2.0
2.5
3.0
3.5
4.0
523
878
1016
450
990
2235
Ad2,5

Table 4-2. Summary of reported results of Sawada and Fujinaga (1980) on mRNA species complementary to region E1 of Ad12 [a]

Region of Ad12 genome	Ad12 infected KB cells			Ad12 transformed rat cell line CY-1	
	nuclease S1 analysis		northern-blot hybridization	nuclease S1 analysis	northern-blot hybridization
	Early	Late	Early		
E1a	980	980	1,000 - 1,200	840	1,000 - 1,200
(0.04 - 4.6 map units)	840	840		930	
	930	930		1,070	
	1,070	1,070			
E1b	none	450	none	2,190	2,300
(4.6 - 11.0 map units)		980			
Total E1	-	-	-	(3,110)[b]	3,200
(0.0 - 11.0 map units)				(3,200)	
				(3,340)	

[a] Two methods were used in identifying mRNA species complementary to region E1, nuclease S1 analysis and northern-blot hybridization.

[b] ( ) indicates a minor species

Fujinaga (1980) to be present only in transformed CY-1 cells and has been shown by nuclease-S1 analysis to consist of a group of continuous transcripts of almost the entire region E1 (Table 4-2). Other minor but larger bands detected late are probably similar, incompletely processed transcripts produced at a low frequency.

In region E1a, the 1.92 kb band found only at the early time is peculiar since the entire region E1a is only about 1.5 kb in size. A similar sized band of 1.9 kb in length is found in transformed GY-1 cells [transformed with Hind III-G (0-6.7%)] and is also specific to region E1a alone (Sawada and Fujinaga, 1980). This band in GY-1 cells is suggested to represent an mRNA which initiates in 5'-flanking cellular sequences and terminates within region E1a. It is not clear how such an mRNA would be generated during early, lytic infection. Alternatively, this 1.92 kb mRNA could represent a smaller mRNA which migrates anomalously slowly under these experimental conditions. The prominent broad band at 1.2 kb detected at both early and late times and the 0.83 kb band detected in greater amounts at the late time may each represent multiple species as suggested by Sawada and Fujinaga (1980). It is tempting to speculate, however, that the 1.92, 1.2 and 0.83 kb region E1a specific RNA bands found here correspond to the three region E1a mRNAs of Ads 2 and 5 (Figure 4-9), though they would appear to have migrated anomalously slowly in these experiments.

The results of this study clearly demonstrated the presence of mRNA species complementary to region E1b early in Ad12 infected KB cells. The major bands were 2.35, 1.32, 0.81 and 0.5 kb in size. However, in all reported studies to date, no region E1b mRNAs have

been directly demonstrated to be present early during lytic infection of KB cells by Ad12. Perricaudet, le Moullec and Tiollais (1980) found no cDNA sequences complementary to region E1b of Ad12 among 10,000 cDNA clones of early mRNA from Ad12 infected KB cells, and no hybridization was detected between single-stranded (^{32}P)-DNA copies of this early mRNA and the Hind III-I fragment (6.7 - 10.8 map units). Sawada and Fujinaga (1980) found no mRNA sequences complementary to region E1b during early infection of KB cells as determined by the methods of northern-blot hybridization and nuclease-S1 analysis. However, both Sawada and Fujinaga (1980) and Bos et al. (1981) have found E1b specific mRNAs in cell lines transformed by Ad12.

The 2.35 kb mRNA species were readily detected by the E1b probes and were present in large amounts. This mRNA may correspond to a 2.19/2.3 kb E1b mRNA detected in transformed CY-1 cells (Table 4-2) and a 2.2 kb E1b mRNA detected in transformed HT3 cells (Fig. 4-9). This mRNA is not detected by Sawada and Fujinaga (1980) even in late infected KB cells.

In this study, a 2.35 kb mRNA was also detected with the E1a probes although less efficiently. There are several possible explanations for this. First, the 2.35 kb mRNA could be initiated in region E1a but is spliced such that the majority of its sequence is encoded in region E1b. Secondly, there could be two populations of molecules, the minor one spanning both regions and the major one fully encoded in region E1b. Thirdly, the early regions may share common nucleotide sequences for example, in areas with regulatory functions. The first proposal is

unlikely considering the detailed mapping of the 2.2. kb mRNA by Bos et al. (1981) showing it to be entirely encoded in E1b, similar to that of Ad5.

The 1.2 kb mRNA may correspond to the 1.0 kb mRNA identified in CY-1 cells and in late infected KB cells by Sawada and Fujinaga (1980) (Table 4-2) and in HT3 cells by Bos et al. (1981)(Fig. 4-9).

The 0.5 kb band detected only late by the Hind III-I probe corresponds in size and location to the mRNA for polypeptide IX (Pettersson and Mathews, 1977). According to the sequence mapping of this mRNA by Bos et al. (1980) (Fig. 6-4), the Hind III-I probe should hybridize only to the 5'-one hundred and twenty-four nucleotides of this short mRNA.

The 0.81 kb band was readily detected late in infection by the Hind III-I probe but less efficiently with the Acc1-J probe, suggesting that the majority of this mRNA coding sequence lies to the right of 6.7 map units. No corresponding mRNA has been reportedly identified in Ad5, Ad2 nor Ad12.

The incomplete correspondence between Ad12 and Ad5 in E1b mRNAs also extends to the suggested E1b encoded polypeptides. Bos et al. (1981) propose that the Ad12 E1b specific 2.2 kb and 1.0 kb mRNAs theoretically encoded polypeptides of 54 kilodaltons (kd) and 19 kd, analogous to Ad5 in which the corresponding mRNAs encode polypeptides of approximately 55 kd and 21 kd. However, Jochemsen et al. (1980) by in vitro translation of mRNA from Ad12

infected KB cells selected by E1b DNA sequences finds three polypeptides of 60 kd, 19 kd, and 17 kd. The 17 kd polypeptide translates most abundantly from mRNA selected by DNA sequences to the right of 6.7 map units. Esche and Siegmann (1982) similarly translate in vitro from mRNA from Ad12 infected KB cells selected by E1b DNA sequences, polypeptides of molecular weights 59 kd, 19 kd and 17 kd. Therefore, these results suggest that the novel 0.81 kb mRNA may encode the 17 kd polypeptide. Since Ad5 hr 6 is defective in the 58 K protein and did not induce DNA degradation, then both the 19 kd and 17 kd polypeptide remain as candidates for the DNase inhibitory function.

It is not clear why other studies have not directly detected mRNAs transcribed early in lytic infection from region E1b of Ad12. It was shown that the drugs cyclohexamide and cytosine arabinoside and a high multiplicity of infection affected the steady state levels of mRNA from this region. Differential effects produced by both of these drugs on the accumulation of different mRNA species from region E1b of Ad5 during lytic infection have been reported (Chow, Broker and Lewis, 1979; Chow and Broker, 1980). The use of high multiplicities of infection [exceeding 20,000 virions/cell in the study of Sawada and Fujinaga (1980)] and drugs are prevalent in transcriptional studies of Ad12 and may account for their unexpected results.

There were no significant differences in mRNA sequences detected between the wild type Ad12 and the cyt mutants H12 cyt 70 and H12 cyt 61.

Therefore, perhaps not surprisingly, the mutations did not appear to affect functions which act in cis or trans in regulating the transcription and processing of mRNAs from region E1. Missense and nonsense mutations would not be detected by this method.

It can be concluded from this study that the region of the Ad12 genome which physically corresponds to region E1b of Ad5 is expressed at both early and late times during lytic infection of KB cells. The mRNAs found to be transcribed from this region include species corresponding to the three region E1b mRNAs of Ad5, and an additional species of 0.81 kb in length. These results in addition to earlier genetic studies in Chapter 3 are consistent with the physical mapping of the DNase inhibitory function in Ad12 in region E1b (4.5-11.0 map units) of the Ad12 genome.

Chapter 5

Regulation of DNA Degradation Induced by Cyt Mutants of Adenovirus Type 12 in KB Cells

INTRODUCTION

Extensive degradation of newly synthesized DNA is induced in KB cells infected with Ad12 cyt mutants and Ad5 d1313, and in hamster embryo cells infected with Ad12. Complementation studies between cyt mutants of Ad12 and early region E1 mutants of Ad5 suggested that an inhibitory factor against an endonuclease activity is encoded in early region E1b of the Ad12 and Ad5 genomes. Furthermore, the dominant character of one mutant, H12 cyt 61, in inducing DNA degradation and the absence of induction of DNA degradation by infection with region E1a mutants of Ad5, suggested that the virus also encodes for a positive acting factor involved in inducing DNA degradation.

In this chapter, the objective was to further elucidate the roles of these and other possible factors which influence the induction of DNA degradation. The approach taken was to investigate the effects of increasing the periods of time between infection with the mutant H12 cyt 70 and superinfection with viruses of homologous and heterologous strains and serotypes on the induction of DNA degradation and virus production.

RESULTS

Degradation of DNA in KB Cells Infected with H12 cyt 70 and Superinfected with Ad12-1131 and Ad12-Huie (M8)

KB cells were infected with the cyt mutant H12 cyt 70 at a multiplicity of 150 virions/cell and at various times afterwards were superinfected with Ad12-1131 or Ad12-Huie (M8) at an equal multiplicity of virions/cell. Each virus was allowed 60 min to adsorb. Infected cells were labelled continuously with (^{3}H)-thymidine (15 μCi/ml, 7.5 μCi/μg) from 14 h after primary infection or immediately after superinfection, whichever is later, till 39 h after primary infection. Cell lysates were analyzed by alkaline sucrose density gradient sedimentation to determine the size distribution of (^{3}H)-labelled DNA (Fig. 5-1). Superinfection at 1 h with both wild type strains of Ad12 resulted in no DNA degradation (Fig. 5-1A,D) as compared to cells infected with the mutant only (see Fig. 2-1, C), demonstrating that both viruses inhibited the induction of DNA degradation by H12 cyt 70. Similar sedimentation profiles were obtained with cells superinfected at 4h, 8h (data not shown) and 12 h (Fig. 5-1, B, E). Cells superinfected at 18 h with both viruses, however, produced a significant DNA breakage (Fig. 5-1, C,F). The DNA fragments produced sedimented as a relatively sharp peak compared to that produced in cells infected with the mutant alone. The cells superinfected with Ad12-1131 at 18h were also pulse-labelled for 60 min at 30 h post-infection, and chased for 6h (Fig. 5-2). A similar sedimentation profile was produced as in

Figure 5-1 Sedimentation profile in alkaline sucrose gradients of intracellular DNA in KB cells infected with H12 cyt 70 and superinfected at various times after infection with Ad12-1131 and Ad12-Huie (M8). Sedimentation is from right to left. Cells superinfected with Ad12-1131 at 1h (A), 12h (B), and 18h (C) and with Ad12-Huie (M8) at 1h (D), 12h (E) and 18h (F) after infection. The multiplicity of infection of each virus was 150 virions/cell. Arrows indicate the positions of [^{14}C]-labelled Ad5 marker DNA.

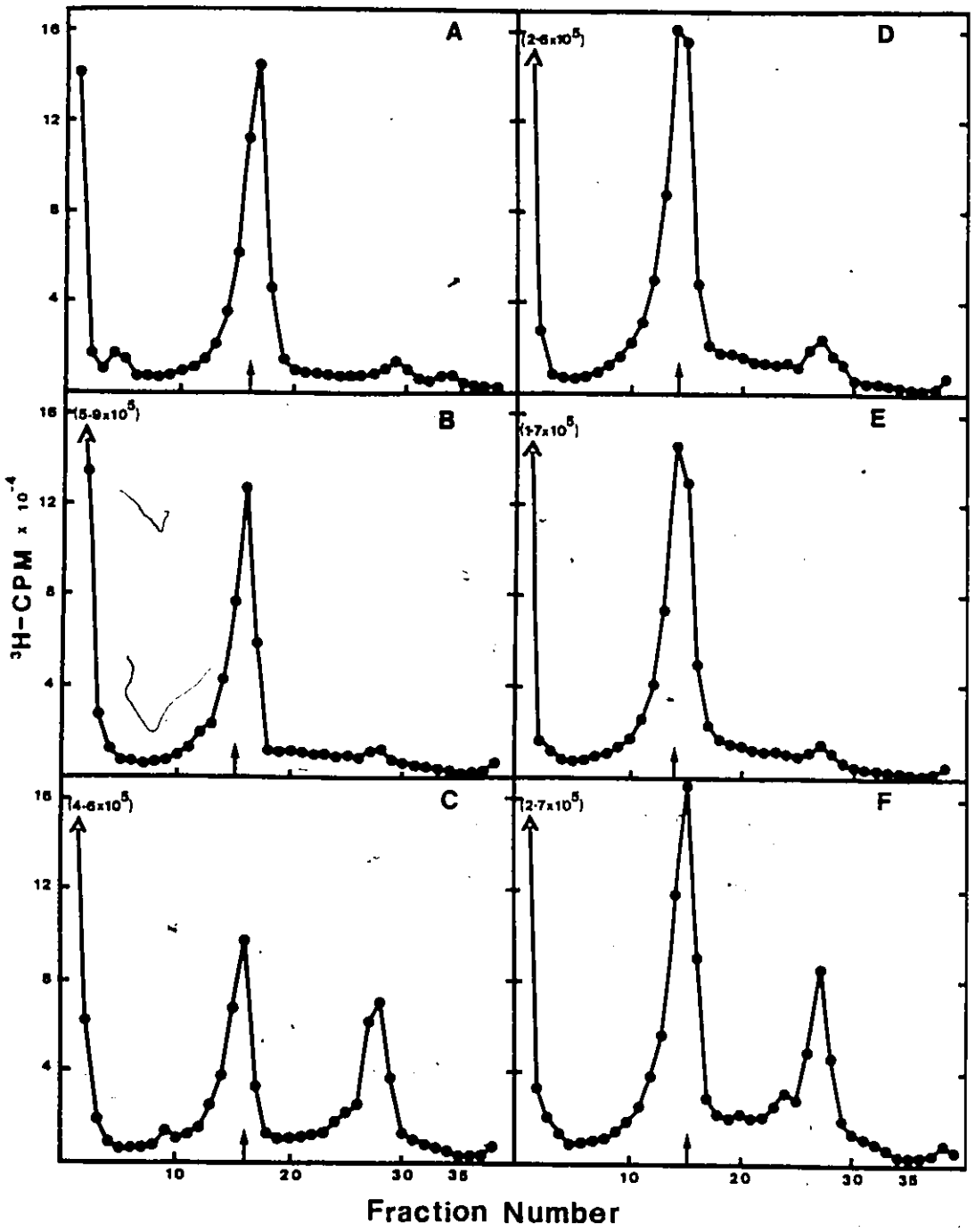
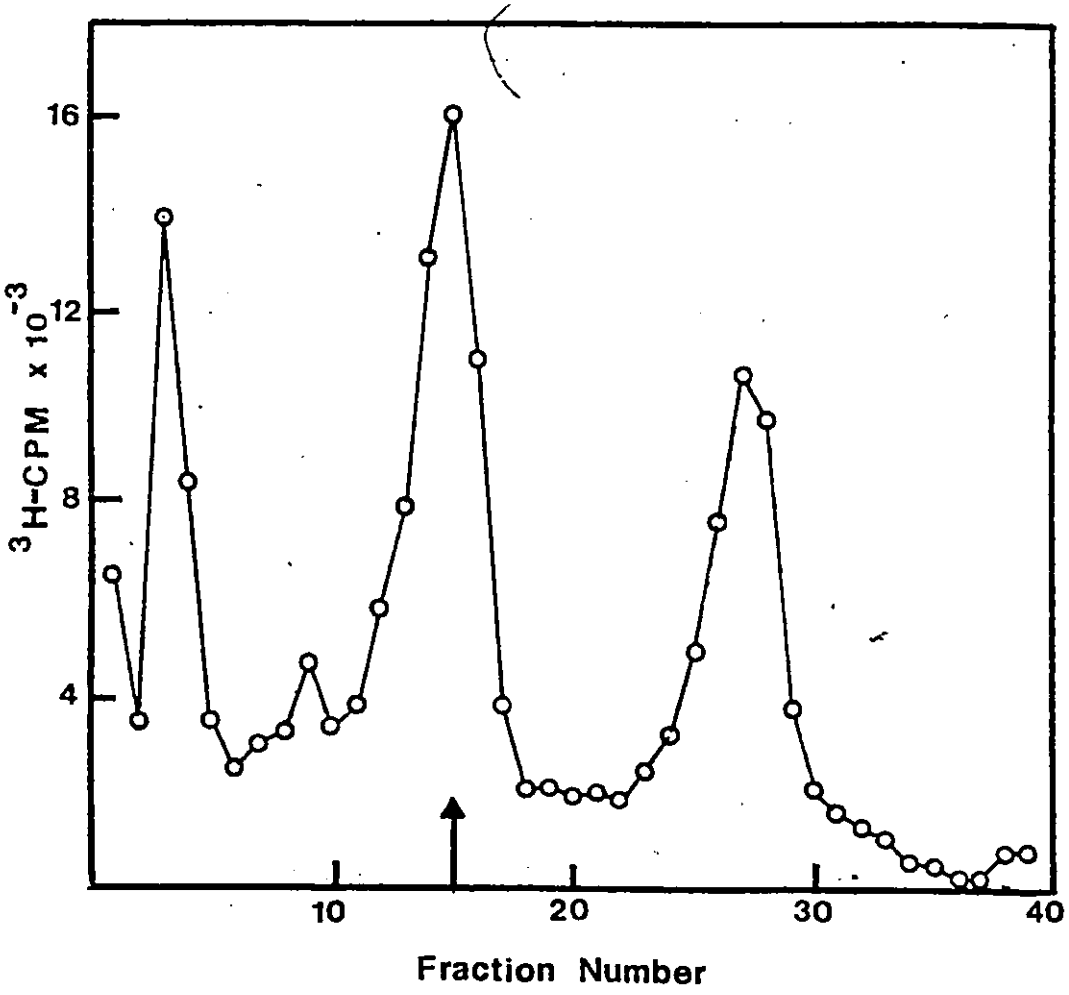

A
B
C
D
E
F
(5·9x10⁵)
(14·6x10⁵)
(2·6x10⁵)
(1·7x10⁵)
(2·7x10⁵)
³H-CPM x 10⁻⁴
16
12
8
4
10
20
30
35
Fraction Number

Figure 5-2 Sedimentation profile in alkaline sucrose gradient of intracellular DNA in KB cells infected with H12 cyt 70 and superinfected at 18 h with Ad12-1131. Infected cells were labelled with [^{3}H]-thymidine (15 µCi/ml, 20 Ci/mM) for 60 min at 30 h after primary infection, and chased for 6 h. Sedimentation is from right to left. Arrow indicates position of [^{14}C]-labelled Ad5-marker DNA.


16
12
8
4
^{3}H-CPM x 10^{-3}
10
20
30
40
Fraction Number

continuous labelling, indicating that DNA degradation was occurring at greater than 12 h after the time of superinfection. The reason for the greater homogeneity in size of the degraded fragments is not clear, although it may reflect a preferential incorporation of label into viral DNA due to the inhibition of host DNA synthesis by the superinfecting virus.

Adsorption Efficiency of the Superinfecting Virus

The inability of the superinfecting virus to suppress the degradation of DNA at late times after H12 cyt 70 infection may be the result of a substantial reduction in the adsorption efficiency of the second virus to the infected cells. Therefore, the adsorption efficiency of the superinfecting virus to H12 cyt 70-infected KB cells was measured at various times after the primary infection. Table 5-1 gives the results using (^{3}H)-labelled Ad12-1131 as the superinfecting virus at an even greater multiplicity of infection than that used in the preceding experiments. There is no significant change in the adsorption efficiency, 80-87 percent, of the ^{3}H-Ad12 virus up till 19 h after H12 cyt 70 infection. Therefore, the expression of DNA degradation in cells superinfected at 18 h was not due to a lack of adsorption of the superinfecting virus. It is also unlikely that the superinfecting virus was somehow non-functional beyond adsorption since the DNA sedimentation profiles produced were different compared to that from non-superinfected cells.

Virus Yields from Superinfected Cells

Virus was extracted by the sodium deoxychplate method from the

Table 5-1. Adsorption of [^{3}H]-labelled Ad12-1131 virus to cyt mutant infected KB cells[a]

Time of super-infection	Cell Associated ^{3}H-CPM	^{3}H-CPM remaining in super-natant	% of Ad12 (1131) Adsorbed
no super-infection	13,550	2,320	85.4
1 h	14,970	2,630	85.1
4 h	16,350	2,540	86.6
12 h	15,010	2,730	84.6
19 h	14,290	3,650	79.7

[a] KB cells were infected with H12 cyt 70 at a multiplicity of 300 virions/cell for 90 min., and superinfected with [^{3}H]-labelled Ad12-1131 at a multiplicity of 2,000 virions/cell for 90 min.

same infected cell cultures as were used in the sucrose gradient assays for DNA degradation. The cells were harvested at 40 hr and the yields are given in Table 5-2. Total virus yields from Ad12-1131 and Ad12-Huie (M8) superinfected cells were about 10-fold greater than that from cells infected with the mutant only. The different times of superinfection did not significantly affect the total yield of virus. These virus yields were further analyzed to determine the relative abundances of the virus types in each.

Virus Types Produced in Cells Superinfected with Ad12-1131

The only assay which distinguishes the cyt mutant from the parental virus is the plaque assay on human embryonic kidney cells as described by Takemori, Riggs and Aldrich (1968). However, repeated efforts in this laboratory have found this not to be a reliable, quantitative assay. Control experiments with each virus type plaqued separately showed slight but overall distinct differences between the two viruses. With a mixture of the two viruses, however, it was difficult to consistently and unambiguously distinguish between the two types of plaques formed. The virus types produced in the cells superinfected with Ad12-1131 therefore, could not be assayed.

Virus Types Produced in Cells Superinfected with Ad12-Huie (M8)

The genomes of Ad12-Huie (M8) and Ad12-1131 can be distinguished using the restriction endonuclease Bam Hl. The cleavage patterns of the two are identical except for the E-fragment, the right-hand 11 percent of the genome. This fragment is undetectable in Ad12-Huie (M8)

Table 5-2. Total virus yields[a] from singly infected and superinfected KB cells

Infecting virus	Yield ($\times 10^3$ virions/cell) from single injection (m.o.i. 300 virions/cell)	Yield ($\times 10^3$ virions/cell) from KB cells infected with H12 cyt 70 (m.o.i. 150 virions/cell) then superinfected with the 2nd virus (m.o.i. 150 virions/cell) at the following times after infection.				
		1 h	8 h	12 h	18 h	24 h
H12 cyt 70	0.28	-	-	-	-	-
Ad12-1131	4.0	3.3	nd[b]	nd	3.4	nd
Ad12-Huie (M8)	4.5	2.6	3.2	nd	2.4	nd
Ad5	9.5	nd	nd	13.0	9.8	12.0

[a] virus yield determined by optical density, one A_{260} absorbance unit = 4×10^{11} virions/ml

[b] not done

DNA under identical conditions of restriction and gel electrophoresis as with Ad12-1131 DNA (Fig. 5-3 i, lanes a and f, respectively). The inability to detect this Bam H1 fragment in the Huie strain is not understood. Both strains have similar EcoR1 restriction patterns, including the A-fragment, the right-hand 36 percent of the genome (Fig. 5-3-ii). Nevertheless this difference in the Bam H1 restriction patterns affords a method to detect and quantitate the presence of H12 cyt 70 DNA, which cleaves identically to that of the parental Ad12-1131 DNA (Fig. 5-5, b,c).

A reconstruction experiment was done using defined mixtures of (^{3}H)-labelled DNA, in terms of amounts of radioactivity, isolated from purified Ad12-1131 and Ad12-Huie virions. Quantitation of the fragments in the fluorogram (Fig. 5-3 -i) by microdensitometry demonstrated that the ratio of the amount of fragment E to the amount of fragment A, a common fragment, is roughly proportional to the percentage of Ad12-1131 DNA in the mixture (Fig. 5-4).

Virions were purified from KB cell cultures infected with H12 cyt 70 and superinfected with Ad12-Huie (M8) and the viral DNA was extracted. These cell cultures had also been labelled with (^{3}H)-thymidine from 14 h after primary infection or immediately after superinfection, till 39 h after primary infection. These (^{3}H)-labelled viral DNA preparations were analyzed with Bam H1 (Fig. 5-5). The relative amounts of fragments E and A in the fluorogram were measured and the approximate percentages of H12 cyt 70 DNA (Table 5-3) determined from the standard curve. These percentages are minimum values since (^{3}H)-thymidine was

Figure 5-3 (i) Bam Hl digestion of (^{3}H)-labelled DNA from Adl2-Huie (M8) and Adl2-1131 in virions. Mixtures of (^{3}H)-DNA (in terms of amounts of radioactivity) from virions of both strains were made before enzyme digestion: a) 100% - Huie (M8), b) 80% - Huie (M8), 20% - 1131, c) 60% - Huie (M8), 40% - 1131 d) 40% - Huie (M8), 60% - 1131, e) 20% - Huie (M8), 80% - 1131 and f) 100% - 1131. (ii) Eco Rl digestion of (^{3}H)-labelled DNA from Adl2-Huie (M8) and Adl2-1131 virions; a) 100% - Huie (M8), b) 50% - Huie (M8), 50% - 1131 and c) 100% - 1131.

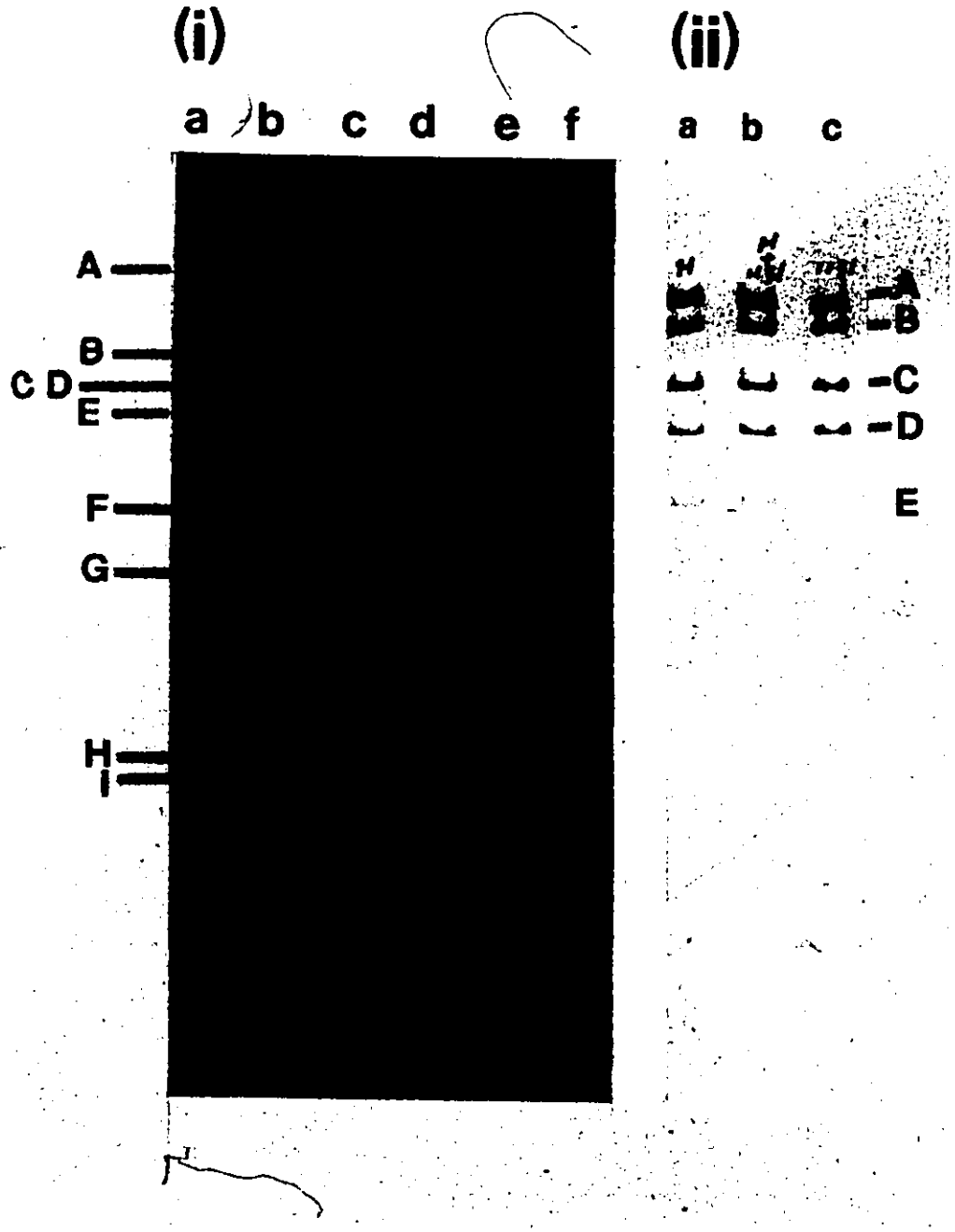

(i)
a b c d e f
A
B
C D
E
F
G
H
I
(ii)
a b c
B
C
D
E

Figure 5-4 A correlation between the ratio of the amount of Bam Hl E-fragment to that of A-fragment and the percentage of Adl2-1131 DNA in a mixture of Adl2-1131 and Adl2-Huie (M8) DNA. The amounts of each fragment was determined from microdensitometer tracings of the fluorograph in Fig. 5-2 by integration of the areas under the respective peaks.

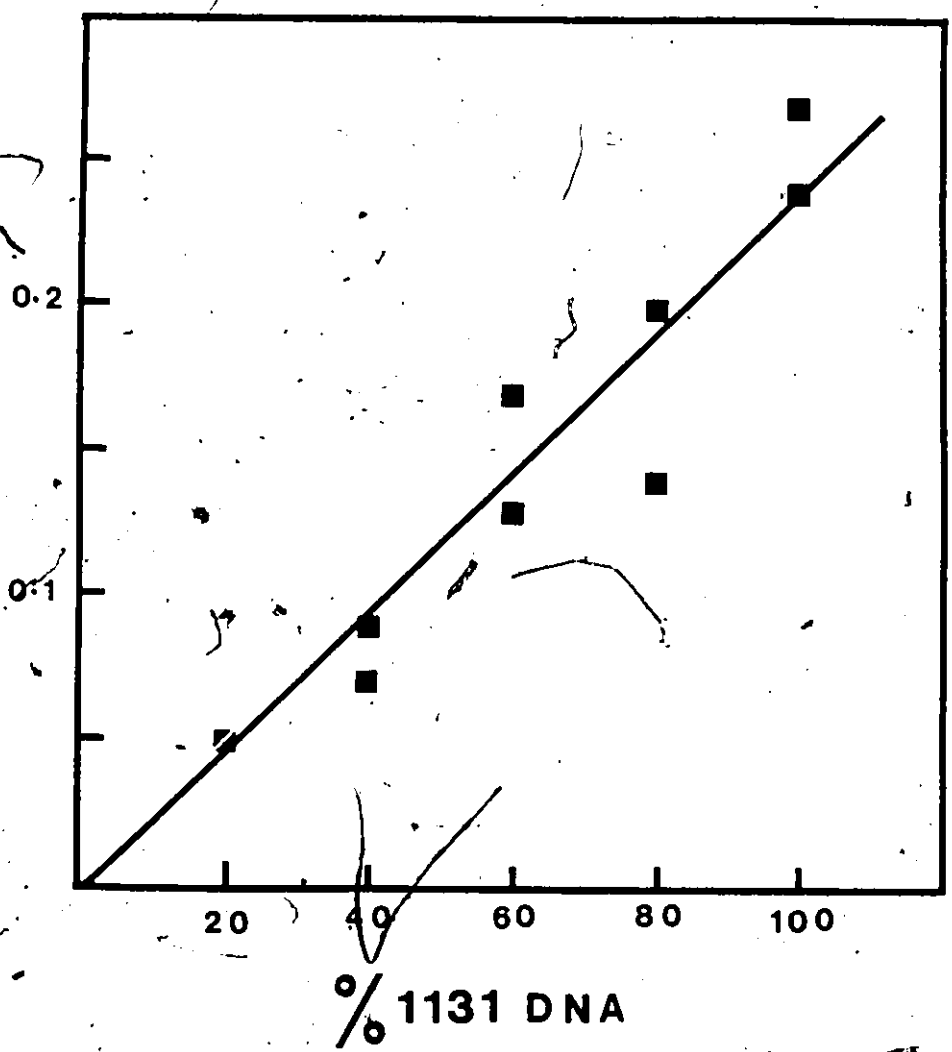
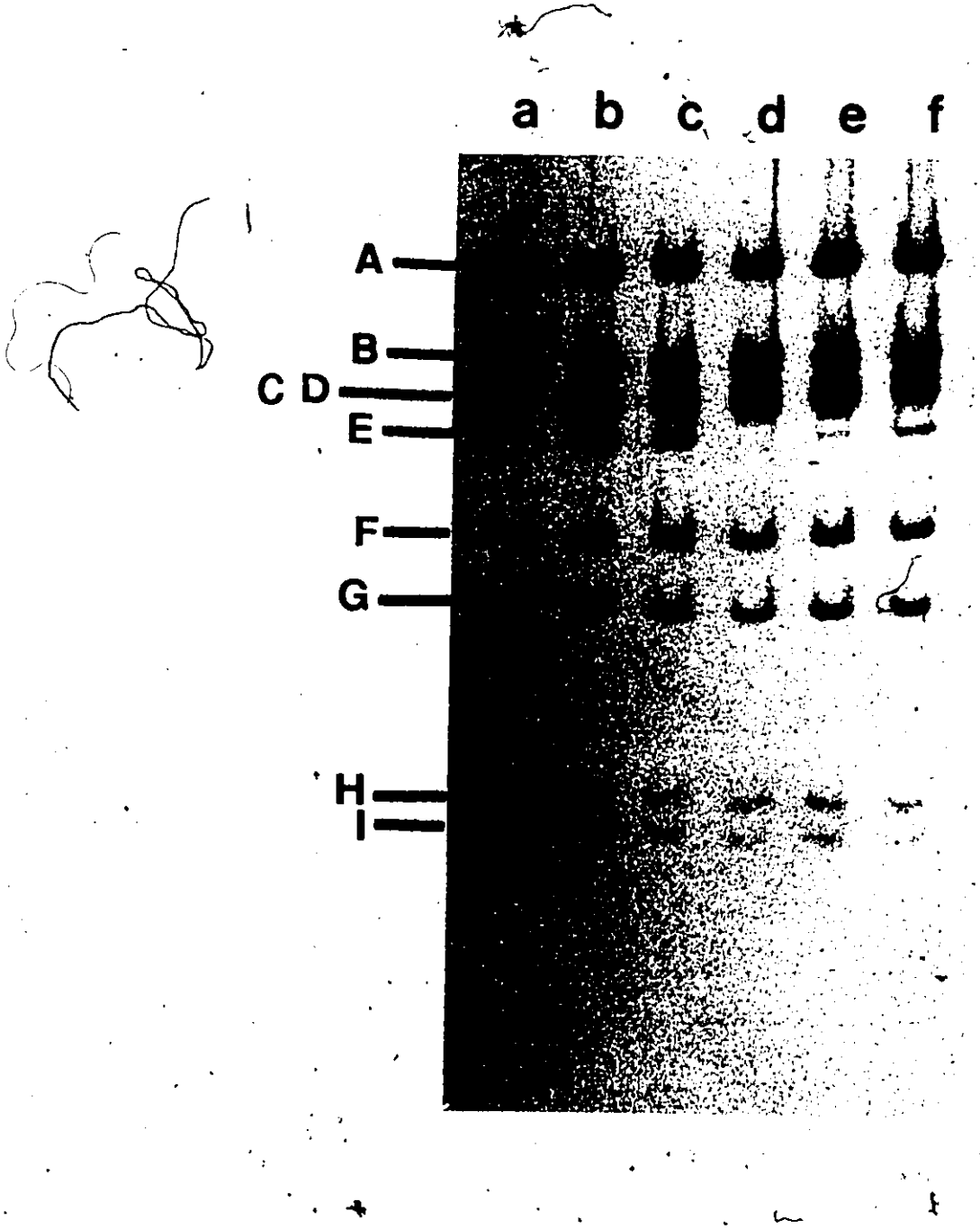

0·2
0·1
E/A
20
40
60
80
100
% 1131 DNA

Figure 5-5 Bam H1 digestion of DNA from virions extracted from KB cells infected with H12 cyt 70 and superinfected with Ad12-Huie (M8). Bam H1 digestion of control a) Ad12-Huie (M8) DNA, b) Ad12-1131 DNA and c) H12 cyt 70 DNA, and of DNA from virions from cells infected with H12 cyt 70 and superinfected with Ad12 Huie (M8) at d) 1h, e) 8h and f) 18 h after infection.


a
b
c
d
e
f
A
B
C D
E
F
G
H
I

added to the cultures only after superinfection and any H12 cyt 70 DNA synthesized prior to labelling was not detectable in the assay. These percentages were in turn used to calculate from the total yields given in Table 5-2, the minimum absolute yield of cyt virions (Table 5-3). From cells superinfected at 1 h, no H12 cyt 70 DNA was detected in the labelled virions. From cells superinfected at 8 h, however, roughly 10 percent of the labelled viral DNA was of the mutant H12 cyt 70. In this instance, the absolute yield of cyt virions was approximately equivalent to that from non-superinfected cells, yet no DNA degradation was evident. In cells superinfected at 18 h which exhibited DNA degradation, roughly 30 percent of the labelled viral DNA was of H12 cyt 70, indicating an approximately 2.5 fold greater absolute yield of H12 cyt 70 virions than from non-superinfected cells.

Superinfection of H12 cyt 70 Infected KB Cells with Ad5

Ad5, a member of the non-oncogenic subgroup C adenoviruses, shares little DNA sequence homology with Ad12 (Sambrook et al., 1980). Therefore it was of interest to investigate the effect of superinfection with Ad5 on the induction of DNA degradation in KB cells infected with mutant H12 cyt 70 and on the virus yields of the two serotypes from these cells.

KB cells were infected with mutant H12 cyt 70 at a multiplicity of 150 virions/cell and at various times afterwards was superinfected with Ad5 at an equal multiplicity of virions/cell. Superinfected cells were labelled with (^{3}H)-thymidine as described earlier for superinfections with Ad12 viruses.

Table 5.3 Types of virions produced in KB cells infected with the cyt mutant and super-infected with Ad12-Huie (M8)

Time of super-infection	Bam H1-E fragment/ Bam H1-A fragment	% H12 cyt 70 DNA	Minimum yield of H12 cyt 70 virions (x 10^3/cell)
1 h	0	-	-
8 h	.02	10	0.32
18 h	.065	29	0.70

i) Degradation of DNA

Alkaline sucrose gradient sedimentation profiles showed that the degradation of DNA was suppressed by superinfection with Ad5 at 1 h and 18 h (Fig. 5-6,a,b) after infection with H12 cyt 70. Similar sedimentation profiles were produced from cells superinfected at 8 h and 12 h (data not shown). Superinfection at 24 h with Ad5 however, resulted in DNA degradation (Fig. 5-6, c) in two of three experiments.

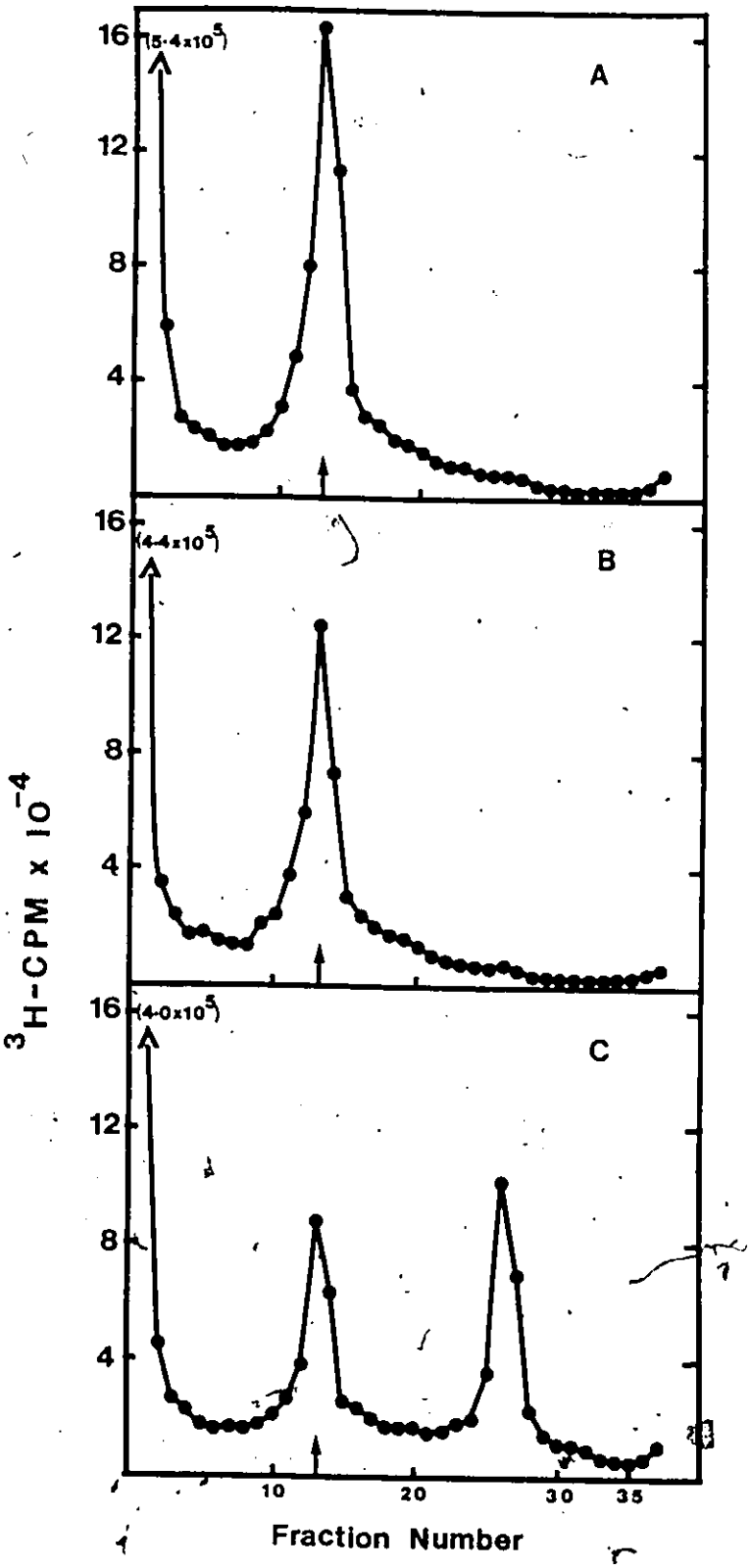
ii) Total Virus Yields

Virus was extracted by the sodium deoxycholate method from the superinfected cells and the yields are given in Table 5-2 shown earlier. Virus yields from the Ad5 superinfected cells were about 30 fold greater than that from cells infected with H12 cyt 70 alone, irrespective of the time at superinfection.

iii) Yields of Each Virus Serotype

Ad12 virions can be distinguished and separated from Ad5 virions by equilibrium density centrifugation due to a difference in DNA content of the virions. The virion density of Ad5 is 0.009 gm/ml greater than that of Ad12, taking the density of Ad12 to be 1.340 gm/ml. There is no detectable difference in density between H12 cyt 70 virions and parental Ad12 virions (Ezoe and Mak, 1974). The (^{3}H)-labelled virus yields from Ad5 superinfected cells described in Table 5-2 were analyzed for the amounts of each serotype present. Each virus preparation plus a (^{14}C)-labelled Ad5 marker was sedimented to equilibrium in a preformed CsCl gradient having a linear density range of 1.32 - 1.36 gm/ml. A control mixture of (^{14}C)-labelled Ad5

Figure 5-6 Sedimentation profiles in alkaline sucrose gradients of intracellular DNA in KB cells infected with H12 cyt 70 and superinfected at various times after infection with Ad5. Sedimentation is from right to left. Cells superinfected at A) 1 h, B) 18 h and C) 24 h after infection. The multiplicity of infection of each virus was 150 virions/cell. Arrows indicate the positions of [^{14}C]-labelled Ad5 marker DNA.


16
12
8
4
(5·4x10^5)
A
(4·4x10^5)
B
(4·0x10^5)
C
^{3}H-CPM x 10^{-4}
10
20
30
35
Fraction Number

and (^{3}H)-labelled Ad12 virions was clearly separated by this method (Fig. 5-7, a). The sedimentation profiles of the virions from the three superinfected cultures are shown in Fig. 5-7 b,c,d. Radioactivity in the peak corresponding to H12 cyt 70 virions was evident only in the virus from cells superinfected at 18 h and 24 h. 70 virions were determined by summation of the (^{3}H)-radioactivity counts in fractions under the respective peaks, and normalization of the counts according to thymine content and molecular weight of the different genomes (Table 5-4). Again the resulting estimates of H12 cyt 70 virus yields are minimum values since (^{3}H)-thymidine was added to the cultures only after superinfection. No labelled H12 cyt 70 virions were detected in the virus yield from cells superinfected at 12 h. Cells superinfected at 18 h and 24 h yielded approximately 10 percent and 40 percent, respectively, of the total labelled yields as H12 cyt 70 virions. The absolute yields of H12 cyt 70 virions in these superinfected cultures were increased 3.5 fold and 16.5 fold compared to the non-superinfected culture.

Figure 5-7 Equilibrium density sedimentation profiles in pre-formed CsCl density gradients of virions from cells infected with H12 cyt 70 and superinfected at various times after infection with Ad5. a) a control mixture [^{14}C]-labelled Ad5 virions (▲) and [^{3}H]-labelled Ad12 virions (●), and [^{3}H]-labelled virions from cells superinfected at b) 12 h, c) 18 h and d) 24 h.

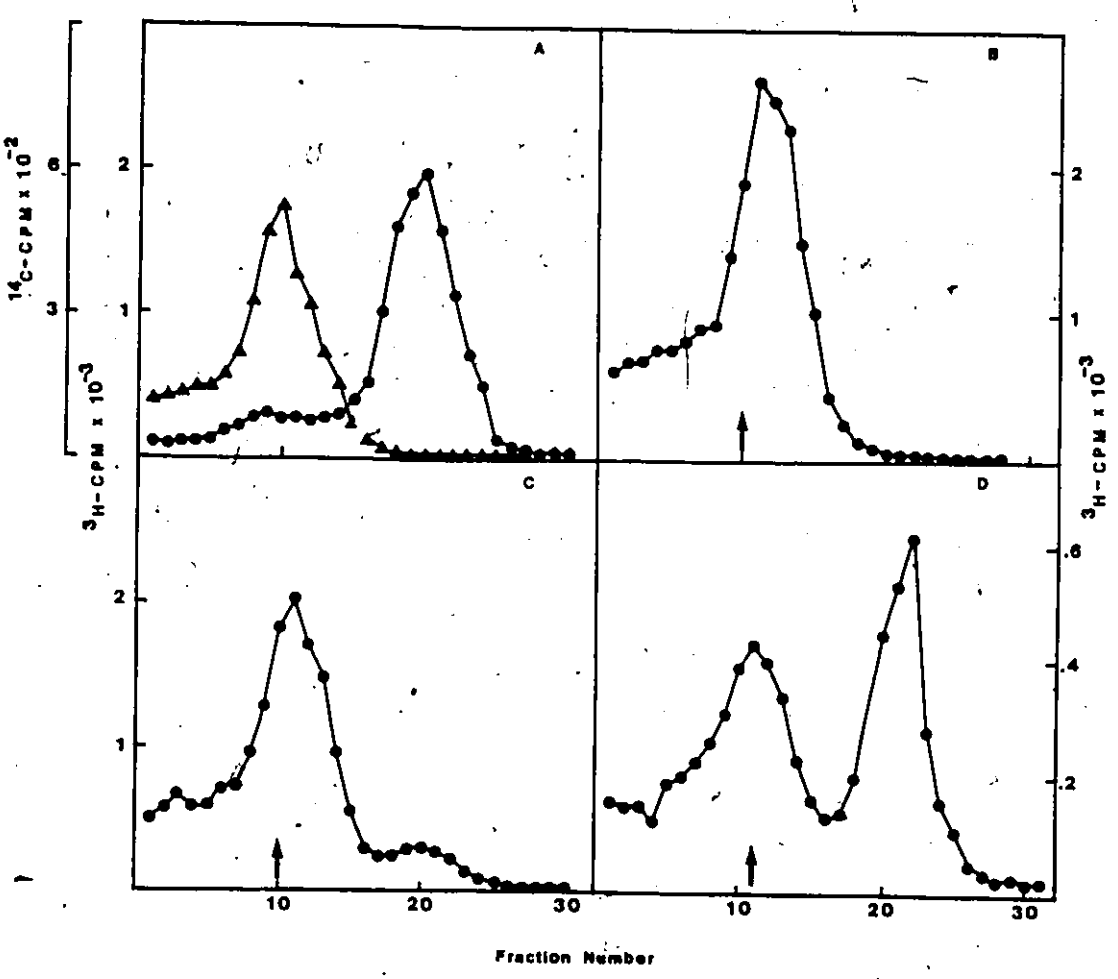

A
B
C
D
^{14}C-CPM x 10^{-2}
^{3}H-CPM x 10^{-3}
^{3}H-CPM x 10^{-3}
10
20
30
Fraction Number

Table 5-4. Types of virions produced in Ad5 superinfected cells

Time of super-infection	^{3}H-CPM in peak corresponding to Ad5	^{3}H-CPM in peak corresponding to H12 cyt 70	% of ^{3}H-CPM in H12 cyt 70 virions [a]	minimum yield of H12 cyt 70 virions (x10^3/cell)
18 h	1.29×10^4	1.79×10^3	9.5	0.93
24 h	3.26×10^3	2.92×10^3	40.4	4.8

[a] ^{3}H-CPM in peaks corresponding to Ad5 and H12 cyt 70 virions were normalized for % thymine content and molecular weight of the respective genomes: Ad12- 25.7% thymine, 21.5×10^6 daltons; Ad5 - 21% thymine, 23×10^6 daltons.

DISCUSSION

Earlier in Chapter 3 it was suggested that at least two viral gene products are involved in the determination of induction of DNA degradation. The first is a DNase-inhibitory function identified to be defective in the recessive mutants H12 cyt 70 and Ad5 dl 313 and which mapped in early region E1b of the genome. The second is a positive acting factor involved in inducing DNA degradation, or a DNase-effecter, based on the observations that mutant H12 cyt 61 was dominant in inducing DNA degradation and that region E1a mutants of Ad5, which do not express most viral genes including the region E1b products, did not induce DNA degradation. It was also found that DNA degradation could be induced in the absence of viral DNA synthesis suggesting that the DNase-effecter is probably expressed early in infected cells which exhibit DNA degradation.

In this chapter, several observations were made which may further elucidate the process of induction of DNA degradation. All three viruses, Ad12-1131, Ad12-Huie (M8) and Ad5, could inhibit the induction of DNA degradation by H12 cyt.70. With late times of superinfection, DNA degradation could still occur subsequent to superinfection. There was not a fixed period of time after H12 cyt 70 infection after which DNA degradation could no longer be inhibited by superinfection; this period of time varied depending upon the serotype of the superinfecting virus. Pulse-chase labelling of cells super-

infected at 18 h with Ad12-1131 demonstrated that the capacity to degrade DNA persisted for greater than twelve hours after superinfection. There was no correlation between a significant yield of cyt mutant virus from superinfected cultures and whether or not DNA synthesized after superinfection was degraded.

Several inferences concerning the process of induction of DNA degradation can be made from these and earlier observations. DNA degradation does not appear to be determined by the expression of the DNase-effecter functioning transiently to induce a cellular nuclease since DNA degradation was not inhibited by superinfection at 18 h with Ad12, but was with Ad5. For this reason, and that high production of cyt mutant did not correlate with DNA degradation, it also appears that DNA degradation is not determined by the absolute accumulation of the DNase-effecter. The nuclease-inhibitory factor apparently does not act directly against an induced cellular factor since induction of DNA degradation by H12 cyt 61 could not be prevented by coinfection with other viruses (see Chapter 3). If it is assumed that some nuclease activity is present at 18 h after infection with H12 cyt 70, yet Ad5 but not Ad12 could inhibit subsequent activity of the nuclease, then it appears that the inhibitory factor does not act at the level of gene expression of the DNase effector.

Based on these premises, a basic model of the functions and process involved in the induction of DNA degradation can be proposed. The nuclease-inhibitory factor is suggested to act directly against

the activity of the viral encoded DNase- effecter. The DNase- effecter could be either a continually required component or inducer of the DNase activity, or the nuclease itself. The degradation of DNA would be determined by the relative amounts of these two viral products. Infection with a cyt mutant alone would result in a net production of the DNase- effecter. Superinfection with wild type virus would provide functional DNase-inhibitory factor, sufficient amounts of which were provided at early times of superinfection to inhibit subsequent DNA degradation. At late times of superinfection, the DNase-inhibitory factor provided by the superinfecting virus may be insufficient to inhibit increased levels of DNase- effecter in the infected cells resulting in continued DNA degradation. It appears that Ad5 was able to provide the DNase-inhibitory factor more efficiently or effectively than Ad12 upon superinfection at 18 h after H12 cyt 70 infection.

It is interesting to speculate that this nuclease induction system may have developed to play a functional role in the reproductive cycle of adenoviruses. During the normal course of productive infection, the "nuclease" function might not be active until late in infection, possibly through the net production in activity of the DNase- effecter over the DNase-inhibitory factor. The viral induced DNA degradation may be conducive to the cytopathic effect and eventual destruction of productively infected cells, facilitating the propagation of infectious virus.

It was found that the replication of the mutant was suppressed in KB cells after superinfection with Ad12-Huie (M8) and Ad5 early after primary infection, yet enhanced by later times of superinfection. Takemori et al. (1969) have reported that cyt mutants of the 1131 strain are complemented in growth in a line of KB cells by coinfection with wild type virus of the homologous strain, but not of the Huie strain nor with Ad2. The reasons for these results are not clear. The inhibition of replication of the primary infecting virus by superinfection with Ad12-Huie (M8) was investigated and discussed in more detail in Chapter 6.

Chapter 6

Interference with DNA Replication of Ad12-1131 by Ad12-Huie (M8)

INTRODUCTION

The virus Ad12-Huie (M8) was shown to interfere with the replication of the cyt mutant H12 cyt 70 in coinfected KB cells. It is not clear, however, if this was a result of the cyt mutation affecting the replication of the mutant, or an innate ability of Ad12-Huie (M8) to interfere with the replication of the virus of the 1131 strain of Ad12. It has been reported that Ad12-Huie (M8) also interferes with the replication of the normally much higher yielding Ad2 in KB cells (Mak, 1969), suggesting that the interference capacity might be a peculiar property of Ad12-Huie (M8).

In this supplementary study, the ability of Ad12-Huie (M8) to interfere with the replication of wild type Ad12-1131 was investigated.

RESULTS AND DISCUSSION

Replication of Ad12-1131 and Ad12-Huie (M8) in Coinfected KB Cells

KB cells were infected first with Ad12-1131 at a multiplicity of 150 virions/cell for 60 min., then superinfected at 1 h or at 18 h post-infection with Ad12-Huie (M8) at an equal multiplicity for 60 min. KB cells were also coinfected with Ad12-1131 at a multiplicity of 250 virions/cell and with Ad12-Huie (M8) at a multiplicity of 50 virions/cell for 90 min. Infected cell cultures were labelled with (^{3}H)-thymidine (2 µCi/ml, 10 µCi/ g) from 14½ h after primary infection or immediately after superinfection, whichever is later, till 36 h after

primary infection for analysis of the (^{3}H)-DNA in virions produced.

Virus was extracted by the sodium deoxycholate method, and yields of virions were determined by optical density (Table 6-1). It is seen that all doubly infected cells produced similar amounts of virus.

DNA was extracted from these virus yields and analyzed by digestion with Bam Hl (Fig. 6-1). The relative amounts of each type of DNA of the two viruses were quantitated as described in Chapter 5 by the relative amounts of fragment E of strain 1131 to that of fragment A of both strains. With cells superinfected at 1 h, the DNA from the virus produced was almost totally of Ad12-Huie (M8) (Fig. 6-1, lane 4), greater than 90 percent. With cells superinfected at 18 h, approximately 85 percent of the DNA from the virus yield was of Ad12-Huie (M8) (Fig. 6-1, lane 5). With the cells coinfected with a 5:1 ratio of Ad12-1131:ad12-Huie (M8), the DNA from the virus yield was about 80 percent Ad12-Huie (M8) (Fig. 6-1, lane 3). These results suggest that Ad12-Huie (M8) interferes strongly with the production of Ad12-1131 in coinfected cells.

To resolve whether these results were due to preferential replication or to preferential packaging of Ad12-Huie (M8), DNA, in the coinfected cell, a portion of the cell culture superinfected at 1 h was also labelled from 24 h to 36 h with (^{3}H)-thymidine (2 µCi/ml, 10 µCi/µg) for analysis of total intracellular (^{3}H)-DNA. Total intracellular DNA was extracted and analyzed by digestion with Bam Hl

Table 6-1. Virus yields from Ad12-1131 and Ad12-Huie (M8) infected KB cells

Infecting virus	Total virus yield ($x10^3$ virions/cell)
Ad12-Huie (M8) [300][a]	4.5
Ad12-1131 [300]	4.0
Ad12-1131 [250] + Ad12-Huie (M8) [50]	3.3
Ad12-1131 [150] and superinfected with Ad12-Huie (M8) [150] at 1 h	3.2
and at 18 h	3.2

[a] multiplicity of infection in [virions/cell]

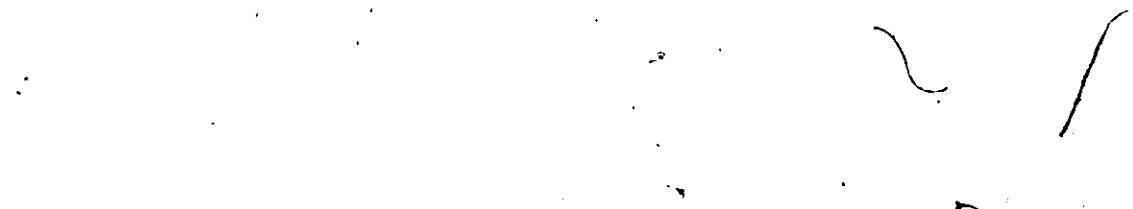
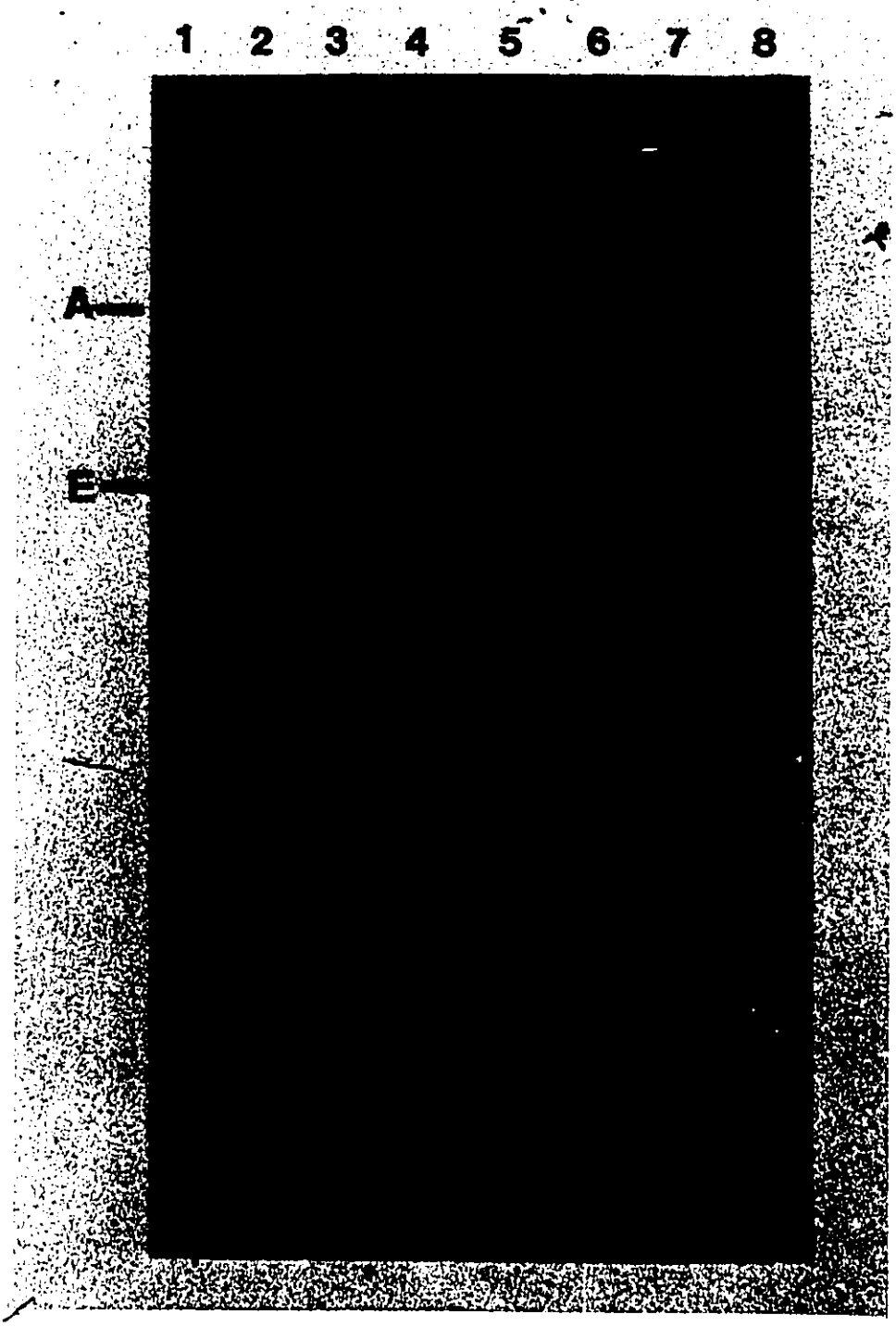


Figure 6-1 Bam H1 digestion of Ad12-1131 and Ad12-Huie (M8) DNA from virions and infected cells. 1,2) Virion DNA of Ad12-Huie (M8) and Ad12-1131 respectively; 3) Virion DNA from cells coinfected with Ad12-1131 at a multiplicity of 250 virions/cell and Ad12-Huie (M8) at a multiplicity of 50 virions/cell; 4,5) Virion DNA from cells infected with Ad12-1131 at a multiplicity of 150 virions/cell and superinfected at 1 h and 18 h respectively, with Ad12-Huie (M8) at an equal multiplicity; 6,7) total intracellular DNA from cells singly infected with Ad12-Huie (M8) and Ad12-1131 respectively; 8) total intracellular DNA from cells infected with Ad12-1131 at a multiplicity of 150 virions/cell and superinfected at 1 h with Ad12-Huie (M8) at an equal multiplicity.


1 2 3 4 5 6 7 8
A
B

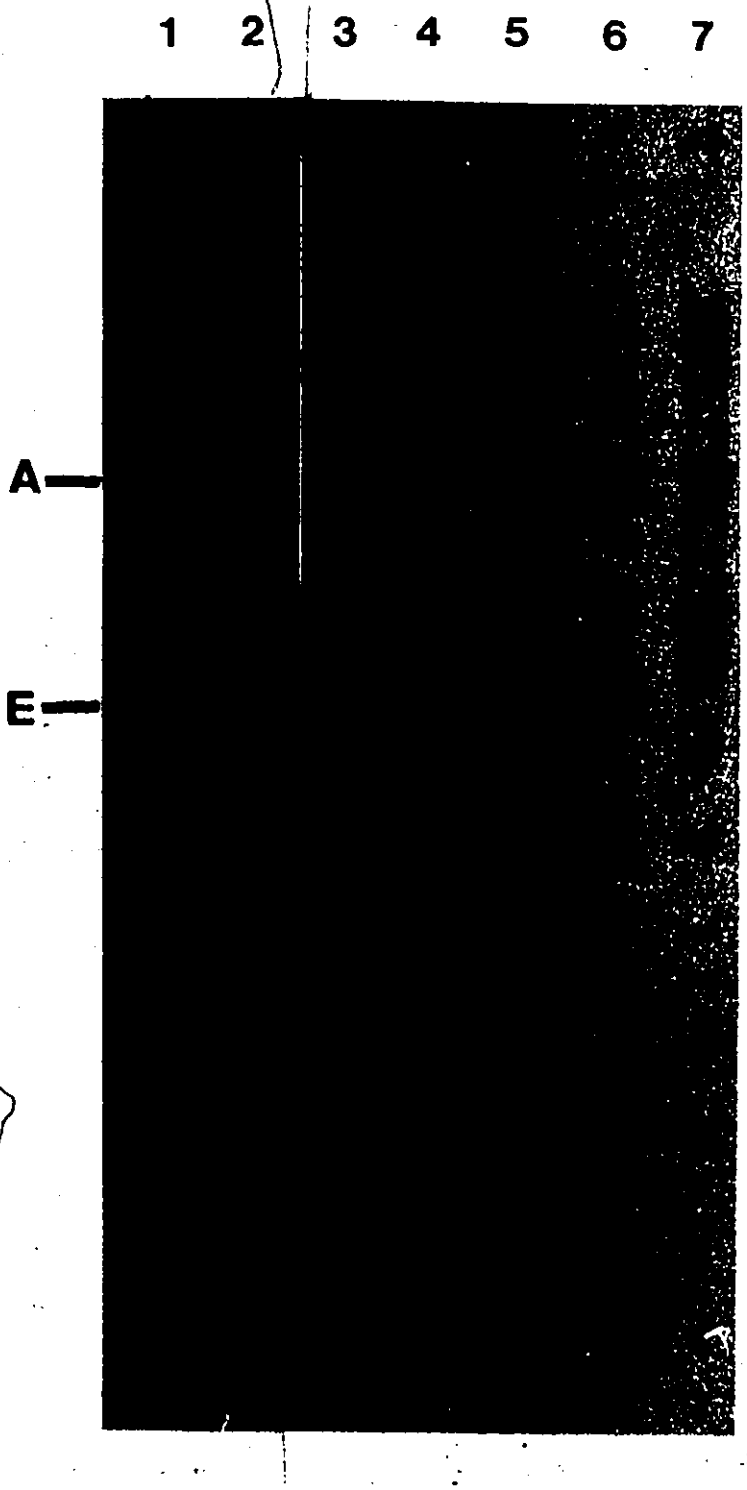
(Fig. 6-1, lane 8). Viral specific DNA bands are clear and indicate incorporation of label almost exclusively into Ad12-Huie (M8) DNA. This suggests that Ad12-Huie (M8) interferes with Ad12-1131 at the level of DNA replication.

In a different experiment KB cells were infected first with Ad12-1131 at a multiplicity of 500 virions/cell, then immediately superinfected with Ad12-Huie (M8) at 100 virions/cell. Portions of the infected culture were labelled with (^{3}H)-thymidine (7.5 µCi/ml, 20 Ci/mM) for 60 min at 18 h and 24 h post-infection for analysis of the total DNA being synthesized in the cells. A portion of the culture was also labelled continuously from 10-40 h with (^{3}H)-thymidine (3 µCi/ml, 10 µCi/µg) for analysis of the DNA in virions produced. Total intracellular DNA was purified from cells labelled at 18 h and 24 h, and from virions produced from cells labelled from 10-40 h. Fig. 6-2 shows the results of digestion of the DNA with Bam Hl. Again it is seen that the virus produced was mostly Ad12-Huie (M8) (Fig. 6-2, lane 3). In the relatively short labelling periods at 18 h and 24 h (Fig. 6-2, lanes 4 and 5, respectively) the viral DNA being synthesized at these times is shown to be also mostly of Ad12-Huie (M8). These results indicate that the interference with replication of Ad12-1131 DNA is occurring fairly early during infection, before viral DNA synthesis normally reaches its maximum rate at about 23 h after infection (Mak, 1969; Ezoe and Mak, 1974).

Mechanism of Interference with DNA Replication

The decisive factor in the mechanism of interference with DNA

Figure 6-2 Bam H1 digestion of Ad12-1131 and Ad12-Huie (M8) DNA from virions and infected cells. 1,2) Virion DNA of Ad12-Huie (M8) and Ad12-1131 respectively; 3) Virion DNA from cells coinfected with Ad12-1131 at a multiplicity of 500 virions/cell and Ad12-Huie (M8) at a multiplicity of 100 virions/cell, and labelled from 10-40 h p.i.; 4,5) total intracellular DNA from cells coinfected with Ad12-1131 at a multiplicity of 500 virions/cell and Ad12-Huie (M8) at a multiplicity of 100 virions/cell and labelled for 60 minutes at 18 h and 24 h p.i. respectively; 6,7) total intracellular DNA from cells singly infected with Ad12-1131 and Ad12-Huie (M8) respectively.


1
2
3
4
5
6
7
A
E

replication of Ad12-1131 by Ad12-Huie (M8) is unlikely to be a trans-acting function since both types of DNA replicating in the same environment would be equally susceptible to such a function. It is more likely to be due to a cis-acting function which allows a DNA molecule to more effectively utilize or compete for factors essential for DNA replication.

Potential candidates are the protein covalently attached at the 5'-termini of the DNA strands, and sequences involved in the initiation of viral DNA synthesis. An altered terminal protein, if it confers an advantage to the parental DNA molecule, would likely affect only the initial round of viral DNA synthesis in the coinfected cell since newly synthesized terminal proteins should be free to associate with replicating DNA (Stillman and Bellet, 1979; Stillman, 1981) of both strains. It is more likely to be determined by a DNA sequence which confers greater efficiency of initiation of DNA synthesis, or multiple copies of a sequence which might allow multiple initiations. Either mechanism could result in a more frequently initiating, and thus more competitive, DNA molecule.

Multiple repeats of DNA sequences have been previously observed to confer interfering capacity in mammalian DNA viruses. In an SV40-like virus of human origin (DAR), defective virions containing a triplication of the sequence encompassing the origin of DNA replication evolve after a few passages at high multiplicity of infection, and these virions will interfere with the replication of normal virus (Fareed, Byrne and Martin, 1974; Khoury et al., 1974). In Herpes

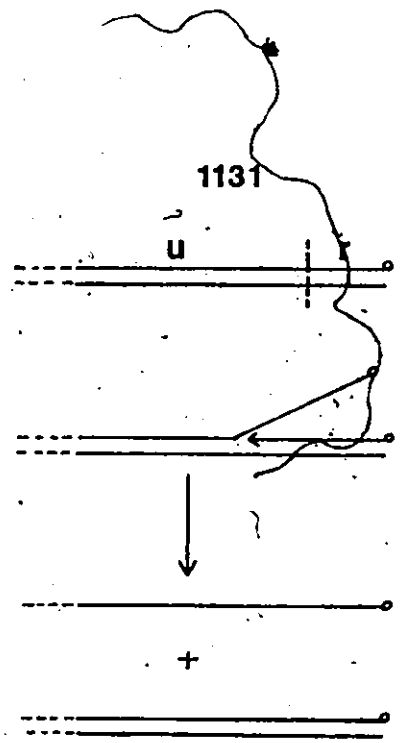
Simplex Virus Type 1, defective interfering virus evolves progressively through serial undiluted passaging of virus in Hey-2 cells, and these defectives have been shown to have acquired head to tail tandem repeats of sequences at the termini of the short segment of the genome (Frenkel et al., 1975; Lockner and Frenkel, 1979).

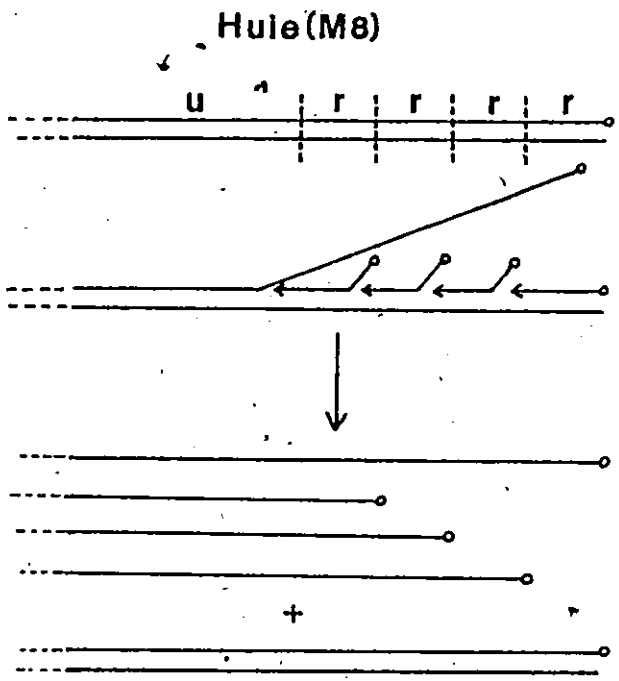
It was of interest to determine if such a mechanism is operating to confer the interference capacity to Ad12-Huie (M8). A clear difference between the genomes of Ad12-Huie (M8) and Ad12-1131 resides in the DNA sequence in the region of the right-end of the molecules. Several studies have suggested that initiation of adenovirus DNA synthesis occurs primarily at the right-end of the d.s. molecule, generating free r-strands, preferentially (Sussenbach, Ellens and Jansz, 1973; Ellens, Sussenbach and Jansz, 1974). Lechner and Kelly (1977) have also observed multibranched replicating molecules indicating that not only initiation at both ends of a single molecule, but multiple initiations at a given end of a parental molecule. Flint, Berget and Sharp (1976) has demonstrated that s.s. DNA molecules in excess of their complement are asymmetrically distributed with r-strands representing sequences from about 35 map units toward the right-end of the genome being more abundant than l-strands representing sequences from about 35 map units toward the left end of the genome. These results suggest that the interference capacity of Ad12-Huie (M8) may reside in the right end of the DNA molecule. A multiple repeated sequence containing a Bam Hl restriction site could also explain the apparent absence of the Bam Hl-E fragment equivalent

of Ad12-1131 in agarose gel-electrophoresis analysis if the fragments generated were very small. Figure 6-3 diagrams a model of proposed structures of the right end of Ad12-Huie (M8) and Ad12-1131 DNA molecules. The structure of the right end of Ad12-Huie (M8) DNA might result in more initiations of DNA synthesis on each parental DNA molecule of Ad12-Huie (M8) compared to Ad12-1131 (Fig. 6-3). Each l-strand could always generate more r-strands with Ad12-Huie (M8) than with Ad12-1131. In coinfected cells, Ad12-Huie (M8) could rapidly generate a greater number of templates and even more available DNA initiation sites than the coinfecting virus, progressively increasing its advantage.

This model of interference predicts that there might be greater amounts of single-stranded (s.s.) viral DNA in Ad12-Huie (M8) infected cells than in Ad12-1131 infected cells. Therefore, to test this model of interference the amount of s.s. DNA relative to the amount of d.s. DNA in a) total infected cell DNA and in b) replicating DNA molecules only, were determined in cells infected with Ad12-1131 or Ad12-Huie (M8) and compared. KB cells were infected at a multiplicity of 600 virions/cell with either Ad12-1131 or Ad12-Huie (M8) and labelled with (^{3}H)-thymidine (20 μCi/ml, 20 Ci/mM) from 23½ - 24½ h post-infection. Host DNA synthesis is almost completely inhibited by this time (Piña and Green, 1969). Viral DNA was then selectively extracted by the Hirt procedure (Hirt, 1967).

Figure 6-9 Model of synthesis of DNA at the right-ends of the genomes of Ad12-1131 and Ad12-Huie (M8). U = unique sequence and r = sequence containing recognition signal for initiation of DNA synthesis.


1131
u
r
+


Huie(M8)
u
r
r
r
r
+

To determine the percentage of the total radioactivity that is in free s.s. molecules, the DNA preparations were fractionated by hydroxylapatite (HAP) column chromatography. As seen in the elution profiles (Fig. 6-4), both preparations of DNA contained approximately the same proportion of the total radioactivity in s.s. molecules, 7.7% and 9.3% for Ad12-1131 and Ad12-Huie (M8) respectively. This does not appear to be a very significant difference as might be expected from the proposed model. To further analyze only the replicating molecules, the DNA eluted by 0.4M PO_4 from the HAP columns was then fractionated by benzoylated,napthoylated, DEAE (BND)-cellulose column chromatography (Fig. 6-5) into replicating molecules (partially s.s.) and d.s. molecules. It is seen that the two DNA preparations both consisted of a similar percentage of total radioactivity in replicating molecules, 73% and 76% for Ad12-1131 and Ad12-Huie (M8), respectively. The replicative intermediates were then digested with nuclease S1 to determine the percentage of the radioactivity in the replicating molecules represented as s.s. DNA. In both preparations of replicating molecules, there was no significant difference in the percentage [15.4% for Ad12-1131 and 15.8% for Ad12-Huie (M8)]of counts susceptible to the nuclease.

These results do not appear to support the predictions of the proposed model for the mechanism of interference by Ad12-Huie (M8). However, recent data of I. Mak (personal communication) show that Ad12-Huie (M8) DNA appears to consist of a heterogeneous population of molecules having variable numbers of repeats of a sequence at the right

Figure 6-4 Hydroxylapatite column chromatography of Hirt-extracted DNA from cells infected with Ad12-1131 and Ad12-Huie (M8). Infected cells were labelled with [^{3}H]-thymidine from 23.5 - 24.5 h, p.i. Cells infected with Ad12-1131 (△) and Ad12-Huie (M8) (○). Fractions to the left of the arrow (↓) were eluted with 0.04 M sodium phosphate, pH 6.8, (eluting s.s. DNA molecules), and to the right of the arrow with 0.4 mM sodium phosphate, pH 6.8, (eluting molecules containing d.s. DNA).

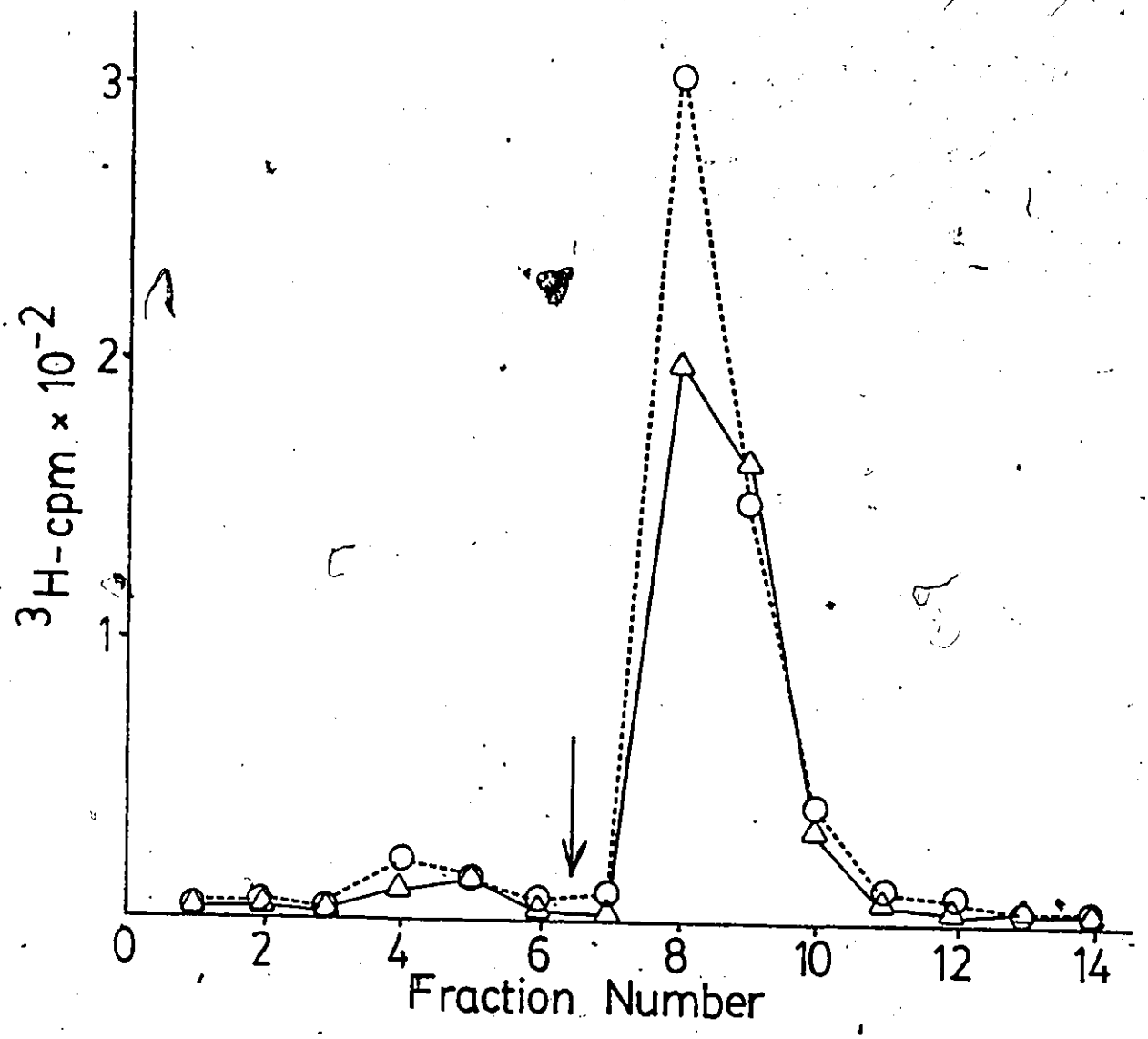
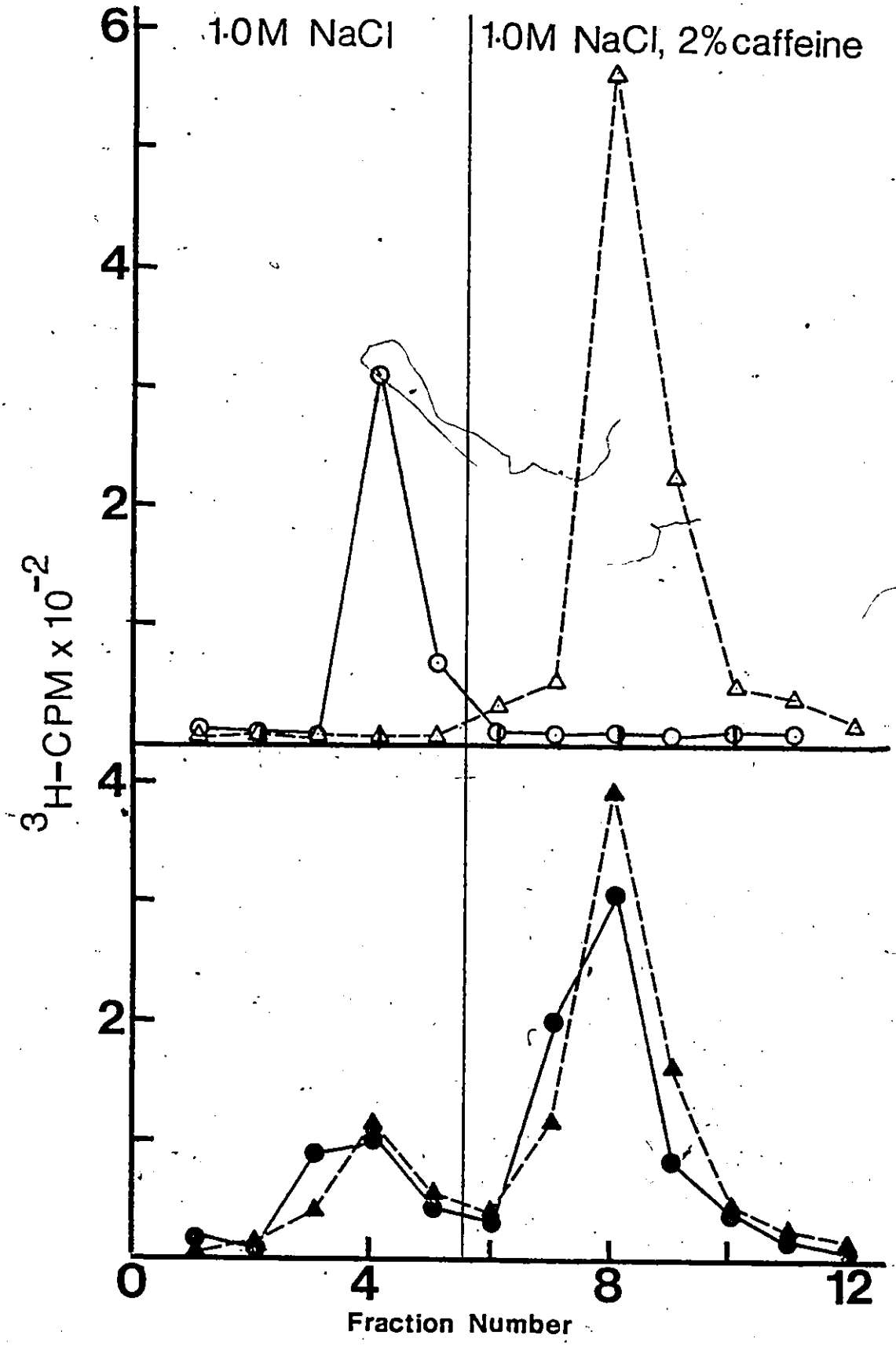

^{3}H-cpm × 10^{-2}
3
2
1
0
2
4
6
8
10
12
14
Fraction Number

Figure 6-5 BND cellulose column chromatography of double-stranded and partially double-stranded DNA from cells infected with Ad12-1131 (●) and Ad12-Huie (M8) (▲). (△) and (○) represent single-stranded and double-stranded DNA controls respectively.


1·0M NaCl
1·0M NaCl, 2% caffeine
^{3}H-CPM x 10^{-2}
6
4
2
4
2
0
4
8
12
Fraction Number

end of the molecules. Several plaques of Ad12-Huie (M8) passaged only twice in KB cells and their viral DNA analyzed with Bam HI produced discrete bands corresponding to the right end of the genome. These bands varied in length from 11 map units, that of the E fragment of Ad12-1131, to 16 map units in increments of 1 map unit. Hybridization of a (^{32}P)-labelled DNA probe containing the sequence of the inverted terminal repeat of Ad12 hybridized equally to the left terminal fragments of the DNA from the different plaques, but increasingly more to the right terminal fragments in proportion to the incremental increase in their lengths. This heterogeneity at the right end of the DNA is rapidly regenerated from a single plaque after only a few passages in KB cells.

Subsequent to this study, two reports were published (Kruczek, Schwarz and zur Hausen, 1981; Schwarz et al., 1982) which describe similar properties of mutants of Ad12-Huie derived from serial passaging in the human cervical carcinoma cell line C4/1 and melanoma cell line Nki. These mutants also have a growth advantage over wild type virus, and by DNA sequence analysis were shown to have at the right end of the genomes, variable repeats (1-6 copies) of units consisting of most of the inverted terminal repeat sequence plus short sequences from the left or right end of the genome. These variants are rapidly generated from single plaques similar to Ad12-Huie (M8). These mutants therefore, appear to be similar to the Ad12-Huie (M8) virus stocks in both physical and biological properties. It was noted (Schwarz et al., 1982) that a conserved sequence of 14 nucleotides from nucleotides 9

to 22 from the ends of the genomes of all human adenoviruses examined so far (Tolun, Alestrom and Petterson, 1979) which might serve as a signal for the initiation of viral DNA synthesis (Tolun, Alestrom and Petterson, 1979; Stillman, 1981), is always present in the repeat units.

This structure of having variable repeats of a specific sequence at the right end of the genome, if this sequence is involved in the initiation of viral DNA synthesis, is not very different from that proposed earlier in this study in terms of function and predicted consequences. The data in this study can be reconciled with either of the two models of multiple initiation sites for DNA synthesis at the right end of the Ad12-Huie (M8) DNA molecule if there exist limiting factors involved in the initiation of DNA synthesis which allow only a certain number of initiation events at any given time during infection. A multiple repeat of a recognition sequence involved in the initiation of DNA synthesis might compete effectively for these factors without necessarily increasing the frequency or number of initiation events. Also, the difference in s.s. DNA might only be detected very early after the onset of viral DNA synthesis since with increasing time, the large amount of r-strands acting as template for l-strand synthesis (which are indistinguishable between the two viruses) and the asynchrony of the replicating molecules might diminish the relative amounts and detectability of molecules of the structure described in Fig. 6-3.

Further competition studies with various plaque purified stocks of Ad12-Huie (M8) and a comparison of r and l strand distribution of s.s. DNA in cells infected with these viruses are therefore warranted.

CONCLUDING REMARKS

These studies identified and characterized functions of adenovirus which are defective in the cyt mutants of Ad12 and are involved in the control of DNA degradation in infected cells. However, there are several other properties of the cyt mutants which are different from those of the wild type Ad12. These include a more intense cytopathic effect (CPE) and early cell destruction, reduced viral DNA synthesis and virus yields, and reduced capacities for transformation and tumorigenesis. All these properties are believed to be affected by a single cistron based on complementation tests and analysis of revertants (Takemori, Riggs and Aldrich, 1969; Takemori, 1972).

With the finding that degradation of both viral and cellular DNA is induced by cyt mutants, it becomes evident how some of these properties could be pleiotropic effects of a single mutation. A mutation affecting a DNase-inhibitory or a DNase-effecter product could result in DNA degradation. The degradation of viral DNA would reduce the number of potential templates for synthesis and thus reduce the total amount of viral DNA synthesized, and both could result in a lower virus yield. The degradation of host DNA understandably could result in a more intense cytopathology and hasten the eventual death and destruction of the cell, and even affect plaque morphology.

The functional relationship between the induction of DNA degradation and the reduction in transforming and tumorigenic abilities

becomes less discernible, however , in light of other studies of I. Mak (pers. commun) in this laboratory on the latter two properties of several cyt mutants. First, the decrease in production of transformed cell colonies in the transformation assays on rat embryo kidney (REK) cells appears not to be due to a reduced survival of potential colony-forming cells. Comparison of cloning efficiencies of REK cells infected with cyt mutants and wild type Ad12 showed no significant differences. Secondly, H12 cyt 61, the only cyt mutant to transform cells efficiently to a level equal to or greater than that of the wild type Ad12, was the most potent cyt mutant at inducing DNA degradation. Thirdly, wild type Ad12 which transforms hamster embryo cells was found to induce extensive DNA degradation in these cells. These observations suggest that the strict ability to induce DNA degradation is not itself responsible for reduced transforming ability. Therefore, functions involved in control of DNA degradation and in transformation appear to be distinct, separable functions. However, since at least one DNA degradation function maps in region E1b, and the mRNA's encoded in this region share overlapping transcribed sequences, it is possible that more than one property can be affected in many cyt mutants.

Two viral functions involved in the control of DNA degradation in adenovirus infected cells were identified and characterized in these studies. One is a DNase-effecter function, apparently an early product which positively influences the induction of DNA degradation. The second is a DNase-inhibitory function residing in a region E1b encoded product. The two products appear from biological studies to interact with each other, and the relative abundance of the two

determines the occurrence of DNA degradation.

These functions were identified in Ad12 and Ad5, two very distantly related human adenoviruses belonging to subgroups A and C, respectively. A temperature-sensitive mutant of Ad2, H2ts 111, also induces DNA degradation in infected KB cells at the non-permissive temperature (D'Halluin et al., 1979). This suggests that these two functions are probably general properties of all adenoviruses.

It is interesting, therefore, to speculate on the possible development and importance of these two viral functions involved in the control of DNA degradation in adenovirus-infected cells. It has been shown in several virus-host systems that in many instances, there is a correlation between CPE, lysosomal enzyme release, and cell breakage (Defendi, 1962; Allison and Sandelin, 1963; De Duve, 1963; Wolff and Bubel, 1964; Halback, Koschel and Jungwirth, 1978). Evidence also suggests that in some cases the lysosomal enzyme release is a cellular response to the expression of specific viral protein(s) (Guskey, Smith and Wolff, 1970; Bablanian, Eggers and Tamm, 1965a,b). It is possible, therefore, that the degradation of DNA induced in certain adenoviruses infected cells is due to the release of lysosomal enzymes, which include acid DNase (Halbach, Koschel and Jungwirth, 1978), in response to a viral product, such as the DNase-effecter product of adenovirus identified in these studies. This suicidal response of the cell, probably to an indispensable viral product, might be considered a defense mechanism to attenuate virus replication and spread of infection in

intact animal. The virus in turn, in adapting to the host, would provide a second factor to negatively regulate the inducing activity of the first factor, at least for a period of time to allow sufficient virus replication after which cell death and lysis might be advantageous for propagation of infectious virus. This negative regulatory factor would correspond to the region E1b product of adenovirus identified in these studies.

REFERENCES

Aiello, L., R. Guilfoyl, K. Huebner and R. Weinmann. 1979. Adenovirus 5 DNA sequences present and RNA sequences transcribed in transformed human embryo kidney cells. Virology 94: 460-469.

Alestrom, P., G. Akusjarvi, M. Perricaudet, M. Matthews, D.F. Klessig and U. Pettersson. 1980. The gene for polypeptide IX of adenovirus type 2 and its unspliced mRNA. Cell 19: 671-681.

Allison, A.C. and K. Sandelin. 1963. Activation of lysosomal enzymes in virus-infected cells and its possible relationship to cytopathic effects. J. Exp. Med. 117: 879-887.

Arrand, J.R. and R.J. Roberts. 1979. The nucleotide sequences at the termini of adenovirus-2 DNA. J. Mol. Biol. 128: 577-594.

Bablanian, R., H.J. Eggers and I. Tamm. 1965a. Studies on the mechanism of poliovirus-induced cell damage. I. The relation between poliovirus-induced metabolic and morphological changes in cells. Virology 26: 100-113.

Bablanian, R., H.J. Eggers and I. Tamm. 1965b. Studies on the mechanism of poliovirus-induced cell damage. II. The relation between poliovirus growth and virus-induced morphological changes in cells. Virology 26: 114-121.

Beladi, I. 1972. Adenoviruses. In, "Strains of human viruses", (Ed. M. Majer and S.A. Plotkin) p. 1, Karger, Basel.

Berk, A.J., F. Lee, T. Harrison, J. Williams and P.A. Sharp. 1979. Pre-early adenovirus 5 gene product regulates synthesis of early viral messenger RNAs. Cell 17: 935-944.

Blanton, R.A. and T.H. Carter. 1979. Autoregulation of adenovirus type 5 early gene expression. III. Transcription studies in isolated nuclei. J. Virol. 29: 458-465.

Bonner, M. and R.A. Laskey. 1974. A film detection method for tritium-labelled proteins and nuclei acids in polyacrylamide gels. Eur. J. Bioch. 46: 83-88.

Bos, J.L., L.J. Polder, R. Bernards, P.I. Schrier, P.J. van den Elsen, A.J. van der Eb and H. van Ormondt. 1981. The 2.2 Kb E1b mRNA of human Ad12 and Ad5 codes for two tumor antigens starting at different AUG triplets. Cell 27: 121-131.

Branton, P.E., N.J. Lassam, J.F. Downey, S.P. Yee, F.L. Graham, S. Mak and S.T. Bayley. 1981. Protein kinase activity immunoprecipitated from adenovirus infected cells by sera from tumor-bearing hamsters. J. Virol. 37: 601-608.

Brawerman, G. 1976. Characteristics and significance of the polyadenylate sequence in mammalian messenger RNA. Progr. Nucl. Acid. Res. Mol. Biol. 17: 117-148.

Brusca, J.S. and G. Chinnadurai. 1981. Transforming genes among three different oncogenic subgroups of human adenoviruses have similar replicative functions. J. Virol. 39: 300-305.

Burlington, B.T. and W. Doerfler. 1972. An endonuclease in cells infected with adenovirus and associated with adenovirions. Virology 48: 1-13.

Cajean-Feroldi, C., Y. Chardonnet and J. Chantepie-Auray. 1977. Deoxyribonuclease activity associated with adenovirus 5 and 7. Eur. J. Biochem. 74: 457-462.

Carter, T.H. and R.A. Blanton. 1978a. Possible role of the 72,000-dalton DNA-binding protein in regulation of adenovirus type 5 early gene expression. J. Virol. 25: 664-674.

Carter, T.H. and R.A. Blanton. 1978b. Autoregulation of adenovirus type 5 early gene expression. II. Effect of temperature-sensitive early mutations on virus RNA accumulation. J. Virol. 28: 450-456.

Casto, B.C. 1973. Biologic parameters of adenovirus transformation. Prog. Exp. Tumor Res. 18: 166-198.

Challberg, M.D., S.V. Desiderio and T.J. Kelly. 1980. Adenovirus DNA replication in vitro: Characterizations of a protein covalently linked to nascent DNA strands. Proc. Nat. Acad. Sci. USA 77: 5105-5109.

Chardonnet, Y. and S. Dales. 1972. Early events in the interaction of adenoviruses with HeLa cells. III. Relationship between an ATPase activity in nuclear envelopes and transfer of core material: A hypothesis. Virology 48: 342-359.

Chow, L.T., T.R. Broker and J.B. Lewis. 1979. Complex splicing patterns of RNAs from the early regions of adenovirus 2. J. Mol. Biol. 134: 265-303.

Chow, L.T. and T.R. Broker. 1980. The elucidation of RNA splicing in the adenoviral system. In, "Gene Structure and Expression"

(ed. D.H. Dean, L.F. Johnson, P.C. Kimball and P.S. Perlman): 175-209.

Colby, W.W. and T. Shenk. 1981. Adenovirus type 5 virions can be assembled in vivo in the absence of detectable polypeptide IX. J. Virol. 39: 977-980.

Daniell, E. 1976. Genome structure of incomplete particles of adenovirus. J. Virol. 19: 685-708.

De Duve, C. 1963. The lysosome concept. In, "The lysosomes". CIBA Foundation Symp. (ed. A.V.S. de Reuck and M.P. Cameron). London: J. and A. Churchill Ltd.

Defendi, V. 1962. Cytopathology of virus infection. Fed. Proc. Amer. Soc. Exp. Biol. 21: 1113-1117.

D'Halluin, J.C., C. Allart, C. Cousin, P.A. Boulanger and G.R. Martin. 1979. Adenovirus early function required for protection of viral and cellular DNA. J. Virol. 32: 61-71.

Doerfler, W. 1969. Nonproductive infection of baby hamster kidney cells (BHK 21) with adenovirus type 12. Virology 38: 587-606.

Doerfler, W. 1970. Integration of the deoxyribase nucleic acid of adenovirus type 12 into the deoxyribonucleic acid of baby hamster kidney cells. J. Virol. 6: 652-666.

Ellens, D.J., J.S. Sussenbach and H.S. Jansz. 1974. Studies on the mechanism of replication of adenovirus DNA. III. Electron microscpy of replicating DNA. Virology 61: 427-442.

Enomoto, T., J.H. Lichy, J.E. Ikeda and J. Hurwitz. 1981. Adenovirus DNA replication in vitro: Purification of the terminal protein in a functional form. Proc. Natl. Acad. Sci. USA 78: 6779-6783.

Ensinger, M.J. and H.S. Ginsberg. 1972. Selection and preliminary characterization of temperature-sensitive mutants of type 5 adenovirus. J. Virol. 10: 328-339.

Esche, H., M.B. Mathews and J.B. Lewis. 1980. Proteins and messenger RNAs of the transforming region of wild-type and mutant adenoviruses. J. Mol. Biol. 142: 399-417.

Esche, H. and B. Siegmann. 1982. Expression of early viral gene products in adenovirus type 12-infected and transformed cells. J. Gen. Virol. 60: 99-113.

Everitt, E., B. Sundquist, U. Pettersson and L. Philipson. 1973. Structural proteins of adenoviruses. X. Isolation and topography of low molecular weight antigens from the virion of adenovirus type 2. Virology 52: 130-147.

Ezoe, H. 1976. Biological and molecular studies of a low oncogenic mutant of human adenovirus type 12. Ph.D. Thesis, McMaster University (Hamilton, Ontario).

Ezoe, H. and S. Mak. 1974. Comparative studies on functions of human adenovirus type 12 and its low oncogenic mutant virions. J. Virol. 14: 713-739.

Ezoe, H., R.B. LaFatt and S. Mak. 1981. Degradation of intracellular DNA in KB cells infected with cyt mutants of human adenovirus type 12. J. Virol. 40: 20-27.

Fareed, G.C., J.C. Byrne and M.A. Martin. 1974. Triplication of unique genetic segment in a simian virus 40 - like virus of human origin and evolution of new viral genomes. J. Mol. Biol. 87: 275-288.

Flint, S.J., S.M. Berget and P.A. Sharp. 1976. Characterization of single-stranded viral DNA sequences present during replication of adenovirus types 2 and 5. Cell 9: 559-571.

Flint, S.J., J. Sambrook, J. Williams and P.A. Sharp. 1976. Viral nucleic acid sequences in transformed cells. IV. A study of the sequences of adenovirus 5 DNA and RNA in four lines of adenovirus 5 transformed rodent cells using specific fragments of the viral genome. Virology 72: 456-470.

Fraser, N.W., J.R. Nevins, E. Ziff and J.E. Darnell. 1979. The major late adenovirus type 2 transcription unit: termination is downstream from the lost poly(A) site. J. Mol. Biol. 129: 634-656.

Frenkel, N., R.J. Jacob, R.W. Honess, G.S. Hayward, H. Locker and B. Roizman. 1975. Anatomy of Herpes Simplex Virus DNA III. Characterization of defective DNA molecules and biological properties of virus populations containing them. J. Virol. 16: 158-167.

Frost, E. and J. Williams. 1978. Mapping temperature-sensitive and host range mutations of adenovirus type 5 by marker rescue. Virology 91: 39-50.

Gallimore, P.H. 1973. Ph.D. Thesis, University of Birmingham, England.

Gallimore, P.H., P.A. Sharp and J. Sambrook. 1974. Viral DNA in transformed cells. I. A study of the sequences of adenovirus 2 in nine lines of transformed rat cells using specific fragments of the viral genome. J. Mol. Biol. 89: 49-72.

Galos, R., J. Williams, M.H. Binger and S.J. Flint. 1979. Location of additional early gene sequences in the adenoviral chromosome. Cell 17: 945-956.

Galos, R.S., J. Williams, T. Shenk and N. Jones. 1980. Physical location of host range mutations of Ad5; deletion and marker-rescue mapping. Virology 104: 510-513.

Geldeblum, H., H. Bauer, H. Krank and R. Wigand. 1967. The structure of group II adenoviruses. J. Gen. Virol. 1: 553-560.

Ginsberg, H.S., H.G. Pereira, R.C. Valentine and W.C. Wilcox. 1966. A proposed terminology for the adenovirus antigens and virion morphological subunits. Virology 28: 782-783.

Ginsberg, H.S., L.J. Bello and A.J. Levine. 1967. Control of biosynthesis of host macromolecules in cells infected with adenovirus. In, "The molecular biology of viruses" (ed. J. S. Colter and W. Parachych), p. 547. Academic Press, New York.

Ginsberg, H.S. and C.S.H. Young. 1975. Genetics of adenoviruses. In, "Comprehensive Virology" (ed. H. Fraenkel-Conrat and R.R. Wagner), vol. 9, p. 91-130, Plenum Press, New York.

Girvitz, S.G., S. Bacchetti, A.J. Rainbow and F.L. Graham. 1980. A rapid and efficient procedure for the purification of DNA from agarose gels. Analytical Biochemistry 106: 492-496.

Graham, F.L., P.J. Abrahams, C. Mulder, H.L. Heijneker, S.O. Warnaar, F.A.J. deVries, W. Fiers and A.J. van der Eb. 1975. Studies on in vitro transformation by DNA and DNA fragments of human adenovirus and SV40. Cold Spring Harbor Symp. Quant. Biol. 39: 637-650.

Graham, F.L., J. Smiley, W.C. Russell and R. Nairn. 1977. Characteristics of a human cell line transformed by DNA from human adenovirus 5. J. Gen. Virol. 36: 59-72.

Graham, F.L., T. Harrison and J. Williams. 1978. Defective transforming capacity of adenovirus 5 host-range mutants. Virology 86: 10-21.

Green, M. and M. Piña. 1963. Biochemical studies on adenovirus multiplication. IV. Isolation, purification and chemical analysis of adenovirus. Virology 20: 199-207.

Green, M., M. Piña and V. Chagoya. 1964. Biochemical studies on adenovirus multiplication. V. Enzymes of DNA synthesis in cells infected by adenovirus and vaccinia virus. J. Biol. Chem. 239: 1188-1197.

Green, M., M. Piña, R. Kimes, P.C. Wensink, L.A. Machattie and C.A. Thomas. 1967. Adenovirus DNA. I. Molecular weight and conformation. Proc. Natl. Acad. Sci. 57: 1302-1309.

Green, M., J.T. Parsons, M. Piña, K. Fugimaza, H. Caffier and M. Landgraph-Leurs. 1971. Transcription of adenovirus genes in productively infected and in transformed cells. Cold Spring Harbor Symp. Quant. Biol. 35: 803-818.

Grodzicker, T., C.W. Anderson, J. Sambrook and M.B. Mathews. 1977. The physical location of structural genes in adenovirus DNA. Virology 80: 111-126.

Guskey, L.E., P.C. Smith and D.A. Wolff. 1970. Patterns of cytopathology and lysosomal enzyme release in poliovirus infected Hep-2 cells treated with either 2-(α-hydroxybenzyl)-benzimidazole or quanidine HCl. J. Gen. Virol. 6: 151-161.

Hallbach, M., K. Koschel and C. Jungwirth. 1978. Interferon enhances the fragility of lysosomes in L-929 mouse fibroblasts. J. Gen. Virol. 39: 387-390.

Halbert, D.N., D.J. Spector and H.J. Raskas. 1979. In vitro translation products specified by the transforming region of adenovirus type 2. J. Virol. 31: 621-629.

Harrison, T., F.L. Graham and J. Williams. 1977. Host-range mutants of adenovirus type 5 defective for growth in HeLa cells. Virology 77: 319-329.

Harrison, T.J. and J.F. Williams. 1978. A host range mutant of human adenovirus type 5 defective for growth in hamster cells. J. Gen. Virol. 40: 219-223.

Hilleman, M.R. 1957. Epidemiology of adenovirus respiratory infections in military recruit populations. Ann. N.Y. Acad. Sci. 67: 262-272.

Hirt, B. 1967. Selective extraction of polyoma DNA from infected mouse cell cultures. J. Mol. Biol. 26: 365-369.

Hope-Simpson, R.E. and P.G. Higgins. 1969. A respiratory virus study in Great Britain: Review and evaluation. Prog. Med. Virol. 11: 354-407.

Jochemsen, H., G.S.G. Daniels, J.H. Lupker and A.J. van der Eb. 1980. Identification and mapping of the early gene products of adenovirus type 12. Virology 105: 551-563.

Jones, N. and T. Shenk. 1978. Isolation of deletion and substitution mutants of adenovirus type 5. Cell 13: 181-188.

Jones, N. and T. Shenk. 1979a. Isolation of adenovirus type 5 host range deletion mutants defective for transformation of rat embryo cells. Cell 17: 683-689.

Jones, N. and T. Shenk. 1979b. An adenovirus type 5 early gene function regulates expression of other early viral genes. Proc. Natl. Acad. Sci. USA 76: 3665-3669.

Kathmann, P., J. Schick, E.L. Winnacker and W. Doerfler. 1976. Isolation and characterization of temperature-sensitive mutants of adenovirus type 2. J. Virol. 19: 43-53.

Keller, E.W., R.H. Rubin, T.H. Black, M.S. Hirsch and J.C. Hierholzer. 1977. Isolation of adenovirus type 34 from a renal transplant recipient with intestinal pneumonia. Transplantation 23: 188-191.

Khoury, G., G.C. Fareed, K. Berry, M.A. Martin, T.H. Lee and D. Nathans. 1974. Characterization of a rearrangement in viral DNA: mapping of the circular simian virus 40-like DNA containing a triplication of a specific one-third of the genome. J. Mol. Biol. 87: 289-301.

Kruczek, I., E. Schwarz and H. zur Hausen. 1981. Mutants of adenovirus

type 12 after adaptation to growth in tumor cell lines. II. Reproducible acquisition of additional sequences after adaptation to a human cervical cancer line. Int. J. Cancer 27: 139-143.

Laskey, R.A. and A.D. Mills. 1975. Quantitative film detection of ^{3}H and ^{14}C in polyacrylamide gels by fluorography. Eur. J. Biochem. 56: 335-341.

Lassam, N.J., S.T. Bayley and F.L. Graham. 1978. Synthesis of DNA, late polypeptides and infectious virus by host-range mutants of Ad5 in non-permissive cells. Virology 87: 463-467.

Lassam, N.J., S.T. Bayley and F.L. Graham. 1979. Tumor antigens of human Ad5 in transformed cells and in cells infected with transformation-defective host-range mutants. Cell 18: 781-791.

Laver, W.G., H.G. Pereira, W.C. Russell and R.C. Valentine. 1968. Isolation of an internal component from adenovirus type 5. J. Mol. Biol. 37: 379-386.

Laver, W.G. 1970. Isolation of an arginine-rich protein from particles of adenovirus type 2. Virology 41: 488-500.

Lawrence, W.C. and H.S. Ginsberg. 1967. Intracellular uncoating of type 5 adenovirus DNA. J. Virol. 1: 851-867.

Lechner, R.L. and T.J. Kelly. 1977. The structure of replicating adenovirus 2 DNA molecules. Cell 12: 1007-1020.

Lehrach, H., D. Diamond, J.M. Wozney and H. Boedtker. 1977. RNA molecular weight determinations by gel electrophoresis under denaturing conditions, a critical re-examination. Biochemistry 16: 4743-4751.

Levine, A.J., P.C. van der Vliet, B. Rosenwirth, J. Rabek, G. Frenkel and M. Ensinger. 1975. Adenovirus-infected, cell-specific DNA-binding proteins. Cold Spring Harbor Symp. Quant. Biol. 39: 559-566.

Lewis, A.M., A.S. Rabson and A.J. Levine. 1974. Studies on non-defective Ad2-SV40 hybrid viruses: Transformation of hamster kidney cells by adenovirus 2 and the non-defective hybrid viruses. J. Virol. 13: 1291-1301.

Lewis, J.B., J.F. Atkins, P.R. Baum, R. Solem, R.F. Gesteland and C.W. Anderson. 1976. Location and identification of the genes for adenovirus type 2 early polypeptides. Cell 7: 141-151.

Lewis, J.B. and M.B. Mathews. 1980. Control of adenovirus early gene expression: a class of immediate early products. Cell 21: 303-313.

Lewis, J.B., B.W. Stillman, L.T. Chow, M.B. Mathews and J.E. Smart. 1981. Identification of the gene and mRNA for the adenovirus terminal protein precursor. Cell 23: 497-508.

Lichy, J.H., M.S. Horwitz and J. Hurwitz. 1981. Formation of a covalent complex between the 80,000-dalton adenovirus terminal protein and 5'-dCMP in vitro. Proc. Nat. Acad. Sci. USA 78: 2678-2682.

Lockner, H. and N. Frenkel. 1979. Structure and origin of defective genomes contained in serially passaged Herpes Simplex Virus Type 1 (Justin). J. Virol. 29: 1065-1077.

Lonberg-Holm, K. and L. Philipson. 1969. Early events of virus-cell interactions in an adenovirus system. J. Virol. 4: 323-338.

Mak, I., and S. Mak. 1983. Transformation of rat cells by cyt mutants of Ad12 and by mutants of Ad5. J. Virology; in press.

Mak, S. 1969. Transcription and replication of viral deoxyribonucleic acid in cells coinfected with adenovirus type 2 and 12. J. Virol. 4: 651-656.

Marusyk, R.G., A.R. Morgan and G. Wadell. 1975. Association of endonuclease activity with serotypes belonging to the three subgroups of human adenoviruses. J. Virol. 16: 456-458.

Minekawa, Y., M. Ishibashi, H. Yause and M. Takahashi. 1976. Characterization of host-range and temperature-sensitive mutants of adenovirus type 5 with particular regard to transformation of a hamster embryo cell line (Nil). Virology 71: 97-110.

Morgan, G., H.S. Rosenkranz and B. Medris. 1969. Structure and development of viruses as observed in the electron microscope. X. Entry and uncoating of adenovirus. J. Virol. 4: 777-796.

Muench, K.H. 1971. Fractionation of transfer ribonucleic acid on columns of hydroxylapatite. In, "Procedures in Nucleic Acid Research" Vol. 2: 515-523, Harper and Row, Publishers.

Nevins, J.R., H.S. Ginsberg, J.M. Blanchard, M.C. Wilson and J.E. Darnell. 1979. Regulation of the primary expression of the early adenovirus transcription units. J. Virol. 32: 727-733.

Nevins, J.R. and J.J. Winkler. 1980. Regulation of early adenovirus transcription: A protein product of early region 2 specifically represses region 4 transcription. Proc. Natl. Acad. Sci. USA 77: 1893-1897.

Nevins, J.R. and M.C. Wilson. 1981. Regulation of adenovirus-2 gene expression at the level of transcriptional termination and RNA processing. Nature 290: 113-118.

Norrby, E. 1966. The relationship between soluble antigens and the virion of adenovirus type 3. I. Morphological characteristics. Virology 28: 236-248.

Ortin, J., K.H. Scheidtmann, R. Greenberg, M. Westphal and W. Doerfler. 1976. Transcription of the genome of adenovirus type 12. III. Maps of stable RNA from productively infected human cells and abortively infected and transformed hamster cells. J. Virol. 20: 355-372.

Padmanabhan, R. and M. Green. 1976. Evidence for palindromic sequences near the terminal of adenovirus.2 DNA. Biochem. Biophys. Res. Commun. 69: 860-867.

Padmanabhan, R., S. Stabel, U. Winterhoff and W. Doerfler. 1979. Specificity and mode of cleavage of pH 4.0 endonuclease from adenovirus type 2-infected KB cells. Nuc. Acids Res. 6; 3161-3176.

Parrott, R.H. 1960. Adenovirus and parainfluenza virus infections. Pediatr. Clin. N. Am. 7: 965.

Pereira, H.G., R.J. Huebner, H.S. Ginsberg and J. van der Veen. 1963. A short description of the adenovirus group. Virology 20: 613-620.

Pereira, M.S. and F.O. MacCallum. 1964. Infection with adenovirus type 12. Lancet 1: 198-199.

Perricaudet, M., G. Akusjärvi, M. Virtanen and U. Pettersson. 1979. Structure of two spliced mRNAs from the transforming region of human subgroup C adenoviruses. Nature 281: 694-696.

Perricaudet, M., J.M. le Moullec and P. Tiollais. 1980. Structure of two adenovirus type 12 transforming polypeptides and their evolutionary implications. Nature 288: 174-176.

Persson, H., H.J. Monstein, G. Akusjärvi and L. Philipson. 1981. Adenovirus early gene products may control viral mRNA accumulation and translation in vivo. Cell 23: 485-496.

Pettersson, U., L. Philipson and S. Höglund. 1968. Structural proteins of adenoviruses. II. Purification and characterization of adenovirus type 2 hexon fibre antigen. Virology 35: 204-215.

Pettersson, W. and M.B. Mathews. 1977. The gene and messenger RNA for adenovirus polypeptide IX. Cell 12: 741-750.

Pettersson, U. and G. Akusjärvi. Molecular biology of adenovirus transformation. Private communication.

Philipson, L., K. Lonberg-Holm and U. Pettersson. 1968. Virus-receptor interaction in an adenovirus system. J. Virol. 2: 1064-1075.

Piña, M. and M. Green. 1968. Base composition of the DNA of oncogenic simian adenovirus SA7 and homology with human adenovirus DNA's. Virology 36: 321-323.

Piña, M. and M. Green. 1969. Biochemical studies on adenovirus multiplication. XIV. Macromolecule and enzyme synthesis in cells replicating oncogenic and non-oncogenic human adenoviruses. Virology 38: 573-586.

Pincus, S., W. Robertson and D. Rekosh. 1981. Characterization of the effect of aphidiocolin on adenovirus DNA replication: evidence in support of a protein primer model of initiation. Nucl. Acids Res. 9: 4919-4938.

Prage, L., U. Pettersson and L. Philipson. 1968. Internal base proteins in adenovirus. Virology 36: 508-511.

Prage, L., U. Pettersson, S. Höglund, K. Lonberg-Holm and L. Philipson. 1970. Structural proteins of adenoviruses. IV. Sequential degradation of the adenovirus type 2 virion. Virology 42: 341-358.

Prage, L. and U. Pettersson. 1971. Structural proteins of adenoviruses. VII. Purification and properties of an arginine-rich core protein from adenovirus type 2 and type 3. Virology 45: 364-

Price, R. and S. Penman. 1972a. Transcription of the adenovirus genome by an α-amanitin sensitive RNA polymerase in HeLa cells. J. Virol. 9: 621-626.

Price, R. and S. Penman. 1972b. A distinct RNA polymerase activity synthesizing 5.5 S and 4S RNA in nuclei isolated from adenovirus 2-infected HeLa cells. J. Mol. Biol. 70: 435-450.

Raska, K. and W. Strohl. 1972. The response of BHK 21 cells to infection with type 21 adenovirus. VI. Synthesis of virus-specific RNA. Virology 47: 734-742.

Redfield, R.J. 1980. Master of Science Thesis. McMaster University, Hamilton, Ontario, Canada.

Reif, U., U. Winterhoff and W. Doerfler. 1977. Characterization of the pH 4.0 endonuclease from adenovirus type 2-infected KB cells. Eur. J. Biochem. 73: 327-333.

Rekosh, D.M., W.C. Russell, A.J.D. Bellett and A.J. Robinson. 1977. Identification of a protein covalently linked to the ends of adenovirus DNA. Cell 11: 283-295.

Richardson, W.D. and H. Westphal. 1981. A cascade of adenovirus early functions is required for expression of adeno-associated virus. Cell 27: 133-141.

Ross, S.R., S.J. Flint and A.J. Levine. 1980. Identification of the adenovirus early proteins and their genomic map positions. Virology 100: 419-432.

Ross, S.R., A.J. Levine, R.S. Galos, J. Williams and T. Shenk. 1980. Early viral proteins in HeLa cells infected with adenovirus type 5 host range mutants. Virology 103: 475-492.

Rowe, D.T. and F.L. Graham. 1981. Complementation of adenovirus type 5 host range mutants by adenovirus type 12 in coinfected HeLa and BHK-21 cells. J. Virol. 38: 191-197.

Russell, W.C., K. McIntosh and J.J. Skehel. 1971. The preparation and properties of adenovirus cores. J. Gen. Virol. 11: 35-46.

Sambrook, J., M. Sleigh, J.A. Engler and T.R. Broker. 1980. The evolution of the adenoviral genome. Annals New York Academy of Sciences 80: 426-452.

Sawada, Y. and K. Fujinaga. 1980. Mapping of adenovirus 12 mRNAs transcribed from the transforming region. J. Virol. 36: 639-651.

Sawicki, S.G., W. Jelinek and J.E. Darnell. 1977. 3'-Terminal addition to HeLa cell nuclear and cytoplasmic poly(A). J. Mol. Biol. 113: 219-235.

Schwarz, E., C. Reinke, N. Yamamoto and H. zur Hausen. 1982. Terminal rearrangements in the genome of adenovirus type 12 mutants adapted to growth in two human tumor cell lines. Virology 116: 284-296.

Sharp, P.A., U. Pettersson and J. Sambrook. 1974. Viral DNA in transformed cells. 1. A study of the sequences of adenovirus 2 DNA in a line of transformed rat cells using specific fragments of the viral genome. J. Mol. Biol. 86: 709-726.

Shenk, T., N. Jones, W. Colby and D. Fowlkes. 1980. Functional analysis of adenovirus type 5 host range deletion mutants defective for transformation of rat embryo cells. Cold Spring Harbor Symp. Quant. Biol. 44: 367-376.

Shimojo, H. and T. Yamashita. 1968. Induction of DNA synthesis by adenoviruses in contact inhibited hamster cells. Virology 36: 422-433.

Shiroki, K. and H. Shimojo. 1974. Analysis of adenovirus 12 temperature-sensitive mutants defective in viral DNA replication. Virology 61: 474-485.

Shiroki, K., K. Maruyama, I. Saito, Y. Fukui and H. Shimojo. 1981. Incomplete transformation of rat cells by a deletion mutant of adenovirus type 5. J. Virol. 38: 1048-1054.

Smiley, J.R. and S. Mak. 1978. Transcription map of adenovirus type 12 DNA. J. Virol. 28: 227-239.

Söderlund, J., U. Pettersson, B. Vennström, L. Philipson and M.B. Mathews. 1976. A new species of virus coded low molecular weight RNA from cells infected with adenovirus type 2. Cell 7: 585-593.

Spector, D.J., D.N. Halbert and H.J. Raskas. 1980. Regulation of integrated adenovirus sequences during adenovirus infection of transformed cells. J. Virol. 36: 860-871.

Stadler, H., J.C. Hierholzer and M.N. Oxman. 1977. New human adenovirus (candidate adenovirus type 35) causing fatal disseminated infection in a renal transplant patient. J. Clin. Microbiol. 6: 257-265.

Steenbergh, P.H., J. Maat, H. van Ormondt and J.S. Sussenbach. 1977. The nucleotide sequence at the terminus of adenovirus type 5 DNA. Nucl. Acids Res. 4: 4371-4389.

Stich, H.F. and D.S. Yohn. 1970. Viruses and chromosomes. Prog. Med. Virol. 12: 78-127.

Stich, H.F. 1973. Oncogenic and non-oncogenic mutants of adenovirus type 12: Induction of chromosome aberrations and cell divison. Prof. Exp. Tumor Res. 18: 260-272.

Stillman, B.W. and A.J.D. Bellet. 1979. Adenovirus protein associated with the ends of replicating DNA molecules. Virology 93: 69-79.

Stillman, B.W. 1981. Adenovirus DNA replication *in vitro*: a protein linked to the 5'-end of nascent DNA strands. J. Virol. 37: 139-147.

Stillman, B.W., J.B. Lewis, L.T. Chow, M.B. Mathews and J.E. Smart. 1981. Identification of the gene and mRNA for the adenovirus terminal protein precursor. Cell 23: 497-508.

Strohl, W.A. 1969a. The response of BHK21 cells to infection with adenovirus 12. I. Cell-killing and T antigen synthesis as correlated viral genome functions. Virology 39: 642-652.

Strohl, W.A. 1969b. The response of BHK21 cells to infection with adenovirus 12. II. The relationship of virus-stimulated DNA synthesis to other viral functions. Virology 39: 653-665.

Sussenbach, J.S., D.J. Ellens and H.J. Jansz. 1973. Studies on the mechanism of replication of adenovirus DNA II: the nature of single-stranded DNA in replicative intermediates. J. Virol. 12: 1131-1138.

Takahashi, M. 1972. Isolation of conditional lethal mutants (temperature-sensitive and host-range mutants) of adenovirus type 5. Virology 49: 815-817.

Takemori, N., J.L. Riggs and C. Aldrich. 1968. Genetic studies with tumorigenic adenoviruses. I. Isolation of cytocidal (cyt) mutants of adenovirus type 12. Virology 36: 575-586.

Takemori, N., J.L. Riggs and C.D. Aldrich. 1969. Genetic studies of tumorigenic adenoviruses. II. Heterogeneity of cyt mutants of adenovirus type 12. Virology 38: 8-15.

Takemori, N. 1972. Genetic studies with tumorigenic adenoviruses. III. Recombination in adenovirus type 12. Virology 47: 157-167.

Thomas, G.P. and M.B. Mathews. 1980. DNA replication and the early to late transition in adenovirus infection. Cell 22: 523-533.

Tolun, AL., P. Alestrom and U. Pettersson. 1978. Sequence analysis of adenovirus DNA. V. Nucleotide sequence of inverted terminal repetition from different adenoviruses: demonstration of conserved sequences and homology between simian adenovirus 7 and SV40 DNA. Cell 17: 705-713.

Trentin, J.J., Y. Yabe and G. Taylor. 1962. The quest for human cancer viruses. Science 137: 835-841.

Tsang, L.W.L. and R.G. Marusyk. 1980. Purification and partial characterization of a 33,000 molecular weight endonuclease associated with human adenovirus type 5. Can. J. Microbiol. 26: 1224-1231.

Tsuro, T., M. Orens, R. Padmanabhan and M. Green. 1978. On endodeoxyribonuclease of human KB cells. J. Biol. Chem. 253: 3400-3407.

Valentine, R.C. and H.G. Pereira. 1965. Antigens and the structure of the adenovirus. J. Mol. Biol. 13: 13-20.

van der Eb, A.J. and L.W. van Kesteren. 1966. Structure and molecular weight of the DNA of adenovirus type 5. Biochim. Biophys. Acta 182: 530-541.

van der Vliet, P.C. and A.J. Levine. 1973. DNA-binding proteins specific for cells infected by adenovirus. Nat. New Biol. 246: 170-174.

van der Vliet, P.C., A.J. Levine, M. Ensinger and H.S. Ginsberg. 1975. Thermolabile DNA-binding proteins from cells infected

with a temperature-sensitive mutant of adenovirus defective in viral DNA synthesis. J. Virol. 15: 348-354.

van der Vliet, P.C. and J.S. Sussenbach. 1975. An adenovirus type 5 gene function required for initiation of viral DNA replication. Virology 67: 415-426.

van der Vliet, P.C., J. Landberg and H.S. Jansz. 1977. Evidence for a function of the adenovirus DNA-binding protein in initiation of DNA synthesis as well as in elongation of nascent DNA chains. Virology 80: 98-110.

Wahl, G.M., M. Stern and G.R. Stark. 1979. Efficient transfer of large DNA fragments from agarose gels to diazolbenzyloxymethyl-paper and rapid hybridization by using dextran sulphate. Proc. Natl. Acad. Sci. USA 76: 3683-3687.

Wallace, R.D. and J. Kates. 1972. State of adenovirus 2 DNA in the nucleus and its mode of transcription: Studies with isolated viral DNA-protein complexes and isolated nuclei. J. Virol. 9: 627-635.

Warnaar, S.O. and J.A. Cohen. 1966. A quantitative assay for DNA-DNA hybrids using membrane filters. Biochem. Biophys. Res. Commun. 24: 554-558.

Weinmann, R., H.J. Raskas and R.G. Roeder. 1974. Role of DNA-dependent RNA polymerases II and III in transcription of the adenovirus genome late in productive infection. Proc. Natl. Acad. Sci. 71: 3426-3430.

Weinmann, R., T.G. Brendler, H.J. Raskas and R.G. Roeder. 1976. Low molecular weight viral RNAs transcribed by RNA polymerase III during adenovirus 2 infection. Cell 7: 557-556.

Weinstock, R., R. Sweet, M. Weiss, H. Cedar and R. Axel. 1978. Intragenic spacers interrupt the ovalbumin gene. Proc. Natl. Acad. Sci. USA 75: 1299-1303.

Wilkie, N.M., S. Ustacelebi and J.F. Williams. 1973. Characterization of temperature-sensitive mutants of adenovirus type 5: Nucleic acid synthesis. Virology 51: 499-503.

Williams, J.F. 1973. Oncogenic transformation of hamster embryo cells in vitro by adenovirus type 5. Nature 243: 162-163.

Williams, J.F., H. Young and P. Austin. 1975. Genetic analysis of human adenovirus type 5 in permissive and non-permissive cells. Cold Spring Harbor Symp. Quant. Biol. 39: 427-437.

Williams, J., U. Ho and R. Galas. 1981. Evidence for functional relatedness of products encoded by the transforming sequences of human adenovirus types 5 and 12. Virology 110: 208-212.

Wincour, E. 1963. Purification of polyoma virus. Virology 19: 158-168.

Wolff, D.A. and J.C. Bubel. 1964. The disposition of lysosomal enzymes as related to specific viral cytopathic effect. Virology 24: 502-505.

Ziff, E.B. 1980. Transcription and RNA processing by the DNA tumor viruses. Nature 287: 491-499.

zur Hausen, H. 1967. Induction of specific chromosomal aberrations by adenovirus type 12 in human embryonic kidney cells. J. Virol. 1: 1174-1185.

zur Hausen, H. 1968. Chromosomal aberrations and cloning efficiency in adenovirus 12 infected hamster cells. J. Virol. 2: 915-924.

zur Hausen, H. 1973. Interactions of adenovirus type 12 with host cell chromosomes. Prog. Exp. Tumor Res. 18: 240-259.