STUDIES ON ADENOVIRUS HETEROTYPIC RECOMBINATION AND CHARACTERISATION OF A SUBGROUP B SPONTANEOUS MUTANT

by

IRIS MURIEL MCDOUGALL BSc

A Thesis presented for the degree of

Master of Science

in

The Faculty of Science

at

The University of Glasgow

Institute of Virology
Church Street
GLASGOW
G11 5JR

February, 1988.

TABLE OF CONTENTS

Acknowledgements

Abbreviations

Summary

CHAPTER 1. INTRODUCTION Page

A. General 1-2

B. Classification of adenovirus

1. Haemagglutination 3
2. Tumourgenicity 3
3. Cellular transformation 4
4. The percentage G+C DNA content 4
5. DNA homology 4-5

C. Morphology of the adenoviruses

1. The capsid 6
   (a) the hexon 6
   (b) penton base 7
   (c) the fibre 7
   (d) polypeptides VI, VIII, IIIa and IX 7-8
2. Core shell 8
3. Core (Nucleoprotein) 8

Page

D. Adenoviral DNA structure and replication

1. Structure 9

2. Replication of DNA 9

(a) The inverted terminal repeat (ITR) 9-10

(b) Terminal protein 10-11

E. Adenoviral DNA infectivity 12-13

F. The lytic cycle of adenovirus in host cells

1. Viral adsorption, penetration and uncoating 14

2. Transcription 15

(I) Early transcription 15

(I)(a) The E1a region (1.3-4.6) m.u. 15-16

(I)(b) The E1b region (4.6-11.2) m.u. 16

(I)(c) The E2a region (61.5-75.1) m.u. 17

(I)(d) The E2b region (11-30) m.u. 17-18

(I)(e) The E3 region (76.6-86.0) m.u. 18-19

(I)(f) The E4 region (91.3-99.1) m.u. 19-20

(I)(g) Virus-associated RNAs 20

(II) Late transcription 20-22

G. Adenovirus mutants

1. Mutagens 23

1(I) *In vitro* 23

1(II) *In vivo* 23

2. Spontaneous mutants 24

3. Cytocidal mutants (*Cyt*) 25-26

4. Temperature sensitive mutants 26-28

5. Host range mutants 28-29

Page

6. The E1a region in cellular transformation 30-31

7. The E1b region in cellular transformation 31-32

8. Deletion and substitution mutants 32-33

9. Large plaque phenotype mutants 33-34

H. Adenoviral recombination

Introduction 35

1. Recombination between full length adenoviral genomes 35-36

2. Recombination by marker rescue 36-37

3. Recombination between overlapping terminal DNA fragments 38

I. Aims of the thesis 39

CHAPTER 2. MATERIALS AND METHODS

A. **Materials**

1. Cell lines 40

(a) 293 cells

(b) Hela cells

(c) KB cells

2. Tissue culture medium 40-41

3. Viruses 41-42

4. Plasmid vector pUC8 43

5. Host bacteria for plasmid propagation 43

6. pARKR 43-44

7. Versine 44

8. Giemsa stain 44

9. "Gelvatol" mounting fluid 44

10. Bisbenzimide H33258 flurochrome stain 45

		Page
11.	Radioisotopes	45
12.	Reagents	45-46
13.	Buffers and solutions	47-50

B. **Methods**

1.	Tissue culture	51
2.	Mycoplasma screening	51
3.	Preparation of viral stocks	51-52
4.	Crude seed stocks	52
5.	Preparation of purified virions	52-53
6.	Viral plaque assay	53-54
7.	Rapid assay technique	54
8.	One step growth curves	54
9.	Preparation of viral DNA	55
10.	Preparation of viral DNA-protein complex	55
11.	Preparation of radioactive labelled viral DNA	56
12.	Extraction of radioactive labelled viral DNA from infected cells	56
13.	Endonuclease analysis of radioactive labelled DNA	56
14.	Nick translation of DNA	57
15.	Elution of DNA from agarose	57
16.	Transfection of viral DNA into 293 or Hela cells	58-59
17.	Isolation of a large plaque fast growing virus	60
18.	Construction of adenovirus 3 wild type BamH1 clones	60
	(i) Spermine precipitation	60
	(ii) Preparation of plasmid vector	61
	(iii) Transformation of host bacteria JM83 to ampicillin resistance	61-62

Page

(iv) Isolation of adenovirus 3 wild type BamH1 clones 62

(v) Rapid isolation of plasmid DNA 63

19. Large scale isolation of plasmid DNA 63-64

20. Cloning of the terminal fragment of adenovirus 3 wild type for construction of plasmid p3A1 64-65

(i) Preparation of pUC8 vector 65

(ii) Transformation of host bacteria DHI to ampicillin resistance 66

(iii) Isolation of positive colonies 66

21. Southern transfer 66-67

22. Extraction of nuclei from viral infected cells 67-68

23. Virion heat stability 68

CHAPTER 3. RESULTS

Section A.

1. Introduction 69-71

2. One step growth curves of adenovirus 7 and 5 wild type viruses 72

3. Co-transfection of overlapping terminal DNA fragments of adenovirus 7 wild type and adenovirus 5 wild type into 293 cells 72-73

4. Analysis of plaque isolates from co-transfection of DNA terminal fragments of adenoviruses 5 and 7 wild type into 293 cells 74-77

5. Infectivity of DNA-protein complexes of adenovirus 7 wild type and adenovirus 5 wild type 78-79

Page

6. BglII restriction enzyme map of adenovirus 7 wild type 79-80

7. Co-transfection of overlapping terminal DNA fragments of dl309 and adenovirus 7 wild type into 293 cells 80-81

8. Analysis of plaque isolates from co-transfection of overlapping DNA terminal fragments of dl309 and adenovirus 7 wild type 81-82

9. DNA transfection comparisons 82-84

10. Isolation of a fast growing large plaque variant (Ad 3var100) of adenovirus 3 strain GB 84-85

11. One step growth curves of adenovirus 3var100, adenovirus 3 wild type and adenovirus 5 wild type 85-86

12. DNA infectivity of adenovirus 3var100, adenovirus 3 wild type, adenovirus 7 wild type and adenovirus 5 wild type 86

13. Co-transfection of unseparated DNA fragments of adenovirus 3var100 with unseparated DNA fragments of adenovirus 5 wild type or adenovirus 2 wild type to give a region of overlap between the terminal fragments 86-88

14. Analysis of plaque isolates from overlapping DNA terminal fragments of adenovirus 3var100 and adenovirus 5 wild type 88-90

15. Analysis of plaque isolates from co-transfection of overlapping DNA terminal fragments of adenovirus 3var100 and adenovirus 2 wild type 90-91

16. Summary 91-93

Page

Section B.

1. Replication of adenovirus 3var100 in Hela cell monolayers 94
2. Replication of adenovirus 3var100 virus at three temperatures in 293 and Hela cell monolayers 94-95
3. Replication of adenovirus 3var100 DNA in 293 and Hela cells 95-96
4. To investigate if cellular DNA will degrade in the presence of adenovirus 3var100 96-97
5. To investigate if adenovirus 3var100 is heat labile 98
6. Restriction enzyme analysis of adenovirus 3var100 DNA 98-100
7. Complementation of adenovirus 3var100 in Hela cells 100-102
8. Location of the E1 lesion in the adenovirus 3var100 genome 102-104
9. Separation of the E1a and E3 lesions of adenovirus 3var100 104
   (1). Cloning of adenovirus 3 wild type DNA fragments 104-105
   (2). Marker rescue of adenovirus 3var100 using cloned adenovirus 3 wild type BamH1 fragments 105-106
   (3). Isolation of adenovirus 3hr102 106
10. Replication of viral isolate in KB cell lines 106
11. Diminished adenovirus 5 wild type DNA replication in the presence of adenovirus 3var100 107-108
12. Replication of adenovirus 5 wild type DNA in 293 cells and Hela cells in the presence of adenovirus 3var100, adenovirus 3dl101 or adenovirus 3hr102 108-110
13. Replication of the subgroup C inverted terminal repeat in the presence of adenovirus 3var100 DNA 111-112

Page

CHAPTER 4. DISCUSSION

Section A.

A. Results obtained from analysis of adenovirus 3var100

1. General properties 113-117

B. Mixed viral infections 117-119

C. Adenovirus type 5 DNA suppression by adenovirus 3var100 119-123

Section B.

A. Recombination - failure to isolate viable progeny between two adenoviral subgroups 124-130

CHAPTER 5. FUTURE PROSPECTS

A. Construction of *in vitro* recombinants between subgroups B and C 131-132

B. Further investigation of adenovirus 3var100 suppression of adenovirus type 5 DNA synthesis 132

C. Sequencing of the E1a and E3 lesions of adenovirus 3var100 133

D. Protein analysis of adenovirus 3var100 133

E. Isolation of revertants from the adenovirus variants 134

REFERENCES

It is written that there is a destiny one must follow, which is reviewed and planned out by our Creator. For some their destiny appears to be easy, but for others there may appear many obstacles to the fulfilment of their desires. To these people, however difficult their destiny may seem, there is always a hope and a longing to achieve that fulfilment.

The following poem, written by David Rokeah (1916-) and published in Modern Hebrew Poetry, edited and translated by Ruth Finer Mintz, University of California Press, Berkeley and Los Angeles, 1968, expresses this hope and desire for fulfilment.

Zealots of Yearning

For the expectant is the glory,
For the future is theirs.
Who stand against the mountain without recoil
Shall ascend its summit.

So hopes the river, pushing to the sea,
For the freeing of its desires in the roar of the ocean.
So hopes the tree, sending a branch toward the sky
To touch the palm of the sun some day.

Therefore we love dawn as certainty of sunrise,
The nightingale's love-song as longings of motherhood,
The bubbling of fountains as beat of dreams becoming real.
Streams pulsing channels for rivers of the future
And not growing weary.

And all who join in the covenant of hope with the universe -

They are the zealots of yearning.

Therefore, forge the future's desires,

As the waves beat out the rocks of the shore,

As the smith forms the white-heated steel to his will.

Form dreams of faithfulness.

The desolation will not vanish from the Negev before it

vanishes from the heart.....

I thank our Creator and Master of the Universe for the guidance and will power to accomplish this work.

Acknowledgements

I wish to thank Professor John H. Subak-Sharpe for the facility of his department to pursue this research, and also for his critical reading of this manuscript.

I acknowledge Dr. Vivien Mautner as project supervisor.

I wish to thank Mr. Paul Clapham for his helpful discussions through my first year of this project.

I wish to thank Mr. James Aitken for helpful photographic discussions.

I wish to thank Dr. Frazer Rixon for carrying out the particle counts on my viruses.

I wish to thank Dr. Joan Harvey for proof reading this manuscript.

I sincerely thank Miss Sonia Faitleson for her persistent encouragement to resume my academic career.

I thank my mother for all the sacrifices she has made on my behalf throughout her life.

I thank Drs. Frazer and Helen Rixon for their sincere friendship.

I thank my dog, Django, for his loyal affection and for welcoming me home at the ends of some very trying days.

I thank Mrs. J. M. Gray for using her excellent skills to type this thesis and for her patience with my inconsistencies.

The author was in employment with the Medical Research Council during the pursuit of this research, which was carried out entirely in the author's own time outwith working hours.

All work in this thesis, unless otherwise stated, was entirely the author's own effort.

Abbreviations

α	alpha
AAV	adeno-associated virus
Ad.	adenovirus
AT	adenine + thymine content of DNA
ATP	adenosine - 5' - triphosphate
β	beta
bp(s)	base pair(s)
°C	degrees centrigrade
ci	curies
CPE	cytopathic effect
CS	cold sensitive
cyt	cytocidal
Δ (delta)	deleted DNA sequences
dl	deletion of DNA
dATP	2-deoxyadenosine-5'-triphosphate
dCTP	2-deoxycytidine-5'-triphosphate
dGPT	2-deoxyguanosine-5'-triphosphate
dTTP	2-deoxythymidine-5'-triphosphate
DNA	deoxyribonucleic acid
dntps	any combination of 2-deoxyribonucleoside-5'-triphosphates (dATP, dCTP, dGTP or dTTP).
dpm/ug	disintegrations per minute/ug DNA
DTT	dithiothreitol
E. coli	Escherichia coli
G + C	guanine + cytosine content of DNA
g/litre	grammes per litre of solution
HEK cells	Human embryonic kidney cells

hr	host range
in	insertion of DNA
ITR(s)	inverted terminal repeat(s)
K (molecular weight) or Kd	1000-dalton molecular weight
K (centrifugal)	1000 revolutions per minute
Kb	1000 base pairs
kBq	kilo Bequerel
log	logarithm
M	molar
mg	milligramme
ml	millilitre
mM	millimolar
mm	millimetre
ul	microlitre
ug	microgram
mRNA	messenger ribonucleic acid
m.u.	map units (for adenovirus 1 m.u. = 359 nucleotides for Ad.2)
M.W.	molecular weight
nm	nanometer
ng	nanogramme
OD_{260}	optical density at 260 nanometers
ORF	open reading frame
p.f.u.	plaque forming units
PBS	phosphate buffered saline
pi	post infection
rpm	revolutions per minute
RSV	Rous Sarcoma Virus
SDS	sodium dodecyl sulphate
SSDNA	salmon sperm deoxyribonucleic acid

sub	substitution of DNA
TM	too many virus plaques to count
ts	temperature sensitive
u/ul	unit per microlitre
v/v	volume to volume (ratio)
var	variant
w/v	weight to volume (ratio)

SUMMARY

Recombination occurs between adenoviruses of closely related serotypes within a subgroup (Williams et al., 1971; 1975; Ensinger and Ginsberg, 1972; Grodzicker et al., 1974) and is homology based, (Wolegemuth and Hsu, 1980; Boursnell and Mautner, 1981; Volkert and Young, 1983; Mautner and Mackay, 1984). Recombination has not been demonstrated between serotypes of different subgroups, although a functional relatedness of subgroups is shown by complementation (Williams et al., 1975; 1981; McDonough and Rekosh, 1981).

The aim of this project was to isolate recombinant viruses by using DNA fragments of adenovirus type 7 (Subgroup B) to marker rescue ts mutants of adenovirus type 5 (Subgroup C); this proved unsuccessful.

Recombination was then approached by co-transfecting heterotypic DNA terminal overlapping fragments into 293 cell monolayers (Chinnadurai et al., 1979), and progeny screened by restriction endonuclease profile (Mulder et al., 1974a;b). Isolates, however, showed no evidence of recombination.

To increase DNA infectivity of adenovirus type 7, DNA-protein complex was prepared (Robinson et al., 1973; Sharp et al., 1976; Chinnadurai et al., 1978). However, while DNA-protein complex from adenovirus type 5 showed increased DNA infectivity, adenovirus 7 DNA-protein complex showed no increase in infectivity over proteinase treated DNA.

The region of overlap between heterotypic DNA terminal fragments was extended to increase the possibility of recombination within the over-

lap; this also failed to produce heterotypic recombinants.

Since adenovirus 7 DNA-protein complex did not increase DNA infectivity, and increasing the region of heterotypic DNA overlap did not yield recombinants, several methods of DNA transfection were assayed, hoping that transfection conditions could be optimised. The method described by Wigler et al., (1978) modified by the method described by Frost and Williams, (1978) showed an increase in number of viral plaques per ug of DNA.

Adenovirus type 5 is a fast growing virus with a large plaque morphology, while adenovirus type 7 yields a small plaque morphology with a final viral titre 2 logs below that of adenovirus type 5.

Several strains of adenovirus types 7 and 3 were screened for a virus with a similar phenotype to adenovirus type 5. A large plaque fast growing variant of adenovirus type 3 (adenovirus 3var100) was isolated from a crude viral seed stock grown in 293 cells at 37°C. This virus grew as well as adenovirus type 5, its DNA yielding plaques on day 4 of DNA transfection. Further recombination experiments were carried out using this variant virus in conjunction with the improved transfection assay, but no heterotypic recombinants were isolated.

Adenovirus 3var100 was found (i) to be host range for growth in Hela cells; (ii) by restriction enzyme profile analysis to have a deletion of approximately 1 Kb lying in the non-essential E3 region. The host range phenotype of adenoviruses has been mapped to the E1 region of the genome, with these viruses growing well in 293 cells which contain and express the E1 region of adenovirus type 5. The E1 lesion of the variant

was mapped to the E1a region by cell lines which express the functions of E1a and E1b separately (Babiss et al., 1983). This data was confirmed by marker rescue of the E1 lesion by a cloned fragment of DNA from adenovirus type 3 spanning map co-ordinates 2 m.u. - 5 m.u. which contains most of the E1a region.

When the variant was co-infected with subgroup C viruses type 2 or type 5 it suppressed their DNA synthesis. This phenomenon was not witnessed when the subgroup C viruses were co-infected with adenovirus type 3 parental. This is contrary to the data published by D'Halluin et al., (1983) who found a hierarchy of transdominance with adenovirus type 3 suppressing the growth of all other subgroups. When the variant was co-infected with its own parent its own DNA replication was suppressed, contrary to data published by Berkner and Sharp, (1983) who found when they co-infected an E3 variant of adenovirus type 5 with its parent, the parental DNA was suppressed.

To define the lesion responsible for DNA suppression, adenovirus 3hr102 containing the E1a lesion and adenovirus 3dl101 containing the E3 lesion were constructed. Adenovirus 3dl101 retained the ability to suppress the DNA synthesis of subgroup C suggesting the E3 lesion is responsible for the suppression. It was not host range for growth in Hela cells, but retained the restriction enzyme profile of adenovirus 3var100. Adenovirus 3hr102 retained the host range phenotype but had the restriction enzyme profile of adenovirus type 3.

Using the sequence data for the adenovirus type 3 E3 region, published by Signäs et al., (1986), it can be predicted what sequences are lost by the E3 deletion within adenovirus 3var100.

Adenovirus 3 wild type has a unique 950 bp AT rich region within the E3 region, which contains two open reading frames with the coding capacity for two polypeptides of 20.1K and 20.5K molecular weight, their function being unknown. The E3 deletion within the variant removes the sequences coding for these polypeptides along with the coding capacity for the polypeptides of 9K and 10.2K molecular weight.

The termination sequence of the 19K glycoprotein and the initiation codon for the 15.2K glycoprotein are also removed, with the remaining coding information lying adjacent on the genome. It is possible that the remaining sequences are read as one open reading frame resulting in a novel protein which could be involved in the suppression of DNA synthesis.

Ho et al., (1982) isolated host range adenovirus type 5 mutants which were also cold sensitive (Cs), growing in 293 cells at 32°C and 37°C, but only in Hela cells at 38.5°C. Two of these mutants mapped in the E1a region and one in the E1b region. Adenovirus 3var100 has only the host range phenotype and is not cold sensitive.

Adenoviruses with a large plaque morphology usually map in the E1b region (Lai Fatt and Mak, 1982) and can cause cellular DNA degradation (Ezoe et al., 1982). Adenovirus 3var100 has a large plaque morphology but does not degrade cellular DNA.

Mutations in structural genes can cause the mutant to be more thermolabile or more thermostable than wild type (Young and Williams, 1975).

Adenovirus 3var100 is less thermostable than either adenovirus type

3 or adenovirus type 5; this may suggest an unmapped mutation in a structural gene.

CHAPTER 1

INTRODUCTION

A. GENERAL

Human adenoviruses were first isolated by Rowe and co-workers, (1953) and Hilleman and Werner, (1954). During the preceding years there had been extensive epidemics of acute respiratory illnesses among the population of America, leading to clinicians attempting the isolation of the causative agents.

Rowe et al., (1953) reported that cells from cultured explants of human adenoid and tonsillar tissue frequently underwent rounding up with the production of grape like clusters. Filtration of culture fluids with serial passage on to established cell lines (eg. Hela cells) showed that the cytopathic effect was transmittable, implicating an unknown viral agent.

During an outbreak of respiratory illnesses among American army recruits, Hilleman and Werner, (1954) isolated an unknown virus from the biopsies of human trachae. Huebner et al., (1954) showed these viruses had similar biological properties and segregated them into six immunological types. To date 41 serotypes of human adenoviruses have been identified, (Pereira et al., 1965; Wigand et al., 1982; de Jong et al., 1981; 1983) and shown to be responsible for a number of ailments, the most common being; (a) Respiratory, Medical Research Council, (1965) study; (b) Urinary, (Numazaki et al., 1968; 1973); (c) Ocular, (Tai et al., 1974; Schaap et al., 1979); (d) Gastrointestinal, (Flewett et al., 1975; White and Stancliffe, 1975; Richmond et al., 1979; Brandt et al., 1980).

Adenoviruses are not unique to the human species. The first recorded

animal adenovirus was isolated in 1926 during a viral epidemic on a fox fur farm in Wisconsin, U.S.A. This virus was named as Fox encephalitis, but later was classified as Canine adenovirus type 1. Adenoviruses can also be isolated from cattle, (Klein et al., 1959; Darbyshire et al., 1965); monkeys, (Hull et al., 1956; Kalter et al., 1980); horses, (McChesney et al., 1973; 1974); mice, (Hartley and Rowe, 1960); fowls, (Kawamura et al., 1964; Zsák and Kisary, 1984); ducks, (Baxendale, 1978).

Table 1

GROUP	Serotypes	DNA homology	DNA(%G+C)	Tumours in Newborn Hamsters	Cell Transformation
A	12,18,31	48-69% within group; 8-20% with other types	Low 47-49%	High : Tumours in most animals in 4 months	+
B	3,7,11,14,16,21	89-94% within group; 9-20% with other types	Intermediate 49-52%	Weak : Tumours in a few animals in 4-18 months	+
C	1,2,5,6.	99-100% within group 10-16% with other types	High 57-59%	Nil	+
D	8,9,10,13,15,17, 19,20,22,23,24,25, 26,27,28,29,30.	94-99% within group 4-17% with other types	High 57-59%	Nil	+
E	4	4-23% with other types	High	Nil	Not done
F+G	40,41 respectively	62-69% with each other 15-22% with Ad5wt	Intermediate 52%	Nil	Not done

Data of subgroups A to E published by Green et al., (1974), Virology 93:481

Data of subgroups F and G published by van Loon et al., (1985), Virology 140:197

B. CLASSIFICATION OF ADENOVIRUSES

The human serotypes of adenoviruses have been classified into subgroups using a number of criteria (see Table 1).

1. Haemagglutination

Rosen, (1958) showed that adenoviruses in vitro agglutinate the erythrocytes of monkeys, mice and rats, but not the erythrocytes of other mammalian species. Rosen, (1960) used this differential haemagglutination, residing in the fibre antigen, to classify adenoviral serotypes into subgroups. Antisera against viruses of the same subgroup will inhibit haemagglutination, but antisera to viruses of heterologous subgroups will not; from these data Rosen proposed four subgroups, (I-IV), three of which were later subdivided by Hierholzer, (1973).

2. Tumourgenicity

Trentin et al., (1962) observed that adenovirus type 12 induced malignant tumours following inoculation into new born hamsters. This approach was extended to other adenoviral serotypes, (Huebner et al., 1962; 1963; 1965; Yabe et al., 1964; Girardi et al., 1964; Rabson et al., 1964; Larsson et al., 1965; Pereira et al., 1965; Trentin et al., 1968) with the result that the serotypes could be divided into three subgroups. Group A (Adenoviruses 12, 18, 31) highly tumourgenic; group B (eg. Adenoviruses 3, 7, 8, 14, 21, 24) weakly tumourgenic after a latent period; group C, the remaining serotypes, classified as non-tumourgenic.

3. Cellular Transformation

All adenoviral serotypes induce, in vitro, cellular transformation of rat cells, (McBride and Wiener, 1964; Freeman et al., 1967; McAllister et al., 1969a). Rodent cells thus transformed do not produce infectious virus, but express virus specific proteins which can be recognised immunologically (Huebner, 1967; McAllister et al., 1969b). There was a strong correlation between the viral antigens synthesised by the transformed cells and the serotype of virus used as the transformant. Thus cells transformed by serotypes within the same subgroup of virus were found to share common antigenic determinants.

4. The percentage G + C DNA content

Piña and Green, (1965) classified the adenoviruses by their percentage G + C DNA content, into high (57%-59%), subgroup C; intermediate (49%-52%), subgroup B and low (47%-49%), subgroup A. Host cell DNA G + C content is low (42%-44%) while oncogenic simian adenovirus G + C content is high (58%-60%), Piña and Green, 1968); therefore the correlation of low G + C content within subgroup A to tumourgenicity could be misleading as host cells which contain a low G + C content are non tumourgenic.

5. DNA homology

Green et al., (1979) further classified the thirty-one adenovirus serotypes into five subgroups (A---E) based on their all over DNA homology by heteroduplex analysis and liquid phase DNA-DNA hybridisation. Previously Garon et al., (1973) had shown two zones of high heterology

within groups mapping between 8 m.u.-- 22 m.u. and 35 m.u. -- 50 m.u.

Serotypes 40 and 41 (Flewett et al., 1975; Schoub et al., 1975; White and Stancliffe, 1975; Brandt et al., 1980; de Jong et al., 1983) have been classified as subgroups F and G respectively. These are infantile enteric adenoviruses which show a restricted host-range by growing in 293 cells but not in HEK cells or human diploid fibroblasts. Their DNA homology throughout the genome is high but little homology with lower numbered adenovirus types is detected.

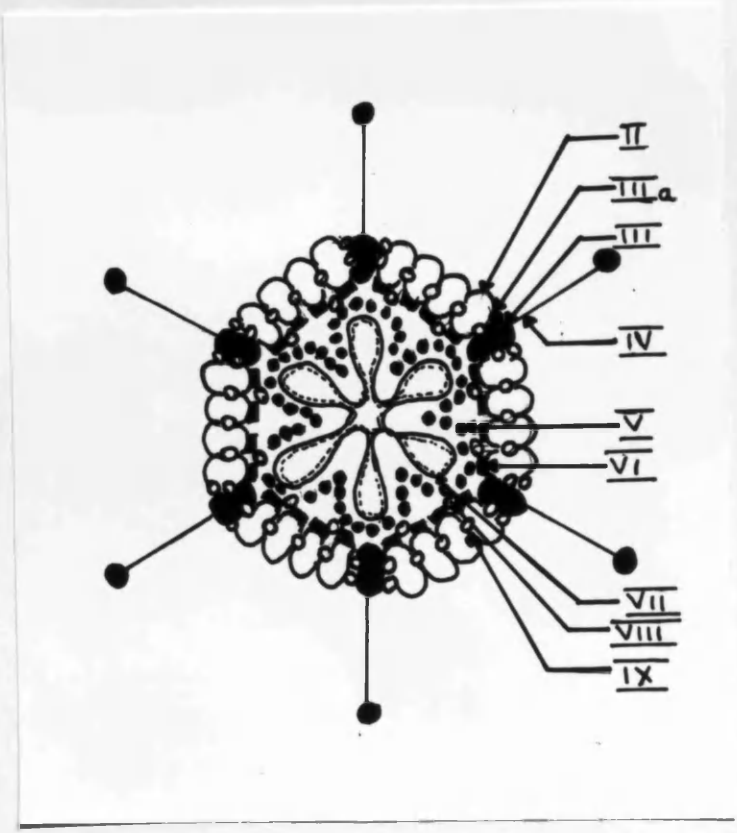

II
III a
III
IV
V
VI
VII
VIII
IX

Figure 1.

Structure of the adenovirus virion

Model of the arrangement of structural proteins and viral DNA within the virion; the structural polypeptides are indicated by Roman numerals and are described in Table II and also in the text of Morphology of the Adenoviruses.

The figure is reproduced from The Adenoviruses; The Molecular Biology of Tumour Viruses Vol. II, TOOZE, J. (Ed.).

II	HEXON .
IIIa	HEXON ASSOCIATED PROTEIN III
III	PENTON BASE
IV	FIBRE
V	MINOR CORE PROTEIN
VI	HEXON ASSOCIATED PROTEIN I
VII	MAJOR CORE PROTEIN
VIII	HEXON ASSOCIATED PROTEIN II
IX	HEXON ASSOCIATED I (GROUPS OF NINE)
	SEE TABLE II

Table II

Protein	Molecular weight	Number per virion	Polypeptide and Molecular weight
Hexon	313K-380K	240	II 90K-120K 3 per hexon
Penton base	600K-515K	12	III 85K 5-6 per penton
Fibre	183K-207K	12	IV 60K-65K 3 per fibre
Major core protein	19K	1000	VII 18.5K
Minor core protein	48K	200	V 48K
Hexon-associated 1 (All)	50K	450	VI 24K
Hexon-associated II	15K	n.k.	VIII 13K
Hexon-associated III	n.k.	n.k.	IIIa 66K
Hexon-associated 1 (groups of nine)	n.k.	n.k.	IX 12.5K

Structural polypeptides of adenovirus 2 wild type. Reproduced from The Adenoviruses; The Molecular Biology of Tumour Viruses Vol. II, TOOZE, J. (Ed.)

C. MORPHOLOGY OF THE ADENOVIRUSES

Adenoviruses are non-enveloped icosahedral virions of diameter 65-88nm, composed of a protein capsid, core shell and a nucleoprotein complex; the ratio of protein to DNA being 87%:13% (Green and Piña, 1963).

1. The Capsid

The icosahedral capsid consists of 240 hexons and 12 pentons each composing a penton base and fibre, (Valentine and Pereira, 1965); see Fig. 1 and table II.

a). The hexon

The hexon which can be crystallised (Pereira et al., 1968; Franklin et al., 1971; Cornick et al., 1971) is a structural protein; that of adenovirus 2 wild type has a molecular weight of 324K (Jörnvall et al., 1981), and consists of three identical subunits (Horwitz et al., 1970; Franklin et al., 1971; Cornick et al., 1973; Gütter and Franklin, 1974; Jörnvall et al., 1981) of molecular weight 108K determined from the deduced hexon amino acid sequence (Jörnvall et al., 1981).

The hexon carries group specific antigenic determinants (Pereira et al., 1959; Wilcox and Ginsberg, 1963; Wilcox et al., 1963; Valentine and Pereira, 1965; Burki et al., 1979; Wigand et al., 1982) and type specific determinants (Norrby et al., 1969a; Willcox and Mautner, 1976). Complement fixation tests on intact and disrupted virions suggest the orientation of the hexon is such as to display only type specific antigens on the surface (Norrby et al., 1969).

b) Penton base

The penton base has a molecular weight of 365K (Devaux et al., 1982) and displays antigenic determinants of group and subgroup specificity (Pettersson and Höglund, 1969; Wadell and Norrby, 1962). The penton base is responsible for the early cytopathic effect of adenoviruses in tissue culture (Pettersson and Höglund, 1969).

c) The fibre

The fibre is a rod like structure composed of two or three identical polypeptides of molecular weight 62K, (Green et al., 1983; Devaux et al., 1983) being responsible for the attachment of the virus to the cell surface (Philipson et al., 1968).

The fibre length varies across subgroups with subgroup B carrying the shortest of 10nm (Norrby, 1968; 1969b). Type specific antigenic determinants are expressed by the fibre knob (Norrby, 1968; 1969). The longer fibres of subgroups C and D carry additional subgroup-specific determinants (Norrby, 1968; 1969; Pettersson et al., 1969).

d) Polypeptides VI; VIII; IIIa and IX

The above proteins are synthesised in minor amounts and are associated with the capsid structure.

Polypeptide VI is a DNA binding protein (Russell and Precious, 1982); polypeptide IIIa is associated with the vertex region of the capsid (Everitt et al., 1973) and involved in virion assembly (Devaux

et al., 1982); polypeptide VIII interacts with the hexon (Everitt and Philipson, 1974); polypeptide IX is a capsid structural protein. Mutants unable to synthesise this protein are less heat stable than the wild type (Colby and Shenk, 1981).

2. Core Shell

Electron microscope freeze fracture studies (Brown et al., 1975; Nermut, 1978; 1979) and treatment of virion cores by deoxycholate or pyridine suggest the presence of a core shell surrounding the nucleoprotein. Polypeptide V may be involved (Nermut, 1979) in this structure as it binds less tightly to adenoviral DNA than polypeptide VII (Brown et al., 1975; Vayda et al., 1983).

3. Core (Nucleoprotein)

Adenoviral DNA is packed into a core structure non-covalently linked to major polypeptides V and VII (Maizel et al., 1968; Laver et al., 1968; Russell et al., 1971; Everitt et al., 1973; Anderson et al., 1973). Micrococcal nuclease protection experiments suggest the nucleoprotein is a discontinuous helix associated with polypeptide VII (Corden et al., 1976; Nermut, 1979; Sato and Hosokawa, 1981; Mirza and Weber, 1982; Vayda et al., 1983).

D. ADENOVIRAL DNA STRUCTURE AND REPLICATION

1. Structure

Virion DNA can be extracted by one of two methods. (a) Proteolytic digestion produces a linear DNA duplex (van der Eb and van Kesteren, 1966; Green et al., 1967; van der Eb et al., 1969; Younghusband and Bellett, 1971), of molecular weight 20-25x10^6 daltons (Green et al., 1967). Nucleotide data infers that the genomes of adenoviruses type 2 and 5 are approximately 36 Kb. (b) Virions disrupted in 4M Guanidinium Hydrochloride and sedimented in a sucrose gradient followed by low salt dialysis yield DNA duplex circles (Robinson et al., 1973; Robinson and Bellett, 1974). Treatment with proteolytic enzymes convert this structure to the linear DNA configuration implying the involvement of a terminal protein in the circular structure.

2. Replication of DNA

DNA replication occurs in the nucleus of lytically infected cells, reaching a maximum at 24 hours post infection. Two structures novel to adenoviruses are involved in DNA replication; (a) the ITR; (b) the terminal protein (55k) and its precursor form (pTP80k).

(a) The inverted terminal repeat (ITR)

Single stranded DNA incubated under renaturing conditions is capable of circularisation (Garon et al., 1972; Wolfson and Dressler, 1972; Robinson and Bellett, 1975) possibly to form the "panhandle structure" proposed by Danniell, (1976). These circles can be converted

to a linear form by exonuclease III or heating to the melting point of native DNA, indicating the presence of inverted terminal repeats. Sequence analysis shows the ITRs vary in length between 103bp--160bp depending on subgroup, (Steenbergh et al., 1977; Arrand and Roberts, 1979; Dijkema and Dekker, 1979; Tolun et al., 1979; Shinagawa and Padmanabhan, 1979; 1980; Sugisaki et al., 1980; Temple et al., 1981; Alestrom et al., 1982; Garon et al., 1982; Stillman et al., 1982). The ITRs show a rich AT base pair homologous compisition within the first 50 base pairs, and within the ITRs of all human adenoviruses, nucleotides 9-22 are totally conserved (Tolun et al., 1979) and involved in the initiation of DNA replication (Stillman, 1981; Bos et al., 1981; Stillman et al., 1982; Byrd et al., 1982).

(b) Terminal protein

The terminal protein (MW,55K) is derived from the COOH terminal end of the precursor protein (MW,80K) which is cleaved by a viral protease late in infection (Challberg and Kelly, 1981). This association has been shown by partial proteolysis (Challberg et al., 1980) and partial amino acid sequence analysis (Smart and Stillman, 1982). The 55K protein is found co-valently linked to the 5' ends of viral DNA via a phosphodiester bond between the β-OH of a serine residue of the protein to the 5'OH group in the deoxycytidine residue in the DNA terminus (Robinson et al., 1973; Robinson and Bellett, 1974; Rekosh et al., 1977; Desiderio and Kelly, 1981).

Replication of adenovirus DNA is initiated at the genomic termini, proceding in a 5' - 3' direction via a strand displacement mechanism completely displacing one of the parental strands. The

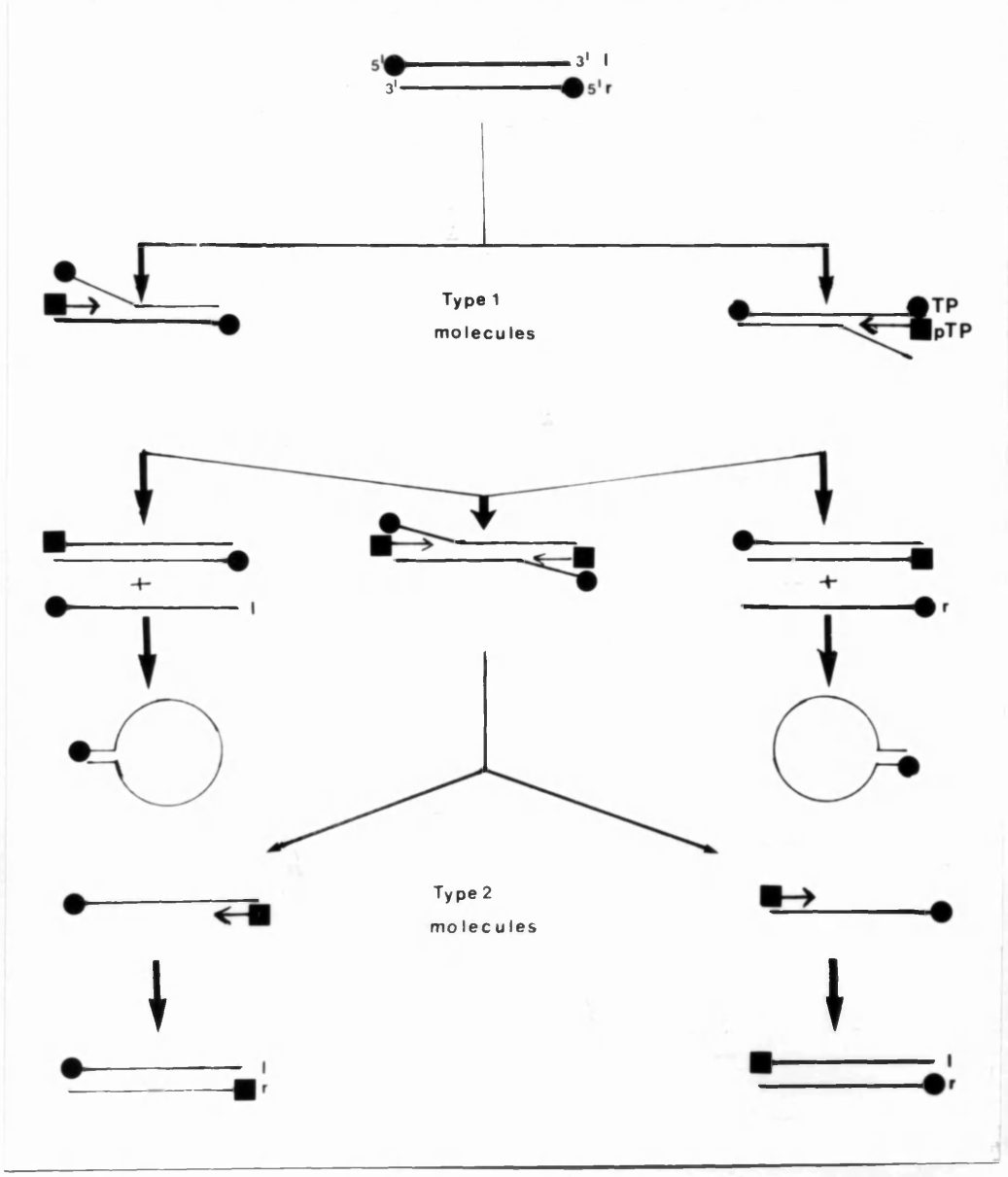

5'
3' l
3'
5' r
Type 1
molecules
TP
pTP
+
l
+
r
Type 2
molecules
l
r
l
r

Figure 2.

Replication of adenovirus DNA

Replication of adenovirus DNA, showing the suggested two intermediate replicative forms.

■ = precursor terminal protein (80K)

● = terminal protein (55K)

Figure reproduced from Futterer, J., and Winnacker, E-L., (1984) Current Topics in Microbiology and Immunology Vol. III : The Molecular Biology of Adenoviruses 3. (Ed. W. Doerfler), pp 41-64, Springer-Verlarg, Berlin, Heidelberg.

pTP80K is used as a primer to initiate synthesis at the molecular ends (Challberg et al., 1980; 1982; Lichy et al., 1981; 1982; Pincus et al., 1981; Stillman et al., 1981; Tamanoi and Stillman, 1982; Ikeda et al., 1982).

EM studies suggest the existence of two replicative intermediates (Ellens et al., 1974; Lechner and Kelly, 1977; Revet and Benichou, 1981) see Fig.2.

E. ADENOVIRAL DNA INFECTIVITY

The ability of adenovirus DNA to produce infectious viral centres in cell monolayers was first demonstrated using simian adenovirus 7 (Burnett and Harrington, 1968); and by Nicolson and MacAllister, (1972) using the DNA-dextran method of transfection which showed a low level of infectivity in HEK cells with adenovirus type 1 DNA.

Graham and van der Eb, (1973) devised a technique for the assay of adenovirus 5 DNA infectivity by the use of calcium, a divalent cation (Spizizen et al., 1966). A fine calcium phosphate-DNA precipitate was formed and inoculated on to cell monolayers. This technique produced a 100 fold increase in plaque number compared to the DEAE-dextran method. A relationship between plaque numbers and concentration of DNA could be correlated, showing that 5ug of native DNA yielded an optimal of 13 plaques per dish on KB cell monolayers.

Sharp et al., (1976) showed that adenovirus 5 DNA-protein complex (Robinson et al., 1973; Robinson and Bellett, 1974) was 100 fold more infectious, yielding 4000 p.f.u. per 1ug DNA, in comparison to pronase treated adenovirus 5 DNA which yielded 46 p.f.u. per 1ug.

Frost and Williams, (1978) modified the DNA transfection method (Graham and van der Eb, 1973) by using 293 cells, and by enhancing cellular uptake of DNA at 4 hours post transfection by exposure of cell monolayers to 20% glycerol, giving 45 p.f.u. per 1ug DNA on Hela cell monolayers and 125 p.f.u. per 1ug on 293 cell monolayers.

Chinnadurai et al., (1978) showed that the infectivity of adenovirus

2 DNA and DNA-protein complex could be enhanced by exposure of cell monolayers to 25% dimethyl sulphoxide (DMSO) at 4 hours post transfection. This method gave between $6x10^3$ p.f.u. and $2x10^4$ p.f.u. per 1ug of DNA-protein complex when assayed on 293 cell monolayers.

F. THE LYTIC CYCLE OF THE ADENOVIRUS IN HOST CELLS

1. Viral adsorption, penetration and uncoating

Human adenoviruses are grown in human embryonic cells (HEK) or immortalized human cell lines, eg. 293, Hela or KB cells.

The virions attach by their fibres to specific receptors which reside in the plasma membrane of the host cell (Levine and Ginsberg, 1967; Lonberg-Holm and Philipson, 1969). How virions enter the host cell is unclear but EM data suggests phagocytosis (Chardonnet and Dales, 1970); direct penetration (Morgan et al., 1969); or absorptive endocytosis (Svensson and Persson, 1984).

Uncoating of the virion begins in the cell cytoplasm (Sussenbach, 1967; Lonberg-Holm and Philipson, 1969; Morgan et al., 1969) and is completed when the virion cores are transported to the cell nucleus (Morgan et al., 1969; Chardonnet and Dales, 1972).

Evidence based on electron microscopic observations and motility inhibition studies of adenoviral infected cells suggests that transportation of virion cores across the cell cytoplasm possibly involves cytoplasmic microtubules (Dales, 1962; Morgan et al., 1969; Luftig and Weihing, 1975; Svensson and Persson, 1984).

Once final uncoating has taken place transcription and viral DNA replication begins within the host cell.

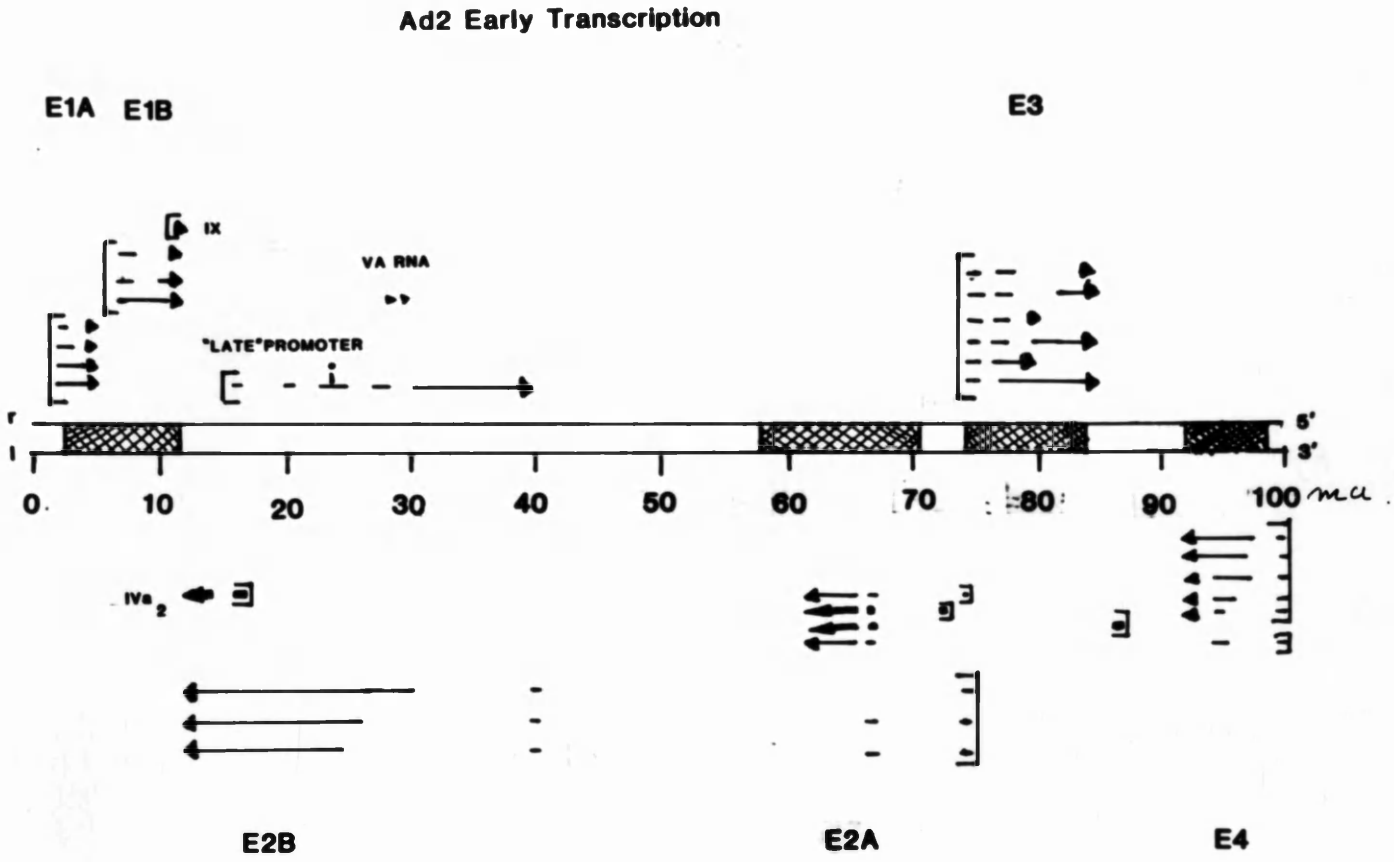

Ad2 Early Transcription
E1A E1B
E3
IX
VA RNA
"LATE" PROMOTER
5'
3'
0
10
20
30
40
50
60
70
80
90
100 mu
IVa2
E2B
E2A
E4

Figure 3.

Adenoviral early transcription as described for the type 2 virus

The figure shows the early transcription of adenovirus type 2. Brackets represent promotor locations; RNA splicing is depicted by interrupted lines; arrows represent the direction of transcription.

The figure is reproduced from Richardson and Westphal, (1981) Cell 27:133-141

The genome is divided into 100 m.u.

2. Transcription

The understanding of adenoviral transcription has come from studies using subgroup C viruses type 2 and type 5, which grow to high titres, have a short replication cycle and are easy to study. The replication cycle is initiated when viral DNA enters the nucleus about 30 minutes after viral absorption into the host cell. The transcription of viral promotors commences in a defined sequence using pre-existing cellular enzymes. Viral transcripts synthesised before the onset of viral DNA replication are classified as early and those after DNA replication as late.

2.(1) Early transcription

The four regions of the adenoviral genome encoding mRNA sequences (Fig. 3), E1, E2, E3 and E4 regions were originally identified by saturation hybridization experiments (Sharp et al., 1975; Tibbets et al., 1974). The E1 region was further divided into two closely related transcriptional units E1a and E1b (Chow et al., 1977; 1979; Evans et al., 1977; Berk and Sharp, 1978; Harrison et al., 1977; Jones and Shenk, 1979a; Ross et al., 1980).

2.(1)(a) The E1a region (1.3-4.6) m.u.

Transcription is from the r strand of viral DNA and encodes three mRNA species with sedimentation co-efficients 13S, 12S and 9S sharing the same 5' and 3' termini and differing only in the size of RNA segment spliced enzymatically during processing of nuclear RNA (Kitchingman et al., 1977; Berk and Sharp, 1977; 1978; Chow et al., 1979a; b; Perricaudet

et al., 1979). DNA sequence analysis allows the prediction of the amino acid sequences of the proteins specified by the 13S and 12S mRNA (van Ormondt et al., 1980); they encode polypeptides of molecular weights 3.8K and 2.3K respectively. Virtanen and Pettersson, (1983) showed by sequencing cDNA transcribed from the 9S mRNA it coded for a polypeptide of molecular weight 6.1K.

Early gene expression is regulated by the product of the 13S mRNA (Berk et al., 1979; Carlock and Jones, 1981; Ricciardi et al., 1981; Montell et al., 1982; Leff et al., 1984; Ferguson et al., 1984; Guilfoyle et al., 1985) while the product of the 12S mRNA is responsible for the repression of the synthesis of the DNA binding protein from its late promotor (Guilfoyle et al., 1985).

2.(1)(b) The E1b region (4.6-11.2) m.u.

Transcription occurs from the r strand of viral DNA, encoding three mRNA species of sedimentation co-efficients 22S, 13S and 9S, detected by electron microscopic heteroduplexing and SI analysis (Berk and Sharp, 1978; Chow et al., 1979). Both 22S and 13S have common 5' and 3' termini and are generated from a common precursor by differential splicing (Berk and Sharp, 1978; Weber et al., 1980). In vitro translation assigns two major polypeptides of molecular weights 55-65K and 15-19K to this region (Lewis et al., 1976; Harter and Lewis, 1978; van der Eb et al., 1979; Brackman et al., 1980). The 55K polypeptide is active in the mechanism which shuts off host cell gene expression (Babiss and Ginsberg, 1984), possibly by the interaction with the 25K protein encoded by the E4 region (Sarnow et al., 1982a), to bring about intracellular modification.

2.(1)(c) The E2a region (61.5-75.1) m.u.

Transcription occurs from the l strand of viral DNA encoding three mRNA species, two of which share a promotor site at 75.1 m.u. (Baker et al., 1979) and differ only by a splice site of 50 nucleotides at 66.3 m.u. Later in infection transcription starts at a promotor lying at 72 m.u.

This region codes for the DNA-binding protein, a phosphoprotein of molecular weight 72K (van der Vliet and Levine, 1973; Levinson et al., 1976; Jeng et al., 1977; Russell and Blair, 1977; Axelrod, 1978) which binds to single strand DNA (van der Vliet and Levine, 1973) and to the ends of double stranded DNA (Fowlkes et al., 1979). The DNA-binding protein is required for elongation during adenoviral DNA synthesis (van der Vliet and Sussenbach, 1975; van der Vliet et al., 1977). A temperature sensitive mutant for the 72K protein (H5ts125) does not replicate its DNA at the non-permissive temperature (van der Vliet and Sussenbach, 1975), but accumulates early viral mRNAs (Babich and Niven, 1981). The 72K DBP can be cleaved with chymotrypsin into two subunits of molecular weights 44K (C-terminal) and 26K (N-terminal) (Klein et al., 1979); the 44K subunit only binds to single strand DNA (van der Vliet and Levine, 1973; Levinson and Levine, 1977).

2.(1)(d) The E2b region (11-30) m.u.

Transcription of this region maps between 11 m.u. - 30 m.u. from the l strand of viral DNA from a promotor lying at 75.1 m.u. (Galos et al., 1979; Stillman et al., 1981). Three transcripts have been identified; a major transcript which maps between 11.3 m.u. - 26 m.u. with three upstream leader sequences, a second, identical to the above except it

spans 11.3 m.u. - 30.3 m.u. and a third mapping between 10.8 m.u. - 23.1 m.u. (Stillman et al., 1981).

In vitro translation has assigned three polypeptides of molecular weight 75K, 87K and 105K to this region (Stillman et al., 1981; Binger et al., 1982). The 87K polypeptide is identical to the 80K precursor of the terminal protein (Challberg et al., 1980; Smart and Stillman, 1982). A polypeptide of molecular weight 140K has been assigned to the region 24.1 m.u. - 14.2 m.u. and is an adenovirus DNA polymerase which can complement the defect in H5ts36, a DNA negative mutant mapping between 18.5 m.u. - 22 m.u. (Galos et al., 1979; Enomoto et al., 1981; Lichy et al., 1982).

2.(1)(e) The E3 region (76.6-86.0) m.u.

This region is non-essential for adenoviral growth in tissue cultures. SV40-adenovirus type 2 hybrids with all the E3 sequences deleted have been shown to produce viable progeny (Flint et al., 1975). Transcription is from the r strand of viral DNA from which four major and four minor mRNA species have been identified (Chow et al., 1979a; Kitchingman and Westphal, 1980; Persson et al., 1980a;b), the most abundant being detected as DNA:RNA heteroduplexes (Chow et al., 1979a). All transcripts share 5' terminal sequences complementary to the region 76.6 m.u. - 77.6 m.u. which are spliced to sequences near 78.6 m.u. Beyond this point the mRNA species differ in both splicing and their 3' terminal sequences. Polypeptides of 13K, 14K, 15.5-16K and 19-21K have been assigned to this region (Lewis et al., 1976; Green et al., 1979b; Harter et al., 1976; Ross et al., 1980).

One of the largest polypeptides is a 19K glycoprotein (Jeng et al., 1978; Persson et al., 1979). Immunoprecipitation with monospecific antibody shows that its synthesis occurs at 2 hours post infection, reaching a maximum at 4 hours post infection. Sequence analysis has shown this polypeptide to have a hydrophobic and a hydrophilic region and it is also shown to be associated with membrane fractions of infected cells. (Chin and Maizel, 1976; Persson et al., 1980a;b; Cladaras and Wold, 1985). Antiserum against either the 19K glycoprotein or the heavy chain of the class 1 histocompatability antigens precipitates a complex of the two components (Kvist et al., 1978; Signäs et al., 1982). Signäs et al., (1982) showed if the 19K glycoprotein was immobilized in a matrix it was possible to bind and release the heavy chain of the human class 1 antigens; this interaction may form the basis for the recognition of adenovirus infected cells by the cytotoxic T-cells produced by the immune system.

2.(1)(f) The E4 region (91.3-99.1) m.u.

Transcription of this region is from the l strand of viral DNA encoding a large set of mRNAs, all of which have a common 5' terminus lying near 99.2 m.u.; a major species has been detected by electron microscopy and biochemical assay (Berk and Sharp, 1978). Eight polypeptides have been assigned to this region having molecular weights between 11K-35K (Matsuo et al., 1982).

The 11K polypeptide is highly conserved between serotypes and is found tightly bound to the nuclear matrix (Sarnow et al., 1982b). Halbert et al., (1985) demonstrated using E4 deletion mutants that most of the gene products are not required for viral growth; however

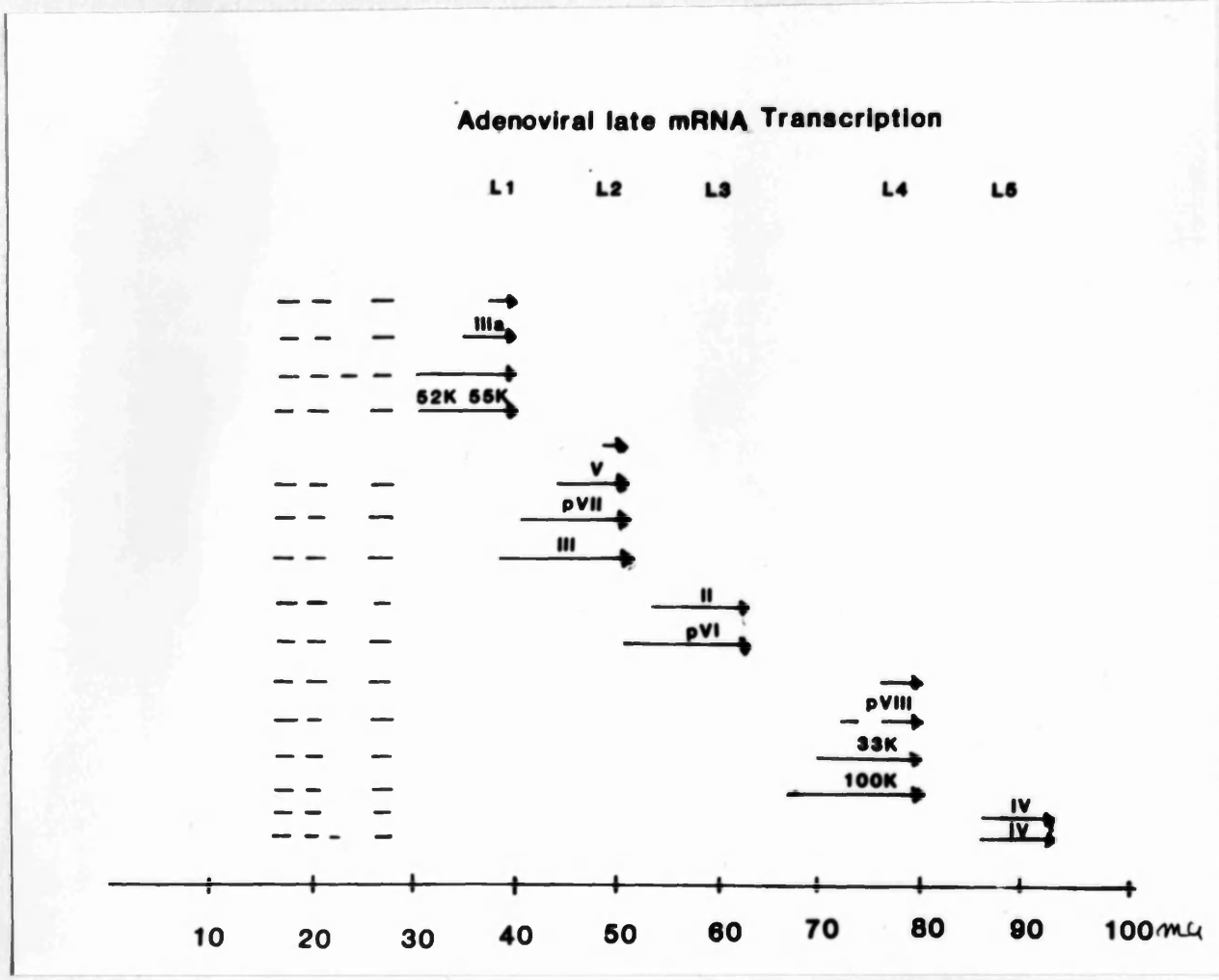

Adenoviral late mRNA Transcription
L1
L2
L3
L4
L5
IIIa
52K 55K
V
pVII
III
II
pVI
pVIII
33K
100K
IV
IV
10
20
30
40
50
60
70
80
90
100 mu

Figure 4.

Adenoviral late transcription as described for the type 2 virus

Late mRNA species are shown by horizontal lines drawn in the direction of transcription; gaps represent sequences removed during splicing; arrowheads represent poly (A) sequences.

Figure is reproduced from Flint, (1982) Biochemica et Biophysica Acta 651:175-208.

The genome is divided into 100 m.u.

H5dl355 and H5dl366, deletion mutants for the 35K polypeptide, show impaired DNA replication, excessive amounts of the E2a 72K DNA-binding protein, reduced levels of late mRNAs and an impairment in the rate of the shut off of host cell metabolism.

2.(1)(g) Virus-associated RNAs

Two major species of small virus-associated mRNAs have been assigned to 29 m.u. on the viral genome, being transcribed from the r strand. Both species are synthesised using host cell RNA polymerase III and are between 157-160 nucleotides long. They contain scattered zones of homology resulting in a secondary structure similar to the cloverleaf of tRNA (Ohe and Weissman, 1970; Mathews, 1975; Söderlund et al., 1976; Celma et al., 1977a;b; Pan et al., 1977; Akusjärvi et al., 1980).

The function of these transcripts is uncertain but data suggests that VA-RNA 1 controls the rate of late adenoviral polypeptide translation (Schneider et al., 1984).

2.(II) Late Transcription

Synthesis of late transcription is initiated after DNA replication from the Major Late Promotor (MLP) at 16.3 m.u. (Evans et al., 1977; Ziff and Evans, 1978) from the r strand of viral DNA. The transcripts are in five defined regions of over-lapping mRNAs, sharing the same tripartite leader sequences generated from 16.3 m.u., 19.6 m.u. and 26.6 m.u. (Berget et al., 1977; Klessig, 1977; Chow et al., 1977a; Dunn and Hassell, 1977; Akusjärvi and Petterson, 1979; Zain et al., 1979), Figure 4.

Eleven proteins have been assigned to the five transcription blocks. Three are non-structural proteins with molecular weights of 23K (L3), 33K and 100K (L4) (Russell and Skehel, 1972; Anderson et al., 1973; Everitt, 1973; Ishibashi and Maizel, 1974; Lewis et al., 1977; Axelrod, 1978; Gamdke and Deppert, 1983; Yeh-Kai et al., 1983; Oosterom-Dragon and Anderson, 1983). Temperature sensitive mutants for late functions of adenoviruses type 2 and type 5 have been mapped by marker rescue and intertypic recombination analysis to four of the defined blocks of mRNA transcripts.

H5ts1 and H5ts115 have mutations in the 100K gene. At the non-permissive temperature they are defective for assembly of hexon monomers into trimers and also for transport of the hexon into the nucleus. This suggests that the 100K polypeptide plays a role in these mechanisms (Russell et al., 1972; 1974; Stinski and Ginsberg, 1974; Lebowitz and Horwitz, 1975; Kauffman and Ginsberg, 1976; Oosterom-Dragon and Ginsberg, 1981). H5ts5, H5ts9, H5ts22 and H5ts125 (D'Hallium et al., 1980; 1982) all express reduced levels of the fibre protein.

The processing of viral polypeptides can be dependent on proteolytic cleavage of a precursor polypeptide, eg. pTP80K to 55K terminal protein. H2ts1 is defective for the cleavage of polypeptides pVI, pVII, pVIII and pTP80K (Weber, 1976; Mirza and Weber, 1977; Challberg and Kelly, 1981; Stillman et al., 1981). Adenovirus type 2 infected cells show a chymotrypsin-like protease activity specific for pVII which is absent in H2ts1 cell infection at the non-permissive temperature (Bhatti and Weber, 1979). Since the H2ts1 mutation lies between 60 m.u. - 61.7 m.u. (Yeh-Kai et al., 1983) with coding capacity for a 23K polypeptide (Kruijer et al., 1980; Akusjärvi et al., 1981),

data suggests the 23K polypeptide may be a protease similar to chymotrypsin.

It is not understood how the switch from early to late transcription is achieved. but late mRNAs can only be detected in significant quantity after viral DNA synthesis has commenced. Thomas and Mathews, (1980) have postulated that late transcription may be invoked by modification of DNA structures, such as methylation, nicking or regional denaturation.

Table III

Phenotypic class	Gross phenotype	Genetic change	Example
1. Temperature restricted			
a. Temperature-sensitive (ts)	Replication inhibited at 38-40°C but not at 32°C	Missense base-pair change	H5ts125
b. Host-range cold-sensitive (hr^{cs})	Replication inhibited in the restrictive host at 32°C but less so at 37°C	Single base-pair deletion	H5hr1
c. Host-range temperature sensitive	Replication inhibited in the restrictive host at 39°C but not at 32°C	Intragenic suppressor mutation caused by single base-pair change	H5r(ts107)202
2. Host range			
a. Restricted	i. Replication in human 293 cells; KB or Hela cells less permissive	Deletions, insertions or substitutions	H5dl312
	ii. Replication in Hela or KB cells; hamster cells less permissive	Unknown	H5hr203
b. Extended	Replication in monkey cells as extensive as in human cells	i. Single base-pair change in 72K DBP gene	H5hr404
		ii. Alterations to genome termini	Vero-adapted Ad12
3. Plaque morphology			
a. Cytocidal	Degeneration of KB cell monolayer and formation of larger plaques	Unknown	H12cyt

b.Large plaque	Larger plaques than those of Wt on KB and Hela cells	i. Single base-pair change in E1b	H2lp3
		ii.Deletion in E3	H5sub304
4. Virion stability			
a.Heat stable	Increased stability to in vitro thermal inactivation	Unknown	H5hs1
b.Heat labile	Decreased stability to in vitro thermal inactivation	i. Deletion of protein IX promotor	H5dl313
		ii.Substitution at EcoR1 site in protein VIII gene	H5RF
5. Virion structural mutants	Defective, isolated in presence of helper virus	m.u. 92.0 - 97.1	dl808
	As above	m.u. 44.0 - 53.0	dl806
6. Drug resistance mutants	-	-	-

Table adapted from Young, C. S. H., The Adenoviruses, Ginsberg (Ed).

G. ADENOVIRUS MUTANTS

The genetics of adenoviruses has been studied extensively using mutants induced by mutagens or arising spontaneously from viral stocks repeatedly passaged at high multiplicity. The classes of mutants isolated are listed in Table III.

1. Mutagens

Mutations can be induced in viruses and their infectious nucleic acids by physical or chemical mutagens. Mutagenic procedures fall into the following categories.

1.(i) In vitro

Nitrous acid, hydroxylamine, ethylmethyl sulphate and U.V. irradiation are directed against viral DNA (Takemori et al., 1968; Williams et al., 1971; Ensinger and Ginsberg, 1972; Shiroki et al., 1972; Suziki et al., 1972).

1.(ii) In vivo

Nitrosoguanidine, 5-bromodeoxyuridine and 5-fluorouracil (Williams et al., 1971; Ensinger and Ginsberg, 1972; Takahashi, 1972; Ledinko, 1974; Rubenstein and Ginsberg, 1974) are used during the viral replication cycle.

2. Spontaneous mutants

During the passage of viral stocks at high multiplicity, diverse mutations accumulate producing virions which are genetically heterogeneous. Mutants can be identified on the basis of altered plaque morphology, altered buoyant density in caesium chloride gradients or altered restriction enzyme profile.

Takemori et al.,(1968) isolated spontaneous mutants of adenovirus type 12 with an altered plaque morphology in HEK cells. Instead of the minute or barely visible wild type morphology a few clear cytocidal (cyt) plaques of .5-2mm were seen, which retained a stable phenotype during successive passages.

Non-defective spontaneous mutants of adenovirus type 12 were isolated by Werner and zur Hausen, (1978). Heteroduplex analysis, restriction enzyme analysis and contour length measurement revealed small insertions within the right hand 12% of the genome.

Groff and Daniell, (1980) isolated a spontaneous deletion mutant from adenovirus 3 strain (H3100) which had undergone passaging at high multiplicity over an extensive period. The DNA restriction enzyme profile showed the isolate (H3dl102) to have a deletion of approximately 2.2Kb mapping between 76.7 m.u. - 86 m.u. in the non-essential E3 region. Growth analysis showed that the mutant could be passaged at low multiplicity to produce infectious virus equivalent to wild type adenovirus 3.

3. Cytocidal mutants (Cyt)

Cytocidal mutants (Cyt) were isolated from adenovirus 12 strains Huie and 1131 (Takemori et al., 1968) as spontaneous or U.V. induced mutants. They gave a distinctive CPE of marked cellular destruction compared to the parental virus. The pH of the medium became very alkaline, possibly due to the massive cellular destruction caused by these mutants.

These mutants showed a marked decrease in tumourigenicity in newborn hamsters compared to the parental virus. When cyt mutant and parental viruses were co-infected into hamsters, neither inhibition nor enhancement of tumour production by the parental virus was observed. Some field strains of adenovirus 12 are less tumourigenic than strains Huie or 1131; co-infection of cyt mutants with these strains into newborn hamsters results in a co-operative effect in tumourigenicity Adenoviruses 7 and 3 wild type will co-operate with cyt mutants to produce tumours, but adenoviruses type 2, type 5 and type 4 will not.

Plaque morphology, CPE and tumourigenicity of cyt mutants remained stable over numerous passages under normal conditions. Revertants isolated from the mutant stocks were from the spontaneous isolates and not those induced by U.V. irradiation.

Ezoe and Mak, (1974) showed cells infected with cyt mutants produced fewer virions than adenovirus 12 wild type. DNA hybridization showed mutant viral DNA was present in reduced amounts; sedimentation profiles of intracellular DNA synthesised in KB cells showed a substantial proportion of the DNA molecules were smaller than the wild type (Ezoe et al., 1981).

Ezoe et al., (1981) showed the newly synthesised DNA from cells infected with H12cyt70 was extensively degraded. To establish if this was a property of the cyt mutation, KB cells were infected with three additional cyt mutants. Alkaline sucrose gradients showed that the DNA extracted from cells infected with cyt mutants was extensively degraded, whereas DNA from cells infected with either wild type or revertants was not.

The location of the cyt mutation was mapped by complementation assay (Lai Fatt and Mak, 1982). KB cells were co-infected with H12cyt70 and H5hr1 (1.3-3.7) m.u., H5hr6 (6.1-8.5) m.u., H5dl312 (Δ 448-1349)bp or H5dl313 (Δ 1334-3639)bp, the latter two viruses thereafter referred to as dl312 or dl313. Alkaline sucrose gradients showed that only co-infection with dl313 resulted in extensive DNA degradation, suggesting the lesion lay between 1334bp and 3639bp in the E1b region. Possibly the 19K glycoprotein encoded by the E1b region was involved in the DNA degradation phenomenon.

Martin et al., (1978) isolated H2ts111, a temperature-sensitive mutant of adenovirus type 2 defective for viral replication, and also extensive cellular DNA degradation (Ezoe et al., 1981). These mutations are independent, mapping in the DNA-binding protein gene (Stillman et al., 1984) and in the E1b 19K glycoprotein (White et al., 1984).

4. Temperature-sensitive mutants

Temperature-sensitive mutants are conditionally lethal, replicating as well as wild type virus at a permissive temperature of 31°C to 32°C, but failing to replicate at a higher non-permissive temperature between

38.5°C to 39.5°C.

Temperature-sensitive (ts) mutants have been isolated from chemically mutagenised viral stocks of adenoviruses type 2, type 5 and type 12.

Seventy ts mutants have been isolated from adenovirus type 5 (Williams et al., 1971; Ensinger and Ginsberg, 1972). These have been classified into 17 non overlapping complementation groups (Ginsberg et al., 1975; Williams et al., 1975a).

Bégin and Weber, (1975) isolated 40 ts mutants from adenovirus type 2, falling into 13 complementation groups and containing mutations in the late lytic cycle (Weber et al., 1975).

Kathmann et al., (1976) isolated a further 14 ts mutants, falling into 7 complementation groups; four of these mutants failed to replicate their DNA at the non-permissive temperature.

Temperature-sensitive mutants are useful for functional studies as their environment (temperature) can be controlled and the mode of action of the mutated gene identified.

Temperature-sensitive mutants falling into William's complementation group N are defective for DNA replication at the non-permissive temperature (Wilkie et al., 1973; Ginsberg et al., 1974; van der Vliet and Sussenbach, 1975; Schutzbank, 1980). Marker rescue experiments (Galos et al., 1979) mapped the mutation to between 18.5 m.u.-22.0 m.u.; in vitro translation showed early adenovirus functions were coded by

this region (Stillman et al., 1981). DNA sequence analysis (Alestrŏm et al., 1982; Gingeras et al., 1982) suggested a gene encoding an adenovirus specific DNA polymerase.

Adenovirus type 2 ts mutant H2ts1 suggested a requirement for an adenoviral specific protease in the proteolytic conversion of precursor proteins, pVI, pVII and pVIII to the specific viral polypeptides (Weber, 1976). Hassel and Weber, (1978) found the mutation to map between positions 57 m.u. and 69 m.u. DNA sequence analysis (Kruijer et al., 1980; Akusjärvi et al., 1981) showed an open reading frame with the capacity to encode a polypeptide with a molecular weight of 23K, possibly a viral protease.

5. Host range mutants

Host range mutants fail to replicate in established human cell lines, eg. Hela or KB cells, but do so in the human embryonic kidney (293) cell line, which is transformed by 12% of the left hand end of the adenovirus type 5 genome (Graham et al., 1977) and expresses adenovirus E1 functions.

Host range mutations have been mapped by recombination within the left end of the genomic map (Harrison et al., 1977) and some have been physically mapped within this region using marker rescue (Frost and Williams, 1978).

Harrison et al., (1977) isolated progeny from chemically mutagenized adenovirus type 5 which were negative for growth in Hela cells. Complementation analysis in Hela cells classified these mutants into

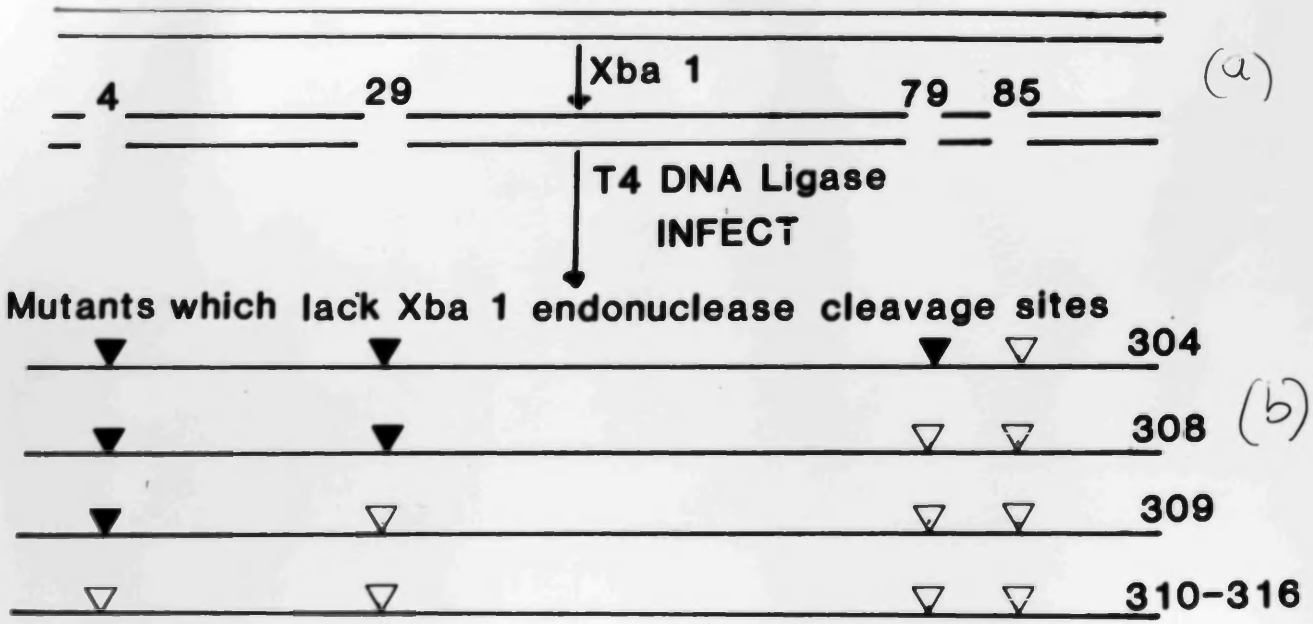

Xba 1
4
29
79
85
(a)
T4 DNA Ligase
INFECT
Mutants which lack Xba 1 endonuclease cleavage sites
304
308
309
310-316
(b)

Figure 5.

Construction of adenovirus 5 deletion mutants

Strategy used to isolate deletion mutants, H5dl304, H5dl308, H5dl309 and H5dl310-H5dl316, of adenovirus 5 wild type lacking Xba1 cleavage sites.

▽ represents Xba1 cleavage sites missing.

Reproduced from Jones and Shenk, (1978) Cell 13:181-188.

(a) Shows the strategy used to manipulate the DNA to produce the isolates listed in (b) .

(b) Shows the initial DNA , sub. 304 which lacked one XbaI site . By further XbaI digestion and re-ligation of the DNA , subsequent XbaI sites are lost . Isolates 310 - 316 have no XbaI sites present .

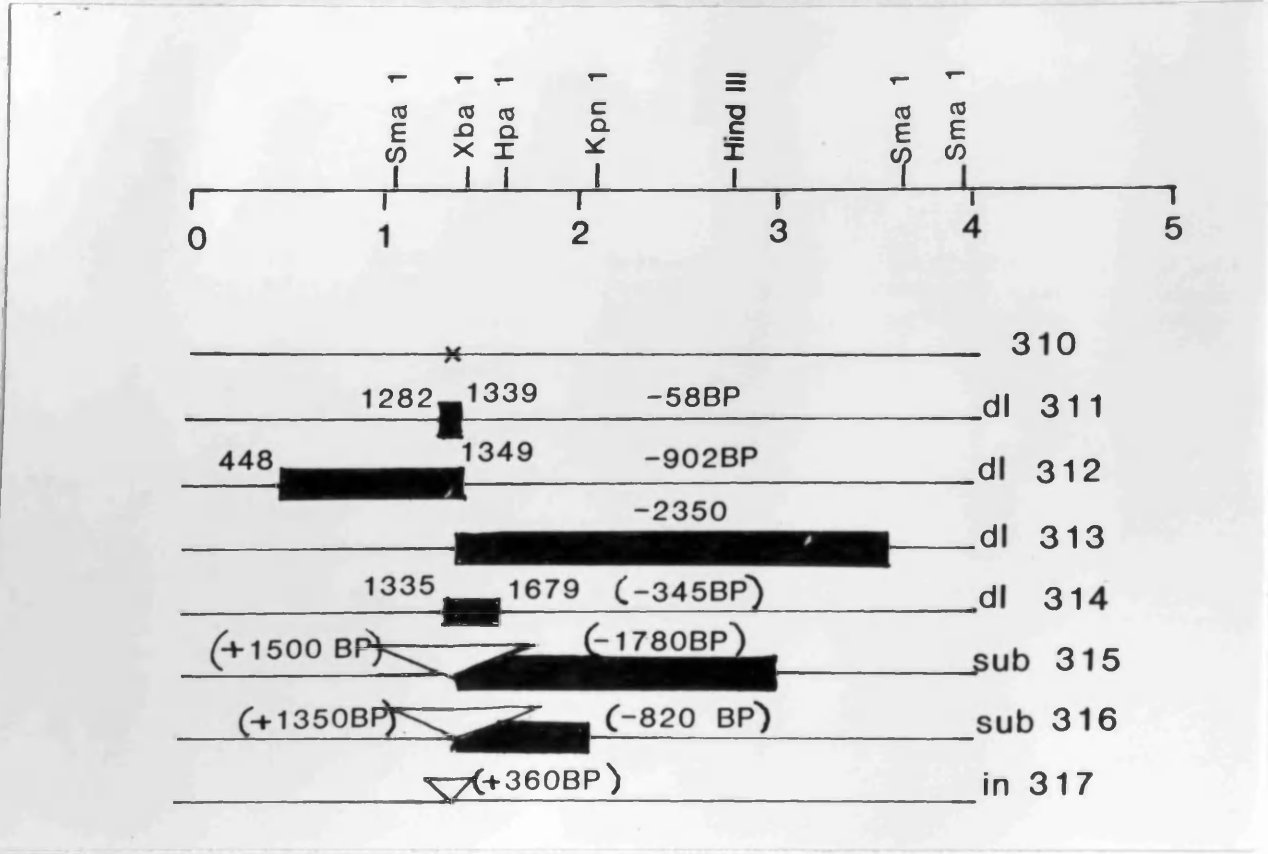

Sma 1
Xba 1
Hpa 1
Kpn 1
Hind III
Sma 1
Sma 1
0
1
2
3
4
5
310
1282
1339
-58BP
dl 311
448
1349
-902BP
dl 312
-2350
dl 313
1335
1679
(-345BP)
dl 314
(+1500 BP)
(-1780BP)
sub 315
(+1350BP)
(-820 BP)
sub 316
(+360BP)
in 317

Figure 6.

Deletion and substitution mutants of adenovirus type 5

Showing the genetic aberrations lying in the left hand end of the host range adenovirus 5 constructed mutants.

Reproduced from Jones and Shenk, (1979) Cell 17:683-689.

 ⇒ The deleted DNA sequences in dl.311-314 sub.315 and sub.316.

 ⇒ The inserted DNA sequences in sub.315-316 and in.317 .

two groups.

Group 1 showed a restrictive host range, only replicating in 293 cells. Representative of this class is H5hr1 which fails to replicate in Hela cells (Esche et al.,1980) and has a single base pair deletion at nucleotide 1055 mapping in the E1a region, resulting in the 51K polypeptide being truncated to a 28K polypeptide (Ricciardi et al., 1981).

Group 2 mutants replicated with equal efficiency in 293 and HEK cells, but not in Hela or Helu cells. Representative of this class is H5hr7 (Harrison et al., 1977; Galos et al., 1980; Ross et al., 1980) mapping between 6.1 m.u. - 8.0 m.u. in the E1b region.

Jones and Shenk, (1979) isolated a series of mutants from adenovirus type 5 deleted in the left hand transforming end of the genome. These mutants propagated in 293 cells but not in Hela cells, (Figs. 5 and 6 show the construction of these mutants).

Growth analysis showed the mutants plaqued as efficiently as wild type virus in 293 cells, but plaquing efficiency in Hela and KEK cells was greatly reduced. At a multiplicity of infection (m.o.i.) between 1-10 p.f.u./cell dl313 and H5sub315 yielded a few plaques in HEK cells; dl312 caused a CPE of the cell monolayer. The host range phenotype of dl312, dl313 and H5dl314 are multiplicity dependent producing substantial yields of virus at m.o.i. between 80-800 p.f.u./cell.

The transformation efficiency of these mutants was assayed by focus formation in rat cells. All were found transformation deficient.

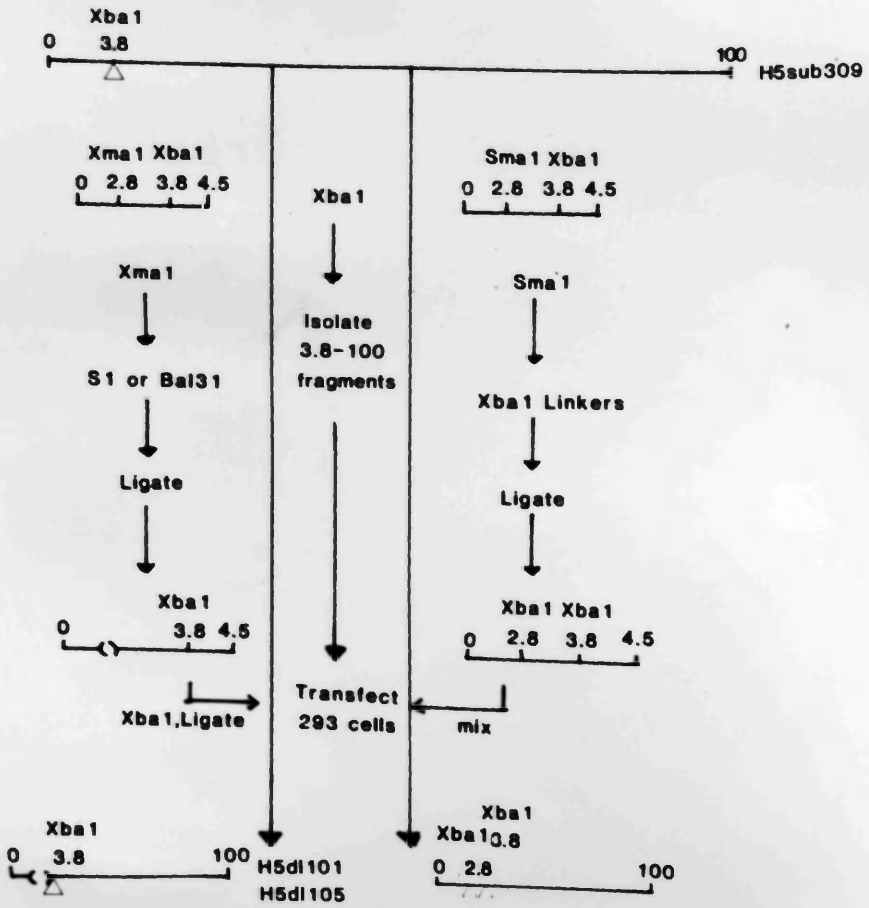

Xba1
0
3.8
100
H5sub309
Xma1 Xba1
0 2.8 3.8 4.5
Xba1
Sma1 Xba1
0 2.8 3.8 4.5
Xma1
S1 or Bal31
Ligate
Isolate
3.8-100
fragments
Sma1
Xba1 Linkers
Ligate
Xba1
0
3.8 4.5
Xba1 Xba1
0 2.8 3.8 4.5
Xba1,Ligate
Transfect
293 cells
mix
Xba1
0
3.8
100
H5dl101
H5dl105
Xba1
Xba1 3.8
0 2.8
100

Figure 7.

Construction of E1a deletion and insertion mutants of adenovirus type 5

Method used to construct H5dl101 and H5dl105 by ligation or overlap recombination. Brackets represent deletion of DNA. △ represents the presence of restriction enzyme sites.

Figure is reproduced from Babiss et al., (1984) J. Virol. 49:731-740.

Dl 101 and dl 105 were constructed by ligating the mutated 0 - 3.8 m.u. fragment of adenovirus 5 to the 3.8 - 100 m.u. fragment of sub. 304 .

In. 106 was constructed by recombination between the mutated 0 - 4.5 m.u. fragment of adenovirus and the 3.8 - 100 m.u. fragment of sub. 304 .

6. The E1a region in cellular transformation

It has been shown that rodent cells can be transformed by the left hand 8% of the adenoviral genome (Graham et al., 1974; Gallimore et al., 1974; Flint et al., 1975; 1976; van der Eb et al., 1977) and that partial transformation can be obtained using only 4.5% of the left hand end of adenovirus type 5 genome (Houweling et al., 1980).

Mutants containing lesions in the E1a region, eg. H5hr1 (Harrison et al., 1977), H5in500 (Carlock and Jones, 1981), H5hr440 (Solnick and Anderson, 1982) are defective for transformation of baby rat kidney cells, suggesting the requirement of a functional E1a region for cellular transformation.

Ho et al., (1982) showed H5hr1 to be cold sensitive for cellular transformation. Babiss et al., (1984) constructed a series of mutants containing deletions or insertions in the E1a 13S message (Fig. 7) to ascertain if the cold sensitive phenotype was a direct result of the E1a mutation and what region of the 13S encoded 51K protein was involved in cellular transformation. Data suggested that the cold sensitive transformation phenotype was a direct result of the altered gene product of the 13S mRNA.

Mutants H5dl101 and H5dl105 encoded a truncated 33K protein in place of the 51K protein encoded by the 13S mRNA transcript; H5dl101 was cold sensitive for cellular transformation. However, H5dl105 would not transform cells at either 37°C or 32°C. DNA sequence analysis showed a 5bp difference mapping between 1002-1007bp, suggesting that the nucleotide difference between a functional truncated protein at 37°C

and a non-functional one at 37°C or 32°C must map in this region and that the E1a 51K protein is necessary for inducing cellular transformation.

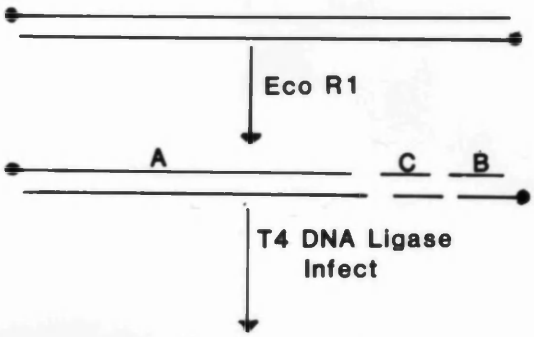
7. The E1b region in cellular transformation

Rodent cells transfected with adenoviral DNA encompassing both the E1a and E1b regions of the genome become transformed (Graham et al., 1974; van der Eb and Houweling, 1977; van der Eb et al., 1977). DNA encompassing the E1a region only is found to transform cells but does not allow for maintained phenotype (Shiroki et al., 1979; Houweling et al., 1980) suggesting that the E1b gene products are necessary for the establishment of transformation.

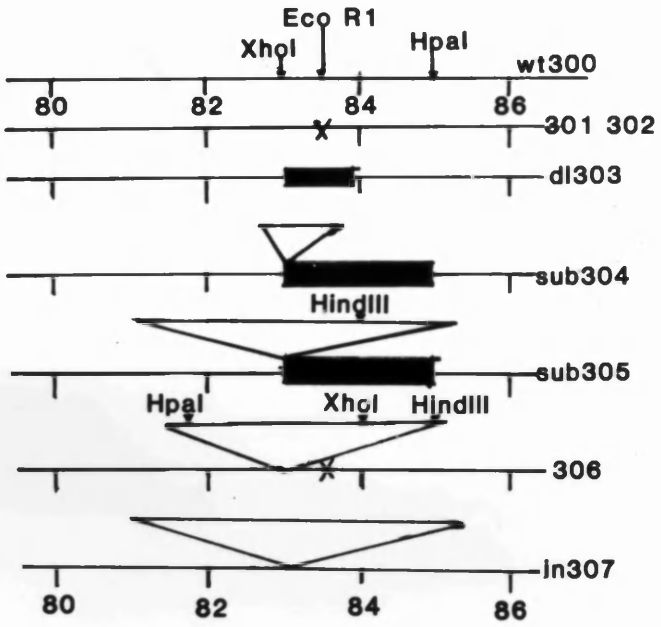
van den Elsen et al., (1982) transformed baby rat kidney cells (BRK) by co-transfection with fragments containing the separated E1a and E1b regions of adenovirus type 5; transfection with the fragments containing single E1 regions showed that the E1a region gave a reduced transformation efficiency and the E1b region showed no transformation activity. Data suggested that the E1b region of adenovirus type 12 could be stimulated by the E1a of adenovirus type 5 and that the E1b viral specific antigens expressed were from adenovirus type 12.

McKinnon et al., (1982) constructed insertion mutations in the 16% left hand end of the genome using the transposable element Tn5. Data showed that transformation was abolished by an insertion of DNA in the E1a region mapping at 2.8 m.u. and in the E1b region mapping near the 5' splice site of the 13S transcript. Further evidence of the involvement of the E1b region in cellular transformation came from Fukui et al., (1984). They constructed insertion and deletion mutants of adenovirus

8 a


Eco R1
A
C
B
T4 DNA Ligase
Infect

8 b


Eco R1
XhoI
HpaI
wt300
80
82
84
86
301 302
dl303
sub304
HindIII
sub305
HpaI
XhoI
HindIII
306
in307
80
82
84
86

Figures 8a and 8b.

Construction and map co-ordinates of E3 mutants of adenovirus type 5

a. Strategy used to construct deletion and substitution mutants of adenovirus type 5 lying in the E3 region listed in (b).

b. Map co-ordinates of mutants lying in the E3 region of the genome.

Figures are reproduced from Jones and Shenk, (1978) Cell 13:181-188.

 ⇒ The deleted DNA sequences in dl 303 , sub. 304 and sub 305

 ⇒ The inserted DNA sequences in sub.304, sub. 305 ,sub. 306 and in 307 .

type 12 mapping in the E1b-19K polypeptide. Data showed that cellular transformation by these mutants was greatly reduced as was their capacity to produce cellular colonies in soft agar. This suggested that the E1b -19K polypeptide was involved in cellular transformation and the formation of transformed cellular colonies in soft agar.

8. Deletion and Substitution mutants

Jones and Shenk, (1978) (Figures 8a and 8b) constructed a series of adenovirus 5 deletion and substitution mutants, mapping at the EcoR1 83 m.u. site in the E3 region. These mutants replicated as well as wild type virus suggesting the E3 region was non-essential for viral replication in tissue culture. Another series of substitution and deletion mutants mapping in the E1 region was constructed using H5dl309 [H5dl309 is called dl309 thereafter] (Jones and Shenk, 1978) as parental virus. These mutants fell into two classes; those which could replicate in Hela cells and those which could not. Included in this class are mutants dl312 and dl313.

A series of eight deletion mutants of adenovirus type 2 was isolated from a high passaged viral stock (Challberg and Ketner, 1981). The yield of mutants isolated was increased by complementing the conditional lethal defects in the adenovirus type 2 mutants with ts mutants of adenovirus type 5. Seven of the mutants were deleted between 78.5 m.u.-97 m.u. One mutant (H2dl807) was deleted in the E3 and fibre region and part of the E4 region. DNA replication was equivalent to wild type. However, protein analysis showed that late viral proteins pII, pV, pVI, pVII and pVIII were present in reduced amounts while pIIIa and the 100K polypeptide were present in normal amounts.

Berkner and Sharp, (1983) constructed a viable E3 deletion mutant of adenovirus type 5 using overlapping cloned DNA fragments. The mutant was deleted between 78.9 m.u.-84.3 m.u., removing most of the mRNAs encoded by this region. This mutant grew as well as wild type adenovirus type 5 synthesising late viral polypeptides to the same level. When the mutant was co-infected with wild type virus or parental dl309 into 293 or Hela cells, it inhibited the DNA replication of both viruses, being found to be in 10 fold excess to the wild type.

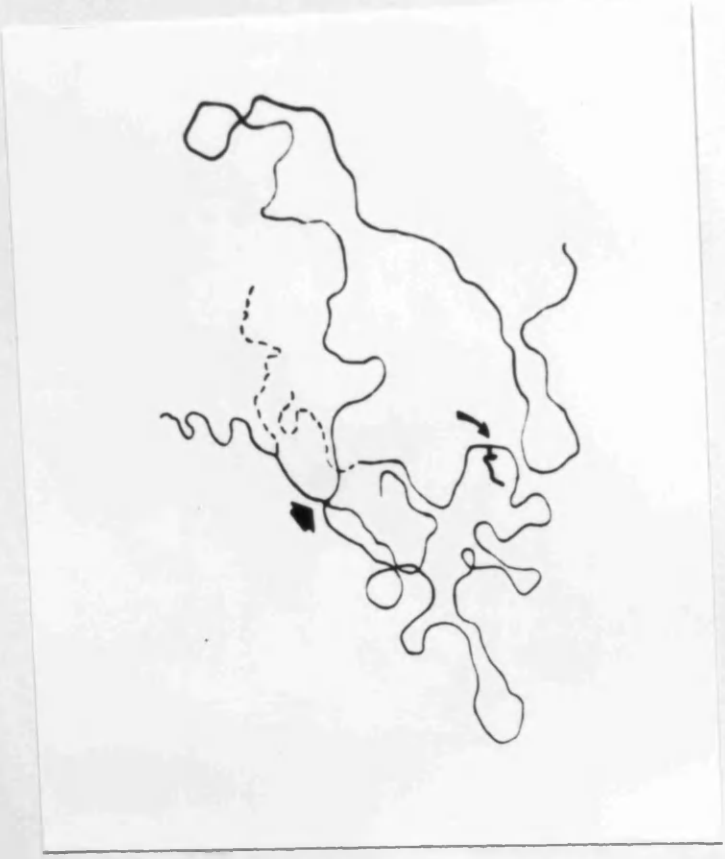
9. Large plaque phenotype mutants

Takemori et al.,(1968) isolated large plaque phenotype mutants of adenovirus 12 strains Huie and 1131 (see Section G:3 cytocidal mutants).

Chinnadurai et al., (1979) mutagenised adenovirus type 2 with hydroxylamine, plated the virus on KB cells and selected for well separated large plaques. By overlapping terminal DNA fragments, recombinants were constructed between the wild type and the large plaque mutants to map this phenotype. Ten recombinants were isolated and found to express the large plaque phenotype which mapped in the region between 0-41% of the genome. Marker rescue and DNA sequence analysis mapped the large plaque phenotype to the E1b region coding for the 19K polypeptide. The mutation was shown to be an amino acid substitution.

Subramanian et al., (1984) constructed a viable deletion mutant of adenovirus type 2 (H2dl250) which lacked most of the 19K polypeptide coding capacity; this mutant had a large plaque phenotype and was also cytocidal. Other large plaque mutants were not cytocidal (Chinnadurai et al., 1983), suggesting the phenotypes to be separate mutations,

although they both code in the 19K region. Like the cyt mutants of adenovirus 12, H2dl250 was defective in cellular transformation; the transformation efficiency of rodent cells being much reduced compared to the wild type. The mutant was also defective in anchorage independent growth in soft agar, suggesting that the adenovirus type 2 E1b-19K polypeptide is involved in transformation and colony formation in soft agar, (See Section G:7).



9a

9b

Figures 9A and 9B.

Recombination intermediate of adenovirus type 2

9A. - Shows an interpretive tracing taken from an electron micrograph of recombination between adenovirus type 2 DNA molecules. The crossover point showing a Holliday type structure is indicated by a large arrowhead. A nascent RNA transcript is shown by the curved arrow and heavy black line; single stranded DNA is indicated by the dashed lines.

Figure is taken from Wolgemuth and Hsu, (1980) Nature 287:168-171.

9B. - Shows enlargement of the crossover point (Holliday structure) which is shaped like the Greek letter Chi, being bilaterally symetrical.

The high concentration of formamide used in E.M. preparation has caused the DNA double helix to partially denature and single stranded DNA connection in the crossover region can be observed.

Figure taken from Dressler and Potter, (1982) Ann. Rev. Biochem. 51:727-761.

The Chi configuration is prepared from plasmid DNA

H. ADENOVIRAL RECOMBINATION

Recombination can take place during the eclipse phase and late phase of viral replication at which time DNA molecules can undergo several rounds of recombination before being encapsidated to produce viral progeny. Thus new strains of adenovirus have been generated and used for genetic mapping and functional studies (Williams et al., 1975a;b; Young and Silverstein, 1980).

Adenovirus recombination is homology based, electron micrographs of adenovirus DNA extracted from infected cells show proposed Holliday Structure (Holliday, 1964; 1968; 1974) with equal length arms on opposite sides of the Chi formation indicating a crossover at equivalent positions on each molecule (Wolgemuth and Hsu, 1980) Fig. 9. Recombination occurs between closely related serotypes but has not been observed between serotypes belonging to different subgroups (Williams et al., 1975a;b; 1981).

Recombination has been observed using three different experimental approaches:-

1. Recombination between full length adenoviral genomes

Adenovirus 5ts mutants readily recombine with each other to produce viable progeny (Williams and Ustacelebi, 1971a; Williams et al., 1971b; Ensinger and Ginsberg, 1972). By two-factor ts x ts crosses a genetic map of the adenovirus ts mutants was constructed (Williams et al., 1975b) on the basis of recombination frequency. The restriction endonuclease profiles of adenoviruses type 2 and 5 are sufficiently different as to

identify each virus (Mulder et al., 1974a;b).

Data showed that recombinant progeny between ts mutants of adenovirus 5 and the non-defective adenovirus - SV40 hybrid, $Ad2^{+}ND1$ (Grodzicker et al., 1974) and also progeny from double infections with ts mutants of adenoviruses type 2 and type 5 (Williams et al., 1975b) contained the restriction enzyme sites of both parental viruses. The location of the crossover sites could be defined and genetic and physical maps could be orientated relative to each other, mapping a mutation to a specific region of the genome.

Restriction endonuclease analysis of these heterotypic recombinants suggested that the genetic and physical maps were co-linear (Grodzicker et al., 1974; Williams et al., 1975b).

Young and Silverstein, (1980) suggested from data of recombination kinetics for ts homotypic and heterotypic recombinants that the recombination frequency increased during DNA replication, implying several rounds of recombination before virion assembly.

Boursnell and Mautner, (1981) showed that recombination within the hexon gene of the closely related serotypes of adenoviruses type 2 and type 5 occurred within regions of DNA homology. This was confirmed by DNA sequence analysis (Mautner and Boursnell, 1983).

2. Recombination by marker rescue

In this approach cell monolayers are co-transfected with full length mutant DNA and a subgenomic fragment bearing the wild type

allele. Resulting progeny selected under non-permissive conditions are usually recombinants exhibiting the wild type allele. This approach has been employed to map the locations of ts and hr mutations.

Lai and Nathans, (1974) mapped the lesions in several ts mutants of SV40 (Tegtmeyer and Ozer, 1971) using wild type subgenomic fragments of known location on the SV40 genomic map (Danna et al., 1973). The strategy used was to mix mutant DNA with wild type fragment DNA under denaturing and renaturing conditions to form heteroduplex structures. This mixture was used to inoculate and infect cell monolayers.

Miller and Fried, (1976) used this approach to map the mutation of several polyoma ts mutants.

Frost and Williams, (1978) mapped the lesions of several ts and hr mutants of adenovirus type 5. Their strategy was to transfect 293 cell monolayers with the DNA of the mutant and subgenomic wild type fragment in the form of a calcium phosphate-DNA precipitate as described by Graham and van der Eb, (1973). Cellular uptake of the precipitate was enhanced by a short exposure to 20% glycerol at four hours post transfection.

Arrand, (1978) mapped the lesions of several ts mutants of adenovirus type 5 by a similar method to the one described above (Frost and Williams, 1978); Hela monolayers were used for transfection. Enhancement of cellular uptake of the calcium phosphate-DNA precipitate was by brief exposure to DMSO at 3-5 hours post transfection.

3. Recombination between overlapping terminal DNA fragments

Chinnadurai et al., (1979) developed a strategy to construct adenovirus recombinants. Recombination was achieved by using overlapping terminal DNA fragments to transfect permissive cells via a calcium phosphate-DNA precipitate (Graham and van der Eb, 1973). Recombination can take place between homologous overlapping regions of DNA to produce viable progeny. They used this approach to construct recombinants between adenovirus type 5 and a large plaque mutant of adenovirus type 2 and were able to physically map the phenotype of the mutant.

Volkert and Young, (1983) used this technique to analyse the genetics of homologous recombination within adenovirus type 5. Data suggested that recombination was not site specific,occurring equally throughout the region of overlap with no preference for molecular termini at the ends of the overlaps. Recombination frequency suggested that within an overlap the recovery of a single mutation was dependent on its location relative to the molecular ends of the overlap.

Mautner and MacKay, (1984) used this method to construct recombinants within the vicinity of the hexon coding region between adenoviruses type 2 and type 5. Data suggested that recombination occurred within the region of overlap with no preference for the termini at the ends of the overlap; the crossover sites within the recombinants lay in zones of homology, the smallest detectable region being 21 nucleotides long.

I. Aims of the Thesis

Adenovirus recombination takes place at a high frequency between serotypes belonging to the same subgroup (Grodzicker et al., 1974; Williams et al., 1975a; b). Recombination has not been demonstrated between serotypes of different subgroups although complementation can occur suggesting a functional relatedness of at least those genes of the subgroups (Williams et al., 1975a; 1981; McDonagh and Rekosh, 1981).

Adenovirus recombination is homology-based; EM analysis of adenovirus DNA extracted from infected cells show the formation of Holliday Structures suggesting recombination to be homology based (Wolgemuth and Hsu, 1980). Boursnell and Mautner, (1981) showed that crossover sites within a recombinant lay in zones of high DNA homology.

The aim of this project was to isolate recombinant viruses between subgroups B and C by

(a) Marker rescue (Lai and Nathans, 1974; Miller and Fried, 1976; and Frost and Williams, 1978) of ts mutants of adenovirus type 5 by defined DNA fragments of adenovirus type 7,

(b) Co-transfection of overlapping terminal DNA fragments into permissive cells (Chinnadurai et al., 1979); Volkert and Young, 1983).

The restriction endonuclease profile of the two serotypes is distinctly different and therefore it is possible to detect recombination within the progeny (Mulder et al., 1974a; b).

Chapter 2.

Materials and Methods

A. MATERIALS

1. Cell lines

The cell lines used were 293, Hela and a family of KB cells.

(a) 293 cells were established by Graham et al., (1977), by transforming in vitro a primary cell culture of human embryonic kidney cells with sheared adenovirus type 5 DNA (Graham and van der Eb, 1973). The integrated viral sequences express both E1a and E1b products.

(b) Hela cells were established by Gey et al., (1952) from an epidermoid cervical carcinoma.

(c) Babiss et al., (1983) modified the plasmid pSV2-gpt containing the XGPRT gene, whose expression is under the control of the SV40 early promotor, to contain the adenovirus type 2 Xho1 C (0-15.5) m.u. DNA fragment. The modified plasmid was transfected into human KB cells via a calcium phosphate precipitate and positive transformants were selected by their ability to grow in xanthine, aminopterin and mycophenolic acid. Four classes of KB cell lines were isolated (i) KB7 expressing no adenoviral products; (ii) KB16 expressing both E1a and E1b products; (iii) KB8 expressing E1a products; (iv) KB18 expressing E1b products.

2. Tissue culture medium

Cell lines were passaged in Glasgow modified Eagles medium (Busby et al., 1964) supplemented with 100 units per ml penicillin, 100ug per ml streptomycin and .002%(w/v) phenol red.

Calf serum or foetal calf serum was added in the following proportions.

EFC10 - 90% Eagles medium and 10% foetal calf serum.

ECS10 - 90% Eagles medium and 10% calf serum.

ECS2 - 98% Eagles medium and 2% calf serum (infection medium).

E(-PO_4) - Eagles medium minus phosphate.

Eagles overlay medium

Eagles medium without phenol red and containing .65% Nobles agar was supplemented with 2% calf serum. 12.5mM $MgCl_2$ was added to the overlay medium (25mM $MgCl_2$ for assays at 31°C) to enhance plaque formation (Williams, 1970).

Tissue culture medium and calf serum were prepared by the Media Department of the Institute of Virology; foetal calf serum was supplied by Gibco, Scotland.

3. Viruses

Background history of adenovirus serotypes used

(i) Serotypes 2 and 5 (subgroup C) were isolated from ademoid cultures from two female patients having undergone adeno-tonsillectomy (Huebner et al., 1954; Rowe et al., 1953; Pereira et al., 1963).

(ii) Serotype 3 strain GB (subgroup B) was isolated from nasal washings of a patient suffering from rhinorrhoea (Huebner et al., 1954; Pereira et al., 1963).

(iii) Serotype 7 Gomen (subgroup B) was isolated from a military recruit suffering from pharyngitis (Berge et al., 1965; Pereira et al., 1963).

(iv) Serotype 7a (subgroup B) was isolated from a throat swab of a patient suffering from an undifferentiated respiratory infection (Rowe et al., 1958).

Freeze dried stocks of adenovirus 7 strain Gomen, adenovirus 7a strain H, adenovirus 7a VR8 and adenovirus 7A Pinkney were obtained from the National Institute for Medical Research, Mill Hill; adenovirus 3 strain GB was obtained from W. C. Russell, Mill Hill. Adenoviruses type 2 and type 5 were current laboratory stocks.

(v) Deletion mutants

Deletion mutants of adenovirus type 5 were dl309 which contains only the 3.8 m.u. Xbal site; dl312 which is deleted in the E1a region between 1.2 m.u.-3.7 m.u. and dl313 which is deleted in the E1a and E1b regions between 3.7 m.u.-10.1 m.u.. Both these viruses are progeny of dl309. All were constructed by Jones and Shenk, 1978; 1979.

(vi) Temperature sensitive mutants

Temperature sensitive mutants of adenovirus type 5 used were:- H5ts1 (100K), H5ts2 (Hexon), H5ts19 (72K or 100K) [Williams et al., 1971]; H5ts14 (Hexon), H5ts125 (72K) [Ensinger and Ginsberg, 1972; Ginsberg et al., 1974; Kruijer et al., 1981; 1982]; H5ts40 (Hexon) [Galos and Williams, 1980].

4. Plasmid vector pUC8

Plasmid vector pUC8 is from the family of pUC vectors (Gronenborn and Messing, 1978; Vieira and Messing, 1982; Messing and Vieira, 1982). They are based on the EcoR1-PvuII ampicillin resistant fragment of pBR322 with the removal of the HincII and PstI sites by mutagenesis and the AccI site by Bal 31 digestion. The resulting plasmid was linearised with HaeII and the HaeII 433bp of bacteriophage M13mp7 inserted. This fragment encodes the sequence required for the α complementation of the β-galactosidase activity and also the multiple cloning site within the polypeptide coding region. The β-galactosidase activity is visualised by the chromogenic indicator 5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside [X-gal, Miller, 1972] which when hydrolysed yields a blue colour. A β-peptide expressed by the bacterial host can be complemented by an intact pUC vector and thus a blue colony will result, whereas a pUC vector with an insert of DNA in the multiple cloning site will be unable to complement and thus the colony will be white.

5. Host bacteria for plasmid propagation.

The host bacteria used to propagate the pUC8 plasmids were E.coli K12 JM83 (Messing, 1979), a β galactosidase deficient strain carrying integrated in the chromosome a phage which codes for the β peptide of this enzyme.

6. pARKR plasmid

Plasmid pARKR (Hay et al., 1984) is constructed to contain two adenovirus termini in the opposite orientation separated by the bacterial

kanamycin resistance gene of pKC7 (Rao and Rogers, 1979). When the plasmid is cleaved with the endonuclease EcoR1 the mini replicon containing the ITRs is released. This replicon is capable of autonomous replication on co-transfection with adenovirus DNA.

7. Versene (EDTA)

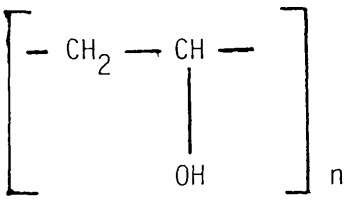
Ethylenediaminetetra-acetic acid (6mM) was dissolved in PBS containing .002% (w/v) phenol red.

8. Giemsa stain

A 1.5% (v/v) suspension of giemsa stain in glycerol was heated to 56°C for 120 minutes, diluted with equal volume of methanol and filtered.

9. Gelvatol mounting fluid

"Gelvatol" is the trade name for polyvinyl alcohol resins. It is a polymer prepared from polyvinyl acetates by the replacement of the acetate group with hydroxyl groups.



To prepare the mounting fluid 20g of "Gelvatol" were dissolved in 80ml of 140mM NaCl, 10mM KH_2PO_4 and 10mM $Na_2HPO_4:12H_2O$ and the solution was agitated at room temperature overnight. 40ml of glycerol were added, followed by overnight agitation at room temperature. The following day the solution was centrifuged at 12K for 15 minutes at room temperature. The supernatant (pH6-7) was collected and stored at 4°C.

10. Bisbenzimide H33258, flurochrome stain

The flurochrome stain was dissolved in PBSA (140mM NaCl, 34mM KCl, 40mM Na_2HPO_4, 2mM KH_2PO_4pH7.4) to a final concentration of 250ng/ml.

11. Radioisotopes

^{32}P inorganic orthophosphate was obtained from the Radiochemical Dispensary of the Western Infirmary, Glasgow. 5' α ^{32}P deoxynucleoside triphosphates (2000-3000ci/m.mol) were purchased from the Radiochemical Centre, Amersham, Bucks., U.K.

12. Reagents

Sigma Chemical Company Ltd., U.K.

Agarose, Boric acid, Sodium dodecyl sulphate, Tris-base, Salmon sperm DNA, Ribonuclease A, Deoxyribonuclease, Ethylenediaminetetra-acetic acid, Spermine, Gelatin (Swine skin), Guanidinium Hydrochloride, Sucrose (ultra pure), Dextrose, Bovine serum albumin, Dithiothreital, Polyvinyl-pyrrolidone, Bromophenol blue, Deoxyribonucleic acid polymerase, Dowex AG 50Wx8, 5-Bromo-4-Chloro-3-Indoyl-β-D-Galactopyranoside (X-gal), Caesium chloride, Rubidium chloride, Sodium azide.

BDH Chemicals Ltd., Poole, England

Sodium chloride, Sodium hydroxide, Magnesium chloride, Magnesium sulphate, Glycerol, Potassium chloride, di-Sodium hydrogen orthophosphate,

tri-Sodium citrate, Calcium chloride, Potassium di-hydrogen orthophosphate, N-2-Hydroxyethylpiperazine-N'-2-ethanesulphonic acid (Hepes), Hydrochloric acid, Sulphuric acid.

Cairns Chemicals Ltd., Bucks.,England

"Gelvatol" 20-30 (Polyvinyl alcohol resin).

Pharmacia Chemicals, Sweden

Sephadex G50 (medium) Ficoll 400

The Boehringer Corporation, London

Bisbenzimide H33258

Difco Laboratories, Surrey, England

Bacto tryptone, Yeast extract.

Bethesda Research Laboratories (BRL)

Restriction endonucleases were purchased from the above company and used for DNA cleavage as specified by the manufacturer with the modification of gelatin being substituted for BSA.

Aldrich Chemical Company

HexamminecobaltCl_3

13. Buffers and Solutions

TE

10mM Tris/HCl (pH8)

1mM EDTA (pH8)

TEN

10mM Tris/HCl (pH8)

1mM EDTA (pH8)

100mM NaCl

TBE

89mM Tris/HCl (pH8)

89mM Boric acid

4mM EDTA

20 x SSC

3M NaCl

.3M $Na_3C_6H_5O_7.2H_2O$

Tris/Saline (pH7)

140mM NaCl

30mM KCl

28mM Na_2HPO_4

1mg/ml. dextrose

25mM Tris

STET buffer

8% (w/v) Sucrose

.25% (w/v) Triton x 100

50mM EDTA

50mM Tris/HCl (pH8)

Hirt buffer

10mM Tris pH7.9

10mM EDTA

.6% SDS

NaCl is added at the end of the 20 minute incubation period to a final concentration of 1M.

Hepes buffer

5g/litre Hepes

8g/litre NaCl

.37g/litre KCl

.125g/litre $Na_2HPO_4.2H_2O$

1g/litre glucose

buffer to pH7.05 with NaOH and sterilise by filtration.

PBS solution

PBSA

.17M NaCl

.0034M KCl

.001M Na_2HPO_4

.002M KH_2PO_4

buffer to pH7.2 in distilled water

PBSB

6.8mM $CaCl_2.2H_2O$

PBSC

4.9mM $MgCl_2.6H_2O$

PBS Solution is 8 parts solution A, 1 part solution B, 1 part solution C.

SOB

2% Bacto tryptone

.5% yeast extract

10mM NaCl

2.5mM KCl

10mM $MgCl_2$

10mM $MgSO_4$

SOB is prepared without magnesium ions and autoclaved. A 2M Mg^{++} stock (1M $MgCl_2.6H_2O$, 1M $MgSO_4.7H_2O$) is sterile filtered and used to make the medium 20mM.

SOC

SOB + 20mM glucose

TFB

10mM K-mes(pH6.2)

100mM RbCl

45mM $MnCl.4H_2O$

10mM $CaCl_2.2H_2O$

3mM $HACoCl_3$

1M Mes is adjusted to pH6.3 using KOH sterile filtered.

Prehybridization buffer

30ml 20 x SSC

60ml distilled water

10ml 50 x Denhardts solution

.5ml 20% SDS

1.0ml denatured salmon sperm DNA at 2mg/ml.

Hybridization buffer

4ml 1M Tris/HCl pH7.5

53ml distilled water

60ml 20 x SSC

40ml 50 x Denhardts solution

.4ml .5M EDTA pH8

10ml 10% SDS

5ml denatured salmon sperm DNA at 2mg/ml

50 x Denhardts solution

1% Bovine serum albumin

1% Ficoll 400

1% Polyvinylpyrollidone

.01% sodium azide

B. METHODS

1. Tissue Culture

Cells were passaged every 4 days as monolayers in 800ml tissue culture flasks for DNA transfections and viral experiments. For growth of viral stocks, monolayers were grown in Winchester roller bottles. Cells were routinely screened for mycoplasma contamination by the method described by Chen, (1974).

2. Mycoplasma Screening (Chen, 1974)

Cells were seeded at low density on to a glass coverslip. When monolayers were 50%-60% confluent they were fixed for 3 minutes at room temperature in methanol:acetone (3:1). One drop of flurochrome stain was added per coverslip and left to stand for 10 minutes at room temperature. Fixed monolayers were washed twice in distilled water and allowed to air dry. For viewing, coverslips were mounted in "Gelvatol" mounting fluid (polyvinyl alcohol resins) and scanned by ultra-violet rays using a x50 oil immersion lens on a Leitz Wetzlar microscope.

3. Preparation of viral stocks

Freeze dried stocks of virus were reconstituted in sterile distilled water. Virus was then passaged through three rounds of plaque purification on 293 cells; isolated plaques were screened by restriction enzyme analysis between each plaque purification step to ensure that isolates were of the original prototype.

Laboratory stocks were plaque purified through three rounds with the restriction enzyme profile of all isolates being screened. On final purification selected plaques were grown-up as crude viral seed stocks.

Virus stocks of dl312 and dl313 were prepared from viral DNA by initially transfecting 293 cell monolayers and selecting plaques which were screened by restriction enzyme analysis. Progeny were plaque purified through three successive rounds as described above.

4. Crude seed stocks

Drained 50mm monolayers of 293 cells were infected from isolated plaques at low multiplicity (i.e. .01 p.f.u./cell in 100ul of ECS2 per plate). Virus was allowed to absorb to cells for 1 hour at 37°C or 1.5 hours at 32°C for temperature sensitive mutants. 4ml of ECS2 medium were added per plate, followed by incubation at the permissive temperature for viral growth. When a CPE was observed monolayers were scraped into the medium and harvested into glass universal containers.

Cells were pelleted by centrifugation at 2K for 20 minutes at room temperature in an MSE bench centrifuge. Supernatants were decanted and the cell pellets resuspended in 2ml of ECS2 medium. Virions were liberated from the cells by three rounds of freezing in methanol/dry ice and thawing at 37°C. Viral supernatants were titrated on 293 monolayers.

5. Preparation of purified virions

Monolayers of 293 cells were grown in Winchester Roller bottles and infected at a low multiplicity of .01 p.f.u./cell with crude seed stocks.

Virus was absorbed to cell monolayers in 5ml of Tris/Saline for 1 hour at 37°C or 1.5 hours at 32°C for temperature sensitive mutants. ECS2 medium (100ml) was added followed by incubation at 37°C or 32°C depending on the viral phenotype. When monolayers showed a CPE, infected cells were shaken off the substratum into the medium and decanted into glass MSE centrifuge bottles. Cells were pelleted using an MSE coolspin centrifuge at 2K for 20 minutes at 4°C. The supernatant was discarded and cell pellets were resuspended in Tris/Saline to a total volume of 10ml. Virus was liberated from cells and cellular debris was pelleted by centrifugation at 2K for 10 minutes in an MSE bench centrifuge. The viral supernatant was decanted into a fresh container and the pellet discarded; the viral suspension was extracted against 2/3 volume of Arcton 113 (trichloro-trifluro-ethane) saturated with Tris/Saline, followed by centrifugation at 2K for 20 minutes at room temperature. The viral suspension was layered on to caesium chloride/glycerol gradients (Mautner and Willcox, 1974). Samples were centrifuged for 1.5 hours at 25K at a temperature of 4°C, using a TST41 swing out rotor in a Sorvall OTD50 Ultracentrifuge. The opalescent virus band was collected by piercing the bottom of the tube and stored at -20°C in 50% glycerol.

6. Viral plaque assay (Williams, 1970)

Serial ten fold dilutions of virus were made in Tris/Saline and 100ul of each dilution was inoculated on to 80% confluent monolayers of 293 cells. Virus was absorbed at the appropriate temperature, followed by two washings per plate of 2ml of Tris/Saline. Monolayers were overlaid with 4ml of Eagles overlay medium and incubated at 32°C, 37°C or 39.5°C in a humid atmosphere containing 5% CO_2. On days 3, 6 and 9 monolayers were supplemented with 1.5ml of Eagles overlay medium;

plaques were counted on days 4 and 7. On day 10 monolayers were fixed using glutaraldehyde and stained with Giemsa stain, with plaque number being recorded.

7. Rapid assay technique (Harrison et al., 1977)

Well isolated plaques were picked from cell monolayers at day 7 of viral infection into 1ml of Tris/Saline and virus liberated from cells. Monolayers of 293 or Hela cells were grown in 50mm petri plates to 80% confluency and overlaid with Eagles overlay medium. When medium had set plates were sectioned and 15ul of viral suspension was used to inoculate a section of the monolayer; plates were incubated at 37°C for 7 days. Cell monolayers were fixed and stained. If virus had produced a zone of CPE on 293 cells, but not on Hela cells, it was scored as being host range for growth in Hela cells.

8. One step growth curves

Semi-confluent monolayers of 293 cells on 50mm petri plates were infected with virus at 10 p.f.u./cell. Virus was absorbed at 37°C for 1 hour, monolayers were washed twice with PBS to remove unadsorbed virus and 4ml of ECS2 medium was added per plate. Infected cell monolayers were harvested at intervals up to 72 hours post infection by scraping monolayers into medium and pelleting cells by centrifugation. Cell pellets were resuspended in 1ml of Tris/Saline and virus liberated, followed by titration on 293 cell monolayers which were fixed and stained on day 7.

9. Preparation of viral DNA (Pettersson and Sambrook, 1973)

Purified virions were dialysed overnight at 4°C against TE buffer followed by digestion with proteinase K at 500ug per ml in the presence of .5% SDS for 3 hours at 37°C.

Samples were extracted at least three times against an equal volume of chloroform saturated phenol; DNA was precipitated out of the solution by the addition of 300mM sodium acetate and 2 volumes of absolute alcohol. DNA was pelleted by centrifugation at 15K for 30 minutes at 0°C in an SS34 fixed angle rotor using a Sorvall Superspeed centrifuge. The pellet was washed with 70% alcohol, dried and resuspended in sterile TE buffer; the final DNA concentration was determined from the optical density at a wavelength of 260 nanometers, (assuming that $1OD_{260}$ = 50ug/ml of DNA).

10. Preparation of viral DNA-protein complex (Robinson et al., 1973)

Dialysed purified virions were diluted with an equal volume of 8M Guanidinium hydrochloride dissolved in TE buffer and layered on top of caesium chloride/guanidinium hydrochloride gradients. DNA-protein complex was banded using a TST41 swing out rotor at 35K for 16 hours at 20°C using a Sorvall OTD50 Ultracentrifuge. Gradients were collected in 500ul fractions by piercing the bottom of the tubes. The optical density of each fraction was read at 260 and 280 nanometers and the results plotted graphically. The peak fractions containing the DNA-protein complex were pooled and dialysed overnight at 4°C against TE buffer; DNA concentration was determined by spectrophotometry.

11. Preparation of radioactive labelled viral DNA

Semi-confluent monolayers of 293 cells or Hela cells grown in linbro wells were infected with either 100ul of viral plaque or 100ul of viral stock at 10 p.f.u./cell. After absorption at 37°C for 1 hour, 1ml of ECS2 was added per well. At six hours post infection, medium was removed and monolayers were washed twice with E(-PO_4) medium; 1ml of E(-PO_4) supplemented with 370 kBq of ^{32}P inorganic orthophosphate (called thereafter inorganic orthophosphate) was added per well. Incubation at 37°C was maintained for 36-48 hours when samples were harvested by a modification of the Hirt method.

12. Extraction of radioactive labelled viral DNA from infected cells

The method used was a modification of Hirt, (1967). At 36-48 hours post infection, medium was drained from monolayers and 200ul of Hirt buffer was added per well, followed by incubation at room temperature for 20 minutes to lyse the cells. Sodium chloride to a final concentration of 1M was added; monolayers were harvested and kept on ice for 18 hours. Hirt supernatants were collected by centrifugation in a microfuge and digested with proteinase K at a final concentration of 500ug/ml for 3 hours at 37°C. Viral DNA was precipitated as described previously and resuspended in 50ul of sterile distilled water.

13. Endonuclease analysis of radioactive labelled DNA

Restriction endonuclease analysis of radioactive labelled DNA was carried out as specified by the suppliers with the modification of substituting gelatin (1mg/ml) where the use of Bovine serum albumin was specified. DNA samples were electrophoresed through a .7% agarose slab

gel at 35 volts overnight in TBE buffer. The gel was dried on to the glass plate and the DNA profile visualised by exposure to pre-flashed Kodak X-Omat S X-ray film (Lasky et al., 1980).

14. Nick translation of DNA

DNA was labelled with ^{32}Pi to specific activity of 3 x 10^8 dpm/ug using the method described by Rigby et al., (1977). 100ng of DNA were labelled in a 25ul reaction mixture of 50mM Tris/HClpH8, 5mM $MgCl_2$, 1mM DTT, 40uM dTTP, 40uM dGTP, .33uM[α^{32}P]dATP, .33uM[α^{32}P]dCTP and 40ug/ml gelatin. DNase at .4ug/ml was added, followed by incubation at 37°C for 5 minutes, 3 units of E.coli DNA polymerase were added and the reaction incubated for 2 hours at 14°C at which time the reaction was stopped by the addition of 100ul of TEN and 12.5ul gel loading buffer (50% sucrose, .2% Bromophenol blue, 100mM EDTA pH8). The nick translated DNA was separated from the unincorporated triphosphates by passage through a 5ml column of Sephadex G50 (medium) in TEN buffer.

15. Elution of DNA from agarose

Adenoviral DNA was cleaved with the required restriction endonuclease and electrophoresed through a .7% agarose slab gel overnight. The DNA endonuclease profile was visualised using the fluorescent dye ethidium bromide (500ng/ml) which intercalates with DNA emitting fluorescent energy when illuminated by transmitted ultra-violet light (Sharp et al., 1973). The required DNA fragments were excised from the gel and the DNA eluted from the agarose on to the dialysis membrane of an electrophoresis chamber in the presence of 400mM Tris/HClpH8, 50mM sodium acetate, 10mM EDTA. The DNA was removed from the dialysis membrane in a total

volume of 350ul and precipitated, the pellets being resuspended in 1 x Hepes buffer.

16. Transfection of viral DNA into 293 cells or Hela cells

Several methods of DNA transfection were explored. Some proved highly toxic to 293 cells (eg. treatment of the cell monolayers with chloroquine or polybrene killed the cells within a few hours of exposure.) Three of the methods explored are described here.

(a) Method as described by Graham and van der Eb, (1973) with modification by Frost and Williams, (1978).

Viral DNA with salmon sperm DNA as carrier was solubilised in Hepes buffer to a final concentration of 20ug/ml. Calcium chloride to a final concentration of 220mM was added, followed by incubation at room temperature for 30 minutes to form a calcium phosphate DNA precipitate. The precipitate was inoculated on to drained cell monolayers, followed by incubation at 37°C for 45 minutes when 4ml of ECS2 medium were added.

At 4 hours post inoculum monolayers were washed with ECS2 medium and exposed briefly to 20% glycerol in Tris/Saline to enhance the cellular uptake of the DNA precipitate. Monolayers were washed twice with ECS2 medium and incubated under the same medium for a minimum of two hours after glycerol exposure before 4ml of Eagles overlay medium were added.

(b) Method as described by Wigler et al., (1978); the method was modified by using 293 or Hela cell monolayers between 30%-80% confluency on 50mm petri plates.

5ug of DNA were solubilised in 210ul of TE buffer (1mM Tris/HClpH7.5, .1mM EDTA) with gentle agitation. 30ul of 2M calcium chloride were added and immediately this solution was added dropwise with gentle agitation to 240ul of 2 x Hepes buffer (280mM NaCl 50mM Hepes 1.5M Na_2HPO_4pH7.12) followed by incubation at room temperature for 30 minutes. The DNA-calcium phosphate precipitate was added dropwise evenly over the cell monolayer without removing the medium. At 24 hours post transfection the monolayers were washed with Tris/Saline and incubated under Eagles overlay medium at 37°C for 7 days when monolayers were fixed and stained.

(c) Method as described by Wigler et al., (1978) with modification from Graham and van der Eb, (1973); Frost and Williams, (1978). Cell monolayers were grown on 50mm petri plates to a confluency between 70%-80%. DNA was solubilised in 175ul of TE buffer with gentle agitation, 25ul of 2M calcium chloride were added and the solution added dropwise to 200ul of 2 x Hepes buffer, again with gentle agitation and incubated at room temperature for 30 minutes to allow the formation of a calcium phosphate DNA precipitate. The precipitate was added evenly to drained cell monolayers followed by incubation at 37°C for 45 minutes when 4ml of ECS2 medium were added. Further incubation at 37°C took place for 4 hours.

At 4 hours post transfection, monolayers were drained and washed with ECS2 medium, followed by exposure to 20% (v/v) glycerol in Tris/Saline for 90 seconds. Monolayers were washed twice with ECS2 medium and incubated at 37°C under Eagles overlay medium for 7 days when monolayers were fixed and stained.

17. Isolation of a large plaque fast growing virus

Viral plaque assays were carried out on semi-confluent monolayers of 293 cells on 50mm petri plates. At 4 days post infection, plaques showing a larger than average phenotype were picked and passaged through several rounds in 293 cells. Isolates which continued to be fast growing and produce large plaques were prepared as crude seed stocks and then as purified virion.

18. Construction of adenovirus 3 wild type BamH1 clones

5ug of adenovirus 3 wild type DNA was cleaved with endonuclease BamH1. After incubation at 37°C for 3 hours, 200ng of digested DNA was examined by gel electrophoresis for complete digestion. The DNA was then extracted with phenol/chloroform followed by precipitation by spermine and resuspended in sterile distilled water to a concentration of 100ng/ul.

(I) Spermine precipitation

Spermine was added to the DNA samples to a final concentration of 10mM followed by incubation at room temperature for 15 minutes. After centrifugation in an Eppendorf centrifuge for 10 minutes, the supernatant was removed and the tubes washed with elution buffer [75% ethanol, .3M sodium acetate, 10mM magnesium acetate] and 500ul of fresh elution buffer were added and samples were incubated for 60 minutes on ice. DNA was washed with 70% ethanol, dried and resuspended in sterile distilled water.

(II) Preparation of plasmid vector

Plasmid vector pUC8 was linearised at the unique BamH1 site and extracted with phenol/chloroform, precipitated by spermine and resuspended in 97ul of Bacterial alkaline phosphatase buffer (50mM Tris/HClpH9, 1mM $MgCl_2$, 1mM $ZnCl_2$, 1mM spermidine). 2ul (15u/ul) of Bacterial alkaline phosphatase were added followed by incubation at 65°C for 30 minutes when a further 1ul (15u/ul) of enzyme was added with further incubation. Bacterial alkaline phosphatase enzyme prevents the cohesive ends of the vector self ligating in the presence of T_4 DNA ligase, by removing the terminal 5' phosphoryl groups. The DNA which is to be inserted into the vector contains its 5' phosphoryl groups and can join to the 3' hydroxyl groups of the vector giving a product which is joined to the vector DNA in one strand only. The other has a nick with 3' and 5' hydroxyl groups which is repaired by the host cell mechanism.

After incubation in Bacterial alkaline phosphatase 1ul of 5mg/ml proteinase K, 2ul of 10% SDS and 5ul of .5M EDTA were added, followed by incubation at 37°C for 60 minutes; samples were extracted, DNA was precipitated by spermine and dissolved in sterile distilled water to a final concentration of 50ng/ul.

DNA fragments and plasmid vector were cohesive end ligated overnight at 12°C in the presence of 50mM Tris/HClpH7.6, 10mM $MgCl_2$, 10mM Dithiothreitol and 1mM ATP with 2.5u of T_4 DNA ligase in a total volume of 10ul.

(III) Transformation of host bacteria JM83 to ampicillin resistance

The method has been described by Hanahan, (1983). Competent JM83

(Messing, 1979) cells were prepared by plating out from glycerol stock for single colonies on an L-Broth agar plate which was incubated at 37°C overnight. A single colony was picked and grown in SOB medium for 2-3 hours at 37°C rotating at 222 rpm. The cells were then placed on ice for 10-15 minutes, then centrifuged at 2.5K at 4°C for 12 minutes. The pellet was resuspended in 2ml TFB with gentle vortexing. After incubation on ice for 10-15 minutes, cells were pelleted by centrifugation at 2.5K at 4°C for 10 minutes and resuspended in 500ul of TFB and 17.5ul of Dimethyl sulphoxide (DMSO) followed by incubation on ice for 5 minutes. 17.5ul of Dithiothreitol were added and incubation on ice for 10 minutes was followed by the addition of 17.5ul of DMSO. After 5 minutes on ice competent cells were aliquoted into 200ul or 20ul amounts; DNA ligation mixes were added with appropriate controls and samples incubated on ice for 30 minutes, followed by a 90 second heat shock at 42°C. After further incubation on ice for 1-2 minutes, 800ul of SOC medium was added per 200ul aliquot of transformed cells. Samples were shaken at 37°C for 60 minutes. Transformed cells were plated on L-Broth agar plates containing 50ug/ml ampicillin with the addition of X-gal.

(IV) Isolation of adenovirus 3 wild type BamH1 clones

White colonies were picked and plated onto a master plate and 5ml of L-Broth medium containing ampicillin (50ug/ml) were inoculated for the preparation of mini lysates for rapid analysis to ascertain colonies containing plasmids with the appropriate inserts of DNA.

(V) Rapid isolation of plasmid DNA (Holmes and Quigley, 1981)

1.5ml of inoculated culture was centrifuged in a microfuge; pellets were drained and resuspended by vortexing in 350ul STET buffer with the addition of 10mg/ml lysozyme.

Samples were boiled for 40 seconds followed by centrifugation at 12K for 15 minutes. Pellets were removed with a toothpick and DNA was precipitated from the supernatant by the addition of 40ul of 2.5M sodium acetate and 420ul of isopropanol at -70°C for 30 minutes. The DNA precipitate was pelleted at 12K for 10 minutes and resuspended in 50ul of TE buffer.

5ul of each sample was then cleaved with BamH1 endonuclease to identify the inserted DNA fragment; positive clones were further identified by double digestion.

19. Large scale isolation of plasmid DNA

Method was as described by Clewel and Helinski, (1970). 10ml of L-Broth containing ampicillin (50ug/ml) were inoculated from a glycerol stock containing the appropriate plasmid and incubated overnight with agitation at 37°C. This was used as an inoculum for a 400ml culture. On reaching OD_{600}.6-.8 the plasmid in the bulk culture was amplified by the addition of chloramphenicol at 100ug/ml and agitated at approximately 200 rpm at 37°C overnight.

The following day the host bacteria were pelleted by centrifugation and resuspended in 5ml of 25% sucrose (w/v) dissolved in 50mM Tris/HClpH8. All further manipulations were carried out on ice. 1ml of 20mg/ml

lysozyme in 250mM Tris/HC1pH8 was added, followed by incubation for 5 minutes when 1ml of 500mM EDTA was added. After 5 minutes 8ml of.2% triton x 100 in 50mM Tris/HC1pH8 and 62.5mM EDTA were added, followed by a 15 minute incubation. The supernatant containing the plasmid DNA was collected by centrifugation at 20K for 30 minutes at 0°C.

Plasmid DNA was banded in a caesium chloride ethidium bromide density gradient by adding 1g of caesium chloride per ml of plasmid supernatant with ethidium bromide at a concentration of 800ug/ml. The DNA was then banded to equilibrium by ultracentrifugation at 45K for 20 hours at a temperature of 20°C in a TV850 vertical rotor. The DNA was collected via a 2ml syringe and rebanded for purity as described above using a TV865 vertical rotor. Plasmid DNA was collected as before and applied to a 10ml packed column of Sephadex-G50 in TEN buffer to remove the caesium chloride. The DNA void volume was passed over a 1ml packed column of Dowex AG50W x 8 to remove the ethidium bromide, the DNA being eluted in 3 column volumes of TEN buffer. The DNA was precipitated and pelleted at 8K for 45 minutes at 4°C and resuspended in sterile TE buffer. The DNA was incubated in the presence of RNaseA at 50ug/ml for 30 minutes at 37°C. This was followed by treatment with proteinase K at 100ug/ml in the presence of .25% SDS at 55°C for 1 hour. DNA was precipitated and pelleted by 5 minutes centrifugation in a microfuge and then resuspended in sterile distilled water. The concentration per ml was determined by spectrophotometry at OD_{260} giving an average yield of 2mg/ml of plasmid DNA per sample.

20. Cloning of the terminal fragment of adenovirus 3 wild type for construction of plasmid p3A1

This method was used to construct p3A1 which contains a single ITR

of adenovirus 3 wild type.

Adenovirus 3 wild type DNA was cleaved with the endonuclease BamH1 at 37°C for 3 hours, at which time 40ul (400mM Tris/HClpH8, 30mM $MgCl_2$), 8ul of 5mMdntps and 1ul of T_4 DNA polymerase were added in a total volume of 400ul with further incubation at 37°C for 1 hour. The DNA sample was electrophoresed through a .6% polyacrylamide gel at 40 volts overnight. The BamH1 I fragment was excised, the DNA eluted from the acrylamide and spermine precipitated followed by quantitation of DNA recovery by comparison to a known standard of DNA.

5ul of the sample was removed to an Eppendorf tube and 2ul of 1.5M NaOH added followed by incubation at 37°C for 90 minutes. The reaction was neutralised by the addition of 1ul of 1M Tris/HClpH8 and 2ul of 1.5M HCl with further incubation at 37°C for 90 minutes. Treatment with sodium hydroxide is necessary to remove the terminal peptide attached to the DNA fragment which makes cloning impossible. Denatured DNA was hybridised in the presence of .3M NaCl at 65°C for 4 hours.

(II) Preparation of pUC8 vector

Plasmid vector pUC8 was cleaved with endonuclease BamH1 and prepared as described previously with the additional step of the 5' overhangs being flush ended by the addition of dntps and T_4 DNA polymerase.

DNA fragment and plasmid vector were blunt end ligated overnight at 20°C in the presence of 50mM Tris/HClpH7.6, 10mM $MgCl_2$, 10mM DTT, 1mM ATP and T_4 DNA ligase in a total volume of 10ul.

(III) Transformation of host bacteria DHI to ampicillin resistance

Method was as described by Hanahan, (1983) to transform competent DHI cells to ampicillin resistance followed by plating on to L-Broth agar plates containing 50ug/ml ampicillin.

(IV) Isolation of positive colonies

Colonies were screened by colony hybridisation. This method allows the analysis of approximately 100-150 colonies per plate. Colonies were picked onto duplicate L-Broth agar plates containing 50ug/ml ampicillin and incubated at 37°C overnight. One plate was stored at 4°C and the colonies of the second plate were transferred to a Schleicher and Schüll nitrocellulose membrane filter. Three layers of Watmans No. 1 filter papers were soaked in 5ml of either .1M HCl, .5M NaOH, 1M Tris/HClpH7.4 or 1.2M NaCl. The nitrocellulose filter was placed colony side up sequentially on to these solutions for 1 minute, 15 minutes, 5 minutes and 15 minutes respectively at room temperature. The filter was air dried and baked in a vacuum oven at 80°C for 2 hours. Colonies were analysed by a nick translated probe of the adenovirus 3 wild type BamH1 fragment as described by Southern, (1975).

21. Southern transfer

Method was essentially as described by Southern, (1975). Restriction endonuclease digests of Hirt supernatant DNA were electrophoresed through an agarose gel overnight. The fractionated DNA was denatured in the presence of 1 litre of .6M NaCl and .2M NaOH at room temperature for 45

minutes. Neutralisation was achieved by soaking 1 litre of 1M Tris/HCl(pH7.5) and 1.2M NaCl at room temperature for 45 minutes.

The denatured DNA was transferred overnight to a Schleicher and Schüll BA85 membrane filter by capillary action in 10 x SSC buffer. After transfer the filter was washed with 10 x SSC and air dried followed by baking at 80°C for 2 hours in a vacuum oven. The filter was prehybridised at 65°C for 2 hours in a shaking water bath in the presence of 6 x SSC, 5 x Denhardts solution (Denhardt, 1966), .1%(v/v)SDS and 2ug/ml denatured salmon sperm DNA. Nick translated DNA was denatured by the addition of sodium hydroxide to a final concentration of .2M and incubated at room temperature for 10 minutes when the reaction was neutralised by the addition of acetic acid to a final concentration of .2M. The denatured nick translated DNA was hybridised to the immobilised DNA on the filter, at 65°C overnight with shaking in 20mM Tris/HCl(pH7.5), 6 x SSC, 10 x Denhardts solution, 1mM EDTA, .1%(v/v)SDS and 50ug/ml denatured salmon sperm DNA. Following hybridisation the filter was washed in two 1 litre washes of 2 x SSC and .25% SDS(v/v) at 65°C for 1 hour with shaking.

Filters were dried and exposed to preflashed X-Omat S X-ray film at -70°C.

22. Extraction of nuclei from viral infected cells

Method was essentially as described by White et al., (1984). Monolayers of 293, Hela or KB cells were infected with virus at 20 p.f.u./cell and absorbed for 1 hour at 37°C when 12ml of ECS2 medium was added per plate. At 2 hours post infection 10mM hydroxyurea was added to block DNA synthesis (Challberg and Kelly, 1979). At 21 hours post

infection monolayers were scraped into the medium and cells pelleted at 2K for 10 minutes at 4°C in an MSE Coolspin centrifuge. Cells were lysed by resuspension in ice cold buffer (150mM NcCl, 10mM Tris/HCl(pH7.2), 2mM $MgCl_2$, 1mM DTT) with the addition of Nonidet-P 40 to .5% followed by incubation on ice for 45 minutes. Nuclei were harvested by centrifugation and resuspended sequentially in ice cold buffers containing .35M or 2M NaCl. Supernatants collected from successive centrifugations were treated with RNaseA and proteinase K before electrophoresis through a 1% agarose gel. DNA was visualised by ethidium bromide staining.

23. Virion heat stability (Young and Williams, 1975)

10^9 p.f.u. of virions prewarmed to 52°C in 100ul were added to 900ul of 50mM Tris/HCl(pH7.4), which was prewarmed to 52°C, in a Tecam TE-7 Tempette shaking water bath.

Every 2 minutes an 100ul aliquot was removed and diluted into ice cold Tris/Saline to a final volume of 1ml. The infectivity of the virus was immediately assayed by titration on monolayers of 293 cells.

Chapter 3.

Results

Section A.

Studies on Adenovirus Heterotypic Recombination

1. INTRODUCTION

Recombination occurs between adenoviruses of closely related serotypes within a subgroup (Williams et al., 1971; Ensinger and Ginsberg, 1972; Grodzicker et al., 1974; Williams et al., 1975) but has not been detected between serotypes of different subgroups, although complementation of gene functions has been shown to occur (Williams et al., 1975; 1981; McDonagh and Rekosh, 1982). It was thought that marker rescue of subgroup C temperature sensitive mutants by DNA fragments of subgroup B was possible.

The aim of this project was to try to isolate recombinants between the two subgroups using the method of marker rescue as described by Frost and Williams, (1978).

The adenovirus type 5 ts mutants used represented three regions of the genome. The hexon coding region was represented by H5ts2(54.8-57) m.u., H5ts14(*) m.u. and H5ts40(51.1-57) m.u. The region coding for the 100K protein was represented by H5ts1(70-71.4) m.u. and H5ts19(63.6-68) m.u. (Williams et al., 1971; Williams and Ustacelebi, 1971); the 72K DNA-binding protein was represented by H5ts125(61.3-62.9) m.u. (Ensinger and Ginsberg, 1972; 1974).

Preliminary attempts were made to rescue the adenovirus type 5 ts mutants using Xho1 DNA fragments of adenovirus type 7. Results from these experiments indicated that in my hands

(i) Some of the ts mutants were leaky

(ii) No evidence of rescue was seen where the temperature sensitive mutants were tight at the non-permissive temperature (39.5°C).

* precise map units not found in literature.

Table IV.

Fragment DNA	Mutant or wild type DNA	p.f.u./dish Day 4		Day 14	
Ad7Xho1 A	H5ts40	8	7	63	52
Ad7Xho1 A	-	0	0	0	0
Ad7Xho1 B	H5ts40	6	4	52	49
Ad7Xho1 B	-	0	0	0	0
Ad7Xho1 C	H5ts40	5	7	54	52
Ad7Xho1C	-	0	0	0	0
Ad7Xho1 D	H5ts40	5	8	51	60
Ad7Xho1 D	-	0	0	0	0
Ad7Xho1 E	H5ts40	6	7	55	61
Ad7Xho1 E	-	0	0	0	0
Ad5EcoR1 A	H5ts40	9	6	62	63
Ad5EcoR1 A	-	0	0	0	0
Ad5EcoR1 B	H5ts40	6	5	59	58
Ad5EcoR1 B	-	0	0	0	0
Ad5EcoR1 C	H5ts40	6	5	61	58
Ad5EcoR1 C	-	0	0	0	0
	H5ts40 (.1ug 32°C)	28	26	276	252
	H5ts40 (.1ug 39.5°C)	8	7	61	67
	Ad7wt (.1ug 39.5°C)	0	0	192	186
	Ad5wt (.1ug 39.5°C)	22	24	226	246
Mock infected	SS DNA (ug/dish)	0	0	0	0

SS DNA = Salmon sperm DNA

Table V.

Fragment DNA	Mutant or wild type DNA	p.f.u./dish Day 4		Day 14	
Ad7Xho1 A	H5ts14	0	0	0	0
Ad7Xho1 A	-	0	0	0	0
Ad7Xho1 B	H5ts14	0	0	0	0
Ad7Xho1 B	-	0	0	0	0
Ad7Xho1 C	H5ts14	0	0	0	0
Ad7Xho1 C	-	0	0	0	0
Ad7Xho1 D	H5ts14	0	0	0	0
Ad7Xho1 D	-	0	0	0	0
Ad7Xho1 E	H5ts14	0	0	0	0
Ad7Xho1 E	-	0	0	0	0
Ad5EcoR1 A	H5ts14	6	6	86	87
Ad5EcoR1 A	-	0	0	0	0
Ad5EcoR1 B	H5ts14	0	0	0	0
Ad5EcoR1 B	-	0	0	0	0
Ad5EcoR1 C	H5ts14	0	0	0	0
Ad5EcoR1 C	-	0	0	0	0
	H5ts14 (.1ug 32°C)	21	22	219	231
	H5ts14 (.1ug 39.5°C)	0	0	0	0
	Ad7wt (.1ug 39.5°C)	0	0	194	199
	Ad5wt (.1ug 39.5°C)	20	18	235	224
Mock infected	SS DNA (1ug/dish)	0	0	0	0

SS DNA = Salmon sperm DNA

Table IV shows the results obtained using H5ts40, a mutant leaky at the non-permissive temperature. The number of plaques per dish where ts mutant has been co-transfected with a wild type fragment is equivalent to the control H5ts40(39.5°C) alone; therefore plaques are most probably the result of breakthrough of the ts mutant and not recombination.

Results (Table V) show that H5ts14 is tight at the non-permissive temperature (39.5°C) by the absence of plaques. The hexon mutation of H5ts14 has been rescued by the EcoR1 A DNA fragment (0-75.9) m.u. of adenovirus type 5, but has not rescued with the appropriate Xho1 A DNA fragment (22.9-76.5) m.u. of adenovirus type 7.

Recombination was then approached by the method described by Chinnadurai et al., (1979). Overlapping terminal DNA fragments of adenoviruses types 7 and 5 were co-transfected into permissive cell monolayers. The restriction enzyme profiles of the two serotypes are significantly different so that progeny can be analysed for recombinants by this method. Recombinant progeny would have the left hand end of adenovirus type 7 (subgroup B) and the right hand end of adenovirus type 5 (subgroup C) with restriction enzyme sites of either parent present within the region of overlap.

The differences between these two recombinational approaches are as follows:-

1(a) Transfected overlapping terminal DNA fragments alone, being subgenomic are non-infectious, therefore are incapable of producing viral plaques; resulting progeny by this method should be recombinants.

1(b) Input DNA used in marker rescue experiments is full length, carrying a temperature sensitive mutation. A drop in the non-permissive temperature during incubation can cause the mutation to become functional resulting in viral replication and the production of viral plaque.

2(a) Marker rescue asks recombination to occur within a small defined region between mutant and a specific wild type DNA fragment of adenovirus type 7.

2(b) Using overlapping terminal DNA fragments the region in which recombination can occur is increased to any homologous DNA zone within the overlap.

3(a) Overlapping terminal DNA fragments have the molecular ends of different subgroups. Recombinants constructed by this method would also have molecular ends of different subgroups and hence heterotypic inverted terminal repeats; these repeated sequences play a role in adenoviral DNA replication.

3(b) Recombinants constructed by marker rescue experiments have the molecular ends of the same serotype and hence have homotypic inverted repeats.

Section A describes the viral growth curves of adenoviruses 5 and 7 wild type, along with the DNA infectivity and data from intertypic recombination experiments.

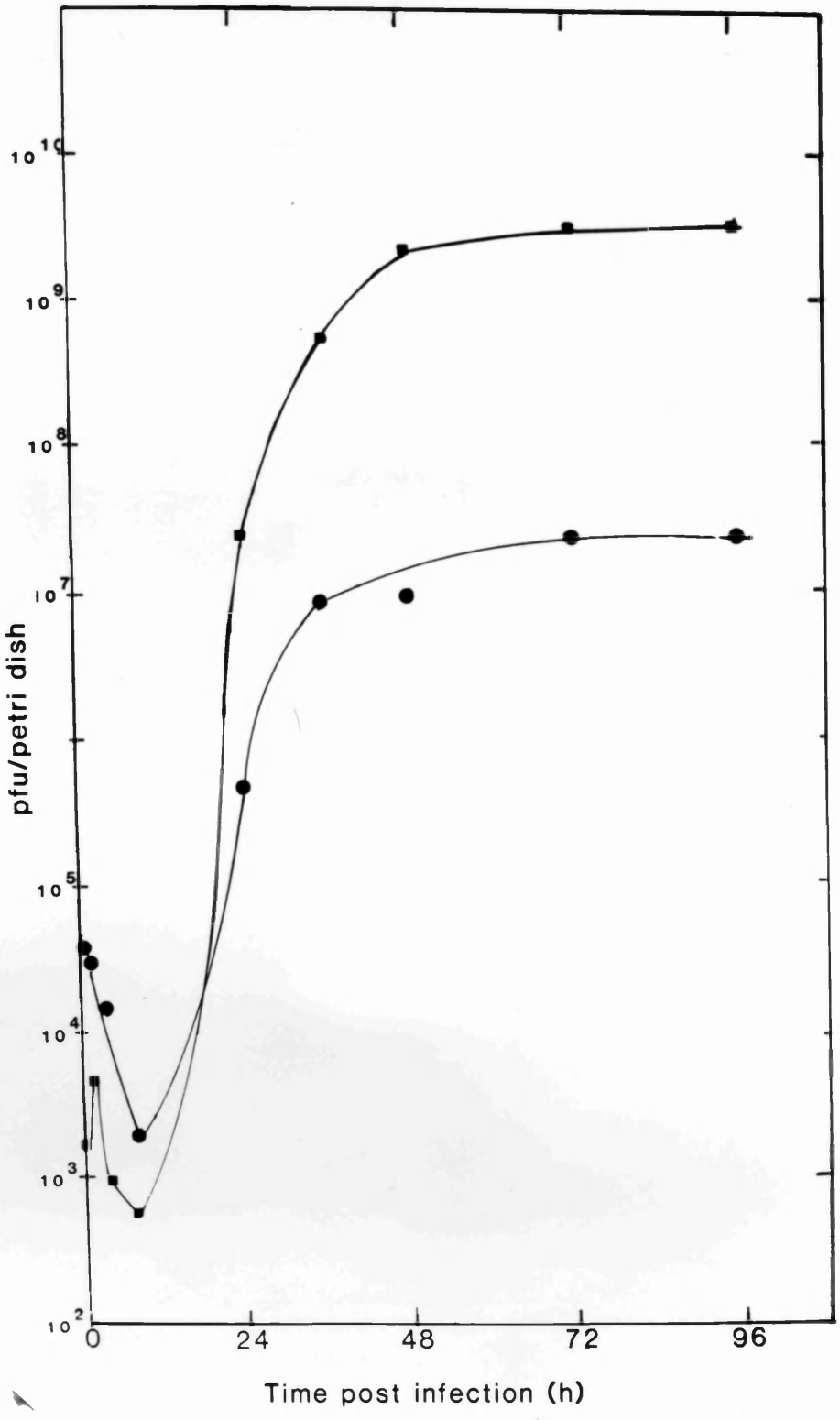

pfu/petri dish
10^{10}
10^{9}
10^{8}
10^{7}
10^{5}
10^{4}
10^{3}
10^{2}
0
24
48
72
96
Time post infection (h)

Figure 10.

One step growth curves of adenovirus type 7 and type 5

Semi-confluent 50mm monolayers of 293 cells were infected with adenovirus 5 wild type or adenovirus 7 wild type at 10 p.f.u./cell.

After 1 hour of viral absorption, monolayers were washed twice with Tris/Saline to remove unadsorbed virus; 4ml of ECS2 medium was added per 50mm petri dish. Time points were taken between zero and 96 hours post absorption and titrated on 293 cell monolayers which were maintained under agar overlay medium at 37°C for 10 days. Cell monolayers were then fixed and stained, the titre being plotted on a logarithmic scale against time of post infection in hours.

■ = Adenovirus 5 wild type

● = Adenovirus 7 wild type

2. One step growth curves of adenoviruses 7 and 5 wild type viruses.

The growth profiles of adenovirus 7 wild type and adenovirus 5 wild type were compared using one step growth curves by the method described in materials and methods. Fig. 10 shows that at 18 hours post infection (pi) the yield of adenovirus 5 wild type is starting to overtake adenovirus 7 wild type and by 24 hours pi there is approximately 2 logs of difference in the yield between the two viruses. At 48 hours the growth rate of both viruses is seen to plateau with the 2 log difference still apparent. This difference is maintained throughout replication to the end point at 96 hours.

The particle to p.f.u. ratio of these two viruses is almost equal (Ad5wt = 12:1; Ad7wt = 17:1). Thus the difference in infectivity is not due to a disproportionate input of infectious particles.

DNA was prepared from the purified virion stocks of both viruses and used in subsequent recombination experiments.

3. Co-transfection of overlapping terminal DNA fragments of adenovirus 7 wild type and adenovirus 5 wild type into 293 cells.

As marker rescue of adenovirus type 5 ts mutants by adenovirus 7 wild type Xho1 DNA fragments was unsuccessful, the method described by Chinnadurai et al., (1979) was used in an attempt to isolate intertypic recombinants.

Overlapping terminal DNA fragments of adenovirus 5 wild type and

Table VIa

DNA terminal fragments		Map co-ordinates (m.u.)		
Ad7 wild type	Ad5 wild type	Map co-ords.	Region of overlap	% overlap
EcoR1 A	Sal1 A	0 - 85.4 m.u. 45.9.m.u.-100 m.u.	45.9 m.u.-85.4 m.u.	39.5%
EcoR1 A Sal1 B		0 - 85.4 m.u. 68 m.u.-100 m.u.	68 m.u.-85.4 m.u.	17.4%
	EcoR1 A Sal1 A	0 - 75.9 m.u. 45.9 m.u.-100 m.u.	45.9 m.u.-75.4 m.u.	30%

Table VIb

DNA terminal fragments (ug/dish)	Plaques/dish		titre at day 14 (average titre p.f.u./ml)
	Day 4	Day 14	
Ad7wt EcoR1 A+Ad5wt Sal1 A 2ug 2ug	0 0	4 0	1.2×10^4
Ad7wt EcoR1 A+Ad7wt Sal1 B 1ug 1ug	0 0	2 0	8×10^2
Ad5wt EcoR1 A+Ad5wt Sal1 A 1ug 1ug	20 15	53 56	6×10^6
Ad7wt EcoR1 A 2ug	0 0	0 0	$<10^1$
Ad5wt Sal1 A 2ug	0 0	0 0	$<10^1$
Ad7wt Sal1 B 2ug	0 0	0 0	$<10^1$
Ad7wt EcoR1 A 2ug	0 0	0 0	$<10^1$
Ad5wt DNA (ug/dish) 1ug .1ug .01ug	 TM TM 30 35 2 1	 CPE CPE 229 214 23 20	
DNA infectivity (p.f.u./ug)	238	2183	
Ad7wt DNA (ug/dish) 1ug .1ug .01ug	 0 0 0 0 0 0	 CPE CPE 198 200 20 20	
DNA infectivity (p.f.u./ug)	0	1995	
Mock infected (SS DNA)	0 0	0 0	$<10^1$

DNA infectivity was calculated by taking the average value of plaques/dish.

adenovirus 7 wild type were co-transfected into 293 cell monolayers.

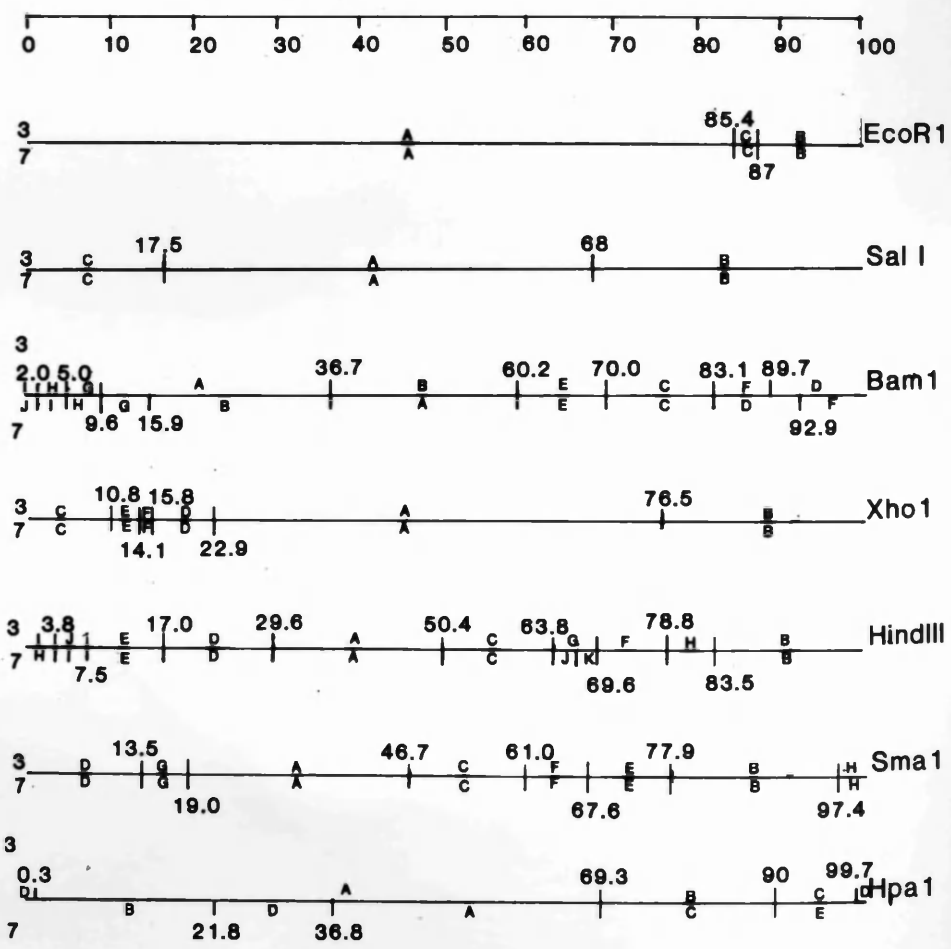
Table VIa lists the DNA fragments used, map co-ordinates and percentage of DNA overlap.

Data (Table VIb) from plates incubated under agar overlay medium suggests that homotypic recombination may have occurred between adenovirus 5 wild type terminal DNA fragments, as plaque number is higher than control plates. Little evidence of homotypic recombination is seen between the terminal DNA fragments of adenovirus 7 wild type. The two resulting plaques gave the restriction enzyme profile of adenovirus type 7. This result can hardly be classified as a good yield of homotypic recombinants, and therefore it is not surprising that heterotypic recombinant plates yielded a low level of plaques.

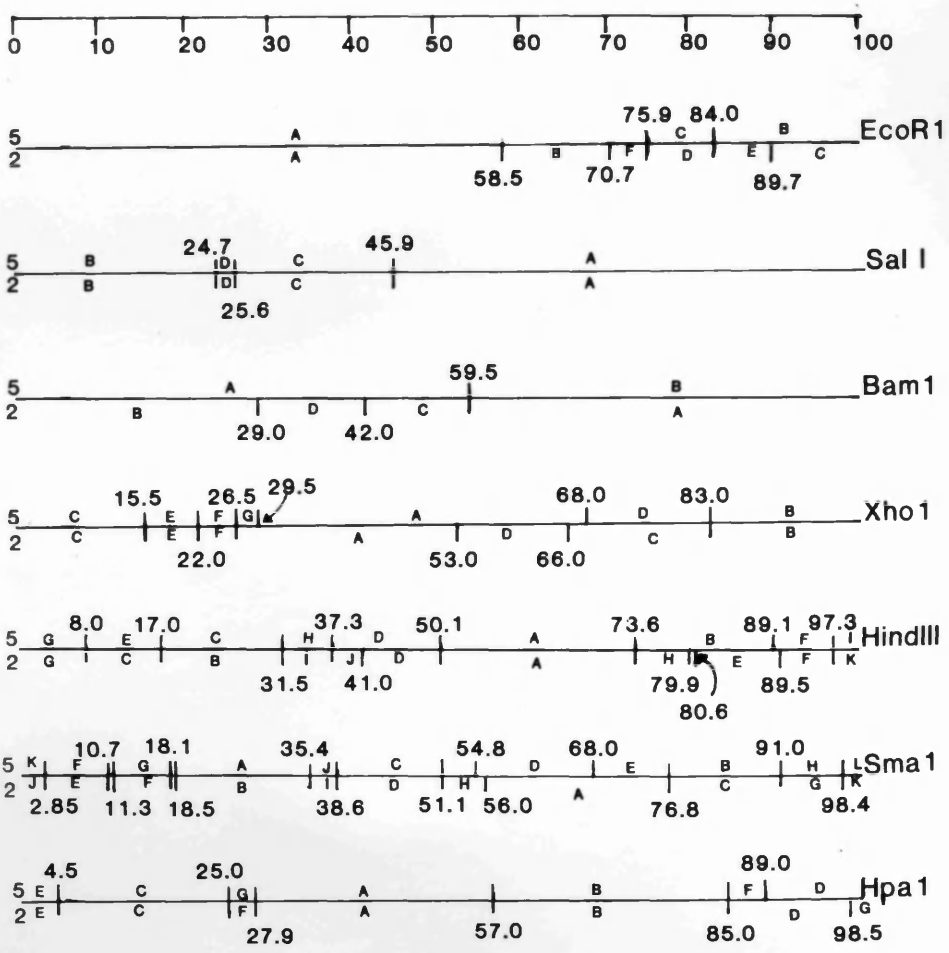
Adenovirus 7 wild type DNA is less infectious than adenovirus 5 wild type DNA at day 4; however, by day 14, their titres are almost equal.

The four plaques from the heterotypic plates were picked, and the infected cell monolayers were harvested for viral yields which were titrated on 293 cell monolayers. Viral yields suggest that homotypic recombination has occurred between the terminal DNA fragments of adenovirus 5 wild type and to a lesser extent between the terminal DNA fragments of adenovirus 7 wild type. Heterotypic yields suggest that recombination may have occurred and well isolated plaques were picked for analysis.

Restriction Endonuclease Maps of Ad.3 and Ad.7



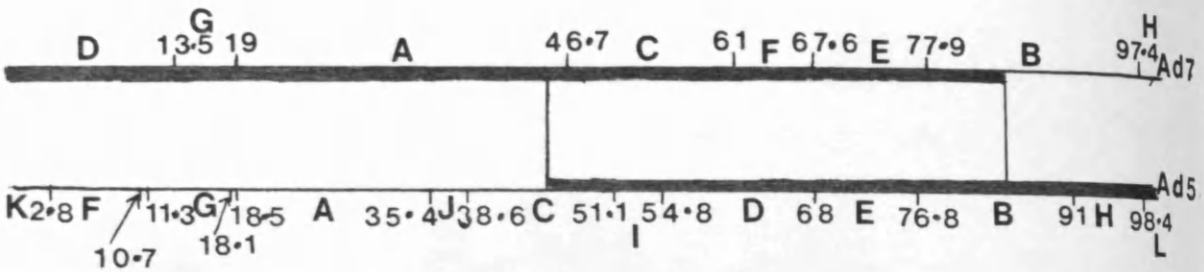
Restriction Endonuclease Maps of Ad.2 and Ad.5

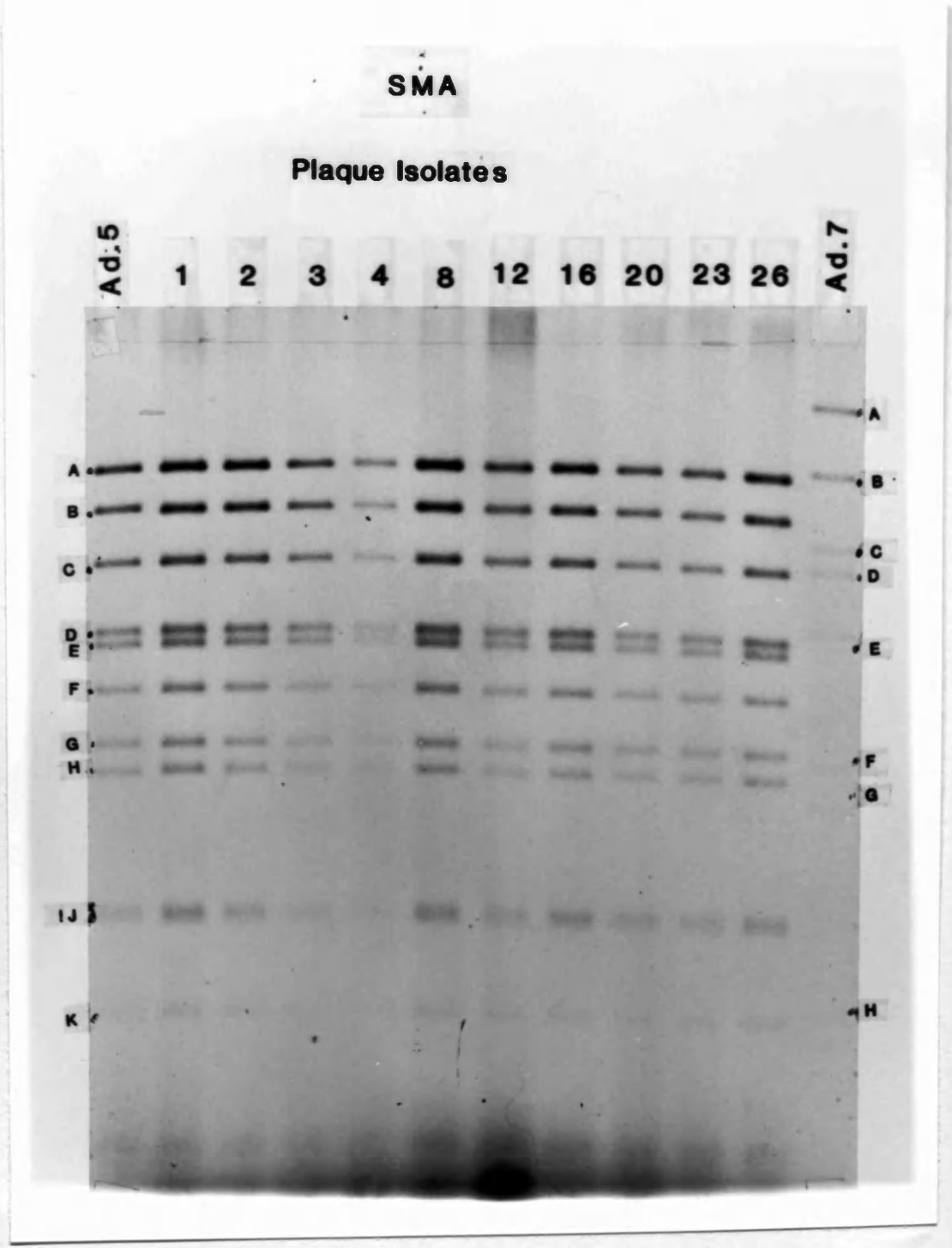


Figures 11 and 12.

Restriction endonuclease maps of subgroup B adenoviruses type 3 and type 7 and subgroup C adenoviruses type 2 and type 5 as published in Molecular Biology of Tumour Viruses (Second edition 1980) Part 2; Edited by John Tooze, Published by Cold Spring Harbor Laboratory, United States of America.

Sma I


D
G
13·5
19
A
46·7
C
61
F
67·6
E
77·9
B
H
97·4
Ad7
Ad5
K2·8
F
10·7
11·3
G
18·1
18·5
A
35·4
J
38·6
C
51·1
I
54·8
D
68
E
76·8
B
91
H
98·4
L


SMA
Plaque Isolates
Ad.5
1
2
3
4
8
12
16
20
23
26
Ad.7
A
B
C
D
E
F
G
H
I J
K

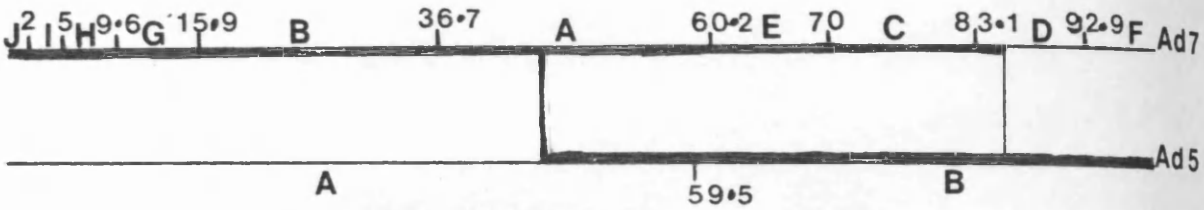
Figures 13,

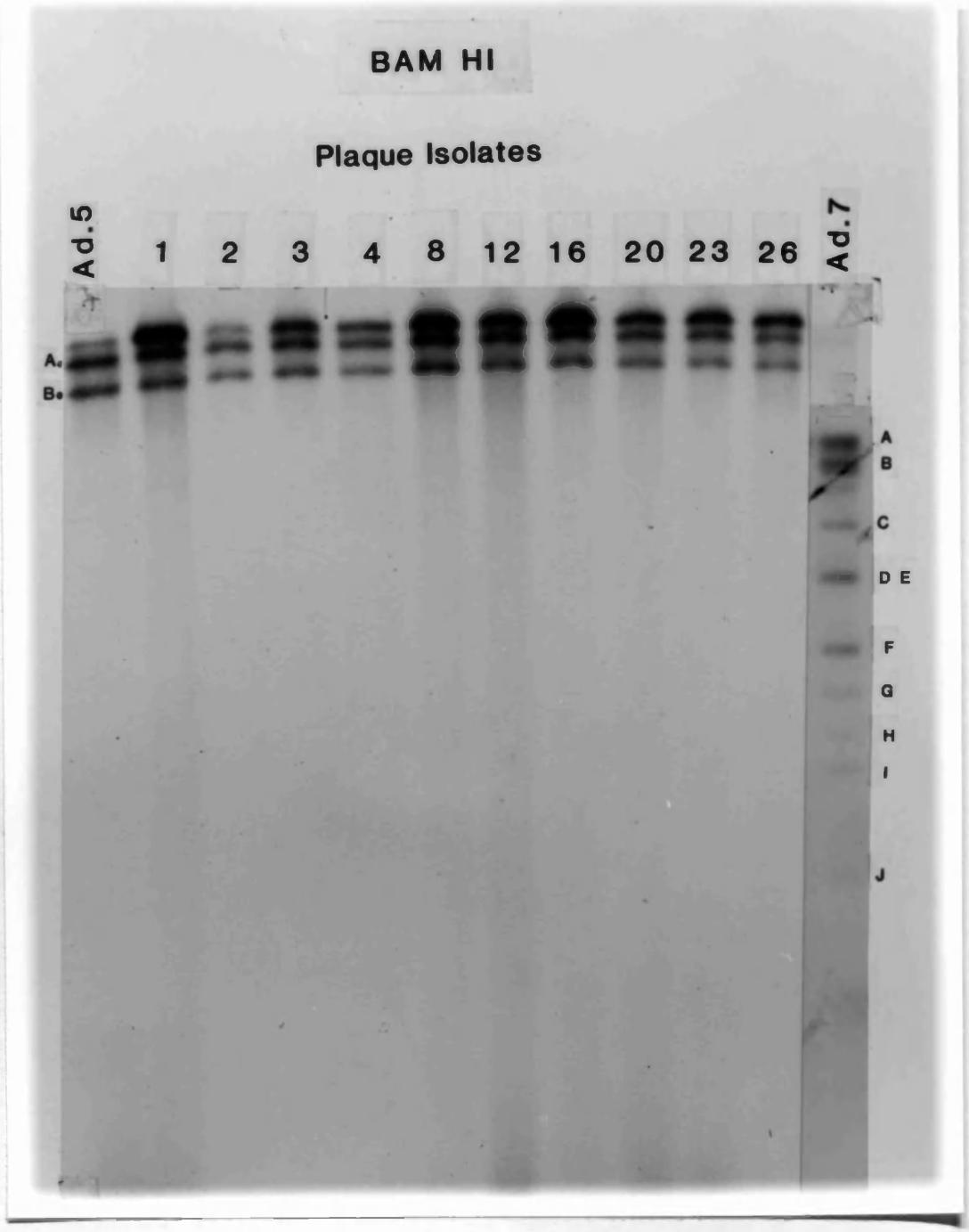
Analysis of plaque isolates from cell monolayers transfected with with heterotypic overlapping terminal DNA fragments

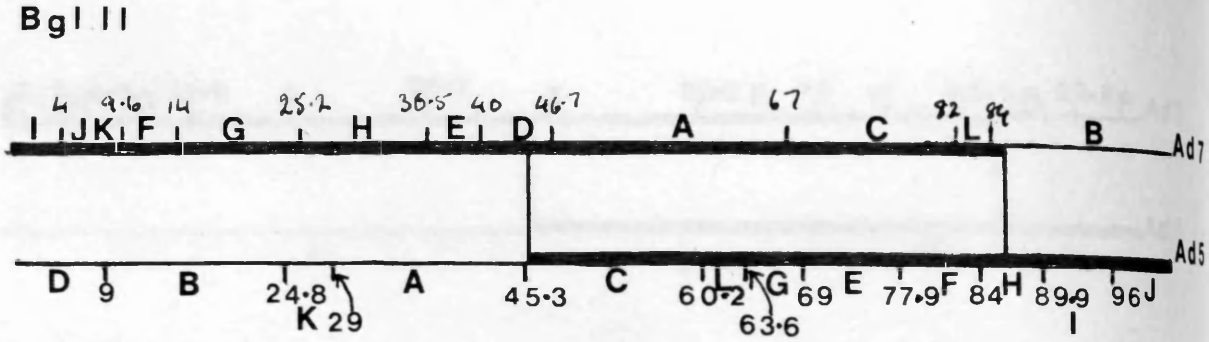
The DNA restriction endonuclease profile was examined by labelling the DNA with inorganic orthophosphate and harvesting as a Hirt supernatant. Equal aliquots of labelled DNA were cleaved as single digests with endonucleases BamH1, HindIII, Sma1 or BglII and electrophoresed through an agarose gel followed by exposure to X-ray film at -70°C.

The restriction enzyme maps for each endonuclease used is shown above the figure; the heavy black lines show the heterotypic terminal DNA fragments and the region of overlap is represented by two vertical lines.

Bam H I

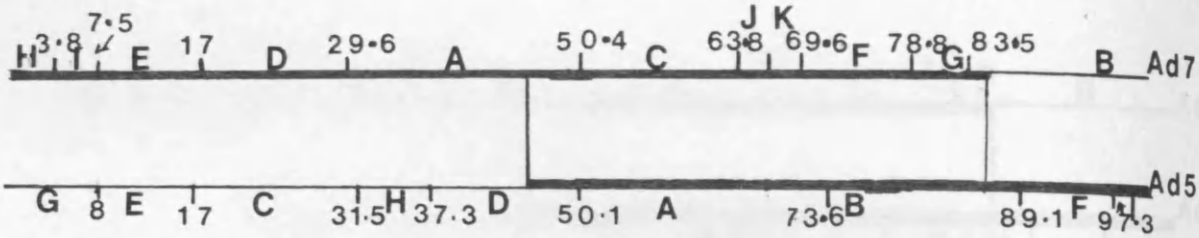

J 2
I 5
H 9·6
G 15·9
B
36·7
A
60·2
E
70
C
83·1
D
92·9
F
Ad7
Ad5
A
59·5
B

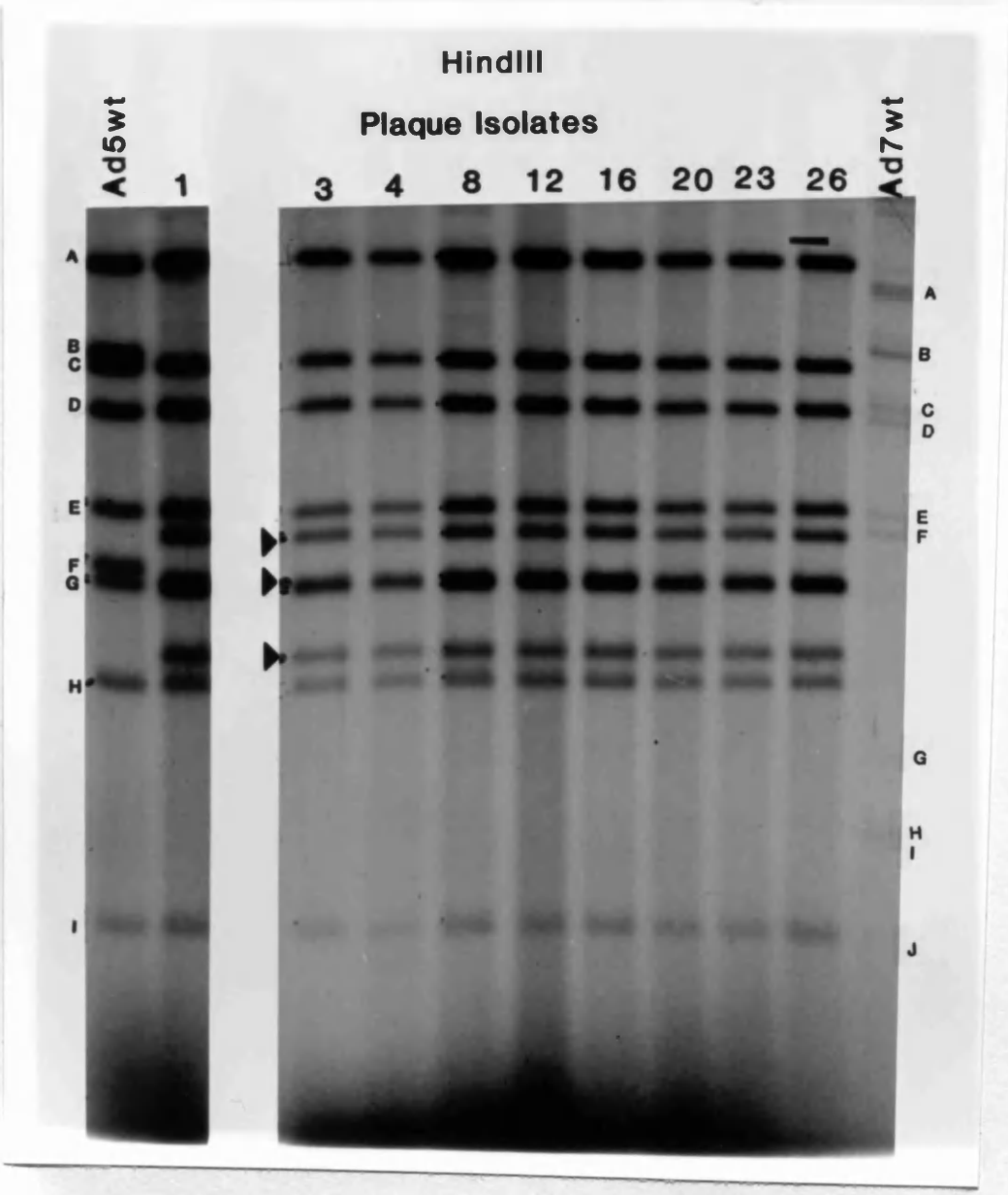

BAM HI
Plaque Isolates
Ad.5
1
2
3
4
8
12
16
20
23
26
Ad.7
A
B
C
D E
F
G
H
I
J


Bgl II
I J K F G H E D A C L B
4 9·6 14 25·2 36·5 40 46·7 67 82 94
Ad7
Ad5
D 9 B 24·8 K 29 A 45·3 C 60·2 63·6 G 69 E 77·9 F 84 H 89·9 I 96 J


Bgl II
Plaque Isolates
Ad5wt
1
3
4
8
12
16
20
23
26
Ad7wt
A
B
C
D
E
F
G
HI
JK
L
H
I
J
K

Hind III


H 3·8 7·5 E 17 D 29·6 A 50·4 C J K 63·8 69·6 F 78·8 G 83·5 B Ad7
G 8 E 17 C 31·5 H 37·3 D 50·1 A 73·6 B 89·1 F 97·3 Ad5


HindIII
Plaque Isolates
Ad5wt
1
3
4
8
12
16
20
23
26
Ad7wt
A
B C
D
E
F G
H
I
A
B
C D
E F
G
H I
J

Figures 13, 14, 15 and 16.

Analysis of plaque isolates from cell monolayers transfected with with heterotypic overlapping terminal DNA fragments

The DNA restriction endonuclease profile was examined by labelling the DNA with inorganic orthophosphate and harvesting as a Hirt supernatant. Equal aliquots of labelled DNA were cleaved as single digests with endonucleases BamH1, HindIII, Sma1 or BglII and electrophoresed through an agarose gel followed by exposure to X-ray film at -70°C.

The restriction enzyme maps for each endonuclease used is shown above the figure; the heavy black lines show the heterotypic terminal DNA fragments and the region of overlap is represented by two vertical lines.

4. Analysis of plaque isolates from co-transfection of DNA terminal fragments of adenoviruses 5 and 7 wild type into 293 cells.

Grodzicker et al., (1974) and Williams et al., (1975) showed that recombinants could be identified from their parentals by restriction enzyme analysis. Since the restriction enzyme profiles of adenoviruses type 5 and type 7 are significantly different (Figs 11 and 12) this strategy could be used to screen for heterotypic recombinants.

Hirt supernatant DNA was prepared from the four original isolates on the plate co-transfected with heterotypic DNA overlapping fragments (isolates 1-4) and also from a random selection of plaque isolates from the heterotypic yield (isolates 8, 12, 16, 20, 23 and 26). The DNA was cleaved with restriction enzymes, HindIII, Sma1, BamH1 and BglII. Figures 13, 14, 15 and 16 show the cleavage profiles obtained by each restriction enzyme; above each figure the diagram shows the restriction enzyme sites of each viral serotype. Heavy black lines represent input terminal DNA fragments and the boxed zone represents the area of expected recombination.

Cleavage with restriction enzymes Sma1 and BamH1 (Figs. 13 and 14) showed that all isolates looked like adenovirus type 5.

Cleavage with the restriction enzyme BglII showed that all the isolates were identical with the absence of adenovirus type 5 fragments H and I. The restriction enzyme map of adenovirus type 5 (Fig. 15) shows that DNA fragments H and I are separated on the genome by a BglII site at 89.9 m.u. and lie outwith the area of expected recombination. The absence of those two fragments suggests the loss of this site. BglII fragments

H and I together make up approximately 10.5% of the genome. There is no evidence of a band of this size migrating in the gel system. There is a new band of about 5.5% and possibly a band of about 4% running as a triplet with bands J and K. This would suggest a deletion of about 1% and hence the loss of the BglII site at 89.9 m.u.

Cleavage with HindIII showed that all the isolates were identical with the absence of adenovirus type 5 DNA fragments B and F. The restriction enzyme map of adenovirus type 5 (Fig. 16) shows that fragments B and F are separated on the genome by a HindIII cleavage site at 89.1 m.u., which lies outwith the zone of expected recombination. The absence of these fragments suggests the loss of the 89.1 m.u. HindIII cleavage site.

Bands B and F together equal 23.7% of the genome. A fragment of this size would migrate in the gel system in the region of the adenovirus 5 wild type band A. No new fragment of this size is present. Instead there are three new fragments of 8.6%, 8% and 6% of the genome - a total of 22.6%. This suggests a deletion of approximately 1.1% and hence the absence of the HindIII site at 89.1 m.u. However, the presence of three new bands suggests the gain of two new HindIII sites.

There are two possible explanations for this.

(a) Heterotypic recombination has occurred between adenoviruses type 5 and 7;

(b) There has been genetic rearrangement within the adenovirus 5 genome to give new HindIII sites.

Figure (17) shows the restriction enzyme map for the isolates within

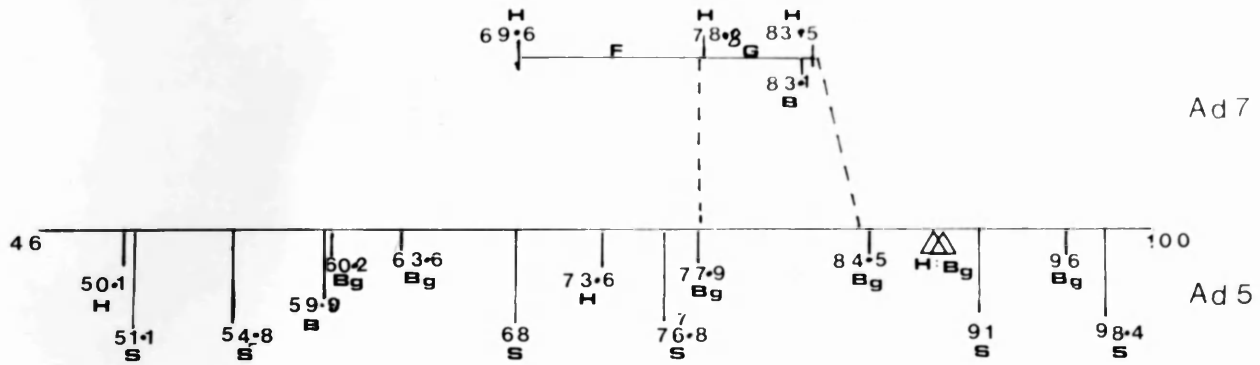

H
69·6
F
H
78·8
G
H
83·5
83·1
B
Ad 7
46
50·1
H
51·1
S
54·8
S
59·9
B
60·2
Bg
63·6
Bg
68
S
73·6
H
76·8
S
77·9
Bg
84·5
Bg
H Bg
91
S
96
Bg
98·4
S
100
Ad 5

Figure 17.

Summary of the adenovirus 5 wild type restriction enzyme sites present in the isolates: and a schematic diagram of a recombinational event with adenovirus 7 wild type which could give rise to two new HindIII sites within the isolates.

the heterotypic overlap, mapping between (46-85.4) m.u. and beyond to 100 m.u.; the data is based on the analysis obtained using BamH1, HindIII, BglII and Sma1. All positively identified sites are those belonging to adenovirus type 5.

The fragment of the adenovirus type 7 genome (Fig. 12) which could recombine to give the new HindIII sites would contain HindIII G (78.8-83.5) m.u., as all isolates have the restriction enzyme profile of adenovirus 5 mapping between 46 m.u. and 77.9 m.u. (Fig. 17).

For recombination to occur to give these sites (Fig. 17),a crossover would have to take place after the BglII 77.9 m.u. site of adenovirus type 5 and before the HindIII site at 78.8 m.u. of adenovirus type 7. To have the BglII site at 84.5 m.u. of adenovirus type 5, the crossover would have to occur after the HindIII 83.5 m.u. site of adenovirus type 7.

There are two lines of evidence against this hypothesis:-

(1) The adenovirus type 7 fragment HindIII G (78.8-83.5) m.u. has a BamH1 site at 83.1 m.u.; cleavage of the isolates shows no evidence of this adenovirus type 7 BamH1 site. If the isolates contained this site, then the proposed restriction enzyme profile would show three bands of 59.5%, 23.6% and 16.9%. The profile of the isolates is distinctly that of adenovirus type 5.

(2) The three new HindIII fragments of the putative recombinants would be 13.8%, 5.2% and 4.7%; the sizes of the new fragments of the isolates are 8.6%, 8% and 6%, suggesting that these fragments are not a result of the proposed recombination.

The above data suggests that no heterotypic recombination has occurred. Therefore the variation of the isolates must be due to genetic rearrangement of adenovirus type 5. Further evidence to support this hypothesis comes from the fact that there is no restriction enzyme site of adenovirus type 7 between 0-46 m.u., as by the construction of the experiment (Table VIa) any heterotypic recombinant should possess this.

Isolates have a deletion of approximately 1.1% mapping in the L5 region (86.0-91.3) m.u., giving rise to the loss of the 89.1 m.u. HindIII and the 89.9 m.u. BglII sites of adenovirus type 5. The presence of the new HindIII sites in the isolates could be either the result of point mutations or the insertion of cellular DNA into the adenovirus type 5 genome.

By the construction of the experiment the only input of adenovirus type 5 was the Sal1 A fragment (45.9-100) m.u. Therefore to justify the isolation of the adenovirus type 5 variants there must have been contamination by full length adenovirus type 5 DNA molecules. When adenovirus type 5 is cleaved with Sal1 four fragments result (Fig. 11); fragments B and C are separated on the genome by the .9% fragment D. If a low level of DNA molecules carried a deletion so as to lose band D, then B and C would form one fragment of 45% which would migrate in the gel system close to the Sal1 band A and hence would be excised along with it. These two fragments would be capable of self ligation to form a viable almost full length molecule of adenovirus type 5 on transfection.

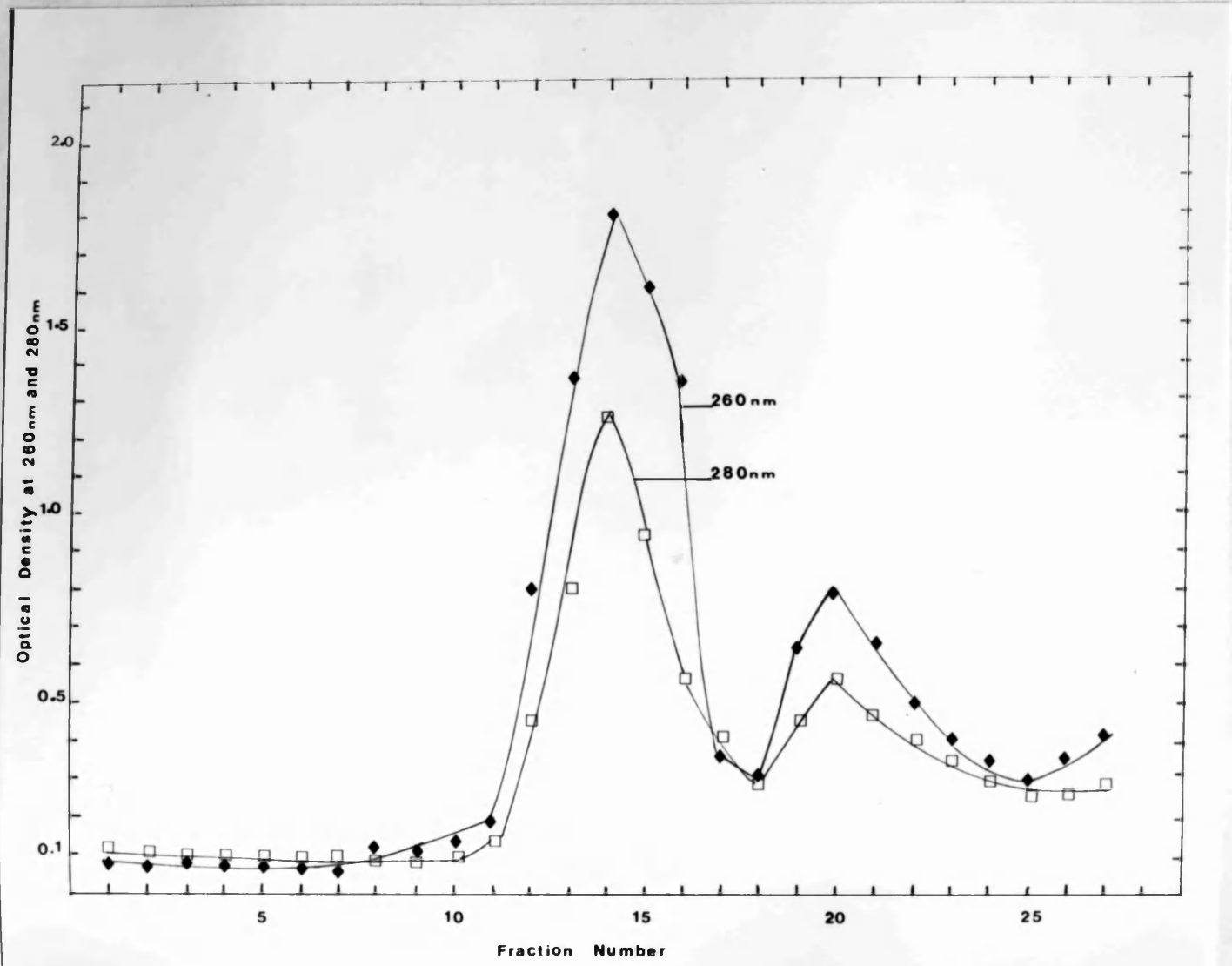

Optical Density at 260nm and 280nm
2.0
1.5
1.0
0.5
0.1
260nm
280nm
5
10
15
20
25
Fraction Number

Figure 18.

Adenovirus 7 DNA-protein complex

Optical density readings of adenovirus 7 wild type DNA-protein complex at wavelengths of 260 and 280 nanometers. The peak fractions 12-17 were pooled as containing the DNA-protein complex. After dialysis the sample was read at OD_{260} to determine the DNA concentrate which was found to be 1ug per 20ul.

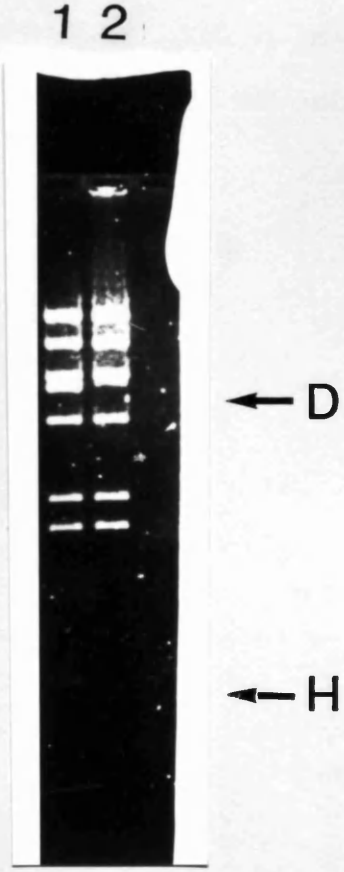

1 2
D
H

Figure 19.

Cleavage of adenovirus 7 DNA-protein complex

One microgramme of adenovirus 7 DNA-protein complex or adenovirus 7 DNA which had been treated with proteinase K was cleaved with the endonuclease Sma1. The DNA samples were electrophoresed through a .7% agarose gel overnight and the DNA visualised by ethidium bromide staining.

Lane 1 : The restriction enzyme profile of proteinase K treated adenovirus 7 DNA, cleaved with Sma1.

Lane 2 : The restriction enzyme profile of adenovirus 7 DNA-protein complex cleaved with Sma1. Arrows indicate the two absent terminal fragments D and H.

5. Infectivity of DNA-protein complexes of adenovirus 7 wild type and adenovirus 5 wild type.

When DNA is extracted from adenovirus virions by treatment with Proteinase K and SDS followed by phenol extraction, the resulting DNA is in the form of a linear molecule.

Robinson et al., (1973) extracted DNA from adenovirus 2 wild type by releasing viral DNA directly into guanidinium hydrochloride followed by sedimentation in a guanidinium hydrochloride gradient; this DNA was found to be in the form of relaxed circularised protein complexes. Sharp et al., (1976) and Chinnadurai et al., (1978) showed that by using DNA-protein complex instead of linearised DNA, the infectivity of transfected DNA could be increased 100 fold.

Previous data showed that adenovirus 7 wild type DNA had a low infectivity at day 4 of transfection; therefore adenovirus 7 wild type DNA-protein complex was prepared in an attempt to increase the DNA infectivity at day 4. Figure 18 shows the O.D. profile of the adenovirus 7 wild type DNA-protein complex guanidinium hydrochloride gradient, the peak between fractions 12-17 being DNA-protein complex. Terminal fragments of adenovirus DNA-protein complexes on enzyme cleavage followed by gel electrophoresis do not migrate into the gel system (Reiter et al., 1980). This method was used to show that the resulting adenovirus type 7 DNA had terminal peptide; cleavage of the DNA with Sma1 followed by gel electrophoresis showed that the terminal fragments D and H were absent from the DNA profile suggesting it to be DNA-protein complex. (Fig.19).

Table VII

DNA (ug/dish)	Average number of plaques from two dishes at days 4 and 14.			
	DNA-protein complex		DNA treated with Proteinase K & SDS	
	Day 4	Day 14	Day 4	Day 14
Ad7wt (100ng)	0	198	0	192
Ad7wt (10ng)	0	20	0	20
Ad7wt (1ng)	0	2	0	2
DNA infectivity (p.f.u./ug)	0	1993	0	1973
Ad5wt (100ng)	42	407	25	225
Ad5wt (10ng)	4	40	2	19
Ad5wt (1ng)	0	4	0	2
DNA infectivity (p.f.u./ug)	273	4023	150	2050

Table VIII

Ad7wt DNA cleaved with BglII (fragment)	Percentage of genome estimated from DNA calibration curve
Fragment A	20%
" B	16.5%
" C	15.5%
" D	10.5%
" E	8.8%
" F	6.5%
" G	6.4%
" H	5.3%
" I	4.2%
" J	3.2%
" K	2.6%
" L	1.8%

Figure 20

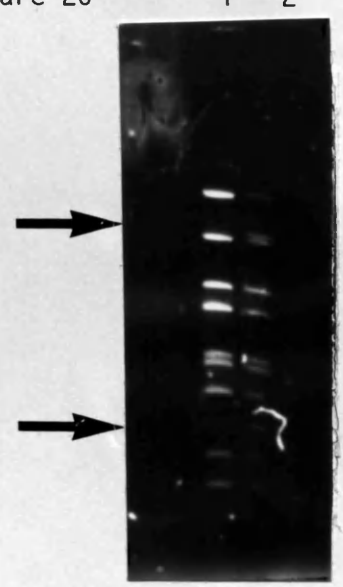


Figure 20.

Cleavage of adenovirus 7 DNA-protein complex to detect the BglII terminal fragments

Adenovirus 7 DNA-protein complex was cleaved with the endonuclease BglII and electrophoresed through a ·7% agarose gel overnight; DNA was visualised by ethidium bromide staining.

Lane 1 : The restriction enzyme profile of adenovirus 7 DNA-protein complex cleaved with BglII.
Arrows indicate the two absent terminal fragments B and I.

Lane 2 : The restriction enzyme profile of proteinase K treated adenovirus 7 DNA, cleaved with BglII.

Data (Table VII) shows the DNA infectivity profiles at days 4 and 14. DNA infectivity was calculated by averaging the number of plaques formed per ug of DNA. There is no difference between the DNA infectivity of adenovirus 7 wild type DNA-protein complex and Proteinase K treated DNA. At day 4 of transfection both forms of adenovirus 7 DNA are of equally low infectivity compared to adenovirus 5, adenovirus 5 DNA being approximately 200 times more infectious. By day 14 both forms of adenovirus 7 DNA are as infectious as Proteinase K treated adenovirus 5, while adenovirus 5 DNA-protein complex is twice as infectious as its linear form and both forms of adenovirus type 7.

Since there was no increase in DNA infectivity at day 4 using adenovirus 7 DNA-protein complex it is unlikely that the use of this would increase the chance of either intertypic or intratypic recombination.

6. A BglII restriction enzyme map of adenovirus 7 wild type DNA.

To evaluate present and future restriction enzyme data it was necessary to construct a BglII map of adenovirus 7 wild type DNA.

When adenovirus type 7 is cleaved with BglII twelve fragments are seen (Table VIII) and by cleaving adenovirus 7 DNA-protein complex it was seen that bands B and I are the terminal fragments containing terminal peptide (Reiter et al., 1980) (Fig. 20).

By single and double digests of BglII along with the following restriction endonucleases, EcoR1, Sal1, Xba1, Hpa1, Kpn1, Xho1, HindIII, Cla1 and BamH1, with appropriate marker fragments ranging in size from

Table IXb

DNA terminal fragments (ug/dish)	Plaques/dish		
	Day 4	Day 7	Day 14
Ad7wt EcoR1 A + dl309 Xba1 A 1ug + 1ug	1 0	3 9	8 15
Ad7wt EcoR1 A + Ad7wt Sal1 B 2ug + 2ug	0 0	0 0	0 0
Ad5wt EcoR1 A + dl309 Xba1 A 1ug + 1ug	16 19	154 120	TM TM
Ad7wt EcoR1 2ug	0 0	0 0	0 0
Ad5wt EcoR1 A 2ug	0 0	0 0	0 0
Ad7wt Sal1 B 2ug	0 0	0 0	0 0
dl309 Xba1 A 2ug	0 0	0 0	0 0
Ad5wt DNA (ug/dish)			
1ug	TM TM	TM TM	CPE CPE
.1ug	29 26	198 214	231 234
.01ug	2 2	20 20	20 21
DNA infectivity (p.f.u./ug)			2187
Ad7wt DNA (ug/dish)			
1ug	0 0	+ +	TM TM
.1ug	0 0	+ +	200 210
.01ug	0 0	0 0	19 22
DNA infectivity (p.f.u./ug)			2025
dl309 DNA (ug/dish)			
1ug	TM TM	TM TM	CPE CPE
.1ug	35 36	238 286	298 309
.01ug	3 3	26 26	30 31
DNA infectivity (p.f.u./ug)			3030
Mock infected (SS DNA)	0 0	0 0	0 0

+ sign represents the faint appearance of plaques which are too difficult to count

TM represents plaques too numerous to count.

23.5% to 4.2%, the DNA fragments were aligned on the genome (Figs. 15 and 22).

7. Co-transfection of overlapping terminal DNA fragments of dl309 and adenovirus 7 wild type into 293 cells.

Data from the previous experiment Sections 3 and 4 showed that no intertypic recombination had occurred using a terminal DNA overlapping region of 39.5%.

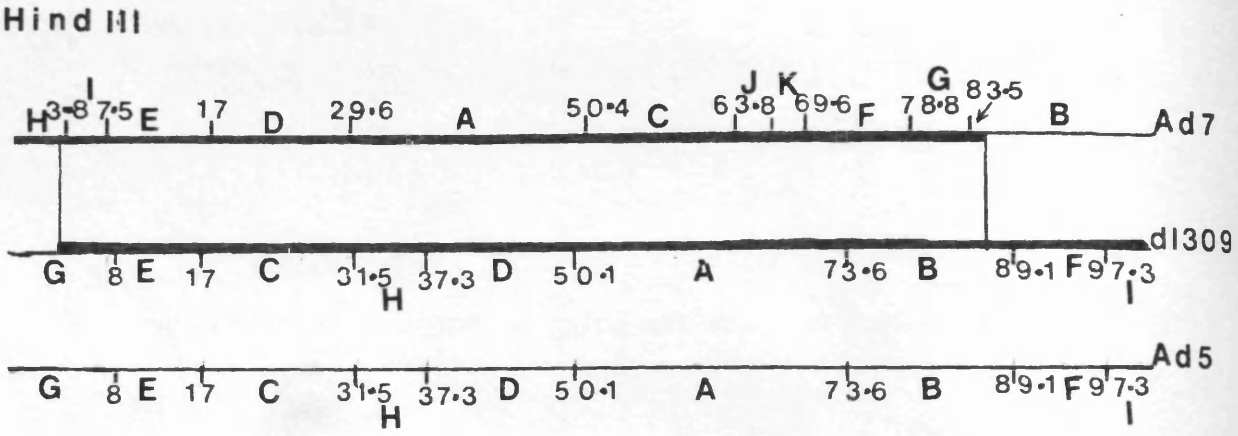
Dl309 (Jones and Shenk, 1979) is a variant of adenovirus type 5 from which all the Xba1 sites but one at 3.8 m.u have been removed; on treatment with Xba1 enzyme it yields two fragments of 3.8% and 96.2% of the genome. Using the Xba1 A(3.8-100) m.u. fragment of dl309 to replace the Sal1 A(45.9-100) m.u. fragment of adenovirus 5 wild type, the region of heterotypic overlap can be increased from 39.5% to 81.6%, thus increasing the chance of recombination by the possible increase in number of homologous zones.

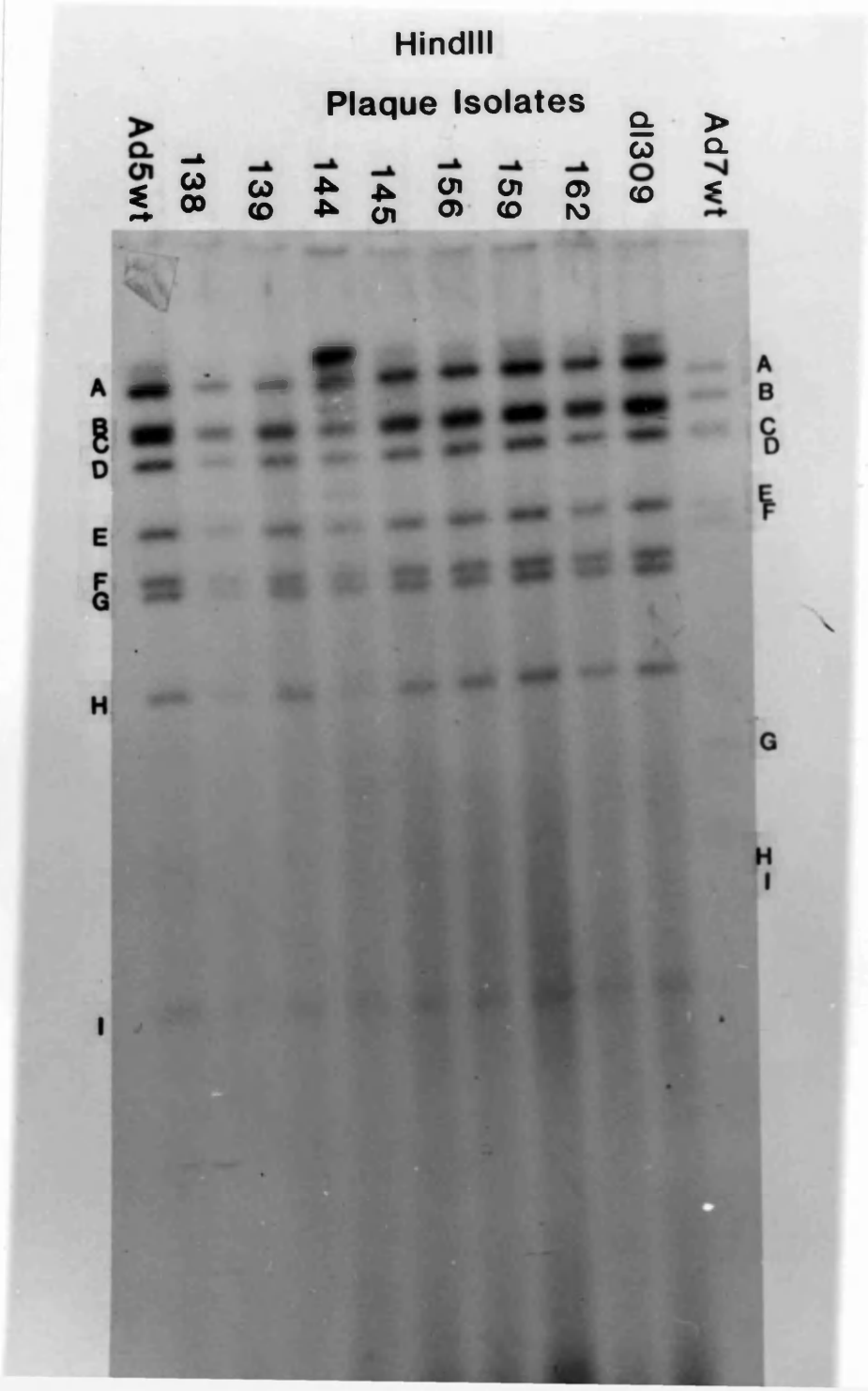
Table IXa shows the DNA fragments used with the map co-ordinates and the percentage of DNA overlap.

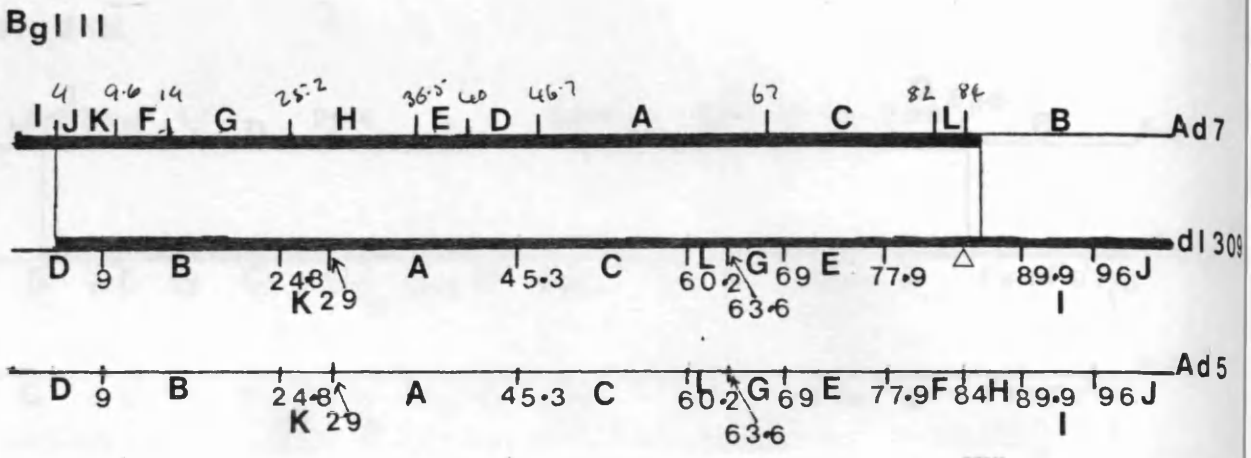
Data (Table IXb) shows that there is an increase in plaque number from intertypic adenovirus 5 wild type recombination compared to previous experiment Section 3.

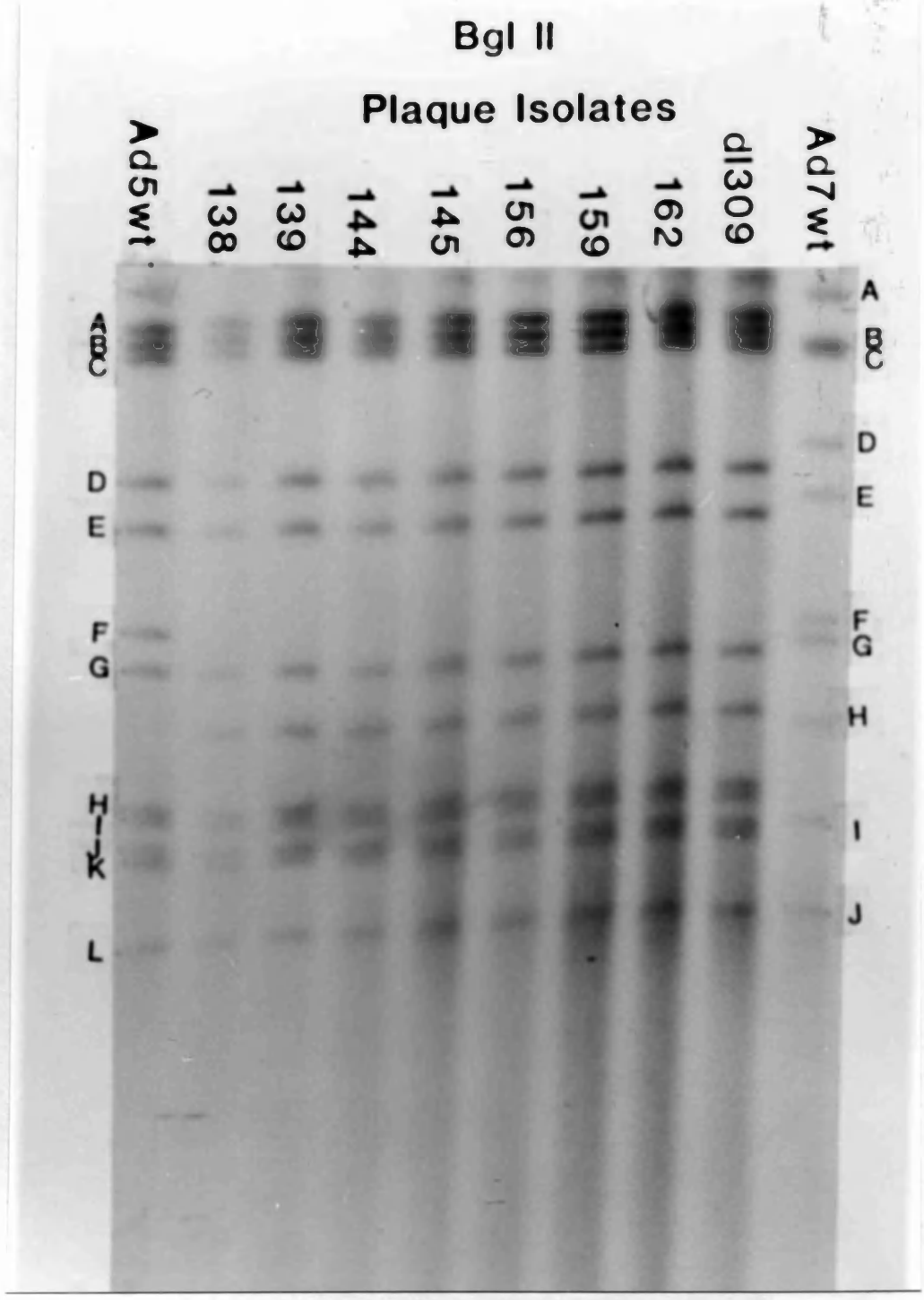
This data could be the result of the following:-

(a) The increase in the region of homotypic overlap may result in a higher frequency of recombination than in the previous experiment.


Hind III
H 3·8 I 7·5 E 17 D 29·6 A 50·4 C J 63·8 K 69·6 F G 78·8 83·5 B Ad7
dl309
G 8 E 17 C 31·5 H 37·3 D 50·1 A 73·6 B 89·1 F 97·3 I
Ad5
G 8 E 17 C 31·5 H 37·3 D 50·1 A 73·6 B 89·1 F 97·3 I


HindIII
Plaque Isolates
Ad5wt
138
139
144
145
156
159
162
dl309
Ad7wt
A
B
C
D
E
F
G
H
I
A
B
C
D
E
F
G
H
I


Bgl II
4
9.6
14
25.2
36.5
40
46.7
67
82
84
I J K F G H E D A C L B
Ad7
dl309
D 9 B 24.8 K 29 A 45.3 C 60.2 L 63.6 G 69 E 77.9 89.9 I 96 J
Ad5
D 9 B 24.8 K 29 A 45.3 C 60.2 L 63.6 G 69 E 77.9 F 84 H 89.9 I 96 J


Bgl II
Plaque Isolates
Ad5wt
138
139
144
145
156
159
162
dl309
Ad7wt
A
B
C
D
E
F
G
H
I
J
K
L

Figures 21 and 22.

Analysis of plaque isolates from cell monolayers transfected with heterotypic overlapping terminal DNA fragments

The DNA restriction enzyme profile was examined by labelling the DNA with inorganic orthophosphate and harvesting as a Hirt supernatant. Equal aliquots of DNA were cleaved with HindIII or BglII and electrophoresed through a .7% agarose gel overnight followed by exposure to X-ray film at -70°C.

The restriction enzyme map for each of the endonucleases used is shown above the figure; the heavy black lines show the heterotypic terminal DNA fragments and the region of overlap is represented by two vertical lines.

△ indicates the deletion of a restriction enzyme site.

(b) A low level of DNA molecules resistant to Xba1 cleavage at 3.8 m.u. would result in a fragment which would migrate with Xba1 A(96.2%) in the gel system and be excised with it. These molecules, on transfection into permissive cells, would be capable of producing viral progeny resulting in an increase in plaque number. Control plates containing Xba1 A fragment alone show no evidence of viral progeny. Hence the observed increase may be a result of (a) above.

Intertypic plates show an increase in plaque number also; plaque isolates were analysed for recombinants.

8. Analysis of plaque isolates from co-transfection of overlapping DNA terminal fragments of dl309 and adenovirus 7 wild type.

Viral DNA was prepared as a Hirt supernatant from the plaque isolates on the plates transfected with heterotypic DNA overlapping terminal fragments.

The restriction enzyme profile of the DNA was examined using endonucleases BamH1, Sma1, HindIII and BglII.

Figure 21 shows that all the isolates cleaved with HindIII have the profile of dl309 which is also the same as adenovirus type 5.

To establish that these isolates were of dl309 origin, they were cleaved with BglII; dl309 carries a deletion of the 84 m.u. BglII site resulting in the absence of band F and the appearance of a smaller band.

Data (Fig. 22) confirms that all the isolates are of dl309 origin and that no heterotypic recombination has taken place.

9. DNA transfection comparisons.

Szybalska and Szybalski, (1962) and Bhargava and Shannugan, (1971) showed that eukaryotic cells could take up DNA and transport it to the cell nucleus. This observation has been used to induce the transformation of eukaryotic cells with viral DNA.

Several methods have been developed to enhance the uptake of foreign DNA into cell systems:- (a) Transfection of a calcium phosphate DNA co-precipitate (Graham and van der Eb, 1973) into 293 cell monolayers followed at 4 hours post transfection by exposure of cell monolayers to glycerol (Frost and Williams, 1978) to enhance DNA uptake; (b) Straus et al., (1981) showed that by trapping adenovirus type 2 DNA in large unilamellar vesicles of phosphatidylserine they could transfect KB cells to an efficiency of about 4×10^3 plaques per 1 ug of encapsulated DNA; (c) Kawai and Nishizawa, (1984) showed that chicken embryo fibroblasts could be transfected with RSV<DNA using polybrene (a polycation) as a mediator to absorb DNA to the cell surface followed at 6 hours post transfection by exposure of the cell monolayer to 20%-30% DMSO at room temperature. DMSO was removed and monolayers were maintained under medium for 7 days at which time transformed foci were scored.

Adenovirus type 7 DNA has consistently shown a low infectivity at day 4 and homotypic recombination has been shown to be almost negligible even at day 14. The restriction endonuclease maps of adenovirus type 7

Table X

Transfection Experiment	1	2	3	4	5	6
Cell line	293	293	293	Hela	Hela	Hela
Monolayer confluency	70% - 80%	70% - 80%	30% - 40%	30% - 40%	70% - 80%	70% - 80%
Method of transfection	Graham and van der Eb modified by Frost and Williams	Wigler et al. modification	Wigler et al.	Wigler et al.	Wigler et al.	Wigler et al.
Plaque count of adenovirus 5wt DNA at day 14						
1ug/plate	CPE CPE	CPE CPE	TM TM	62 68	TM TM	102 101
.1ug/plate	238 239	CPE CPE	67 69	7 7	126 129	10 10
.01ug/plate	23 23	106 107	7 6	0 0	12 13	0 0
DNA infectivity (p.f.u./ug)	2342	10650	665	66	1262	100

(Fig. 12) show that the maximum region of homotypic overlap which has been used in previous experiments is 17.4%. Section 5 shows that adenovirus type 7 DNA infectivity at day 4 cannot be increased in my hands even using DNA-protein complex. Therefore an attempt was made to find a method of transfection which would increase the DNA infectivity at day 4.

Initially adenovirus 5 wild type DNA was used as it was more infectious than adenovirus 7 wild type and could be used as a standard comparison.

The method described by Kawai and Nishizawa, (1984) was modified. Adenovirus 5 wild type DNA was mixed with 20 ug/dish of polybrene and absorbed to drained 293 cell monolayers for 30 minutes at 37°C. 4ml of ECS2 medium was added, with the intention of enhancing DNA uptake at 4 hours post transfection by exposure to glycerol; however, polybrene proved to be highly toxic to 293 cell monolayers even at a very low concentration. Therefore this line of transfection was discontinued.

Three transfection methods were compared:- (a) Method described by Graham and van der Eb, (1973) with modification by Frost and Williams, (1978). (b) Method described by Wigler, (1978). (c) Method described by Wigler with modification described in Materials and Methods.

Data (Table X) shows that method number 2 [modification of Wigler, (1978)] yields the highest p.f.u./ug. This method is ten times more infectious than Method 6 and four times more infectious than Method 1, the transfection method used in previous experiments. Methods 1 and 2 were compared using adenovirus 7 wild type.

Table XI

Transfection experiment	1	2
Cell line	293	293
Cell monolayer confluency	70% - 80%	70% - 80%
Method used for transfection	Graham and van der Eb with modification by Frost and Williams	Wigler et al., with modification
Plaque count of Ad7wt DNA at day 14		
1ug/dish	TM TM	TM TM
.1ug/dish	189 191	275 271
.01ug/dish	18 18	25 26
DNA infectivity (p.f.u./ug)	1850	2640
Plaque count of Ad5wt DNA at day 14		
1ug/dish	CPE CPE	CPE CPE
.1ug/dish	216 221	CPE CPE
.01ug/dish	22 25	107 109
DNA infectivity (p.f.u./ug)	2267	1080

Table XI shows that adenovirus 7 wild type DNA infectivity is increased 1.4 times by method 2. It was noted that adenovirus 7 wild type DNA is still less infectious than adenovirus 5 wild type at day 4 by the absence of plaques. This method of DNA transfection was, however, used in subsequent experiments unless otherwise stated.

10. Isolation of a fast growing large plaque variant (Ad3var100) of adenovirus 3 strain GB.

Previous data has shown that adenovirus 7 wild type DNA is less infectious than adenovirus 5 wild type DNA on day 4 of transfection. At day 14 the titres are almost comparable with adenovirus 7 wild type yielding plaques which are very much smaller than adenovirus 5 wild type.

One step growth curves show that adenovirus 7 wild type virus is 2 logs less infectious than adenovirus 5 wild type virus. This difference of 2 logs is apparent by 24 hours post infection and is maintained throughout the growth cycle.

An attempt was made to isolate a fast growing large plaque virus of subgroup B, similar to adenovirus 5 wild type subgroup C. The following stocks of subgroup B viruses were passaged on 293 cell monolayers: (1) Adenovirus 7a strain H; (2) Adenovirus 7 Pinkhey HEK1; (3) Adenovirus 7A VR8 K1114; (4) Adenovirus 3 strain GB. Plaque morphology and growth rates were noted. Plaques which appeared to be fast growing with a large plaque morphology were selected for further analysis. The numbers were as follows:-

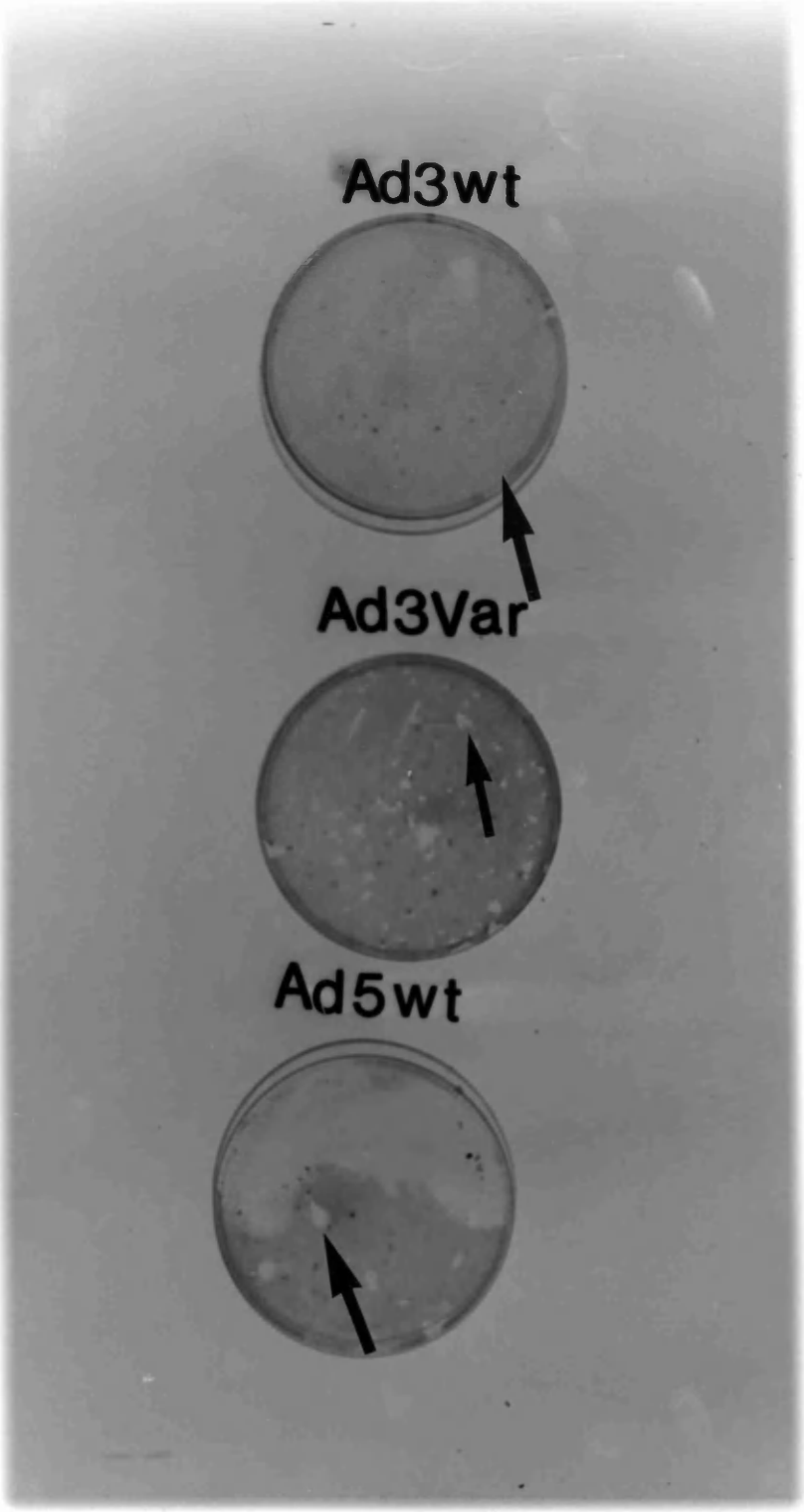

Ad3wt
Ad3Var
Ad5wt

Figure 23.

Plaque morphology of adenovirus 3var100, adenovirus 3 wild type and adenovirus 5 wild type.

Monolayers of 293 cells were infected with adenoviruses 3var100, 3 wild type or 5 wild type at a dilution such that isolated plaques could be discerned, and maintained under agar overlay medium for 10 days.

Monolayers were fixed and stained; isolated plaques are arrowed to show the difference in size between adenovirus 3var100, adenovirus 3 wild type and adenovirus 5 wild type.

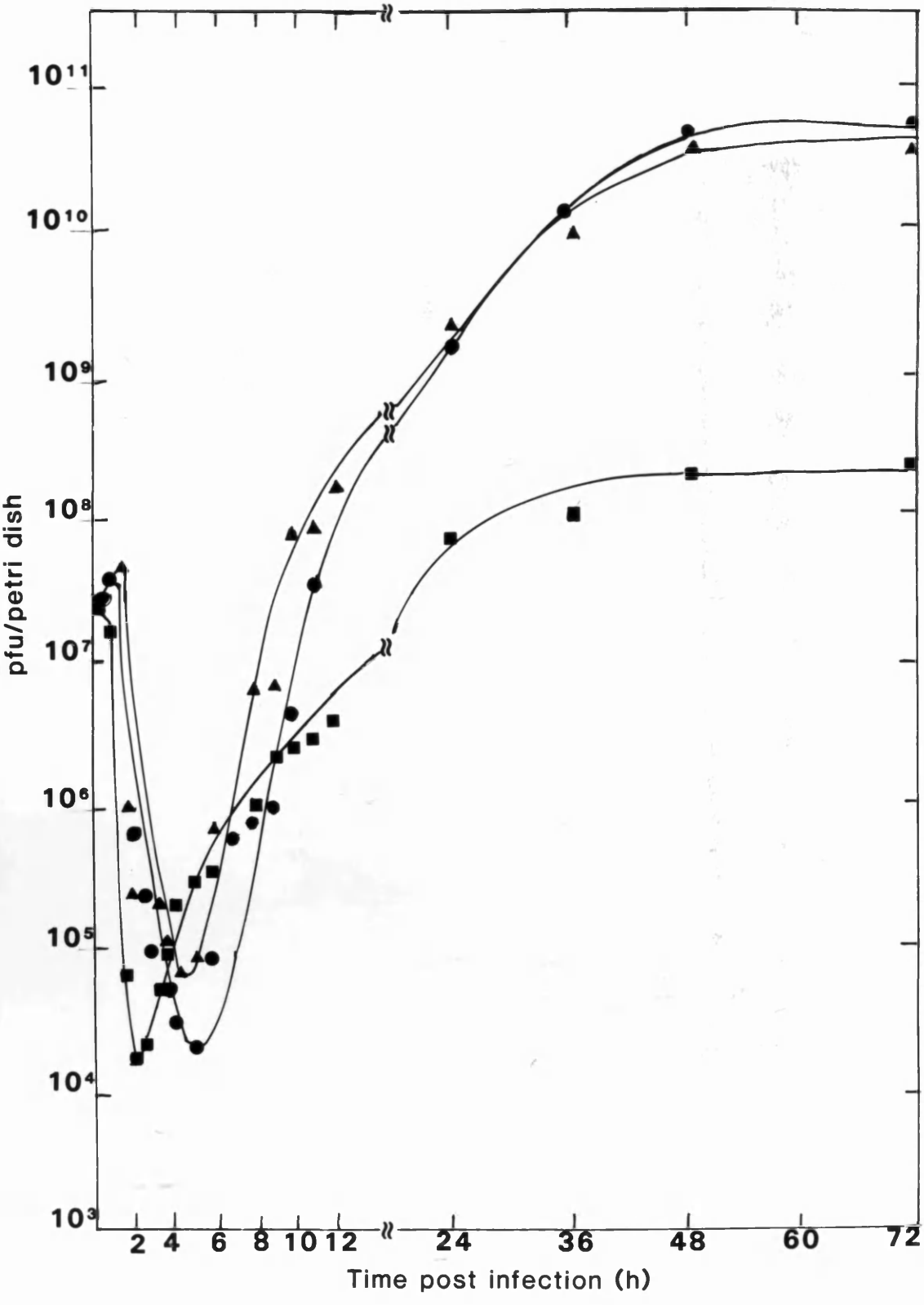

pfu/petri dish
10^11
10^10
10^9
10^8
10^7
10^6
10^5
10^4
10^3
2 4 6 8 10 12 24 36 48 60 72
Time post infection (h)

Figure 24.

One step growth curves of adenovirus 3var100, adenovirus 3 wild type and adenovirus 5 wild type

Semi confluent 50mm monolayers of 293 cells were infected with adenovirus 3var100, adenovirus 3 wild type or adenovirus 5 wild type viruses at 10 p.f.u./cell. After 1 hour of viral absorption, monolayers were washed with Tris/Saline to remove unadsorbed virus. Four millilitres of ECS2 medium was added per dish and time points taken between zero and 72 hours post infection. The time points were titrated on monolayers of 293 cells which were maintained under agar overlay medium for 7 days at 37°C, supplemented at days 3 and 6 with 1ml of the above medium. Monolayers were fi xed and stained, the titres being plotted on a log scale against time of post infection.

● = Adenovirus 5 wild type

▲ = Adenovirus 3var100

■ = Adenovirus 3 wild type

(1) Adenovirus 7a strain H : 22 isolates

(2) Adenovirus Pinkhey HEK1 : 0 isolates

(3) Adenovirus 7A VR8 K1114 : 11 isolates

(4) Adenovirus 3 strain GB : 0 isolates

On repeated passage on 293 cell monolayers all selected isolates reverted back to the normal small plaque morphology and growth rate of subgroup B.

A crude seed stock of adenovirus 3 strain GB (obtained from W. C. R. Russell), independent from the stock (4) above, was titrated on 293 cell monolayers and maintained under agar overlay medium at 37°C. Large plaques which appeared by day 4 of infection were isolated and screened for a stable large plaque phenotype and fast growth rate. Of twenty isolates screened, one (adenovirus 3var100) maintained its original large plaque phenotype and fast growth rate. Figure 23 illustrates that the plaque morphology of adenovirus 3var100 is more comparable to that of adenovirus 5 wild type than the minute size of adenovirus 3 wild type.

11. One step growth curves of adenovirus 3var100, adenovirus 3 wild type and adenovirus 5 wild type.

The growth profiles of adenovirus 3var100, adenovirus 3 wild type and adenovirus 5 wild type were analysed by one step growth curves in 293 cell monolayers.

The final yields (Fig. 24) of virus at 72 hours were approximately the same for adenovirus 3var100 and adenovirus 5 wild type (1.1×10^3 p.f.u./cell) whereas the yield for adenovirus 3 wild type was 2 logs lower (1.6×10^1 p.f.u./cell). Adenovirus 3var100 and adenovirus 5 wild

Table XII

Sample \ Time	Day 4		Day 14	
Ad5wt DNA				
1ug/dish	TM	TM	CPE	CPE
.1ug/dish	23	19	211	219
.01ug/dish	2	3	22	22
DNA infectivity (p.f.u./ug)	230		2178	
Ad3var100				
1ug/dish	16	17	TM	TM
.1ug/dish	2	1	121	122
.01ug/dish	0	0	11	12
DNA infectivity (p.f.u./ug)	16		~~1080~~ 1182	
Ad3wt DNA				
1ug/dish	0	0	TM	TM
.1ug/dish	0	0	67	69
.01ug/dish	0	0	6	5
DNA infectivity (p.f.u./ug)	0		615	
Ad7wt DNA				
1ug/dish	0	0	TM	TM
.1ug/dish	0	0	100	98
.01ug/dish	0	0	9	9
DNA infectivity (p.f.u./ug)	0		945	

type have a relatively long lag phase (5-6 hours) compared to adenovirus 3 wild type which displays an unusually short lag phase (2-3 hours).

12. DNA infectivity of adenovirus 3var100, adenovirus 3 wild type and adenovirus 5 wild type.

DNA prepared from the above virus stocks was tested for infectivity in 293 cell monolayers. As the DNA transfection experiments were running concurrently with the isolation of a large plaque fast growing virus of subgroup B, the method used for DNA transfection was that described by Graham and van der Eb, (1977) with modification by Frost and Williams, (1978).

Adenovirus 3var100 DNA, unlike adenoviruses 7 and 3 wild type, yields plaques about 1 log less than adenovirus 5 wild type (Table XII) at day 4 of transfection. At day 14 adenovirus 3var100 DNA is about 1.7 times more infectious than its parental adenovirus 3 wild type and slightly more infectious than adenovirus 7 wild type DNA. Adenovirus 5 wild type DNA is 2 times more infectious than adenovirus 3var100 and 4 times more infectious than adenovirus 3 wild type DNA.

13. Co-transfection of unseparated DNA fragments of adenovirus 3var100 with unseparated DNA fragments of adenovirus 5 wild type or adenovirus 2 wild type to give a region of overlap between the terminal fragments.

D'Hallium et al., (1983) have shown that Cla1 enzyme will cleave adenovirus 3 wild type once at 52.2 m.u., yielding two fragments of 52.2%

Table XIIIa

DNA terminal fragments		Map co-ordinates		
Ad3var100	Ad5wt/or Ad2wt	Map co-ordinates of terminal fragment	Region of overlap	% overlap
Cla1 B	Ad5wt EcoR1 A	52.2 m.u. - 100 m.u. 0 - 75.9 m.u.	52.2 m.u. - 75.9 m.u.	23.7%
EcoR1 A	Ad2wt BamH1 B	0 - 85.4 m.u. 59 m.u. - 100 m.u.	59 m.u. - 85.1 m.u.	26.1%
EcoR1 A Cla1 B	- -	0 - 85.1 m.u. 52.2 m.u. - 100 m.u.	52.2 m.u. - 85.1 m.u.	30%
-	Ad5wt EcoR1 A Ad5wt Sal1 A	0 - 75.9 m.u. 45.9 m.u. - 100 m.u.	45.9 m.u. - 75.9 m.u.	30%

Table XIIIb

Co-tranfected DNA digests (ug/plate)	plaques/dish				p.f.u./ug DNA
	Day 4		Day 14		
Ad3var100/Cla1 + Ad5wt/EcoR1 1ug + 1ug	3	3	14	16	7.5
Ad3var100/Cla1 + Ad2wt/BamH1 1ug + 1ug	1	1	18	18	9
Ad5wt/EcoR1 + Ad5wt/Sal1 1ug + 1ug	16	15	151	152	75
Ad3var100/EcoR1 + Ad3var100/Cla1 1ug + 1ug	6	8	124	126	63
Ad3var100/EcoR1 2ug	1	0	5	5	2.5
Ad5wt/EcoR1 2ug	3	2	5	2	1.7
Ad3var100/Cla1 2ug	0	0	1	2	.8
Ad2wt/BamH1 2ug	0	0	0	0	0
Ad5wt/Sal1 2ug	0	0	0	0	0
Ad5wt DNA					
1ug/dish	TM	TM	CPE	CPE	
.1ug/dish	159	145	TM	TM	8650
.01ug/dish	14	15	82	91	
Ad3var100 DNA					
1ug/dish	137	157	TM	TM	2287
.1ug/dish	15	16	266	259	
.01ug/dish	1	0	20	19	
Ad2wt DNA					
1ug/dish	TM	TM	TM	TM	
.1ug/dish	71	57	271	240	2502
.01ug/dish	6	3	26	23	
Mock infected SS DNA 1ug/dish	0	0	0	0	0

and 47.8% of the genome. Cla1 will also cleave adenovirus 3var100 to give two fragments of 52.2% and approximately 44.8% of the genome. A recombination experiment was designed with the following modifications:

(a) In previous co-transfections, DNA fragments were separated in an agarose gel system with DNA being eluted from an agarose gel slice. In this approach unseparated DNA fragments were used for co-transfections; this omitted the preparative steps that the DNA terminal fragments were subjected to previously.

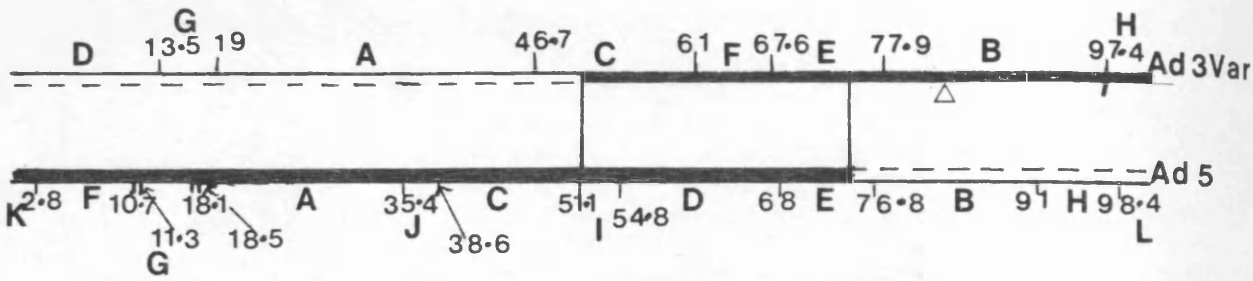
(b) Adenovirus 3var100 DNA was used to represent subgroup B instead of adenovirus 7 wild type.

(c) The method of DNA transfection was the Wigler modification described in section 9 instead of that described by Graham and van der Eb, (1973) with modification by Frost and Williams, (1978) used previously.

The DNA was cleaved so as to produce a terminal fragment from each subgroup capable of forming a region of heterotypic overlap. Table XIIIa gives the map co-ordinates of the terminal fragments and their region of overlap.

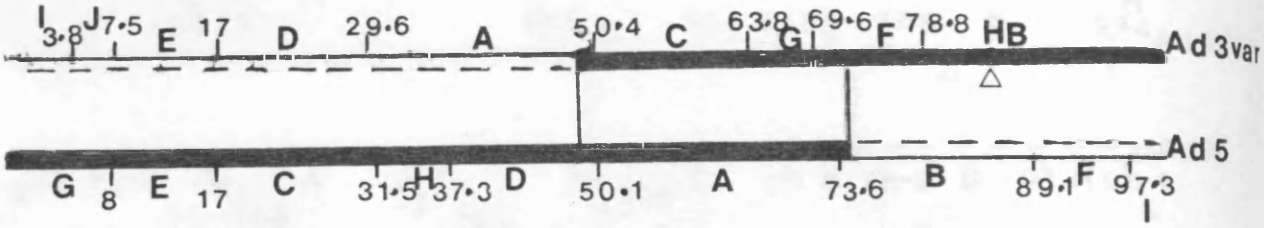
Table XIIIb lists data obtained; there is an increase in plaque number from homotypic subgroup B recombination compared to previous experiments, the number being almost as high as that obtained by homotypic adenovirus type 5 recombination. This section also shows that the Wigler modification method of DNA transfection has enhanced the number of plaques per ug of adenovirus 3var100 DNA compared with previous results, section 12,

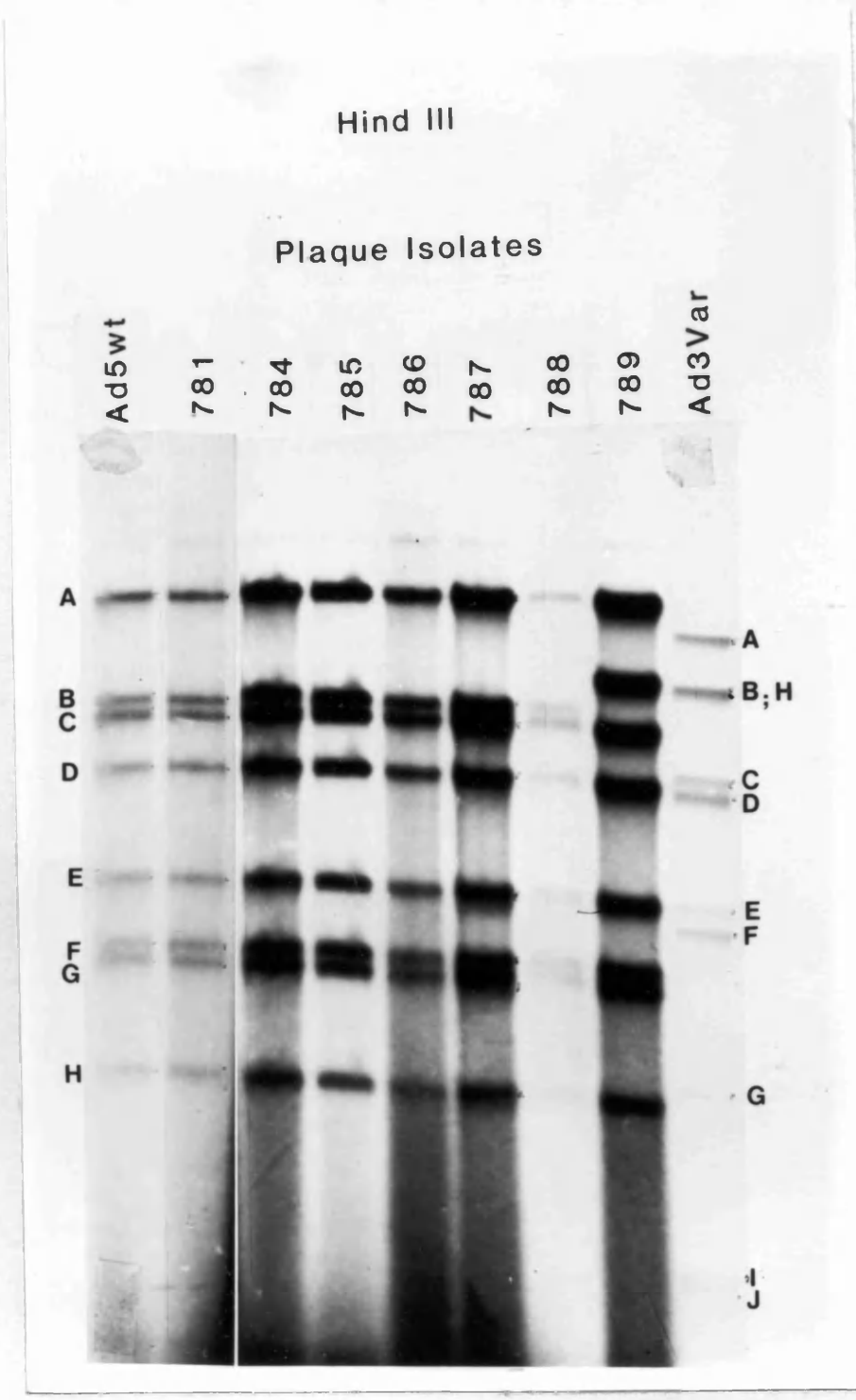
Sma I


G
D
13·5
19
A
46·7
C
61
F
67·6
E
77·9
B
97·4
H
Ad 3Var
Ad 5
K
2·8
F
10·7
11·3
G
18·1
18·5
A
35·4
J
38·6
C
51·1
I
54·8
D
68
E
76·8
B
91
H
98·4
L


Sma1
Plaque Isolates
781
782
783
784
785
786
787
788
789
Ad3Var
Ad5wt

Hind III


I
3·8
J
7·5
E
17
D
29·6
A
50·4
C
63·8
G
69·6
F
78·8
HB
Ad 3var
Ad 5
G
8
E
17
C
31·5
H
37·3
D
50·1
A
73·6
B
89·1
F
97·3
I


Hind III
Plaque Isolates
Ad5wt
781
784
785
786
787
788
789
Ad3Var
A
B
C
D
E
F
G
H
A
B;H
C
D
E
F
G
I
J

Figures 25 and 26.

Analysis of plaque isolates from cell monolayers transfected with digested heterotypic DNA

The DNA restriction enzyme profile was examined by labelling the DNA with inorganic orthophosphate and harvesting as a Hirt supernatant. Equal aliquots of labelled DNA were cleaved with Sma1 or HindIII as single digests and electrophoresed through an agarose gel followed by exposure to X-ray film at -70°C.

The restriction enzyme map for each endonuclease used is shown above the figure; the heavy black lines show the heterotypic terminal DNA fragments and the region of overlap is represented by two vertical lines. The dotted lines represent DNA fragments which were present in the transfection of full length digested DNA.

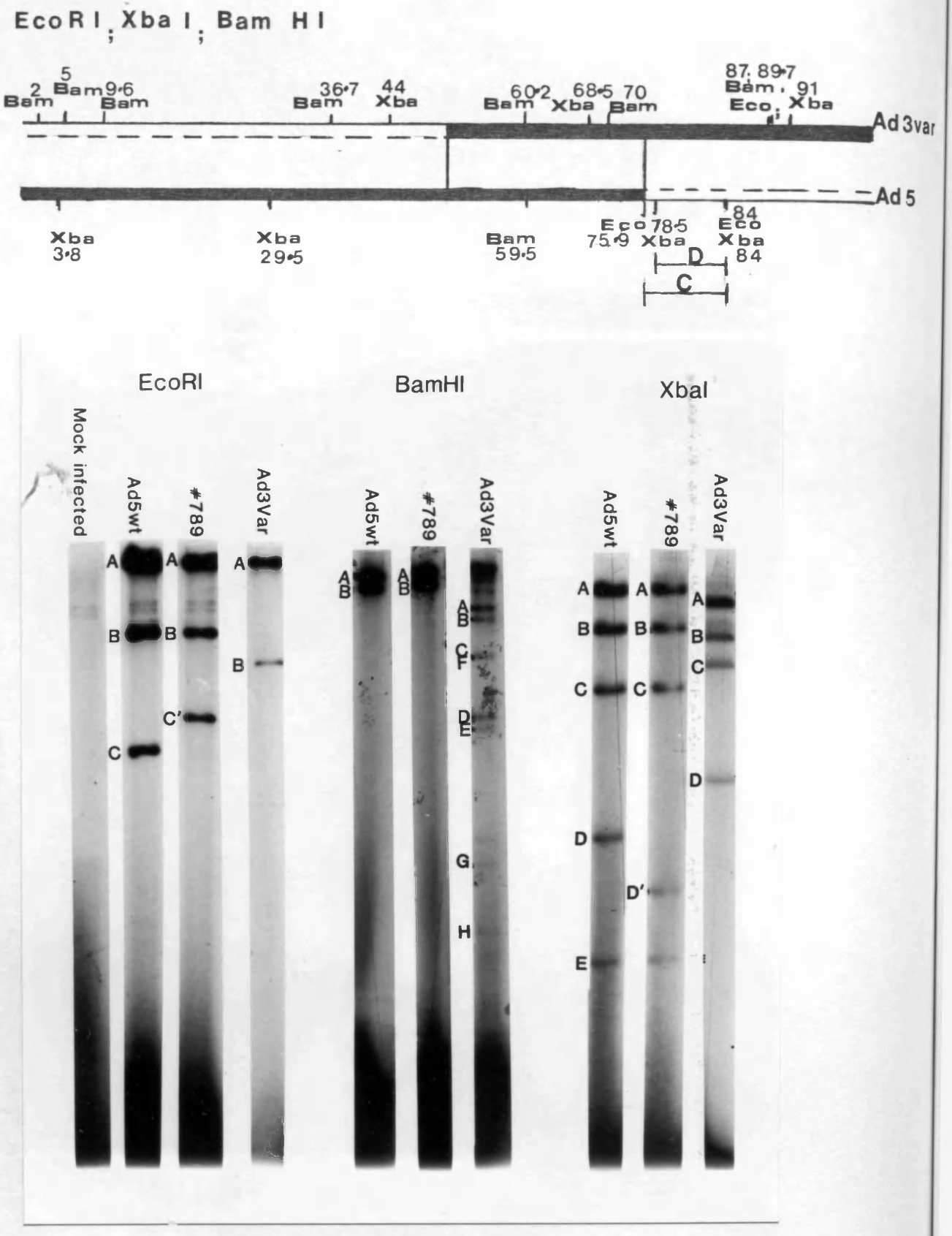
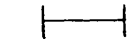

EcoR I ; Xba I ; Bam H I
Bam 2
5
Bam 9·6
Bam
Bam 36·7
44 Xba
Bam 60·2
Xba 68·5
70 Bam
87, 89·7
Bam , 91
Eco , Xba
Ad 3var
Ad 5
Xba 3·8
Xba 29·5
Bam 59·5
Eco 75·9
78·5 Xba
84
Eco Xba 84
D
C
EcoRI
BamHI
XbaI
Mock infected
Ad5wt
#789
Ad3Var
A
B
C
C'
D
E
F
G
H
D'

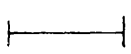
Figure 27.

Analysis of Isolate 789

The DNA of isolate 789 was labelled by inorganic orthophosphate and harvested as a Hirt supernatant. The restriction endonuclease profiles were examined by cleaving equal aliquots of labelled DNA with EcoR1, BamH1 or Xba1, followed by electrophoresis through an agarose gel overnight. DNA was visualised by exposing the agarose gel to X-ray film at -70°C.

The diagram above the figure shows the restriction enzyme maps for the endonucleases used. The heavy black lines represent the terminal DNA fragments.


= XbaI D fragment 78.5 - 84 m.u.


= EcoR I C fragment 75.9 - 84 m.u.

using the method described by Frost and Williams, (1978). The number of plaques on heterotypic DNA co-transfection plates were significantly above background suggesting that recombination may have occurred. Plaques were picked for screening.

14. Analysis of plaque isolates from overlapping DNA terminal fragments of adenovirus 3var100 and adenovirus 5 wild type.

Viral DNA was prepared as a Hirt supernatant from the isolates obtained from heterotypic DNA overlap plates. The DNA was cleaved with HindIII, Sma1, BamH1, Xba1 and EcoR1. Figure 25 shows that eight of the nine isolates exhibit the restriction enzyme profile of adenovirus 5 wild type cleaved with Sma1. Isolate 789 shows the loss of the adenovirus type 5 Sma1 B fragment with the appearance of a new band about 2% larger. This alteration maps between 76.8 m.u. and 91 m.u.

Figure 26 shows the restriction enzyme profile of the isolates cleaved with HindIII. Eight of the isolates have the DNA profile of adenovirus type 5. However, isolate 789 shows the absence of an adenovirus 5 HindIII B fragment and the presence of a new band about 2% larger mapping between 73.6 m.u and 89.1 m.u.

Figure 27 shows the restriction enzyme profiles of isolate 789 cleaved with BamH1, Xba1 and EcoR1. The other isolates have been omitted from the figure as they all have the DNA profile of adenovirus type 5.

BamH1 cleavage yields a DNA profile indistinguishable from adenovirus type 5. Cleavage with EcoR1 shows that the adenovirus type 5 band C is

absent, with a new band approximately 2% larger being present; this alteration maps between 75.9 m.u. and 84 m.u. Xba1 cleavage shows the absence of adenovirus type 5 band D. However, in this case there is no new larger band, but the appearance of a smaller band.

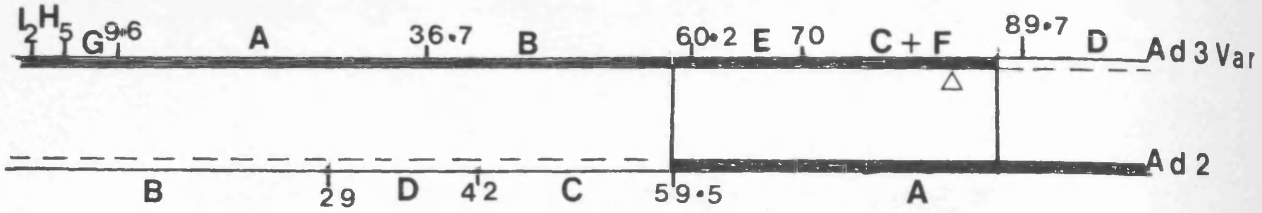
The above data suggests that isolate 789 has a DNA insert of approximately 2% mapping between the adenovirus 5 Sma1 site at 76.8 m.u. and the adenovirus EcoR1 84 m.u. site. Xba1 cleavage shows that band D is smaller. The presence of the Xba1 C fragment implies that the site at 84 m.u. is present, therefore the alteration must map to the left of this site and contain a new Xba1 site.

The new site must lie closer to 84 m.u than the site at 78.5 m.u. to give a smaller D band. This is possibly a consequence of the inserted DNA between 76.5 m.u. and 84 m.u. containing a Xba1 site. If the inserted DNA spanned 78.5 m.u., then the Xba1 site 78.5 m.u. between fragments A and D would be lost with part of the inserted DNA being joined to Xba1 A. As Xba1 A is 40% of the genome, additional DNA would not be detected on migration in an agarose gel system.

The heterotypic overlap of the terminal DNA fragments maps between 52.2 m.u. and 75.9 m.u. The genetic alteration of isolate 789 maps outwith these co-ordinates and is not a result of recombination within the overlap.

To explain why the isolates showed the DNA profile of adenovirus type 5, the adenovirus type 5 EcoR1 digest perhaps contained DNA molecules resistant to enzyme cleavage, or DNA molecules which were only partially

Bam H I


I
H
2
5
G
9·6
A
36·7
B
60·2
E
70
C + F
89·7
D
Ad 3 Var
Ad 2
B
29
D
42
C
59·5
A

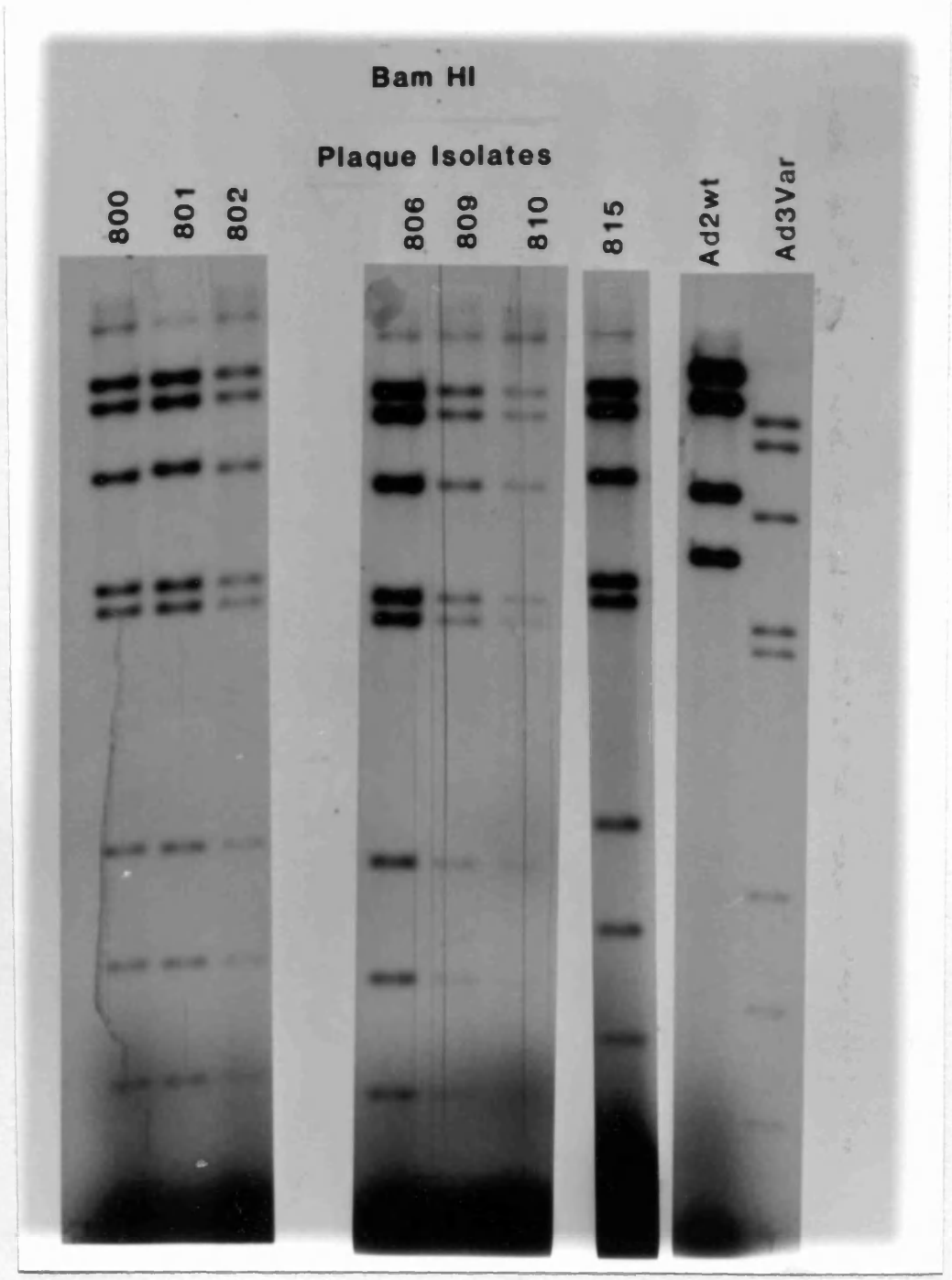

Bam HI
Plaque Isolates
800
801
802
806
809
810
815
Ad2wt
Ad3Var

Figure 28.

Analysis of plaque isolates from cell monolayers transfected with digested heterotypic DNA

DNA which had been labelled with inorganic orthophosphate and harvested as a Hirt supernatant was cleaved with BamH1 to analyse the endonuclease profile. The digested DNA was electrophoresed through an agarose gel and visualised by exposure to X-ray film at -70°C.

The restriction enzyme map for cleavage with BamH1 is shown above the figure; The heterotypic DNA terminal fragments are represented by heavy black lines and the region of overlap is represented by vertical lines. The dotted lines represent DNA fragments which were present in the transfection of full length digested DNA.

digested by EcoR1.

Isolates show both EcoR1 sites at 75.9 m.u. and 84 m.u. of adenovirus type 5. This favours the presence of partially digested molecules. These molecules could self-ligate to form full length molecules of adenovirus 5 DNA and hence viable progeny on transfection. DNA molecules genetically resistant to EcoR1 cleavage would give rise to progeny lacking EcoR1 sites.

The DNA insertion of isolate 789 may be the result of recombination between (a) cellular DNA or (b) adenovirus 3var100 DNA. Isolate 789 has gained a new Xba1 site mapping between 78.5 m.u. and 84 m.u. to give rise to a smaller D fragment. Adenovirus 3var100 input DNA consists of two fragments - (1) Cla1 A = 52.2% of the genome and contains one Xba1 site at 44 m.u.; (2) Cla1 B = 44.8% of the genome and contains two Xba1 sites at 68.5 m.u. and 91 m.u. Either of these fragments may be able to recombine with enzyme resistant input molecules of adenovirus type 5 DNA. However, considering the low DNA homology with adenovirus 5 wild type, it is only remotely possible that this DNA insertion and the new Xba1 site are the result of recombination between adenovirus 3var100 and adenovirus type 5.

15. Analysis of plaque isolates from co-transfection of overlapping DNA terminal fragments of adenovirus 3var100 and adenovirus 2 wild type.

Viral DNA was prepared as a Hirt supernatant from isolates obtained from the plates containing heterotypic DNA overlaps. The DNA was cleaved with BamH1. Fig. 28 shows all isolates have the restriction enzyme

profile of adenovirus 3var100. This result may be explained as follows- adenovirus 3var100 DNA was cleaved with EcoR1 to give two terminal fragments of approximately 84% and 13% respectively. These unseparated fragments would be capable of self-ligation to form full length DNA molecules and hence viable progeny on transfection.

16. Summary of Section A.

One step growth curves and DNA infectivity assays showed that adenovirus 7 wild type was less infectious than adenovirus 5 wild type.

Recombination between the two serotypes was attempted by co-transfection of DNA terminal overlapping fragments. Results showed that homotypic recombination of adenovirus 7 wild type was very low and heterotypic recombination between adenoviruses 5 and 7 was not detected.

The region of overlap between the terminal fragments was increased by substitution of the dl309 Xba1 A fragment for that of adenovirus 5 wild type Sal1 B. Data suggested adenovirus type 5 recombination had increased. However, recombination between dl309 and adenovirus type 7 was not detected.

An attempt to increase the infectivity of adenovirus type 7 DNA by the use of DNA-protein complex proved unsuccessful, although adenovirus type 5 DNA-protein complex increased DNA infectivity two fold.

Since adenovirus type 7 DNA-protein in my hands failed to increase DNA infectivity an attempt was made to improve the method of DNA

transfection. A modified method of Wigler et al., (1978) was found to produce a threefold plaque increase over the previous method used. However, the DNA infectivity of adenovirus type 7 was still below that of adenovirus type 5 on day 4 of transfection.

In parallel an attempt was made to isolate a subgroup B virus which was fast growing with a large plaque morphology similar to adenovirus 5 wild type. A stable fast growing large plaque variant of adenovirus 3 strain GB was isolated. One step growth curves showed it to grow as well as adenovirus 5 wild type. Transfection of DNA showed it to produce large plaques on day 4, however, the DNA was less infectious than adenovirus type 5.

D'Hallium et al., (1983) showed that adenovirus type 3 had a unique Cla1 site at 52.2 m.u. Adenovirus 3var100 also has a unique Cla1 site at 52.2 m.u. A recombination experiment (13) was attempted by co-transfecting adenovirus 3var100 unseparated fragments of either Cla1 or EcoR1 with unseparated fragments of adenovirus 5 wild type cleaved with EcoR1 or adenovirus 2 wild type cleaved with BamH1 to give a region of overlap between the terminal fragments.

Controls suggested that homotypic recombination had occurred with adenovirus 5 wild type and also with adenovirus 3var100; heterotypic recombination between subgroup B and subgroup C was undetected.

I decided to discontinue this line of research and concentrate on the properties of adenovirus 3var100. In parallel with the previous experiments I had been looking at the characteristics of this virus,

some of which looked interesting and perhaps could be used as a model to explain at least in part why no recombination had been detected between the two subgroups.

Section B.

Characterisation of a Subgroup B Spontaneous Mutant

Table XIV

Virus	Grown in	Virus yield titrated on	
		293 cells	Hela cells
Ad3wt	293 cells	9.5×10^6	8.5×10^6
Ad3wt	Hela cells	9.5×10^6	1.1×10^7
Ad3var100	293 cells	2.5×10^9	$<10^1$
Ad3var100	Hela cells	7.5×10^5	$<10^1$
Ad5wt	293 cells	8×10^8	5.5×10^8
Ad5wt	Hela cells	8.5×10^8	5.5×10^8

Input multiplicity 10 p.f.u./cell each virus.

1. Replication of adenovirus 3var100 in Hela cell monolayers

Replication of adenovirus 3var100 in Hela cells showed a reduced yield of virus when titrated by plaque formation on 293 cells compared to the yield obtained from 293 cells and also compared to the yields obtained from adenovirus 3 wild type and adenovirus 5 wild type from Hela cells (Table XIV). A yield from adenovirus 3var100 infected 293 cells titrated on 293 and Hela cell monolayers gave greater than eight log difference in plaque titre in the two cell lines, while a yield from Hela cells gave a greater than five log difference. The reduced viral yield from Hela cells suggested that adenovirus 3var100 may be a host range mutant of adenovirus 3 wild type, dependent on one of the adenovirus 5 wild type E1 functions expressed by 293 cells.

2. Replication of adenovirus 3var100 virus at three temperatures in 293 and Hela cell monolayers

Ho et al., (1982) isolated a series of adenovirus 5 host range mutants which also showed a cold sensitive phenotype.

These mutants $hr^{CS}11$, $hr^{CS}12$ and $hr^{CS}13$ were isolated at 37°C from monolayers of 293 cells; they would grow equally well at 32°C and 38.5°C in 293 cell monolayers but were host range on Hela cells at 32°C and 37°C but not at 38.5°C. The 293/Hela cell yield ratios at 32°C and 37°C were almost equal and greater than the ratio obtained at 38.5°C.

Complementation and recombination experiments showed that $hr^{CS}11$ and $hr^{CS}12$ mutations lay in the E1a region while that of $hr^{CS}13$ lay in the E1b region.

Table XV

Virus titre of adenovirus 3var100 at three temperatures

Temperature / Virus	Titre of Virus on 293 cells			Titre of Virus on Hela cells		
	32°C	37°C	38.5°C	32°C	37°C	38.5°C
Adenovirus 3var100	5.5×10^{10}	6.2×10^{10}	6.6×10^{10}	$<10^2$	$<10^2$	$<10^2$
Adenovirus 3wt	3×10^9	3.7×10^9	3.9×10^9	3.5×10^9	3.5×10^9	3.5×10^9
Adenovirus 5wt	1.2×10^{11}	1.5×10^{11}	1.4×10^{11}	1.2×10^{11}	1×10^{11}	1.1×10^{11}
Adenovirus H5ts2	6.5×10^7	$<10^2$	$<10^2$	4.5×10^7	$<10^2$	$<10^2$

As adenovirus 3var100 was isolated at 37°C on 293 cells and shows a host range phenotype at this temperature on Hela cells, the probability of its also having a cold sensitive phenotype was investigated.

Adenovirus 3var100, adenovirus 3 wild type or adenovirus 5 wild type were titrated on 293 or Hela cell monolayers at 32°C, 37°C or 38.5°C. As I had no $\underline{hr}^{cs}$ mutant to use as a control I used H5ts2, a temperature sensitive mutant positive for growth in both 293 and Hela cells at 32°C.

Data (Table XV) shows that adenovirus 3var100 is defective for growth in Hela cells at 32°C, 37°C and 38.5°C, suggesting that it is host range only.

3. Replication of adenovirus 3var100 DNA in 293 and Hela cells

If adenovirus 3var100 cannot grow in Hela cells because it lacks an essential function which can be complemented by an adenovirus type 5 E1 function expressed by 293 cells, it is possible that it may not be able to replicate its DNA in Hela cells.

Hela cells were infected with adenovirus 3var100 and labelled with inorganic orthophosphate. At 36 hours post infection, viral DNA was extracted by a modification of the Hirt method.

Restriction endonuclease digestions were carried out and the DNA fragments separated in an agarose gel system. It was necessary to examine the DNA restriction profile rather than uncleaved viral DNA as there is a DNA band of cellular origin in Hirt extracts which co-migrates

293cells
Hela cells
Ad3wt
Ad3var
Ad5wt
uninfected
Ad3wt
Ad3var
Ad5wt
uninfected
A
B
A
B
C
D
E
F
G
H
I

Figure 29.

Replication of adenovirus 3var100 DNA in 293 and Hela cells

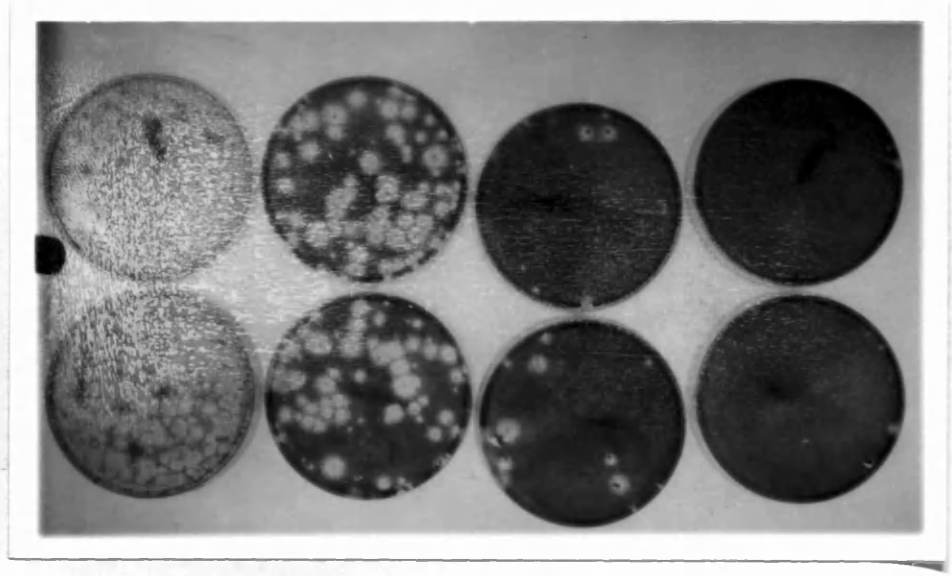
Monolayers of 293 and Hela cells were infected at 10 p.f.u./cell with adenovirus 3var100, adenovirus 3wt or adenovirus 5wt. DNA was labelled with inorganic orthophosphate and harvested as a Hirt supernatant. The DNA was cleaved with the endonuclease BamH1 and electrophoresed through a .7% agarose gel overnight; DNA was visualised by exposure to X-ray film at -70°C.

◀ indicates the new DNA band formed by a fused C + F product of the wild profile.

Ad.3var

293 Cells

(a)



Ad.3var

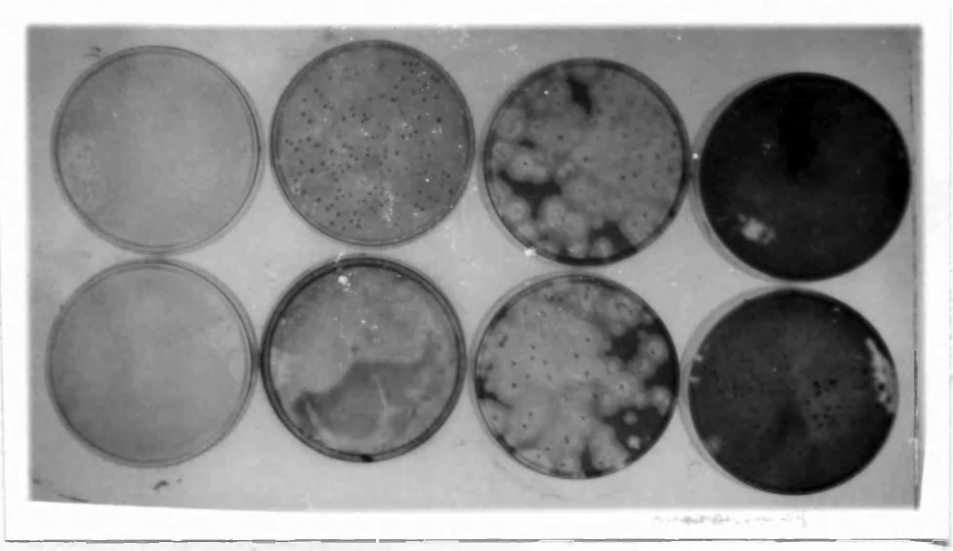
Hela Cells

(b)

Ad.5wt

293 Cells

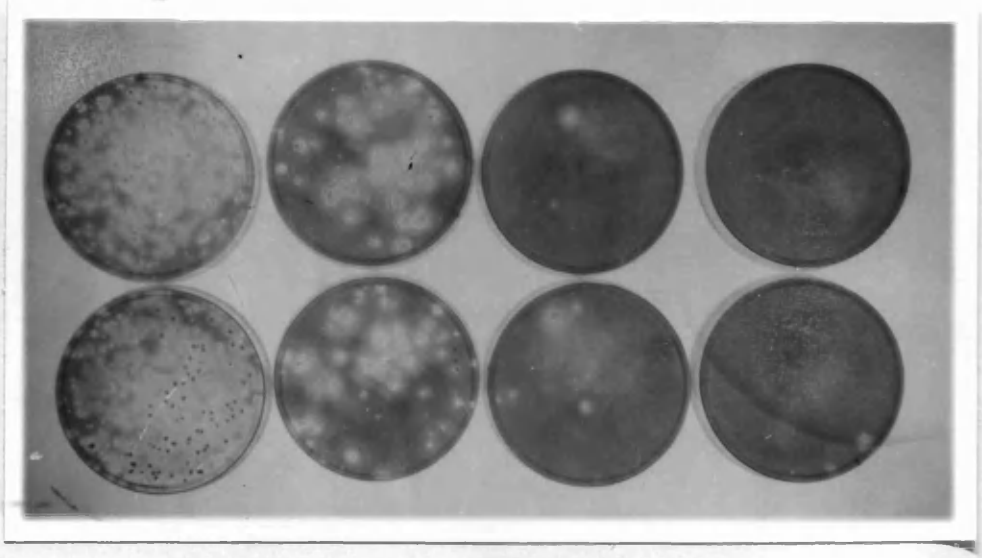
(c)



Ad.5wt

Hela Cells

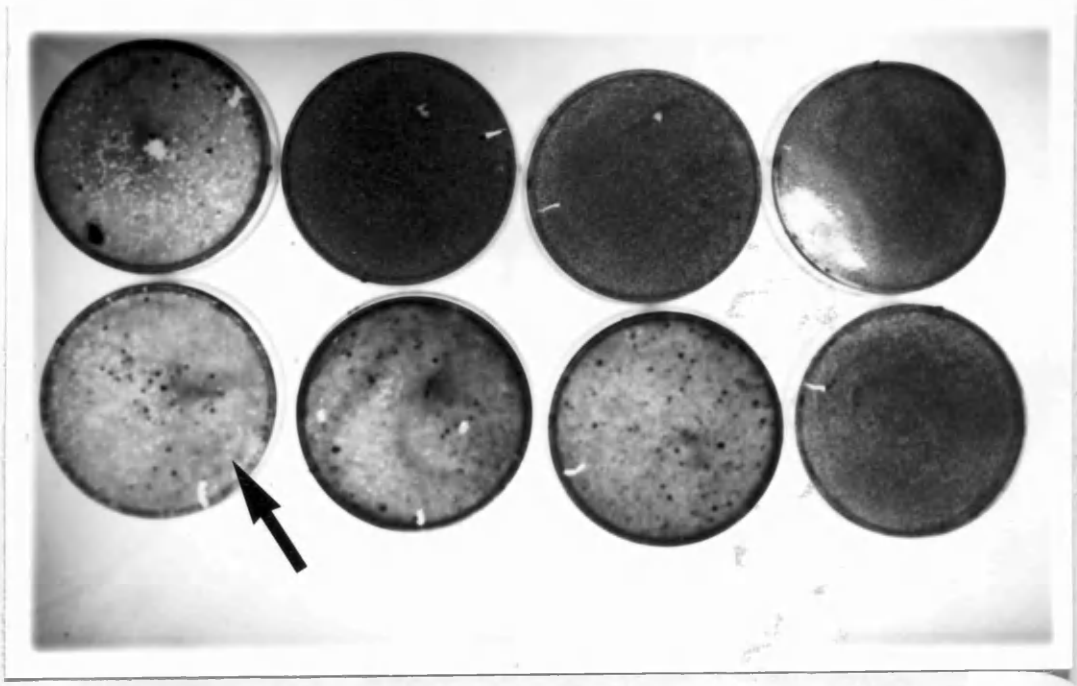
(d)



Ad.3wt

293 Cells

(e)



Ad.3wt

Hela Cells

(f)

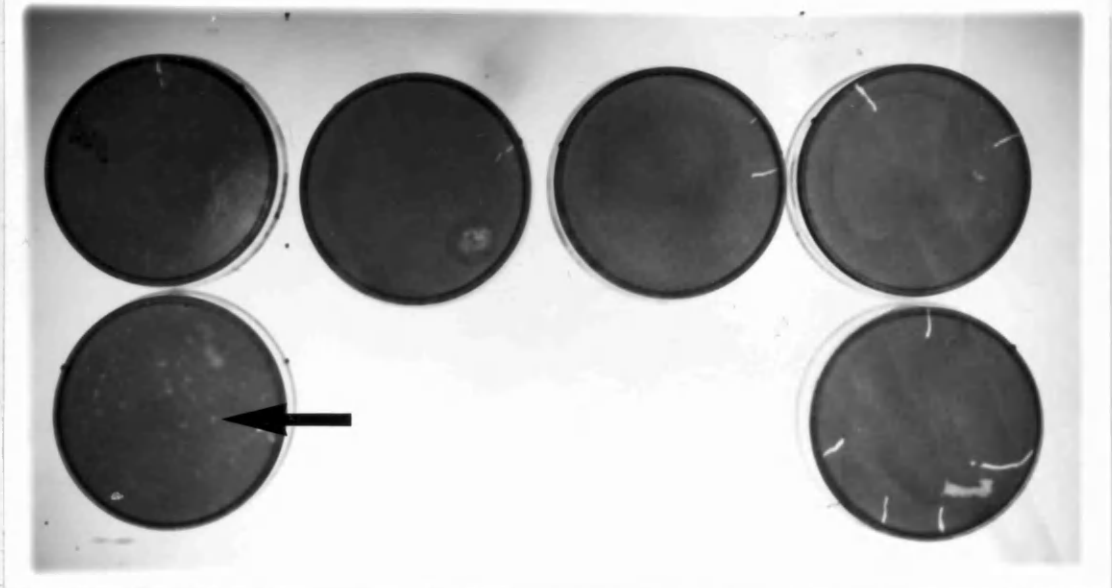


Figure 30.

Adenovirus 3var100 DNA transfection into 293 or Hela cells

Monolayers of 293 or Hela cells were transfected with DNA from adenovirus 3var100, adenovirus 3wt or adenovirus 5wt and maintained under agar overlay medium for 14 days. Agar medium was removed and cell monolayers were fixed and stained using Giemsa Stain.

Figure a - 293 monolayers infected with Ad.3var100

Figure b - Hela monolayers infected with Ad.3var100

Figure c - 293 monolayers infected with Ad.5wt

Figure d - Hela monolayers infected with Ad.5wt

Figure e - 293 monolayers infected with Ad.3wt

Figure f - Hela monolayers infected with Ad.3wt

DNA concentrations used were 1ug., .1ug or .01ug per dish.

Ad.3wt plaques are arrowed Figs. E and F.

Table XVI

Cell line transfected	DNA used in transfection	Viral titre at Day 14 in p.f.u./ml.	
		293 cells	Hela cells
293	Ad3var100	1.5×10^7	$< 10^1$
Hela	Ad3var100	6×10^2	$< 10^1$
293	Ad3wt	2.3×10^4	2.1×10^4
Hela	Ad3wt	2.1×10^4	1.9×10^4
293	Ad5wt	1×10^6	1.6×10^6
Hela	Ad5wt	1.7×10^6	2×10^6
293	Mock infected	$<10^1$	$<10^1$
Hela	Mock infected	$<10^1$	$<10^1$

with adenovirus full length DNA and makes quantitation impossible.

Data (fig.29) shows that adenovirus 3 wild type, 3var100 and 5 wild type all replicate their DNA in 293 cells; in Hela cells only adenovirus 3 wild type and 5 wild type replicate their DNA. Adenovirus 3var100 DNA replication is not detectable.

To show that the block to adenovirus 3var100 replication was not a consequence of the virus being unable to enter Hela cells, they were transfected with purified virion DNA. This approach was intended to by-pass any potential block to the virion at the attachment or uncoating level. Monolayers of 293 and Hela cells were transfected and after 14 days were fixed and stained; in parallel viral yields were harvested and titrated on monolayers of 293 and Hela cells. Figs. 30 (a, b, c, d, e, f) and Table XVI show that this approach did not suffice to overcome the block to adenovirus 3var100 replication in Hela cells.

4. To investigate if cellular DNA will degrade in the presence of adenovirus 3var100

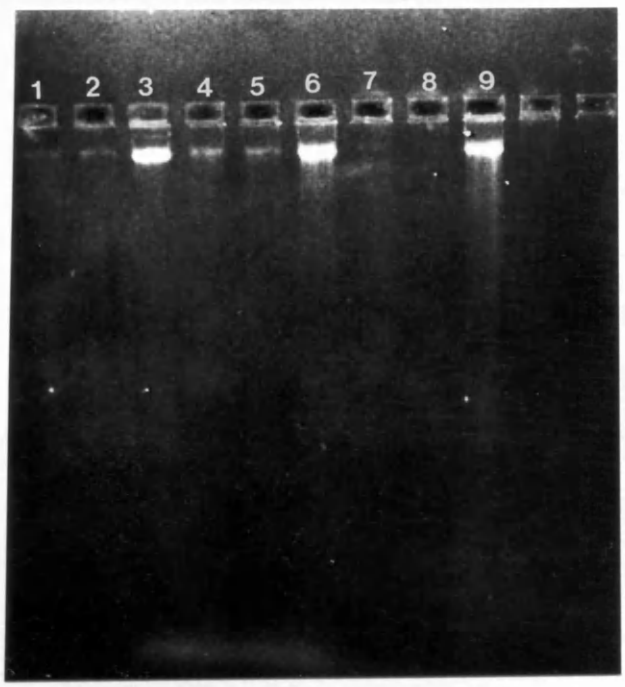
Takemori et al., (1968) isolated a class of adenovirus type 12 mutants (Cyt mutants) which produced large clear plaques on human embryonic kidney cells compared to wild type adenovirus 12 which produces small fuzzy plaques. These mutants caused extensive cellular destruction and produced a low viral yield.

Ezoe et al., (1982) showed that these large plaque mutants caused extensive cellular DNA degradation in KB cells. Lai Fatt and Mak, (1982) showed that the function involved in the inhibition of cellular DNA

Ad.3var

293 Hela KB

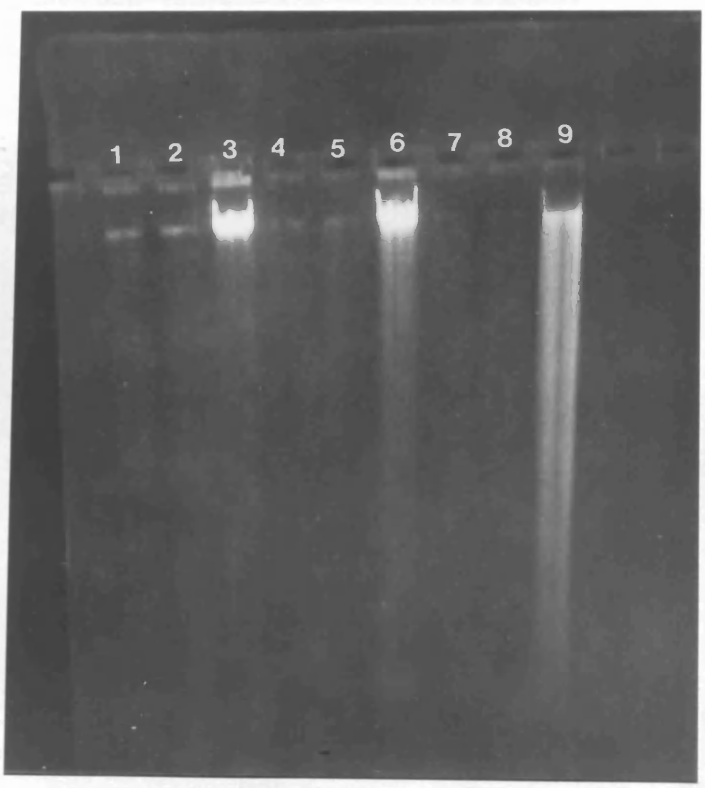
(a)



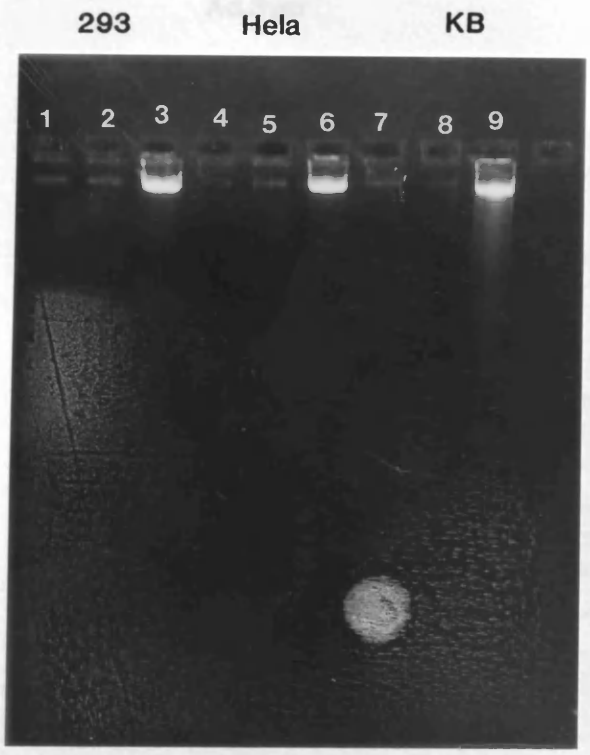
dl313

293 Hela KB

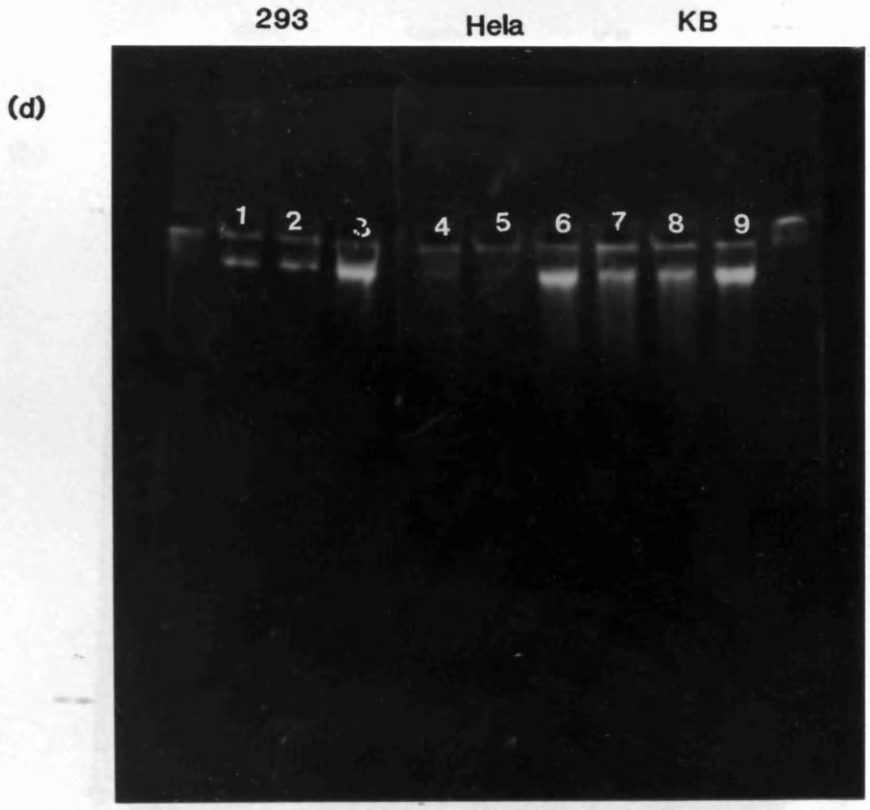
(b)



dl312



Ad.3wt



(e)

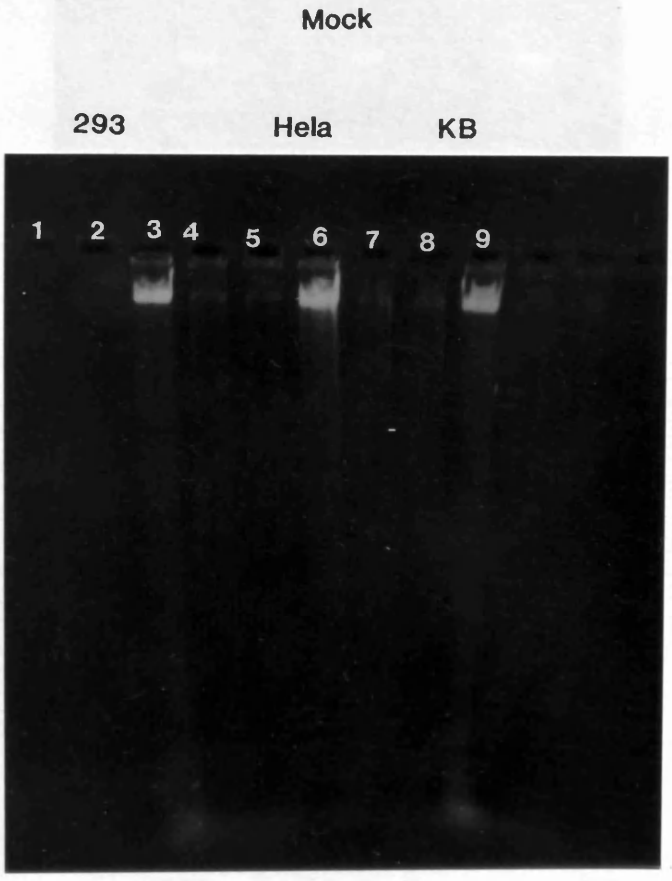


Figure 31 (a, b, c, d and e)

The effect of adenovirus 3var100 on the cellular DNA of KB, Hela and 293 cell monolayers

Monolayers of KB7, 293 or Hela cells were infected at 20 p.f.u./cell with adenoviruses 3var100, 3wt, dl312 and dl313.

DNA synthesis was blocked at 2 hours post infection with 10mM hydroxyurea and at 21 hours post infection cell nuclei were harvested. DNA was extracted by treatment with successive salt concentrations of 100mM, 350mM and 2M, being then treated with RNaseA and proteinase K. 100ul aliquots were electrophoresed through a 1% agarose gel overnight at 30 volts. DNA was visualised by ethidium bromide staining and transillumination by an ultra-violet light source.

Photograph (a)

Cell monolayers infected with adenovirus 3var100

Lanes 1, 2 and 3 = 293 cell monolayers

Lanes 4, 5 and 6 = Hela cell monolayers

Lanes 7, 8 and 9 = KB7 cell monolayers

Salt concentrations

Lanes 1, 4 and 7 = 100mM salt.

Lanes 2, 5 and 8 = 350mM salt

Lanes 3, 6 and 9 = 2M salt.

Photograph (b)

Cell monolayers infected with adenovirus dl313

Lanes 1, 2 and 3 = 293 cell monolayers

Lanes 4, 5 and 6 = Hela cell monolayers

Lanes 7, 8 and 9 = KB7 cell monolayers

Salt concentrations

Lanes 1, 4 and 7 = 100mM salt

Lanes 2, 5 and 8 = 350mM salt

Lanes 3, 6 and 9 = 2M salt

Photograph (c)

Cell monolayers infected with adenovirus dl312

Lanes 1, 2 and 3 = 293 cell monolayers

Lanes 4, 5 and 6 = Hela cell monolayers

Lanes 7, 8 and 9 = KB7 cell monolayers

Salt concentrations

Lanes 1, 4 and 7 = 100mM salt

Lanes 2, 5 and 8 = 350mM salt

Lanes 3, 6 and 9 = 2M salt

Photograph (d)

Cell monolayers infected with adenovirus 3 wild type

Lanes 1, 2 and 3 = 293 cell monolayers

Lanes 4, 5 and 6 = Hela cell monolayers

Lanes 7, 8 and 9 = KB7 cell monolayers

Salt concentrations

Lanes 1, 4 and 7 = 100mM salt

Lanes 2, 5 and 8 = 350mM salt

Lanes 3, 6 and 9 = 2M salt

Photograph (e)

Cell monolayers mock infected

Lanes 1, 2 and 3 = 293 cell monolayers

Lanes 4, 5 and 6 = Hela cell monolayers

Lanes 7, 8 and 9 = KB7 cell monolayers

Salt concentrations

Lanes 1, 4 and 7 = 100mM salt

Lanes 2, 5 and 8 = 350mM salt

Lanes 3, 6 and 9 = 2M salt

degradation mapped in the E1b region of the genome. When KB cells were infected with dl313 (Jones and Shenk, 1979) which lacks all the E1b region, extensive cellular DNA degradation was found.

Since adenovirus 3var100 has a large plaque phenotype and viral DNA is not detected in Hela cells, it is possible that, like the Cyt mutants, it is involved in cellular and viral DNA degradation.

293, Hela and KB cell monolayers were infected with adenovirus 3var100, 3 wild type, dl313 (ΔE1b) and dl312 (ΔE1a) and exposed to the presence of hydroxyurea for 21 hours. DNA was released from infected cell nuclei as described by Challberg and Kelly, 1979; Stillman et al., 1984; White et al., 1984. Aliquots of the DNA samples were electrophoresed through an agarose gel and visualised by ethidium bromide staining. Figs. 31 (a, b, c, d and e).

Figure 31b shows that the DNA released from the nuclei of KB cells infected with dl313 is extensively degraded (Lai Fatt and Mak, 1982) while that from 293 cells is not. The result that the DNA released from infected Hela cells is slightly degraded compared to that from KB cells is surprising. Hela cells, unlike 293 cells, have no adenoviral expression and therefore are unable to complement dl313. Hence one would expect extensive degradation of DNA. DNA released from KB, Hela or 293 cells infected with adenovirus 3var100, 3 wild type or dl312 shows no degradation (Figs. a, c and d); therefore adenovirus 3var100 is not involved in DNA degradation.

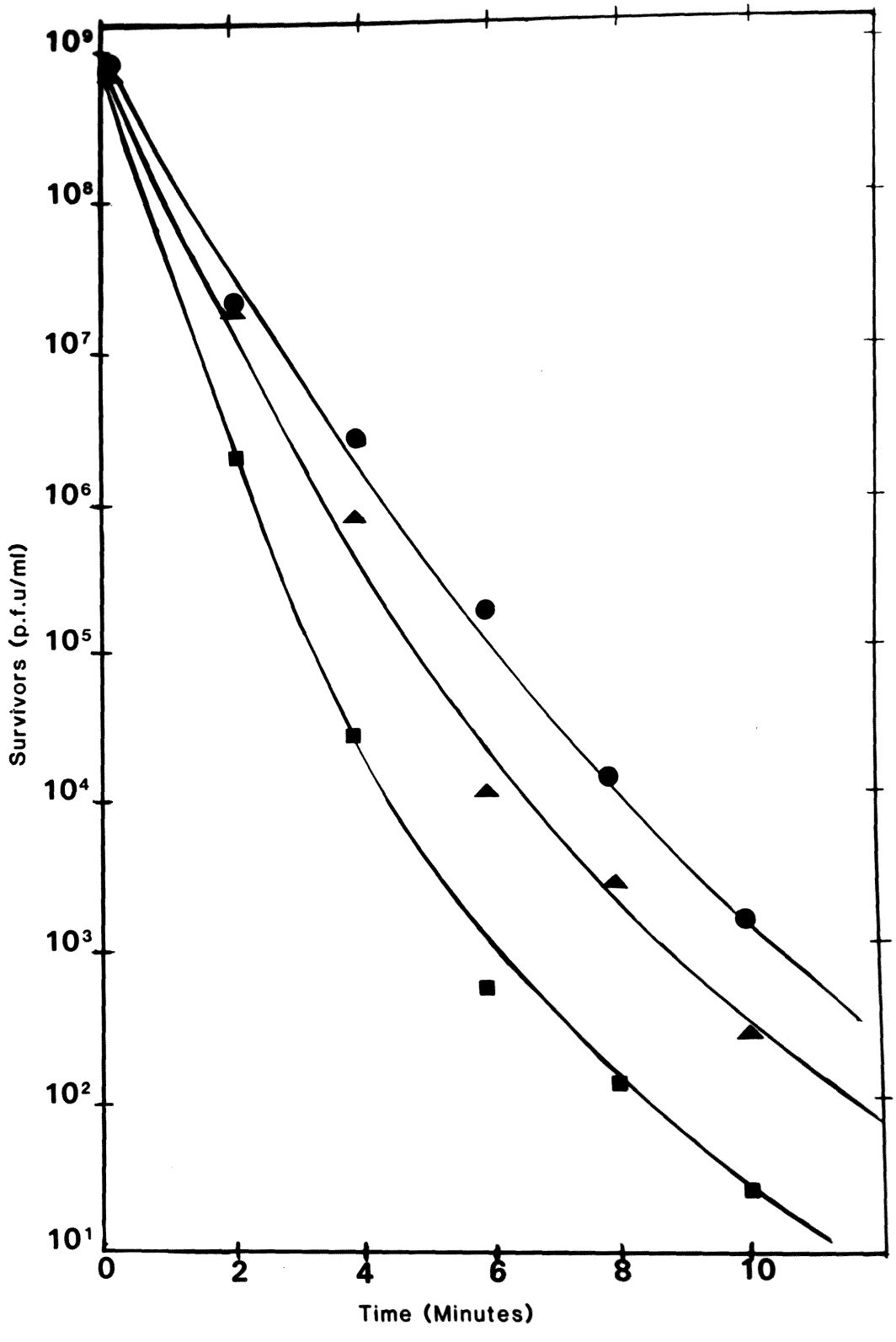

10^9
10^8
10^7
10^6
10^5
10^4
10^3
10^2
10^1
Survivors (p.f.u/ml)
0
2
4
6
8
10
Time (Minutes)

Figure 32.

Heat stability/lability of adenovirus 3var100, adenovirus 3 wild type and adenovirus 5 wild type

10^9 p.f.u./ml of each virus was heated at 52°C in .05M Tris/Hcl (pH7.4) with gentle agitation.

At 2 minute time intervals 100 ul aliquots were removed and diluted to 1ml in ice cold Tris/Saline. Infectivity was assayed on monolayers of 293 cells immediately and at day 10 monolayers were fixed and stained. Survivors were plotted against time.

● = adenovirus type 5

▲ = adenovirus type 3

■ = adenovirus 3var100

5. To investigate if adenovirus 3var100 is heat labile

Mutations affecting the structural components of a virion may result in an increase in the heat lability of the virion *in vitro*. Adenovirus mutants H5*ts*18 and H5*ts*19 which are temperature sensitive for the 100K protein are inactivated faster when heated to 52°C than wild type adenovirus 5. It is also possible to isolate heat stable (*hs*) mutants which are less rapidly inactivated than wild type virus *in vitro* (Young and Williams, 1975).

Adenovirus 3var100 was investigated for heat lability/stability by heating 10^9 p.f.u. of virions at 52°C in .05M Tris/HClpH7.4 with gentle agitation.

At time intervals 100ul aliquots were removed and diluted to 1ml in ice cold Tris/Saline. Infectivity was assayed immediately by titration on 293 cell monolayers which were maintained under agar overlay medium for 10 days; monolayers were then fixed and stained.

Results (Fig. 32) show that adenovirus 3var100 is less thermostable than either adenovirus 3 wild type or adenovirus 5 wild type. At every time point taken adenovirus 3var100 has 1 log less survivors than parental adenovirus 3 wild type and 2 log less survivors than adenovirus 5 wild type.

6. Restriction enzyme analysis of adenovirus 3var100 DNA

An alteration in the E1 region of the adenovirus 3var100 genome

Sma I
Ad3wt
Ad3var
A
B
C
D
E
F
G
H
Hind III
Ad3wt
Ad3var
A
B
C
D
E
F
G
H
I
J
Xho I
Ad3wt
Ad3var
A
B
C
D
E
Sal I
Ad3wt
Ad3var
A
B
C

Figure 33.

Restriction enzyme profile of adenovirus 3var100 with Sma1, HindIII, Xho1 and Sal1

Adenovirus 3var100 or adenovirus 3wt DNA was nick translated and equal aliquots were digested with Sma1, HindIII, Xho1 or Sal1 as single digests. Samples were electrophoresed through a .7% agarose gel overnight. DNA was visualised by exposure to X-ray film at -70°C.

may be detectable as an alteration in a restriction enzyme site or by a change in mobility of a restriction enzyme fragment.

The restriction enzyme profile of adenovirus 3var100 (Fig. 29) digested with BamH1 showed that it differed from the parental adenovirus 3 wild type by the absence of fragments C and F. Instead there is a new fragment which is approximately 3% smaller than a C + F fusion product suggesting that the BamH1 site at 83.1 m.u. has been lost as a result of a deletion spanning this region.

Cleavage of adenovirus 3var100 DNA with HindIII (Fig. 33) shows that fragments B and H are absent compared to the parental. Instead there is a new fragment 3% smaller than a B + H fusion product indicating that the 3% deletion also spans the HindIII site at 83.5 m.u. Cleavage with EcoR1 (Section A:Figure 27) shows that EcoR1 band C is absent compared to the parental suggesting the deletion also spans the 85.4 m.u. EcoR1 site. Analysis of adenovirus 3var100 with restriction endonucleases Xho1, Sal1 and Sma1 (Fig. 33) support the evidence that there is a 3% (~1000bp) deletion in the right hand end of the genome. Sma1 band B', Xho1 band B' and Sal1 band B' are all smaller than the corresponding parental bands. These fragments lie in the right hand end of the genome and contain the deletion spanning map co-ordinates 83.1-85.4, which maps in the E3 region (76.6-86) m.u. of the genome.

Although restriction enzyme analysis has shown that adenovirus 3var100 carries a deletion in the right hand end of the genome, there is no indication of a loss of restriction enzyme site or altered fragment mobility within the left hand end.

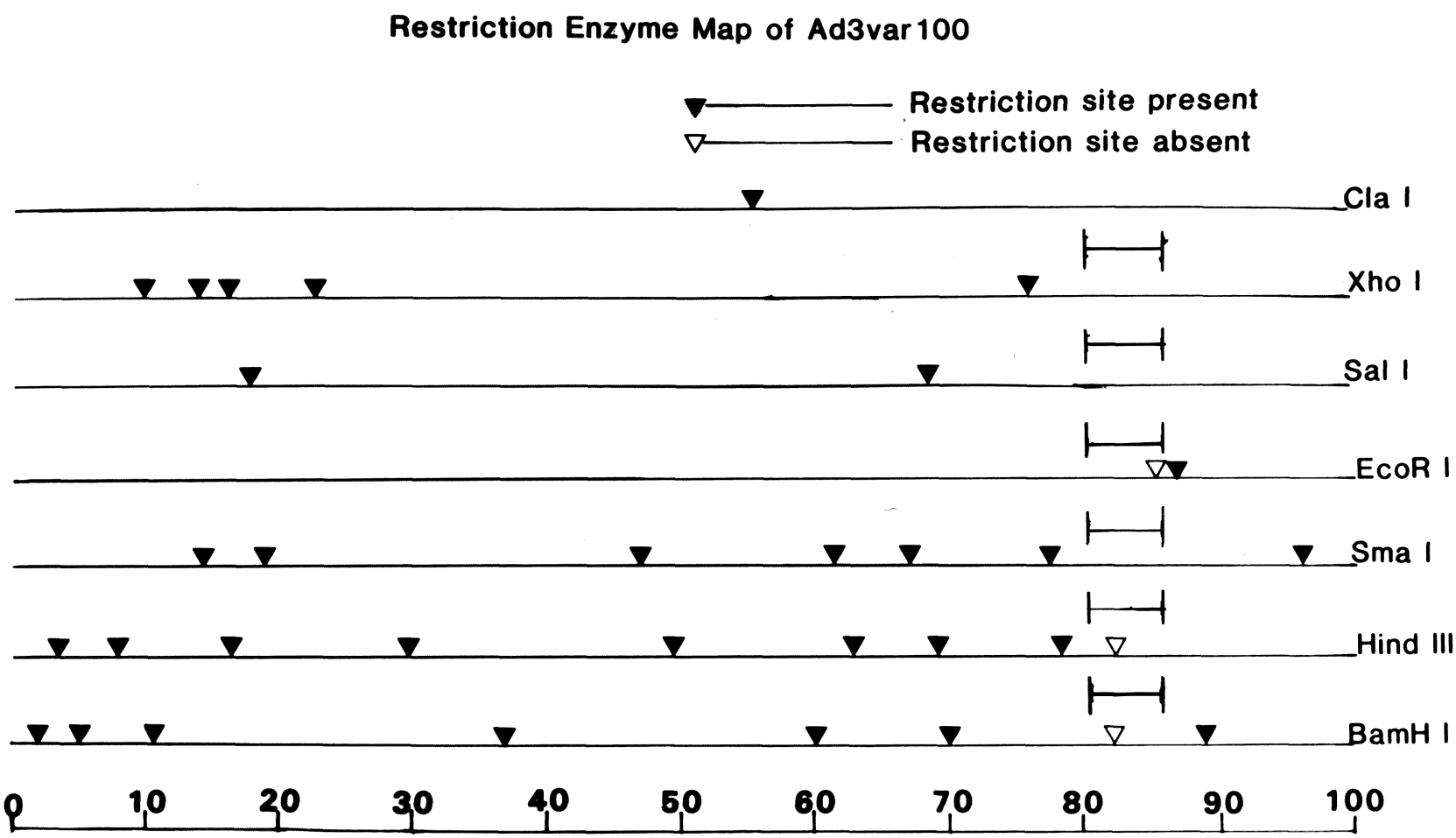

Restriction Enzyme Map of Ad3var100
Restriction site present
Restriction site absent
Cla I
Xho I
Sal I
EcoR I
Sma I
Hind III
BamH I
0
10
20
30
40
50
60
70
80
90
100

Figure 34.

DNA restriction enzyme maps of adenovirus 3var100

Data from figure 33 was transposed onto a linear DNA map; data from restriction enzyme profiles with EcoR1 and Cla1 are also added.

Data is based on the restriction endonuclease cleavage maps of adenovirus 3 wild type as determined by Tibbetts (1977).

⊢——⊣ => DNA segment deleted from adenovirus 3var100.

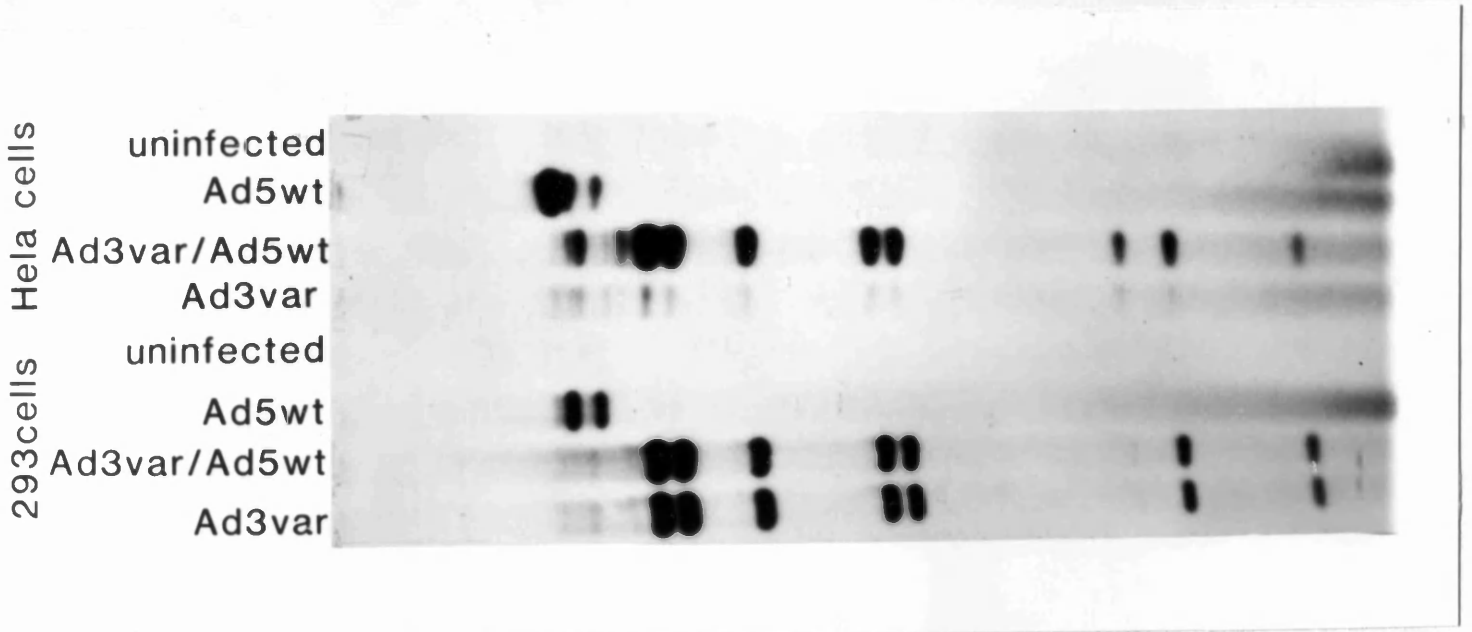

Hela cells
uninfected
Ad5wt
Ad3var/Ad5wt
Ad3var
293cells
uninfected
Ad5wt
Ad3var/Ad5wt
Ad3var

Figure 35.

Co-infection of adenovirus 3var100 and adenovirus 5 wild type in 293 and Hela cells

Monolayers of 293 and Hela cells were co-infected with adenovirus 3var100 and adenovirus 5 wild type at 5 p.f.u./cell each virus; single infections were at 10 p.f.u./cell. DNA was labelled with inorganic orthophosphate and harvested as a Hirt supernatant. Equal aliquots of DNA were treated with the endonuclease BamH1 and electrophoresed through a .7% agarose gel at 30 volts overnight; DNA was visualised by autoradiography.

Figure 34 shows the proposed restriction enzyme maps for adenovirus 3var100 based on restriction enzyme data Fig. (29) and Fig. 33.

7. Complementation of adenovirus 3var100 in Hela cells

Since adenovirus 3var100 will replicate in 293 cells which contain and express adenovirus type 5 E1 functions, but not in Hela cells, it seemed possible that one of the expressed functions could complement its growth. Therefore it would also be expected that adenovirus 5 wild type virus would also complement the defect in the variant and allow replication in Hela cells. Hela cell monolayers were co-infected with adenovirus 3var100 and adenovirus 5 wild type at 10 p.f.u./cell and assayed for complementation at the level of DNA replication, as it has been previously shown (Fig. 29) that adenovirus 3var100 does not replicate its DNA in Hela cells. Viral DNA was labelled with inorganic orthophosphate and extracted as a Hirt supernatant as before.

To distinguish between adenovirus 3var100 and adenovirus 5 wild type, the DNA was cleaved with the restriction endonuclease BamH1; the two viral serotypes have distinctive BamH1 profiles. (Figs. 11 and 12).

When adenovirus type 5 and adenovirus 3var100 were present together (Fig. 35) in the Hela cells, there was clear evidence of replication of adenovirus 3var100 DNA, but, in addition, the DNA replication of adenovirus 5 wild type was substantially reduced compared to a single infection with adenovirus 5 wild type. A similar reduction in adenovirus 5 wild type DNA replication in the presence of adenovirus 3var100 was seen in 293 cells (Fig. 35) which are equally permissive to both viruses in single

infection.

The possibility that this phenomenon may have been related to the input multiplicities of the viruses was examined by co-infecting 293 cells with adenoviruses 3var100 and 5 wild type at various ratios and different total multiplicities (10 p.f.u./cell - 100 p.f.u./cell).

At ratios of adenovirus 3var100 to adenovirus 5 wild type ranging from 1:1 to 1:100 there was no difference in the ability of adenovirus 3var100 to suppress adenovirus 5 DNA replication. The particle to p.f.u. ratios of the two seed stocks were determined to ensure that one was not contributing a disproportionate level of non-infectious particles, but the values were 10:1 for adenovirus 3var100 and 12:1 for adenovirus 5 wild type which is unlikely to alter the input ratios.

The requirement for a functional adenovirus 3var100 for the suppression of adenovirus 5 wild type DNA replication was examined by co-infecting Hela cells with adenovirus 5 wild type virus and adenovirus 3var100 virus which had been heat inactivated for 30 minutes at 56°C. This resulted in detectable replication of adenovirus 5 wild type DNA; however, no replication of adenovirus 3var100 was detected, suggesting that the suppression of adenovirus type 5 DNA replication is dependent on adenovirus 3var100 in an active state.

To see if this apparently trans-dominant effect of adenovirus 3var100 on adenovirus type 5 was a general property of the variant, co-infections with other wild type viruses were carried out.

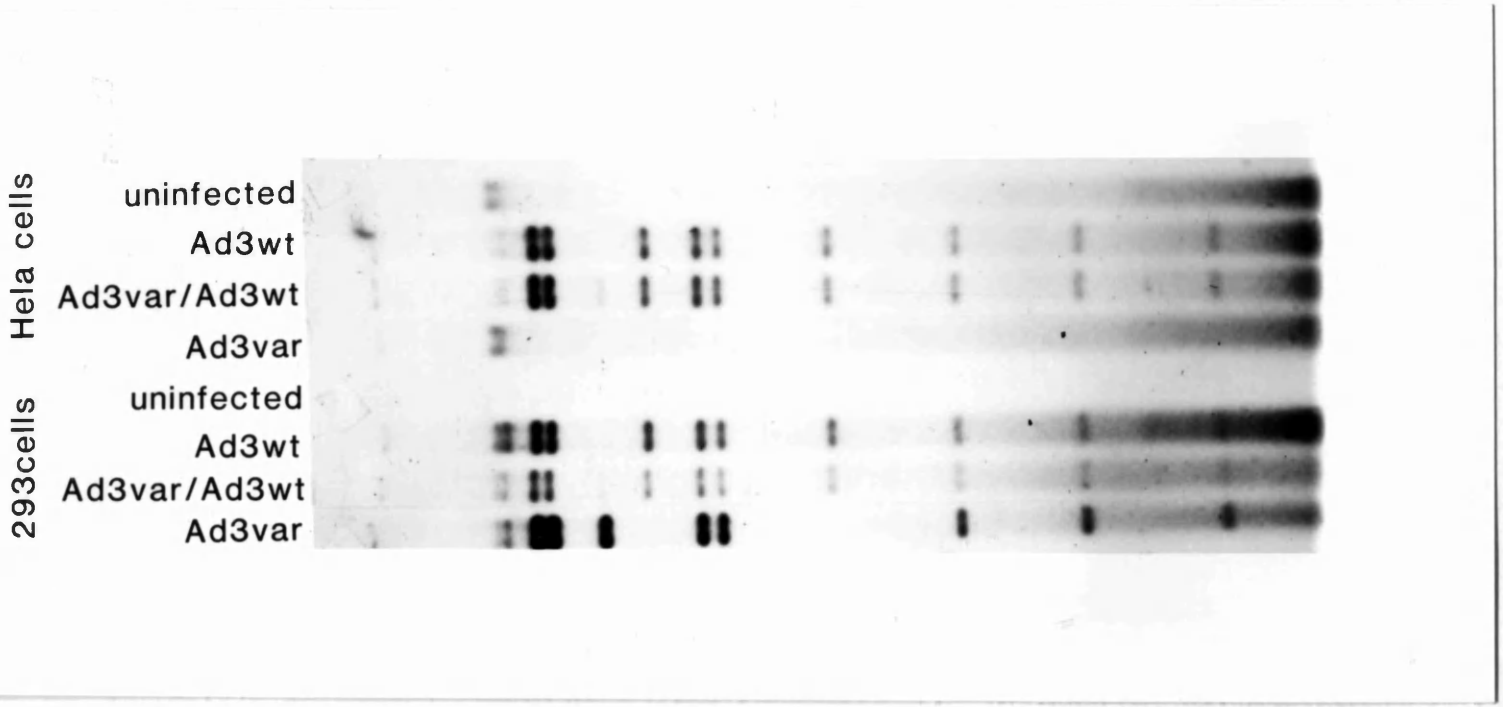

Hela cells
uninfected
Ad3wt
Ad3var/Ad3wt
Ad3var
293cells
uninfected
Ad3wt
Ad3var/Ad3wt
Ad3var

Figure 36.

Co-infection of adenovirus 3var100 and adenovirus 3 wild type in 293 and Hela cells

Monolayers of 293 and Hela cells were co-infected with adenovirus 3var100 and adenovirus 3 wild type (parental) at 5 p.f.u./cell each virus; single infections were at 10 p.f.u./cell. DNA was labelled with inorganic orthophosphate and harvested as a Hirt supernatant; equal aliquots were cleaved with the endonuclease BamH1 and electrophoresed overnight at 30 volts through a .7% agarose gel. DNA was visualised by autoradiography.

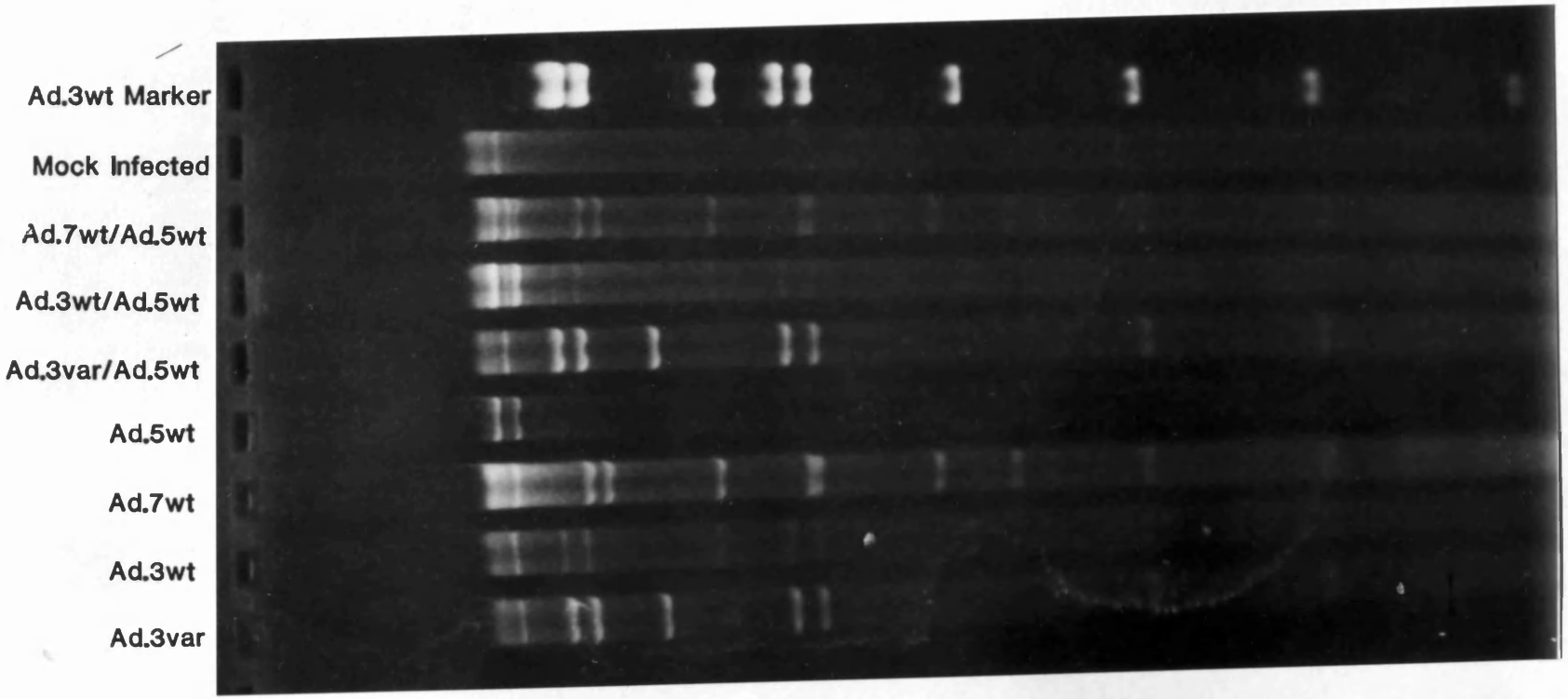

Ad.3wt Marker
Mock Infected
Ad.7wt/Ad.5wt
Ad.3wt/Ad.5wt
Ad.3var/Ad.5wt
Ad.5wt
Ad.7wt
Ad.3wt
Ad.3var

Figure 37.

Co-infection of adenovirus 3var100, adenovirus 3 wild type or adenovirus 7 wild type with adenovirus 5 wild type.

Monolayers of 293 cells were co-infected at 5 p.f.u./cell each virus for double infection and at 10 p.f.u./cell for single infections. DNA was labelled with inorganic orthophosphate and harvested as a Hirt supernatant. Equal aliquots were cleaved with the endonuclease BamH1 and electrophoresed overnight at 30 volts. DNA was visualised by autoradiography.

data for hela cells is not shown

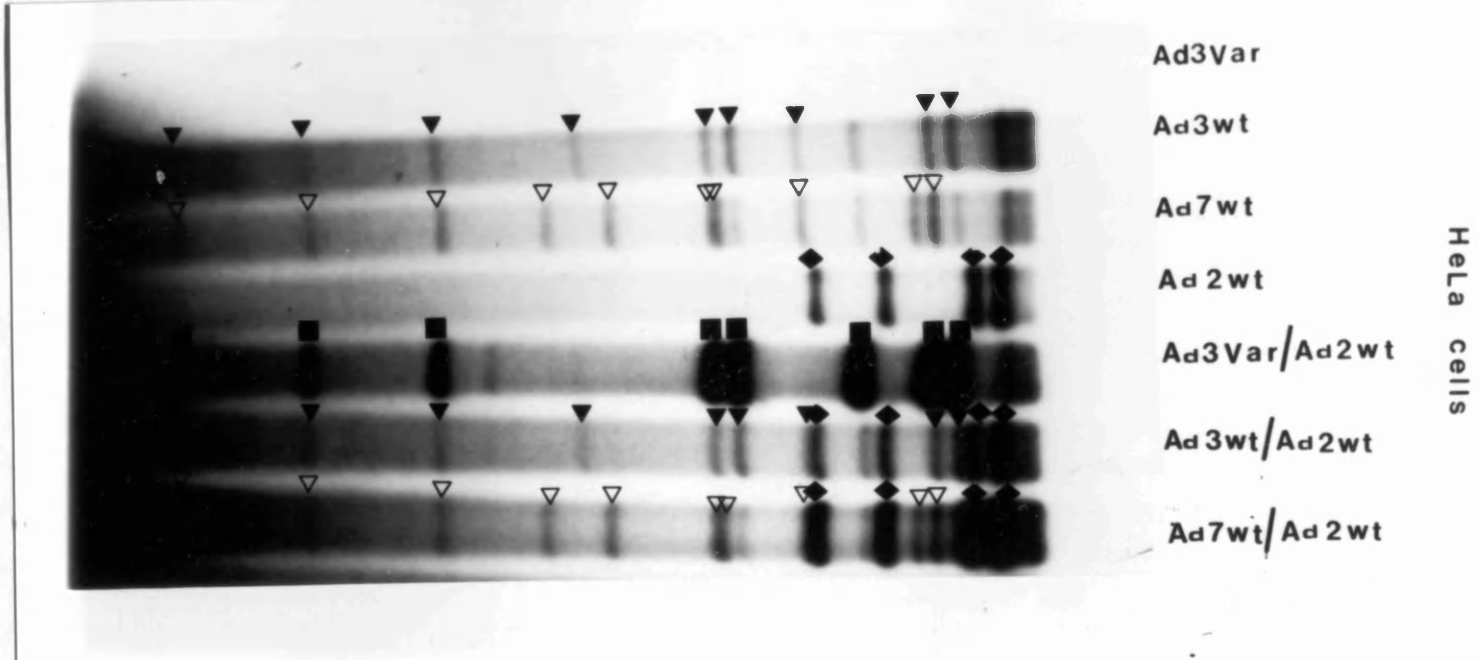

Ad3Var
Ad3wt
Ad7wt
Ad 2wt
Ad3Var/Ad2wt
Ad 3wt/Ad2wt
Ad7wt/Ad 2wt
HeLa cells

Figure 38.

Co-infection of adenovirus 3var100, adenovirus 3 wild type or adenovirus 7 wild type with adenovirus 2 wild type

Monolayers of Hela cells were co-infected at 5 p.f.u./cell each virus for double infections and at 10 p.f.u./cell for single infections. DNA was labelled with inorganic orthophosphate and harvested as a Hirt supernatant. Equal aliquots were cleaved with BamH1 and electrophoresed overnight at 30 volts. DNA was visualised by autoradiography.

Monolayers of 293 and Hela cells were co-infected with adenovirus 3var100 and its parental, adenovirus type 3. DNA was labelled and extracted as a Hirt supernatant and cleaved with BamH1. Replication of DNA was monitored by the presence or absence of fragments of adenovirus 3 wild type band C, or C' which is the C + F fusion seen in the variant. Replication of the parental DNA was not affected by the presence of the variant in 293 cells (Fig. 36). Instead it appeared to suppress DNA replication of the variant. A similar effect is seen in Hela cells (Fig. 36) where adenovirus 3 wild type grew normally and only a faint trace of adenovirus 3var100 DNA was seen.

When adenovirus 3 wild type or adenovirus 7 wild type were co-infected with adenovirus 5 wild type into 293 or Hela cells, their DNA replication appeared very slightly reduced; however, adenovirus 5 wild type had replicated its own DNA. (Fig. 37). data for hela cells is not shown

Adenovirus 3var100 was seen to suppress the DNA replication of adenovirus 2 wild type while adenovirus 3 wild type or adenovirus 7 wild type did not. (Fig. 38)

8. Location of the E1 lesion in the adenovirus 3var100 genome

Adenovirus 3var100 has approximately a 1Kb deletion at the right hand end of the genome mapping in the E3 region (Fig. 34) detectable as an alteration in the restriction enzyme profile, but no alteration is seen in the left hand end of the E1 region (Fig. 33). The host range phenomenon in Hela cells described suggests the variant to have an alteration in the E1 region of the genome.

Table XVII

Location of the E1 Lesion in the Adenovirus 3var100 Genome

Cell line	Adenovirus E1 expression	Adenovirus 5wt titre (p.f.u./ml)	Adenovirus 3var100 titre (p.f.u./ml)	Adenovirus 3wt titre (p.f.u./ml)
KB7	No expression	1.8×10^{11}	3×10^{3}	7.2×10^{9}
KB16	E1a+E1b(Ad2wt)	2.65×10^{11}	9.8×10^{9}	7.5×10^{9}
KB18	E1b(Ad2wt)	2.5×10^{11}	6×10^{3}	7.9×10^{9}
KB8	E1a(Ad2wt)	2.1×10^{11}	7×10^{8}	8.4×10^{9}
293	E1a+E1b(Ad5wt)	4.5×10^{11}	2.9×10^{10}	9×10^{9}
Hela	No expression	2×10^{10}	$< 10^{1}$	8.2×10^{9}

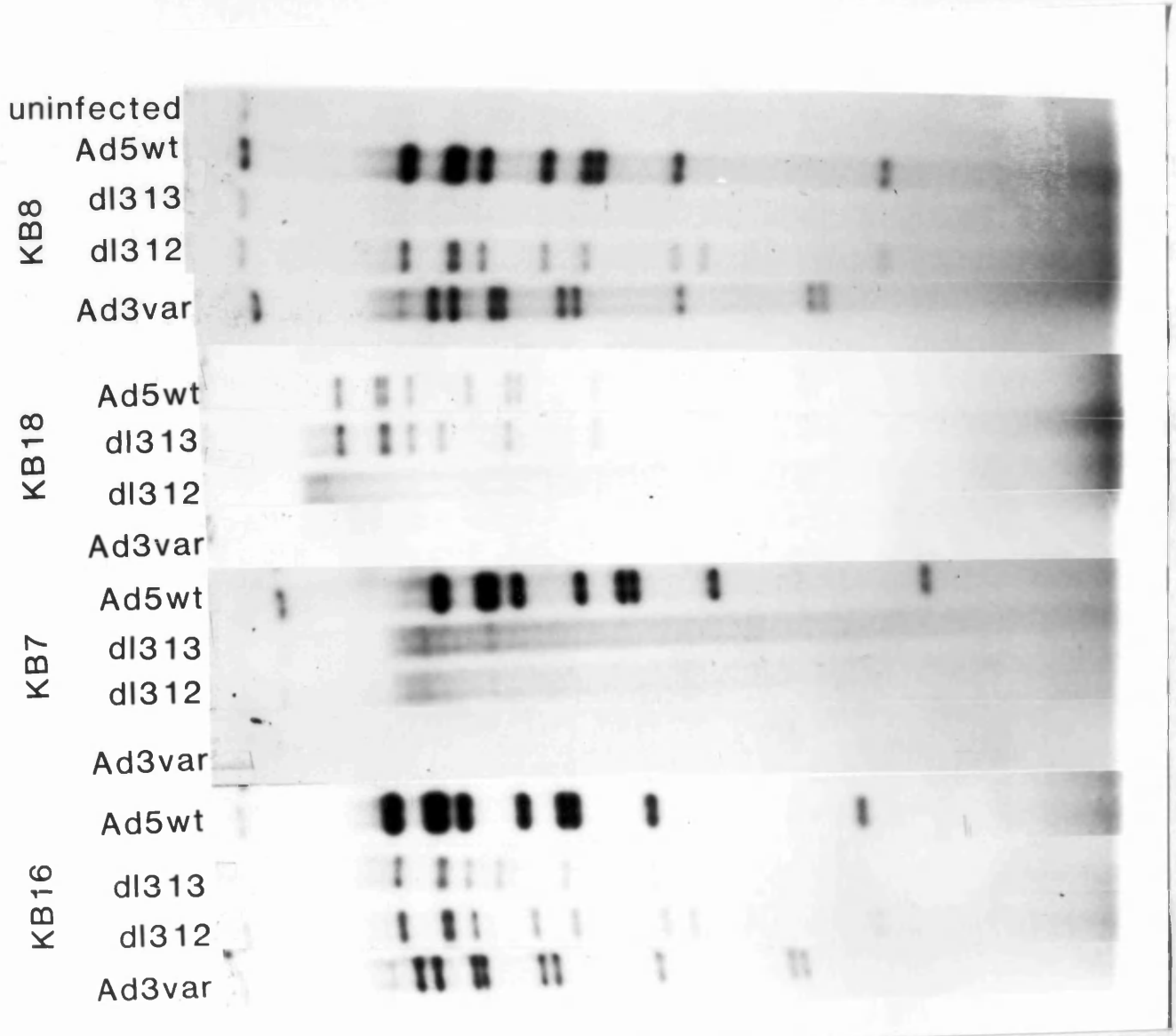

uninfected
Ad5wt
dl313
dl312
Ad3var
KB8
Ad5wt
dl313
dl312
Ad3var
KB18
Ad5wt
dl313
dl312
Ad3var
KB7
Ad5wt
dl313
dl312
Ad3var
KB16

Figure 39.

DNA replication of adenovirus 3var100 in KB cell lines expressing early functions of adenovirus 2 wild type

Monolayers of KB7, KB8(E1a), KB18(E1b) or KB16(E1a+E1b) cells were infected with adenovirus 3var100, adenovirus 5 wild type, dl312 (ΔE1a) or dl313(ΔE1b) at 10 p.f.u./cell. DNA was labelled with inorganic orthophosphate and harvested as a Hirt supernatant. DNA was visualised by autoradiography.

To detect the location of the E1 lesion the family of KB cells constructed by Babiss et al., (1983) which contain and express the E1 functions of adenovirus 2 wild type, was used. Adenovirus 3var100, adenovirus 3 wild type and adenovirus 5 wild type viruses were titrated on these cell lines and maintained under agar overlay medium for 14 days; monolayers were fixed and stained. Viral titres are recorded in Table (XVII). Adenovirus 3 wild type and adenovirus 5 wild type grow equally well in all cell lines. However, adenovirus 3var100 grows with reduced titres in cell lines which do not express adenoviral functions and also poorly in cell line KB18 which expresses the E1b region only. The titre of adenovirus 3var100 in cell line KB8 which expresses the E1a region of the genome is comparable to the titre where both the E1a and E1b regions are expressed, suggesting the virus has a defect in the E1a region.

DNA replication of adenovirus 3var100 was looked at in these KB cell lines; adenovirus 3var100 was used to infect all four cell lines and, as controls, deletion mutants dl312 (ΔE1a) and dl313 (ΔE1b) (Jones and Shenk, 1979) were also used. Viral DNA was labelled with inorganic orthophosphate and extracted as a Hirt supernatant; DNA was cleaved with HindIII. This enzymatic cleavage distinguishes between adenovirus 5 wild type and the deletion mutant.

Figure (39) shows that adenovirus 3var100 replicates its DNA equally well in cell lines KB16 (E1a+E1b) and KB8 (E1a) as does dl312 (E1a). Adenovirus 3var100 DNA is not detectable in cell lines KB7, which does not express any adenoviral functions, and also KB18, which expresses only the E1b region. This data also suggests that the lesion maps within the E1a region of the genome.

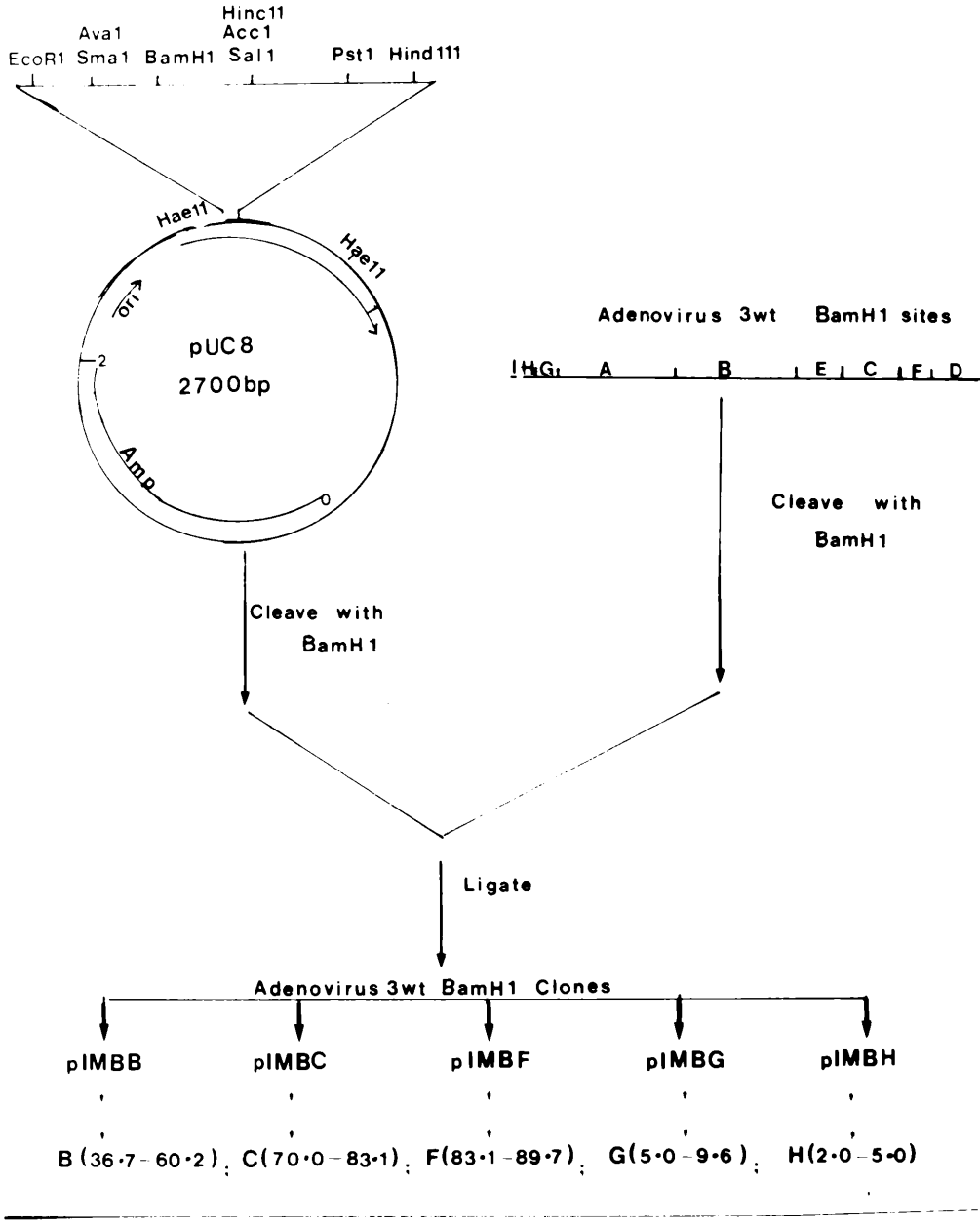

Hinc11
Ava1
Acc1
EcoR1
Sma1
BamH1
Sal1
Pst1
Hind111
Hae11
Hae11
ori
pUC8
2700bp
Amp
Adenovirus 3wt BamH1 sites
A
B
E
C
F
D
Cleave with BamH1
Cleave with BamH1
Ligate
Adenovirus 3wt BamH1 Clones
pIMBB
pIMBC
pIMBF
pIMBG
pIMBH
B (36·7 - 60·2)
C (70·0 - 83·1)
F (83·1 - 89·7)
G (5·0 - 9·6)
H (2·0 - 5·0)

Figure 40.

Strategy used to clone the BamH1 wild type fragments of adenovirus 3 wild type.

Plasmid pUC 8 was linearised at the unique BamHI site and prepared as described in Materials and Methods , page 61 .

Adenovirus 3 wild type DNA was cleaved with endonuclease BamHI and co-hesive end ligated into pUC8 as described in Materials and Methods pages 60 and 61 .

The ligated DNA was used to transform JM 83 host bacteria to ampicillin resistance as described in Materials and Methods pages 61 - 64 .

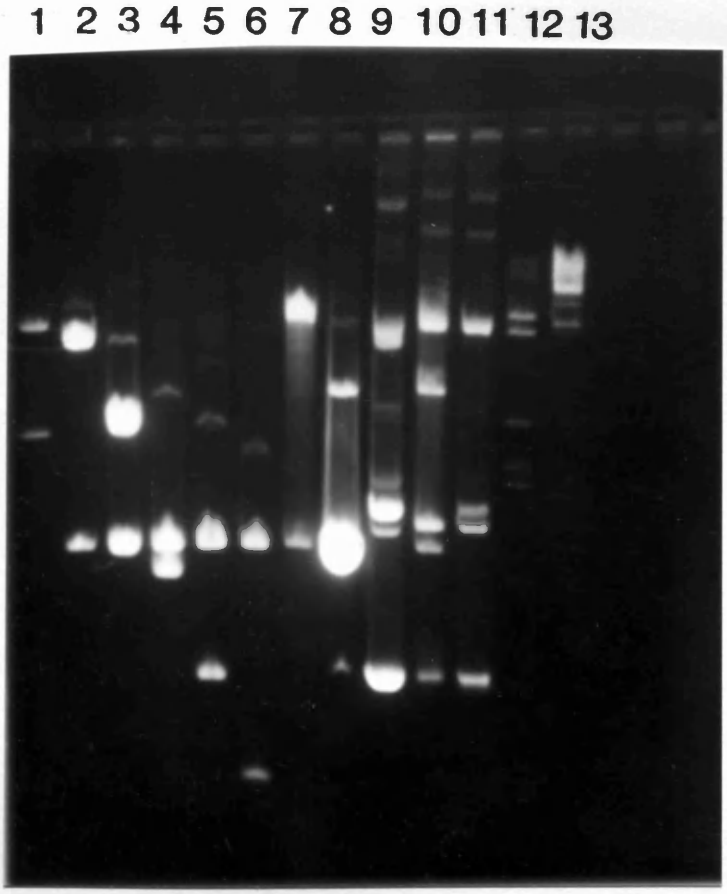

1 2 3 4 5 6 7 8 9 10 11 12 13

Figure 41.

BamH1 clones of adenovirus 3 wild type

One microgram of DNA from each of the adenovirus 3 wild type BamH1 clones was cleaved with the endonuclease BamH1 to release the BamH1 insert and electrophoresed overnight through a .7% agarose at 30 volts. DNA was visualised by ethidium bromide staining and UV transillumination.

Lane	Clone	Ad. 3wt DNA insert
1	pJB329* [Engler and Kilpatrick,(1981)]	BamH1 A
2	pIMBB	BamH1 B
3	pIMBC	BamH1 C
4	pIMBF	BamH1 F
5	pIMBG*	BamH1 G
6	pIMBH*	BamH1 H
8	pUC8 cut BamH1	
9	pUC8 uncut	
12	Ad. 3wt DNA cut BamH1	

Clones marked with * were used to marker rescue adenovirus 3var100

Adenovirus 3var100 has two lesions; a deletion of about 1.5Kb mapping between 81.1 m.u. to 87 m.u. within the E3 region at the right hand end of the genome and a lesion mapping in the E1 region to within the E1a, which is possibly responsible for the host range phenomenon in cell lines which do not express the left hand adenoviral functions.

9. Separation of the E1a and E3 lesions of adenovirus 3var100.

Since adenovirus 3var100 has two lesions it was desirable to separate them for future experiments. This was achieved by (a) marker rescue using adenovirus 3 wild type BamH1 cloned fragments, and (b) re-ligation of adenovirus 3var100 and 3 wild type DNA each cleaved with Cla1. These methods gave rise to adenovirus 3dl101 and adenovirus 3hr102 whose origins will be described.

9.1 Cloning of adenovirus 3 wild type DNA fragments

Adenovirus 3 wild type DNA was cleaved with BamH1 and the complete digest was co-hesive end ligated into pUC8 linearised at the unique BamH1 site. When adenovirus 3 wild type DNA is cleaved with BamH1 nine fragments result, seven being capable of co-hesive end ligation into the vector plasmid; terminal fragments D and I containing terminal peptide will not ligate by this method. Figure 40 shows the construction of the adenovirus 3 wild type BamH1 clones; Figure 41 illustrates the resulting clones.

The clones were further identified by restriction endonuclease digestion; fragment BamH1 B can be distinguished from fragment BamH1 A by cleavage with Xho1. Xho1 cleaves BamH1 A at four sites to yield five

Table XVIII

Yields of Marker Rescue of Adenovirus 3var100 Left-Hand End Lesion by Cloned DNA Fragments of Adenovirus 3 wild type.

DNA co-transfection / Cell line	Ad3var100/ pIMBG (5-9.6) m.u.	Ad3var100/ pIMBH (2-5) m.u.	Ad3var100/ pJB329* (9.6-36.7) m.u.	Ad3var100 DNA
293	3.5×10^9	1×10^9	1.9×10^9	3×10^9
Hela	$< 10^1$	2×10^3	$\sim 10^1$	$\sim 10^1$

* Engler and Kilpatrick, (1984). GENE 13 : 125-132.

fragments, while BamH1 B remains uncleaved. Fragment BamH1 B will cleave with HindIII to yield two fragments.

BamH1 C fragment cleaves with Xho1 producing two fragments of about 6% which co-migrate as a doublet. BamH1 F fragment cleaves with EcoR1 into three fragments of about 2.3% each. As they co-migrate together in an agarose gel system it is impossible to distinguish three fragments. Fragments BamH1 G and H are cleaved with HindIII; BamH1 H yields two fragments approximately 1.2% and 1.8% and BamH1 G is cleaved into two fragments of 2.9% and 2.5%. BamH1 G and H fragments span the E1 region and can be used to marker rescue adenovirus 3var100.

9.2 Marker rescue of adenovirus 3var100 using cloned adenovirus 3 wild type BamH1 fragments

To marker rescue the left hand E1 lesion adenovirus 3var100 DNA was co-transfected into 293 cell monolayers in 50mm petri plates along with equimolar amounts of linearised plasmids pIMBH or pIMBG or pJB329 which contains the adenovirus 3 wild type fragment A (Engler and Kilpatrick, 1981).

Monolayers were maintained under liquid medium for 7 days when viral yields were harvested and titrated on 293 and Hela cell monolayers. The titre (p.f.u./ml) of the viral yield from co-transfection of adenovirus 3var100 with pIMBH in Hela cells (Table XVIII) suggests that rescue may have occurred as this figure is significantly greater than the control. Isolated plaques from Hela plates were analysed for the loss of host range phenotype by rapid assay on 293 and Hela cell monolayers.

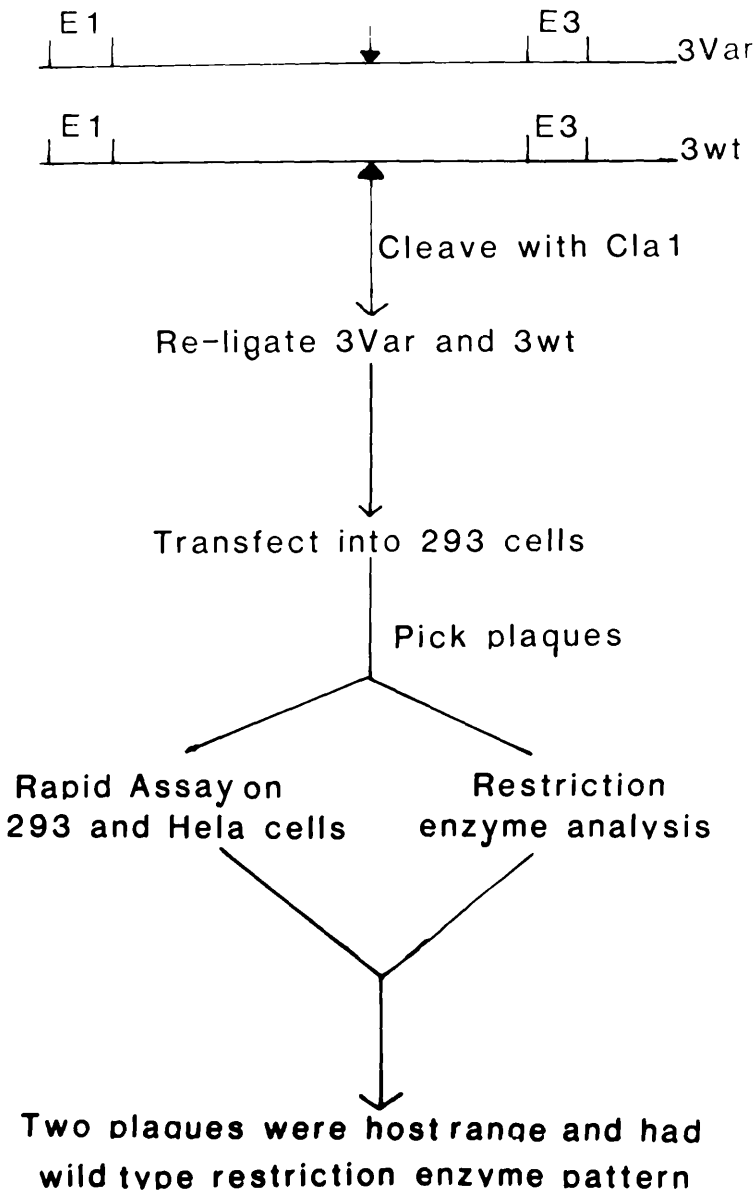

E1
E3
3Var
E1
E3
3wt
Cleave with Cla1
Re-ligate 3Var and 3wt
Transfect into 293 cells
Pick plaques
Rapid Assay on 293 and Hela cells
Restriction enzyme analysis
Two plaques were host range and had wild type restriction enzyme pattern

Figure 42.

Construction of adenovirus 3hr102

Table XIX

Isolates screened by Rapid Assay	293 cells	Hela cells
9	+	-
8	+	+
3	-	-

Figure 43.

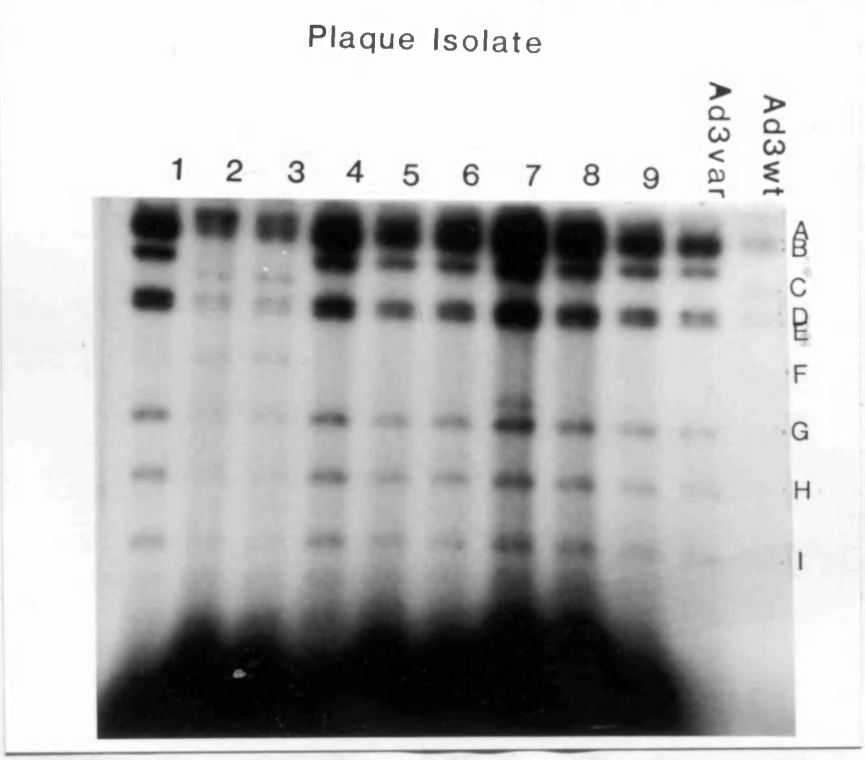


Figure 43.

Shows the restriction endonuclease profile of the nine progeny expressing a host range phenotype.

The DNA was labelled with inorganic orthophosphate and harvested as a Hirt supernatant. Equal aliquots of labelled DNA were cleaved with BamH1 and electrophoresed through a .7% agarose gel followed by exposure to X-ray film at -70°C.

Isolates 2 and 3 show the restriction enzyme profile of adenovirus type 3.

Isolates which scored positive on both cell lines were further characterised by restriction enzyme profile. These isolates showed the DNA restriction profile of adenovirus 3var100. They were no longer host range and were therefore classified as rescued at the left hand end. One isolate, adenovirus 3dl101 was grown through three rounds of plaque purification and prepared as a crude viral seed stock.

9.3 Isolation of adenovirus 3hr102

The right hand lesion of adenovirus 3var100 failed to marker rescue in my hands and was therefore rescued as follows.

Adenovirus 3var100 DNA and adenovirus 3 wild type DNA were each cleaved with the restriction enzyme Cla1 (cutting each at 52.2 m.u.). The complete digests were then mixed and re-ligated by co-hesive end ligation (Fig. 42) and transfected into 293 cell monolayers.

Monolayers were maintained at 37°C under agar overlay medium for 10 days. Isolates were screened by rapid assay (Table XIX). The nine progeny expressing a host range phenotype in Hela cells were further characterised by restriction enzyme analysis (Fig. 43) for an adenovirus 3 wild type E3 region. Two isolates were shown to have an adenovirus 3 wild type E3 region and to be host range in Hela cells. One isolate, adenovirus 3hr102 was grown through three rounds of plaque purification and prepared as a crude viral seed stock.

10. Replication of viral isolates in KB cell lines

Adenovirus 3dl101 and adenovirus 3hr102 were titrated on the family

Table XX

Replication of Viral Isolates in the KB cell lines

Cell line \ Virus	Ad3wt	Ad3var100	Ad3dl101	Ad3hr102	Ad5wt
293	9×10^9	2×10^{10}	1.8×10^{10}	1.2×10^9	2.9×10^{11}
Hela	8.5×10^9	10^1	1.1×10^9	10^1	2.7×10^{10}
KB7	6.5×10^9	2×10^3	5×10^9	4×10^3	1.1×10^{11}
KB8(E1a)	6×10^9	2.5×10^8	2.7×10^9	2×10^9	1.1×10^{11}
KB16(E1a+E1b)	7×10^9	8×10^9	6.5×10^9	1.6×10^9	1.1×10^{11}
KB18(E1b)	7×10^9	8.5×10^2	6.5×10^9	2×10^3	1×10^{11}

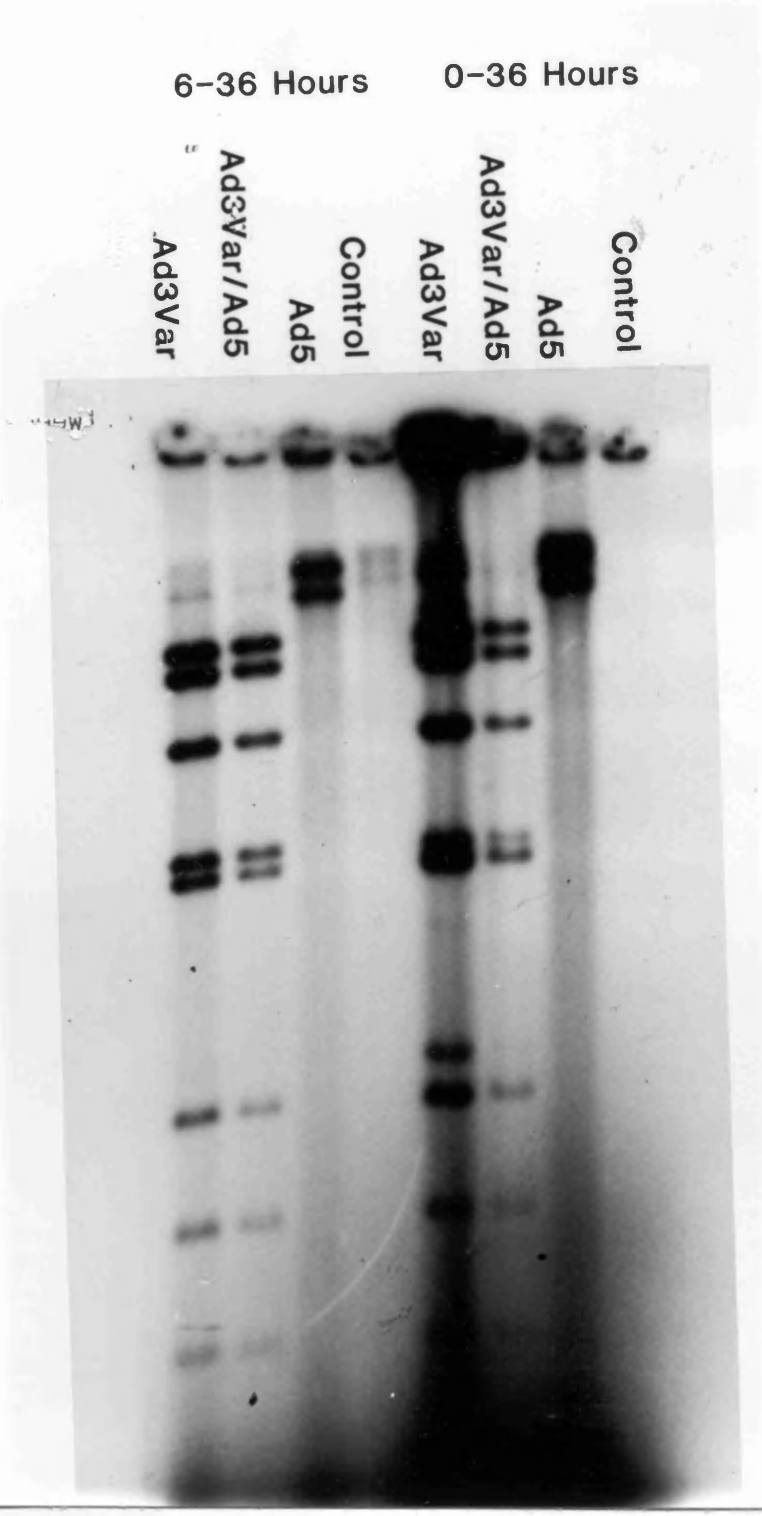

6-36 Hours
0-36 Hours
Ad3Var
Ad3Var/Ad5
Ad5
Control
Ad3Var
Ad3Var/Ad5
Ad5
Control

Figure 44.

Diminished adenovirus 5 wild type DNA synthesis in the presence of adenovirus 3var100

Monolayers of 293 cells were infected with adenovirus 5 wild type at 5 p.f.u/cell where double infection would occur and at 10 p.f.u./cell for single infection. At zero hour, co-infection took place with adenovirus 3var100 at 5 p.f.u./cell and at 6 hours and superinfection took place with adenovirus 3var100 at 5 p.f.u./cell; single infection was at 10 p.f.u./cell. DNA was labelled with inorganic orthophosphate and time points extracted as a Hirt supernatant at 36 hours. DNA was cleaved with the endonuclease BamH1 and electrophoresed through a .7% agarose gel overnight at 30 volts. DNA was visualised by autoradiography.

of KB cells (Babiss et al., 1983) described previously, and maintained under agar overlay medium at 37°C for 10 days. Monolayers were fixed and stained with virus titres being recorded.

Data (Table XX) suggests that adenovirus 3hr102 is host range and like its parental adenovirus 3var100 can be complemented to replicate by cell lines expressing the E1a functions of adenovirus 5 wild type or adenovirus 2 wild type. Adenovirus 3dl101 is not host range as it replicates comparably to wild type virus in cell lines which do not express any adenovirus E1 functions.

11. Diminished adenovirus 5 wild type DNA replication in the presence of adenovirus 3var100

During complementation assays between adenovirus 3var100 and adenovirus 5 wild type viruses, it was noted that although adenovirus type 5 would complement the variant to grow in non-permissive Hela cells, its own DNA synthesis was suppressed.

To investigate at which time in the adenovirus replication cycle adenovirus 3var100 induced suppression, pulse labelling of co-infected monolayers was carried out.

Monolayers of 293 cells were infected with adenovirus 5 wild type and co-infected or superinfected with adenovirus 3var100 at times between zero and 12 hours; inorganic orthophosphate was also added at these times to label viral DNA; samples were harvested between 24 and 36 hours.

Data (Fig. 44) shows that when adenovirus 5 wild type virus is

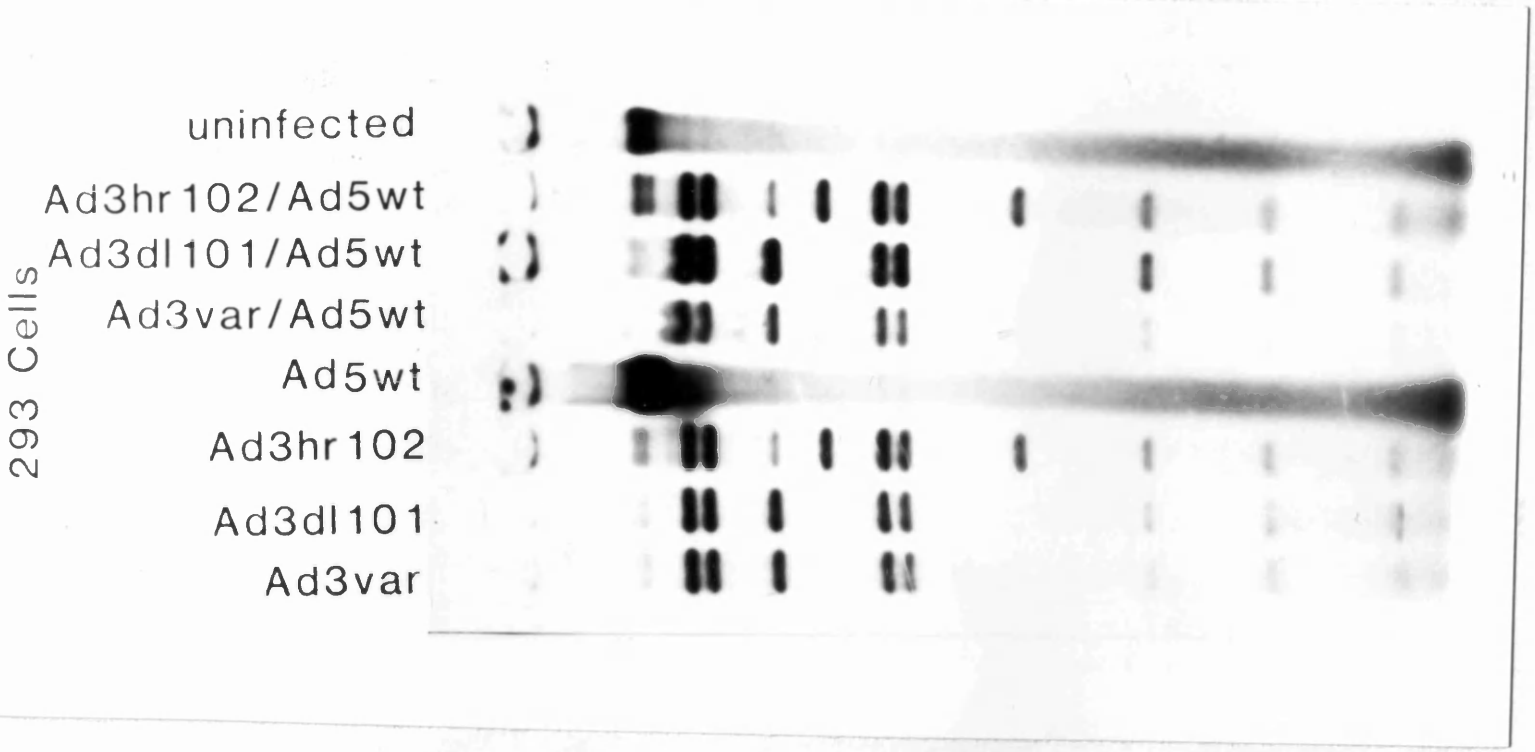
co-infected or superinfected with adenovirus 3var100 virus at zero or 6 hours post infection, and viral DNA extracted as a Hirt supernatant at 36 hours, no adenovirus 5 wild type DNA replication is detectable in co-infected or superinfected tracts. This suggests that if cell monolayers are first infected with adenovirus type 5 and then co-infected or superinfected with adenovirus 3var100, between zero and 6 hours, DNA synthesis of adenovirus type 5 is suppressed.

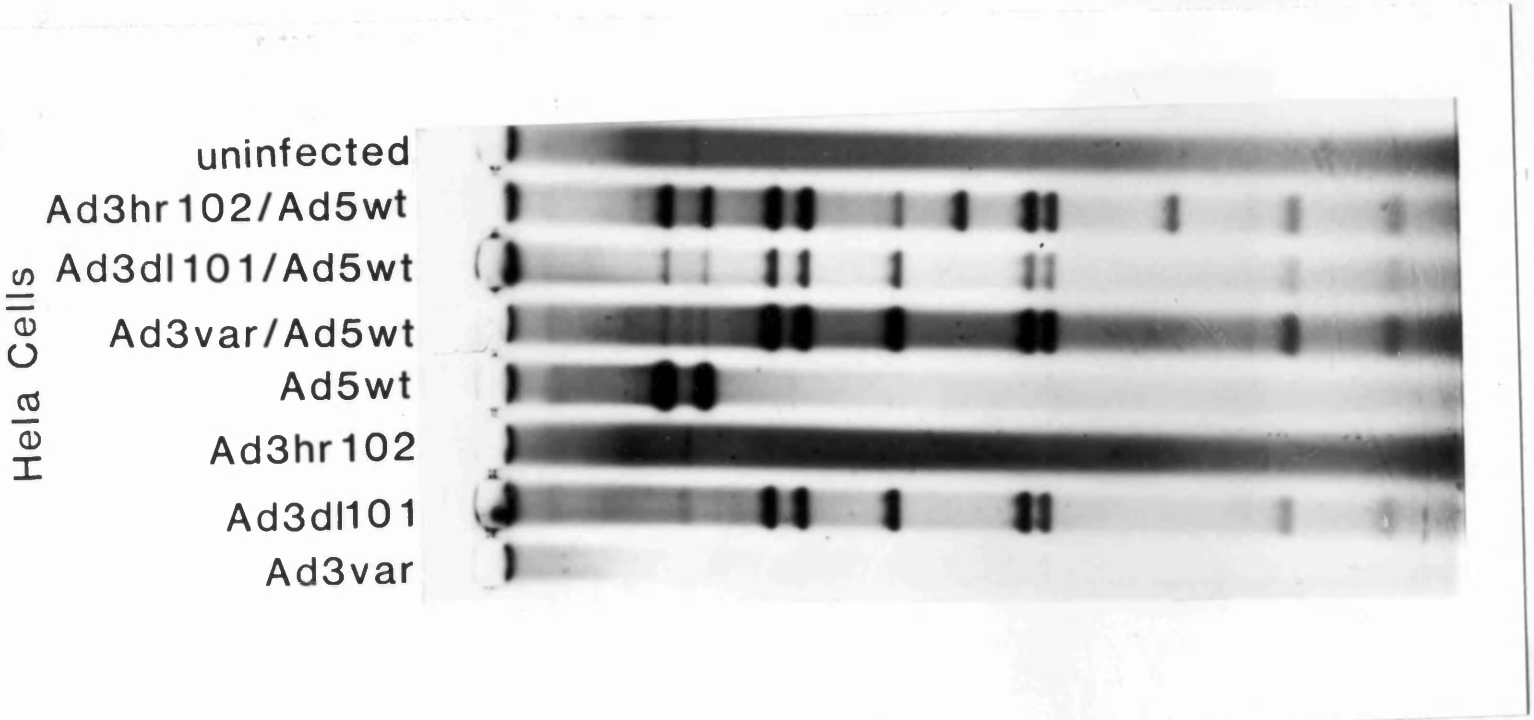
The pulse labelling was extended to the following time intervals; 6-12 hours, 6-24 hours and 12-24 hours, but quantitation of the resulting data was impossible due to a persistent problem with the inefficiency of labelling the adenovirus type 5 DNA.

This could be overcome in future experiments by the omission of the radioactive label from the protocol and electrophorese the Hirt supernatant DNA through a polyacrylamide gel system visualising the DNA by silver staining (Whitton et al., 1983).

12. Replication of adenovirus 5 wild type DNA in 293 cells and Hela cells in the presence of adenovirus 3var100, adenovirus 3dl101 or adenovirus 3hr102

To investigate if adenovirus 5 wild type DNA suppression was a co-operative effect between the two lesions or the result of the E3 lesion alone (Beckner and Sharp, 1983), adenovirus 5 wild type virus was co-infected with adenovirus 3dl101 or adenovirus 3hr102 in 293 or Hela cell monolayers. Viral DNA was labelled with inorganic orthophosphate and extracted at 36 hours post infection as a Hirt supernatant followed by cleavage with the restriction endonuclease BamH1.


293 Cells
uninfected
Ad3hr102/Ad5wt
Ad3dl101/Ad5wt
Ad3var/Ad5wt
Ad5wt
Ad3hr102
Ad3dl101
Ad3var


Hela Cells
uninfected
Ad3hr102/Ad5wt
Ad3dl101/Ad5wt
Ad3var/Ad5wt
Ad5wt
Ad3hr102
Ad3dl101
Ad3var

Figures 45 and 46.

Replication of adenovirus 5 wild type DNA in 293 and Hela cells in the presence of adenovirus 3var100, adenovirus 3dl101 or adenovirus 3hr102

Monolayers of 293 or Hela cells were infected with adenovirus 5 wild type in the presence of adenovirus 3var100, adenovirus 3dl101 or adenovirus 3hr102 at 5 p.f.u./cell each virus for double infections and 10 p.f.u./cell for single infections. DNA was labelled with inorganic orthophosphate and harvested as a Hirt supernatant. Equal aliquots of DNA samples were cleaved with BamH1 and electrophoresed through a .7% agarose gel overnight at 30 volts. DNA was visualised by autoradiography.

Adenovirus 3hr102 has a band which migrates in the gel system to the same position as adenovirus 3var100 band C'. Adenovirus 3hr102 has been plaque purified before the growth of the seed stock, therefore should not contain adenovirus 3var100; contaminant band is possibly a partial band.

In 293 cells (Fig. 45) adenovirus 5 wild type DNA synthesis is suppressed by adenovirus 3dl101 which carries the E3 lesion and also by the parental virus adenovirus 3var100. Adenovirus 5 wild type DNA synthesis is detectable when co-infected by adenovirus 3hr102 which carries the wild type E3 region.

When the experiment was repeated in Hela cells (Fig.46) data shows that adenovirus 3hr102 is host range but can be complemented to replicate its DNA by co-infection with adenovirus 5 wild type virus. Adenovirus 3dl101 is capable of growth in Hela cells as it has a rescued E1a lesion and is therefore no longer host range. Adenovirus 5 wild type replicates its DNA in the presence of adenovirus 3hr102 but is barely detectable in the presence of adenoviruses 3dl101 and 3var100. Both of these viruses contain the lesion in the E3 region.

The above results suggest that the DNA suppression phenomenon of adenovirus 5 wild type by adenovirus 3var100 during co-infection is due to the lesion in the E3 region and not a consequence of the E1a lesion or a co-operative effect between the two lesions.

Previous data has shown that when adenovirus 3var100 is co-infected with its parental adenovirus type 3, the variant DNA replication is suppressed and the parental does not complement its replication in Hela cells.

On co-infection of adenovirus 3dl101 with adenovirus type 3 in 293 and Hela cell monolayers, the DNA profile of both viruses was detectable, although the DNA replication of adenovirus 3dl101 was slightly reduced

from a single infection. The replication of adenovirus 3dl101 would be expected in Hela cells as this virus is not host range.

Adenoviruses 3hr102 and type 3 both yield the wild type DNA restriction enzyme profile. Therefore on co-infection with these viruses into 293 or Hela cell monolayers nothing can be deduced about either DNA suppression or complementation.

The replication of adenovirus 3dl101 DNA in the presence of adenovirus type 3 could suggest that the altered E1a region is involved in the suppression of adenovirus 3var100 DNA replication, or there is a requirement of both the E1a and the E3 lesions.

Adenovirus 3var100 has been shown to have a large plaque morphology. During titration in 293 cells it was noted that adenovirus 3hr102 showed a plaque morphology similar to adenovirus type 3, but did not plaque in Hela cells.

Adenovirus 3dl101 showed a plaque morphology of imtermediate size between adenovirus 3var100 and adenovirus type 3 in both 293 and Hela cells.

This suggests that the large plaque morphology of adenovirus 3var100 is dependent on the two lesions in E1a and E3, although it may be postulated that the E3 lesion governs the plaque morphology more than the E1a lesion, as adenovirus 3dl101 shows plaques of intermediate size.

pARKR

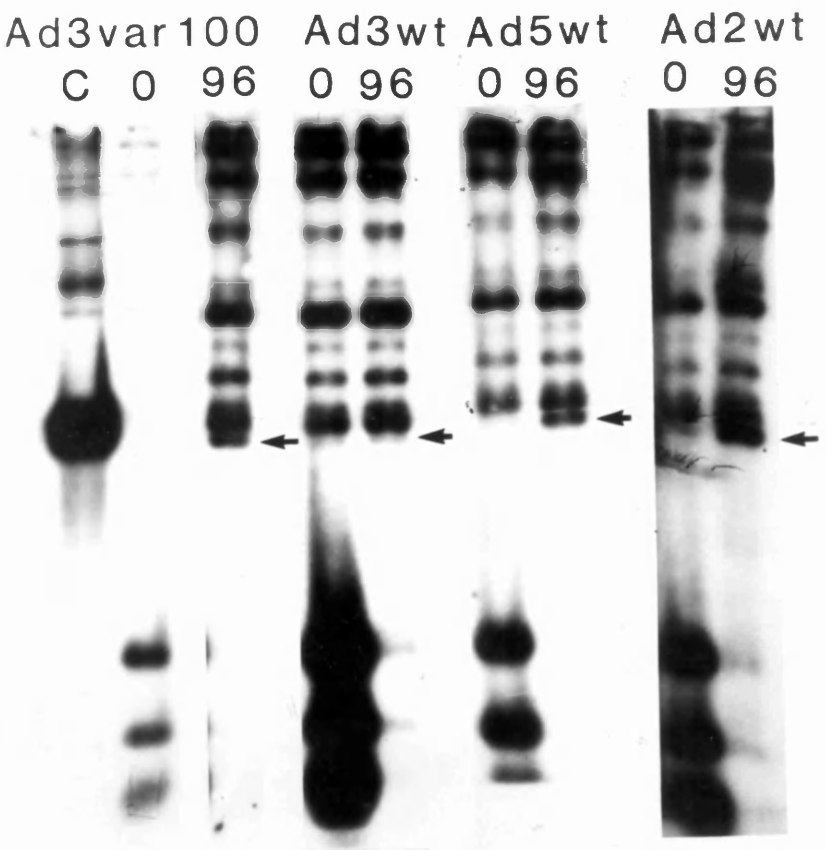


Figure 47.

Replication of adenoviral ITRs in the presence of adenovirus 3var100, adenovirus 3 wild type, adenovirus 5 wild type

pARKR was cleaved with endonuclease EcoR1 and co-transfected into 293 cells with helper adenovirus DNA in the form of adenovirus 3var100, adenovirus 3 wild type, adenovirus 2 wild type or adenovirus 5 wild type. DNA was harvested at time intervals as a Hirt supernatant; aliquots were cleaved with DpnI and electrophoresed through a .7% agarose gel overnight at 30 volts. DNA was analysed by Southern blotting.

C = Control pARKR uncut

0 = Zero hours

96 = 96 hours

Replicating band is arrowed.

The plasmid pARKR (Hay et.al., 1984) contains a pair of inverted 570 base pair right terminal fragments of adenovirus type 2 .These termini are separated by the HindIII/ SmaI fragment of pKC7 (Rao and Rogers , 1979) which contains the bacterial neomycin phosphotransferase gene which confers kanamycin resistance .Thus nick translated pKC7 DNA can be used as a probe for pARKR .

13. Replication of the subgroup C Inverted Terminal Repeat in the presence of adenovirus 3var100 DNA

Hay et al., (1984) showed that when mini replicons consisting of a linear molecule with subgroup C adenoviral type 2 termini at each end were co-transfected into 293 cells with full length adenovirus 2 wild type DNA as helper, they are capable of autonomous replication. The products of replication are known to have co-valently bound protein at the 5' ends and initiate synthesis at the same nucleotide as that used in DNA replication.

The E3 lesion of adenovirus 3var100 has been shown to suppress DNA synthesis of subgroup C adenovirus type 5. Since adenovirus 2 wild type DNA replication is also suppressed by adenovirus 3var100 it would be reasonable to conclude that the E3 lesion is responsible. Therefore is adenovirus 3var100 also capable of the suppression of the mini replicons which contain only the subgroup C adenovirus ITRs?

The experiment was carried out as described by Hay et al., (1984); plasmid pARKR was cleaved with the restriction endonuclease EcoR1 to release the mini replicon and co-transfected with helper DNA into 293 cells. Although 293 cells express the E1 functions of subgroup C, this should not affect the experiment as it has been shown that the E3 lesion is responsible for DNA suppression and not the E1a lesion. At time intervals between zero and 96 hours post transfection, DNA was extracted as a Hirt supernatant and analysed by Southern blotting.

Figure 47 shows that the mini replicon will replicate in the presence

p3AI

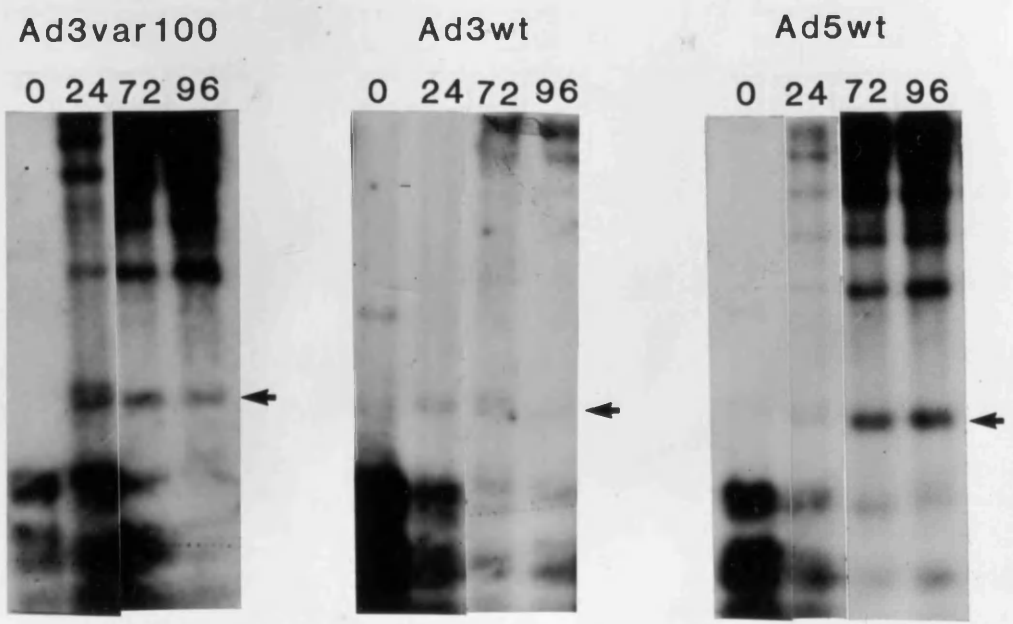


Figure 48.

Replication of p3A1 in the presence of adenovirus 3var100, adenovirus 3 wild type and adenovirus 5 wild type

p3A1 was cleaved with EcoR I and co-transfected into 293 cells with helper adenovirus DNA in the form of adenovirus 3var100, adenovirus 3 wild type or adenovirus 2 wild type. DNA was harvested at time intervals as a Hirt supernatant; aliquots were electrophoresed through a .7% agarose gel overnight at 30 volts. DNA was analysed by Southern blotting.

0 = Zero hour

24 = 24 hours

72 = 72 hours

96 = 96 hours

Replicating plasmid is arrowed.

of DNA from adenoviruses type 2, type 5 and 3var100 as helper; therefore adenovirus 3var100 does not suppress replication of the mini replicons. This suggests that the suppression of subgroup C DNA by adenovirus 3var100 does not involve the ITR containing the origin of replication.

Adenovirus 3 wild type DNA acts poorly as a helper for the replication of the mini replicons. This may be due to the poor infectivity of the DNA rather than a heterotypic subgroup problem, as it requires highly infectious DNA to achieve replication of the mini replicons.

Adenovirus 3 wild type DNA was therefore tested against plasmid p3A1 (described in Materials and Methods) which contains a single adenovirus 3 wild type ITR. When the plasmid is cleaved to expose the adenoviral terminal sequences at the end of the linear DNA, a single round of replication can be detected in the presence of adenovirus helper DNA (Hay, 1985).

Plasmid p3A1 was cleaved with the restriction endonuclease EcoR I and co-transfected into 293 cells with helper DNA. At time intervals between zero and 96 hours, DNA was extracted as a Hirt supernatant and analysed by Southern blotting.

Figure 48 shows that adenovirus 3var100 and adenovirus 5 wild type DNA will act as helper for the replication of plasmid p3A1, while no replication is detected in the presence of adenovirus 3 wild type DNA as helper. As the cloned ITR of p3A1 is homotypic with the helper DNA this would suggest that the lack of observed replication is due to the low DNA infectivity of adenovirus type 3.

Chapter 4.

DISCUSSION

Section A.

A. Results obtained from the analysis of adenovirus 3var100.

   1. General properties

B. Mixed viral infections

C. Adenovirus type 5 DNA suppression by adenovirus 3var100.

Section B.

A. Recombination - failure to isolate viable progeny between two adenoviral subgroups.

Section A.

A. Results obtained from the analysis of adenovirus 3var100

1. General Properties

(a) Adenovirus 3var100 was isolated from a crude seed stock of adenovirus 3 wild type. It has a large plaque phenotype compared to the wild type (Fig 23) and is a fast growing virus giving a final yield comparable to adenovirus 5 wild type over the same time range. This is demonstrated by one step growth curves. (Fig 24).

When assayed for growth in Hela or KB cells the variant was found to have a host range phenotype for these cell lines.(Table XX).

Adenovirus type 5 host range mutants for growth in Hela cells have been isolated by Harrison et al., (1977); Jones and Shenk, (1978). Complementation assays (Harrison et al., 1977); marker rescue experiments (Frost and Williams, 1979) and recombination experiments between deletion and host range mutants (Galos et al., 1980) have defined the host range phenotype to map within the E1 region of the genome. Experiment 3, Section B, shows that adenovirus 3var100 synthesises its DNA in 293 cells which express adenovirus 5 wild type E1 functions, but not in Hela cells, suggesting a lesion in the E1 region.

Restriction enzyme analysis (Experiment 5, Section B) showed that the variant had a deletion of approximately 1Kb mapping in the non-essential E3 region, but there is no detectable difference in the restriction enzyme profile of the E1 region suggesting either a very

small DNA deletion, insertion or a point mutation. Growth of adenovirus 3var100 in KB cell lines expressing the E1 functions of adenovirus 2 wild type (Babiss et al., 1983) experiment 8, section B, suggests that the host range lesion lies in the E1a region, as cell lines expressing E1a functions are seen to complement its growth. Further evidence that the lesion maps in the E1a region comes from marker rescue of adenovirus 3var100 with the adenovirus 3 wild type BamH1 H fragment which maps between 2 m.u. - 5 m.u., spanning most of the E1a region.

(b) Ho et al., (1982) isolated host range mutants of adenovirus type 5 which also showed a cold sensitive phenotype. Two of these mutants were mapped to the E1a region and one to the E1b region.

Since adenovirus 3var100 was isolated in 293 cell monolayers at 37°C, showing a restricted growth in Hela cells and also shown to map in the E1a region, it was possible that the variant also had a cold sensitive phenotype. The variant was plaque assayed on Hela cells at 32°C, 37°C and 38.5°C (Experiment 2, Section B) and found to be host range at all temperatures. Thus the mutant is not cold sensitive, unlike the mutants of Ho et al., (1982).

(c) Adenovirus 3var100 has a large plaque phenotype and such mutants have been shown to map in the E1b region of the genome, e.g. Cyt mutants of adenovirus type 12 (Takemori, 1968; Lai Fatt and Mak, 1982) and large plaque mutants of adenovirus type 2 (Chinnadurai, 1983; Subramanian et al., 1984a). The location of the lesion conferring the large plaque phenotype has been mapped to the E1b region and shown to be a single amino acid substitution within the 19Kd tumour antigen (Chinnadurai, 1983; Subramanian et al., 1984a).

D'Hallium et al., (1979) showed that adenovirus H2ts111 (Martin et al., 1978) a DNA negative mutant, degraded cellular DNA. Their results suggested that an early viral function prevented DNA degradation. Ezoe et al., (1981) described extensive cellular DNA degradation by Cyt mutants and also by E1b deletion mutant dl313 (Jones and Shenk, 1978). Subramanian et al., (1984b) describe extensive degradation of cellular DNA by adenovirus H2dl250 which has a large plaque phenotype and a specific lesion within the 19Kd tumour antigen.

Adenovirus 3var100 was analysed by the method described by Stillman et al., (1984); White et al., (1984) for the phenotype of cellular DNA degradation. Data shows that cellular DNA from KB or Hela cell lines in the presence of adenovirus 3var100 is not degraded, being like dl312, an E1a deletion mutant (Jones and Shenk, 1978). Dl313, an E1b deletion mutant described by Ezoe et al., (1981), produces cellular DNA degradation in KB and Hela cell lines, but not in the 293 cell line which expresses adenovirus type 5 E1b functions. Thus adenovirus 3var100 has a large plaque phenotype but not the phenotype of cellular DNA degradation.

Lui et al., (1985) have described a large plaque phenotype displayed in Hela cells by sub304 (Jones and Shenk, 1978). This mutant has a substitution of foreign DNA between 83.2 m.u and 85.1 m.u. in the non-essential E3 region.

Adenovirus 3var100 has a deletion between 83.1 m.u. and 85.4 m.u. and a large plaque phenotype. This may suggest that the absence of these sequences allows the formation of large plaques. However, while adenovirus 3hr102 regains the small plaque morphology of the wild type, adenovirus 3dl101 displays plaques of intermediate size suggesting that

the large plaque phenotype is not a direct result of the E3 lesion alone.

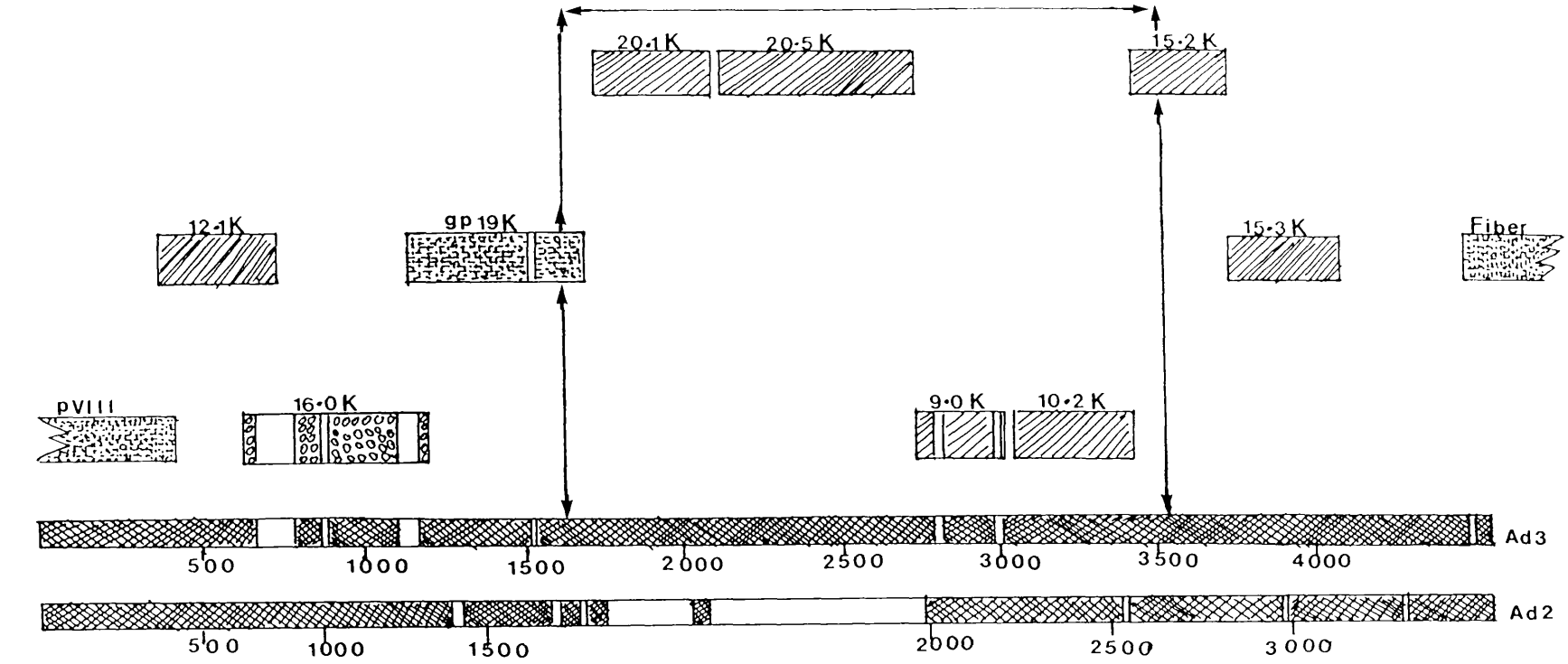
(d) Adenoviruses containing mutations in structural genes can be inactivated faster than wild type virus when heated within the temperature range of 52°C-56°C (Russell et al., 1967; Brown et al., 1975; Colby and Shenk, 1981).

Colby and Shenk, (1981) showed dl312, which carries lesions in both the E1a and E3 regions, to be more thermolabile by a half log than adenovirus type 5.

Adenovirus 3var100 was heat treated at 52°C and surviving progeny assayed. Data showed that the variant was more thermolabile by 1 log than its parental and 2 logs more thermolabile than adenovirus type 5.

Little is known about the function of the E3 region except that it possibly codes for membrane proteins. If this is the case, then it is unlikely that the absence of any of the polypeptides conferred by the deletion is responsible for the thermolability of the variant.

Little is known about the lesion within the E1a region except that it confers the host range phenotype to the variant. No viral structural proteins are coded by the E1a region. Its function has been shown to be the regulation of gene expression from the other early regions and to be involved in the process of oncogenic transformation. The lesion may fall within the coding sequence of the 289 amino acid residue regulatory protein. This would suggest therefore that the variant has an undetected mutation within a gene coding for one of the structural proteins. By screening the thermolability of adenovirus 3dl101 consisting of adenovirus


20·1K
20·5K
15·2K
12·1K
gp19K
15·3K
Fiber
pVIII
16·0K
9·0K
10·2K
Ad3
500
1000
1500
2000
2500
3000
3500
4000
Ad2
500
1000
1500
2000
2500
3000

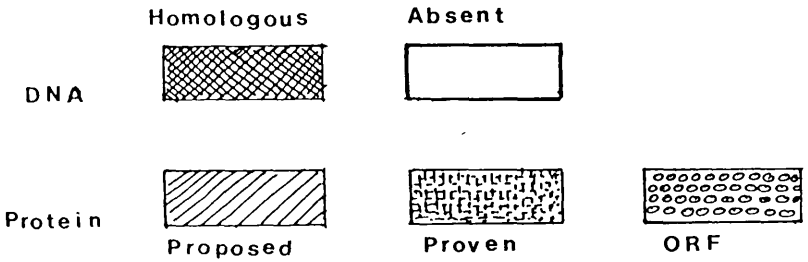

Homologous
Absent
DNA
Protein
Proposed
Proven
ORF

Figure 49.

Depicts the E3 region of adenovirus type 3. The proteins encoded by this region are shown above the DNA segment.

The DNA of the E3 region of adenovirus type 3 is compared with the DNA from the E3 region of adenovirus type 2, for homologous and absent zones.

The arrowed zone shows the proteins which are absent in adenovirus 3var100 owing to the deletion.

The figure is adapted from Signäs et al., (1987) Gene.

3var100 between 52.1 m.u. - 100 m.u. and adenovirus type 3 between 0-52.1 m.u., the thermolabile phenotype could be assigned to one half of the genome.

The E3 region of adenovirus 3 wild type is thought to be non-essential for viral growth and to code for membrane proteins (Signäs et al., 1987). The E3 regions are well conserved between subgroups (Signäs et al., 1987). However, it has been shown that unique to adenovirus 3 wild type is a 950bp AT rich region thought to be of host cell origin. This region contains two open reading frames with the capacity to encode two polypeptides of unknown function, with molecular weights of 20.1K and 20.5K.

I have shown by restriction enzyme analysis that the deletion within the adenovirus 3var100 E3 region maps between 83.1 m.u. - 87 m.u. However, by personal communication from V. Mautner it also extends leftwards from 83.1 m.u. to 81.1 m.u. but not beyond. Using the sequencing data of Signäs et al., (1987) the extent of DNA coding sequences removed in full or part by the deletion can be predicted. (See Fig 49).

B. Mixed Viral infections

Suppression of one virus by a co-infecting partner is well documented.

Castro et al.,(1967) showed that AAV type 1 would replicate in tissue culture in the presence of adenovirus acting as helper virus; however, the yield of adenovirus was decreased.

Dales and Silverberg, (1968) showed co-infection of animal cells with unrelated viruses could result in the complete suppression of one or the other virus. They postulated that the virus with the shorter latent period would be dominant over the one with the longer latent period. My results (Fig 24) show that adenovirus 3 wild type has a short latent period compared to adenovirus 3var100 or adenovirus type 5. However, further experiments have shown that it is only dominant over the progeny virus adenovirus 3var100 and not over other adenovirus serotypes. Unlike the viruses used in the experiments of Dales and Silverberg, these viruses are all related, although belonging to different subgroups.

Rowe and Graham, (1981) complemented host range mutants of adenovirus type 5 to replicate in non-permissive cells by co-infection with adenovirus type 12. At high multiplicities of infection with adenovirus type 12, the level of adenovirus type 5 and host range mutant virus was reduced. Results presented in this thesis (Fig 35) show that adenovirus type 5 will complement host range adenovirus 3var100 to replicate in non-permissive cells, but DNA synthesis of adenovirus type 5 is suppressed. This suppression, which later data attribute to the lesion in the E3 region is seen to act at low m.o.i. with adenovirus 3var100. Rowe and Graham found that the suppression of their adenovirus type 2 took place only in the presence of adenovirus type 12 at high m.o.i. Co-infection of the host range variant with its parental virus into non-permissive cells showed little complementation; however, it was noted that in 293 cells equally permissive for both viruses, the DNA synthesis of adenovirus 3var100 is suppressed by the parental adenovirus type 3.

Adenovirus type 2 or type 5 in the presence of adenovirus type 3 or

type 7 were seen to replicate their DNA to the level of a single infection (Figs 37 and 38). This result is contrary to Delsert and D'Hallium, (1984) who showed a hierarchy of dominance by representatives of subgroup B over representatives of other subgroups, i.e. adenovirus type 3 was dominant over adenovirus type 2 or type 5.

Different experimental procedures from those used by Delsert and D'Hallium were used in this work. The method used here was to label early in infection and harvest late between 36-48 hours. The viral DNA was harvested as a Hirt supernatant and assayed by autoradiography of the restriction enzyme profile.

Delsert and D'Hallium labelled late in infection over a short period and assayed the viral DNA on caesium chloride gradients.

It is difficult to offer a possible explanation to account for these contradictory results as both sets of data are equally convincing. However, throughout the literature there are equally conflicting sets of results. Mak, (1969) showed that on co-infection of KB cells with adenovirus type 2 and adenovirus type 12 the infectious yield of adenovirus type 2 was greatly reduced, adenovirus type 12 producing a yield equal to a single infection. Contrary to this data, Williams et al., (1975); Brusca and Chinnadurai, (1981) co-infected KB cells with adenovirus type 2 and adenovirus type 12, finding no transdominant effect by either virus.

C. Adenovirus type 5 DNA suppression by adenovirus 3var100

Berkner and Sharp, (1983) constructed an E3 deletion mutant of adenovirus type 5 mapping between 78.9 m.u. and 84.3 m.u. On co-infection

this mutant inhibited the DNA synthesis of its parental dl309 and of adenovirus type 5, leading to the postulation that the E3 lesion was responsible for the observed suppression.

Adenovirus 3dl101 containing the E3 lesion of adenovirus 3var100 suppresses the DNA synthesis of adenovirus 5 while adenovirus 3hr102 does not,(Figs 45 and 46) suggesting that the E3 lesion is responsible for the observed DNA suppression. In contrast to Berkner and Sharp, (1983) the parental strain of virus that gave rise to adenovirus 3var100 is seen to suppress its DNA synthesis. It is not known at what stage within the replication cycle of the adenovirus type 3 crude seed stock the variant arose. However, one explanation for the adenovirus type 3 parental suppression observed could be to control the variant progeny in the crude seed stock. Without such control the variant with its accelerated growth rate would outgrow the parental. The variant of Berkner and Sharp was constructed by them and hence no natural selection took place for precedence of parental suppression over progeny.

The deletion in the mutant constructed by Berkner and Sharp (78.9-84.3) m.u. removes most of the eight mRNAs which are found in the E3 transcription unit, leaving the E3 initiation site, the polyadenylation site and one set of 5' and 3' splice sites. The sequences removed and remaining within the variant can be predicted by the DNA sequencing data of Signäs et al., (1987). Fig 49.

Remaining sequences are the E3 transcription initiation site lying at position 121, the polyadenylation signal specifiying the end of the L4 mRNA and the polyadenylation signal at position 4186-4191 preceding the polyadenylation site at position 4209.

Sequences lost by the deletion are; the termination site, at least from the open reading frame of the 19K glycoprotein; the two open reading frames lying in the adenovirus type 3 unique 950 bp AT rich region encoding polypeptides of 20.1K and 20.5K. Also lying within this region at position 2008 is a polyadenylation signal, but it is not known if this is used for polyadenylation of mRNA. The two open reading frames for the 9K and 10.2K polypeptides are also lost, as is approximately 67 bp, including the initiation codon, of the 15.2K polypeptide.

With so little known about the function of the E3 region it is impossible to predict any role that the lack of these polypeptides has in DNA suppression. It may even be that the transdominant DNA suppression is the result of a more complex regulation.

When 293 or Hela cell monolayers were co-infected with adenovirus 3dl101 and adenovirus 3 wild type, the DNA restriction enzyme profile of both viruses was present. Thus adenovirus 3 wild type is not dominant over adenovirus 3dl101. Since adenovirus 3hr102 and adenovirus 3 wild type both have the same DNA restriction enzyme profile, complementation or suppression cannot be assayed at the DNA level.

The yields of mixed infections from 293 cells for suppression, or Hela cells for complementation, could be assayed for the percentage of progeny showing a host range phenotype. However, one must be aware of the probability that (a) the number of recombinants between adenovirus 3hr102 and adenovirus 3 wild type will be high, because of DNA homology and (b) if the lesion in the E1a region is a base pair change, then there is the possibility of revertants of adenovirus 3hr102 to the wild type

Table XXI

Virus	Dominant to	Not Dominant to
Ad3var100 (ΔE1a+ΔE3)	Ad5wt	Ad3wt
Ad3dl101 ΔE3	Ad5wt	Ad3wt
Ad3hr102 ΔE1a	-	Ad5wt
Ad3wt	Ad3var100	Ad3dl101, Ad5wt, Ad2wt
Ad5wt	-	Ad3var100, Ad3dl101
Ad7wt	-	Ad5wt, Ad2wt
Ad2wt	-	Ad3wt, Ad7wt

phenotype during the replication cycle. These viruses will be indistinguishable from the input adenovirus type 3, and, as there are no internal controls which can be added to assay their level, experimental quantitation will be inaccurate.

To investigate if adenovirus 3var100 would only suppress adenovirus 5 wild type DNA synthesis if co-infection of both viruses occurred at zero time, a pulse labelling experiment was carried out (Experiment 11, Section B). The only positive data from this experiment is that adenovirus 3var100 can suppress adenovirus 5 wild type DNA synthesis if co-infected or superinfected between zero and six hours post infection of adenovirus type 5, with viral DNA extracted at 36 hours post infection of adenovirus 5 wild type. The other time intervals of superinfection could not be quantitated because of a persistent radioactive labelling problem with adenovirus type 5. Labelled adenovirus 5 wild type DNA was detectable as a weak signal at 18 hours after addition of label whereas adenovirus 3var100 DNA was detectable at 6 hours after addition of label. This problem could be overcome as described in Results (Experiment 11, Section B).

Table (XXI) summarises the dominance and non-dominance found with the viruses used in this work.

It is difficult to hypothesise why the DNA replication of adenovirus 5 wild type should be suppressed by variants of adenovirus type 3 carrying a lesion in the E3 region.

The following points must be considered within any hypothesis:-

(a) The E3 region is non-essential for viral growth in tissue culture and

is proposed to code for membrane proteins.

(b) The lesion in the E3 region is a deletion of around 1.5Kb mapping approximately between 83.1 m.u. - 86 m.u. The coding sequences removed by this deletion and polypeptides affected have been discussed previously in this section. (Pages 117, 120, 121).

(c) The trans-dominant suppression exhibited by the adenovirus type 3 variants possibly recognises the subgroup C replication cycle. Replication of adenovirus 3 wild type is not suppressed in the presence of these viruses, although it itself may be able to regulate the gene suppression from these variants.

Experimental data from this work shows only that subgroup C DNA synthesis is suppressed in the presence of the variants. Adenoviral gene expression is regulated at multiple levels prior to DNA replication. On the basis of the data published in this work it is difficult to hypothesise a mechanism to rationalise the transdominant DNA suppression observed.

There is one possibility to be considered; it is possible that the deletion removes the termination sequence from the 19K polypeptide and the initiation sequence from the 15.2K polypeptide along with the intervening sequences between. It is possible that the remaining sequences are read as a continuous open reading frame resulting in a novel protein which in turn could be responsible for the transdominant suppression of DNA synthesis.

Section B

A. Recombination - failure to isolate viable progeny between two adenoviral subgroups

Adenovirus homotypic recombination has been demonstrated by Williams et al., (1975); Frost and Williams, (1978); Boursnell and Mautner, (1981); Volkert and Young, (1983); Mautner and MacKay (1984).

Complementation between subgroups has been demonstrated, (Williams et al.,1975; 1981; McDonough and Rekosh, 1982) but recombination between subgroups has not (Williams et al., 1981). Shiroki et al., (1982) have isolated recombinants between adenovirus type 12 and dl312, the E1a deletion mutant of adenovirus type 5 (Jones and Shenk, 1979). These recombinants are not progeny of homologous recombination, but the result of illegitimate recombination. The recombinants are predominantly dl312; one isolate was found to have a deletion of 20% between 5 m.u. and 15 m.u. with a 10% insert of adenovirus type 12 sequences carrying the E1a and part of the E1b regions. The other isolate was deleted in the E3 region with a 10% insertion of adenovirus type 12 as described above.

Singer et al., (1982) showed that 50bp homology was required for recombination within the r11 cistron of the bacteriophage T_4.

Gonda and Redding, (1983) showed in vitro that the E. coli Rec A protein (Clarke, 1973) which promotes homologous pairing of double or partially single stranded DNA molecules did not pair where the zone of

homology was 30bp, but was fully efficient when the zone of homology was 151bp.

Various DNA hybridization techniques have shown that within any adenovirus subgroup the homology can be greater than 96%, e.g. adenovirus 3:7 = 99.5%, but across subgroups the homology is approximately 10%-12%, e.g. adenoviruses 2:7 = 10%; adenoviruses 5:7 = 12.8%, (Garon et al., 1973; Fujinaga et al., 1975; Green et al., 1979).

Heterotypic recombination was attempted by co-transfecting DNA terminal overlapping fragments of subgroups B and C into 293 cells and picking resultant plaques. By restriction enzyme analysis, the progeny were shown to be:

(a) Predominantly of subgroup C phenotype

(b) Isolates with an altered restriction enzyme profile mapping in the right hand end of the genome

(c) Of adenovirus 3var100 phenotype in one experiment.

Homotypic recombination between the adenovirus 5 DNA fragments was much higher than that seen between the fragments of adenovirus type 7. This may be the result of the poor infectivity of subgroup B DNA, which even as a DNA-protein complex is not highly infectious. Homotypic recombination between adenovirus 3var100 DNA terminal overlapping fragments is almost as high as that between adenovirus 5 wild type DNA terminal fragments. Data shows that adenovirus 3var100 DNA infectivity on day 4 of transfection is 1 log less than adenovirus type 5, and more infectious than adenovirus 7 wild type DNA which does not show plaque formation until day 7 of transfection. Thus the homotypic recombination between the DNA

fragments of adenovirus 3var100, a subgroup B virus, may be the result of a higher DNA infectivity at day 4.

DNA homology between adenoviruses type 5 and type 7 is approximately 10%, being distributed throughout the genomes.

Since crossovers have been detected in small zones of DNA homology of 21bp and 50bp, it may be reasonable to assume that recombination can occur between the two serotypes and therefore one must explain why no viable progeny were isolated.

During bacteriophage T_4 recombination, branch migration allows crossovers to take place at a distance from the site of interaction. However, branch migration cannot traverse zones of DNA heterology (Singer et al., 1982). Das Gupta and Redding, (1982) have shown in vitro that the branch migration, mediated by the E. coli Rec A protein (Clarke, 1973), between the recombinant DNAs of phages M13 and fd, will be inhibited if there is one base pair mis-match within ten base pairs.

If such limits are functional within the mechanism involved in adenovirus recombination, then the number of heterologous base pairs between serotypes 7 and 5 (9 heterologous bps to 1 homologous bp) would probably inhibit recombination. It may be argued that mis-match base repair would convert zones of heterology to zones of homology. However, there appears to be a limit to the number of base pairs which can be corrected during DNA excision and repair.

In mammalian cells Cleaver, (1974) found that the number of mis-matched nucleotides which could be repaired successfully was 100, although

Hurst et al., (1972) have found evidence to suggest a 700 nucleotide tract within fungi. Miller et al., (1976) have shown that correction of mis-matched base pairs within polyoma DNA during recombination does not exceed 600 nucleotides.

If mis-match repair is limited to between 100 and 700 nucleotides, and the zones of heterology between the two adenoviral serotypes are probably greater than the upper limit above, it may be difficult or impossible for mis-match repair to take place.

Recombinants arising from transfection of heterotypic overlapping terminal DNA fragments would have an ITR from each subgroup; to date no recombinant adenovirus has been isolated containing the ITRs of two different subgroups.

DNA synthesis occurs via a strand displacement mechanism producing one double stranded DNA molecule and one single strand DNA molecule. The ITRs of the single strand molecule allow the formation of a short duplex of DNA ("panhandle structure") joined by a single strand loop. From this intermediate a duplex DNA molecule is synthesised.

The first 50bp from the termini are highly conserved between subgroups, which would allow homologous pairing in this region. The second region shows little conservation between subgroups and is variable in length. It is possible that a panhandle structure could not be formed successfully from a hybrid DNA molecule between subgroups B and C, resulting in the loss of the intermediate used for DNA synthesis.

Hay, (1985a,b) showed the DNA sequences required for the in vivo

replication of adenovirus type 2 lay within the first terminal 45bp. For adenovirus type 4 the first terminal 18bp are required for the initiation of DNA replication; these sequences containing the origin of replication.

Hay and McDougall,(1986) showed that transfection of viral genomes containing a right ITR of adenovirus type 5 and a left ITR of adenovirus type 2 containing internal deletions could produce infectious progeny containing the deleted ITR; however progeny were only recovered when the viral genomes contained a minimum of 45bp from the terminus.

These results suggest that DNA replication can be initiated provided the origin of replication is present in the terminal sequences. A recombinant DNA molecule would have a left hand ITR of 136bp (Subgroup B) and a right hand ITR of 103bp (Subgroup C), each containing the origin of DNA replication. Viral genomes containing an ITR with an internal deletion, but containing the origin of replication, are able to produce viable progeny. This suggests that the difference in ITR base pair size should not be a barrier to DNA replication, and implies that progeny containing heterotypic ITRs should in theory be able to initiate DNA replication.

My results have not shown that the block to recombination is at the DNA level. It is possible that it is at the transcription level.

Heterotypic recombinants would be a chimera of two subgroups containing at least heterologous E1a and E4 regions. Adenovirus gene expression is regulated at multiple levels within an infectious cycle, and it is possible transcription from such a virus described above may be impaired.

The E1a region is expressed first during the infectious cycle (Nevins, 1981) encoding the 32K protein which is responsible for the stimulation of the other early regions.

Williams et al., (1981) showed adenovirus type 12 could complement adenovirus type 5 host range and deletion mutants; thus the E1 region of adenovirus type 12 could substitute the missing functions of adenovirus type 5.

Guilfoyle et al., (1985) showed that recombinant plasmids containing the E2a DBP gene plus the early and late promotors or early promotor alone, required E1a products for full expression. The DBP gene in the presence of the late promotor alone was inhibited by the E1a products.

In vitro transcription from the E4 region can be inhibited by the addition of purified DBP, Handa et al., (1983). A protein-complex between the E4-32K protein and the E1b-55K protein is thought to shut off cellular gene expression during viral infection, Sarnow et al., (1984). Kaufman, (1985) using recombinant plasmids, demonstrated the necessity for having the VA RNA genes in cis or trans plus the tripartite leader sequences expressed by the MLP for efficient translation of mRNAs late in infection.

Leite et al., (1986) demonstrated heterotypic stimulation of plasmids containing the E2a or E3 promotors from adenovirus type 5 fused to the CAT gene, by co-infection with subgroups A, B, D and E. A plasmid containing the E1a of adenovirus 3 wild type could also stimulate the above plasmids. However, when adenovirus 3 E1a and adenovirus 2 E1a were present together the adenovirus 3 E1a region was transdominant causing the inhibition of the plasmids.

These data demonstrate some of the complicated regulatory steps which may function during adenovirus infection, and show that heterotypic stimulation of transcription may be possible.

The progeny isolated from the heterotypic recombination experiments described were variants of adenovirus type 5 containing genetic rearrangements in the E3 region and must have been present on co-transfection. In light of the data (Section B results) that adenovirus 3var100 and adenovirus 3dl101 (which have altered E3 regions) can suppress the DNA synthesis of adenovirus type 5, it may be reasonable to wonder if these variants of adenovirus type 5 (also with altered E3 regions) are able to suppress co-infecting progeny with subgroup B sequences.

Chapter 5.

Future Prospects.

Future Prospects

A. Construction of *in vitro* recombinants between subgroups B and C.

B. Further investigation of adenovirus 3var100 suppression of adenovirus type 5 DNA synthesis.

C. Sequencing of the E1a and E3 lesions of adenovirus 3var100.

D. Protein analysis of adenovirus 3var100.

E. Isolation of revertants from the adenovirus variants.

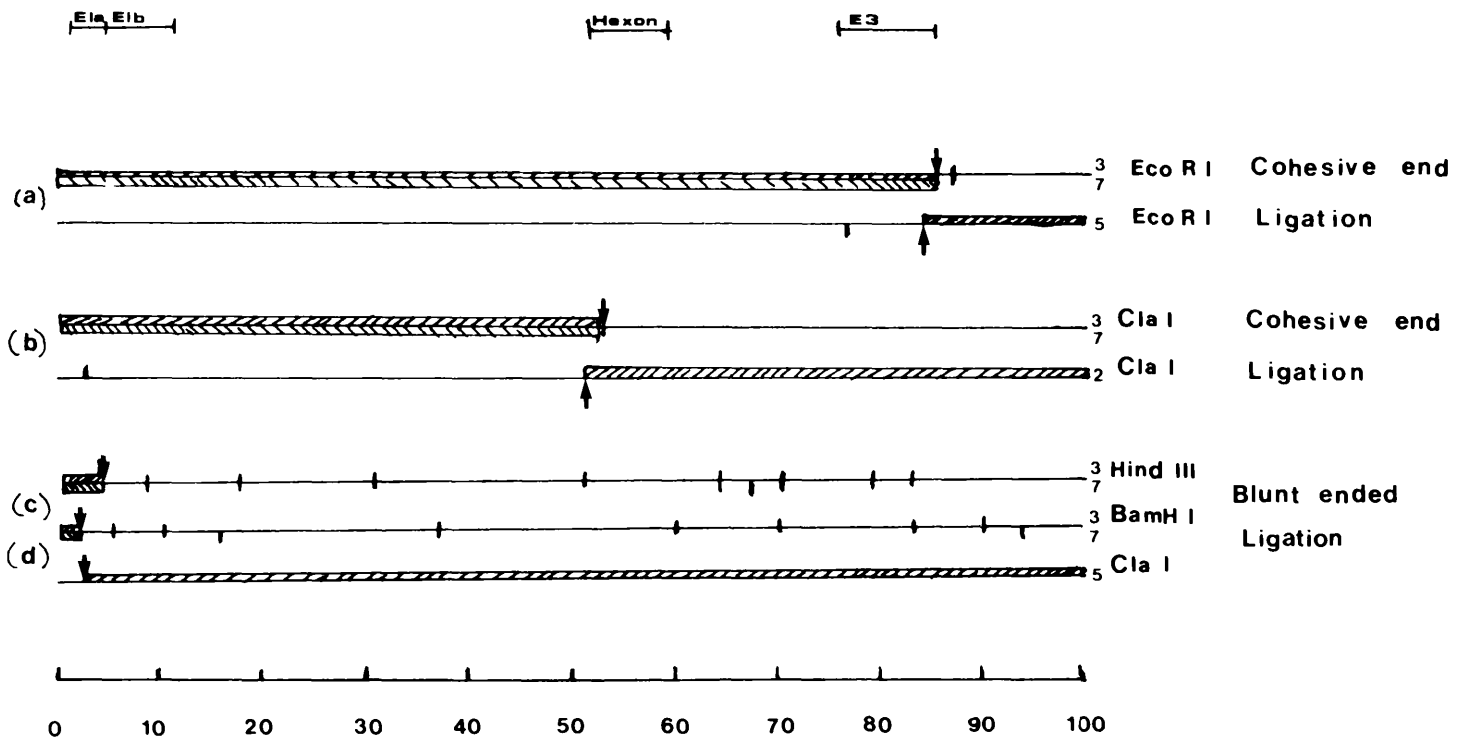

E1a E1b
Hexon
E3
(a)
3
7
Eco R I
Cohesive end
5
Eco R I
Ligation
(b)
3
7
Cla I
Cohesive end
2
Cla I
Ligation
(c)
3
7
Hind III
Blunt ended
(d)
3
7
BamH I
Ligation
5
Cla I
0
10
20
30
40
50
60
70
80
90
100

Figure 50.

Postulation of in vitro recombinants between adenoviruses subgroup B and subgroup C by DNA enzymatic cleavage and re-ligation

Heavy arrows show the sites of cleavage which would give rise to fragments which could be used in ligation. Vertical lines show other restriction enzyme sites within the genome.

(a) EcoR1 cuts adenoviruses 3 and 7 at 85.4 m.u.

⟹ ligate fragments 0-85.4 m.u. + 84 m.u.-100 m.u.

(b) Cla1 cuts adenoviruses 3 and 7 at 52.2 m.u.

Cla1 cuts adenovirus 2 at 51.6 m.u.

⟹ ligate fragments 0-52.2 m.u. + 51.2 m.u.-100 m.u.

(c) HindIII cuts adenoviruses 3 and 7 at 3.8 m.u.

Cla1 cuts adenovirus 5 at 2.6 m.u.

⟹ ligate fragments 0-3.8 m.u. + 2.6 m.u.-100 m.u.

(d) BamH1 cuts adenoviruses 3 and 7 at 2 m.u.

Cla1 cuts adenovirus 5 at 2.6 m.u.

⟹ ligate fragments 0-2 m.u. + 2.6 m.u.-100 m.u.

Future Prospects

A. Construction of in vitro recombinants between subgroups B and C.

It should be possible to construct in vitro recombinants between subgroups B and C by cleavage and religation of full length genomic DNA, followed by transfection into 293 cells. Figure 50 shows representation of four such constructs.

(a) EcoR1 cleaves adenoviruses type 3 or type 7 at 85.4 m.u. yielding a fragment 0-85.4 m.u.; EcoR1 cleaves adenovirus type 5 at 84 m.u. giving a fragment spanning between 84 m.u. - 100 m.u. The ligation site of these two fragments would lie in the non-essential E3 region.

(b) Cleavage of adenoviruses type 3 and type 7 with Cla1 gives a fragment spanning between 0-52.2 m.u.; while cleavage of adenovirus type 2 with the same enzyme yields a fragment of map co-ordinates 51.2 m.u. - 100 m.u. The 51.2 m.u. site within adenovirus type 2 lies to the left of the hexon gene; therefore recombinants would have an adenovirus type 2 hexon.

(c) The restriction enzyme Cla1 cleaves adenovirus type 5 at map position 2.6 to give a fragment spanning between 2.6 m.u - 100 m.u. This results in the loss of the E1a TATA box and the start site for transcription. HindIII cleaves adenoviruses type 3 and type 7 at map position 3.8 to give a fragment spanning between 0-3.8 m.u. This fragment retains the TATA box, the initiation site for transcription and the information for the 13S, 12S and 9S transcripts but not the poly-adenylation signal. On ligation of these fragments it may be possible

to have a construct containing the initiation and transcription information of adenovirus type 3 E1a and also E1a transcriptional information of adenovirus type 5, including the polyadenylation signal for correct splicing of transcripts before polyadenylation.

(d) The restriction enzyme BamH1 cleaves adenoviruses type 3 and type 7 at map position 2 giving a fragment spanning between 0-2 m.u. This results in the loss of most of the transcriptional information for products 12S and 13S, but the retention of the TATA box and the transcriptional initiation site. On ligation of the adenovirus type 5 fragment described above, possible constructs would be similar to those previously described, except that most of the transcriptional information from adenovirus type 3 would be lost.

B. Further investigation of adenovirus 3var100 suppression of adenovirus type 5 DNA synthesis.

Recently the expression of the chloramphenicol acetyl transferase gene under the control of adenoviral promotors has been used to look at cis-acting and trans acting dominance by the early adenoviral regions (Leite et al., 1986; Glenn and Ricciardi, 1987). It may be possible to exploit this method to study the suppression of adenovirus type 5 DNA synthesis by adenovirus 3var100 and adenovirus 3dl101. The E3 regions of both adenovirus 3 wild type and adenovirus 3var100 could be cloned as expression plasmids. These recombinant plasmids could be transfected into Hela cells along with plasmid constructs containing different adenovirus type 5 early promotors fused to the CAT gene. By assaying the level of CAT activity it would be possible to detect suppression by the E3 products.

C. Sequencing of E1a and E3 lesions of adenovirus 3var100

The cloning of adenovirus 3var100 DNA containing the E1a and E3 regions would be desirable for sequencing.

Since the adenovirus 3 wild type BamH1 H fragment (2-5) m.u. has been shown to marker rescue the E1a lesion of adenovirus 3var100 it would be reasonable to clone out this fragment from the variant.

It is possible the E1a lesion maps in the gene coding for the protein analogous to that from the 289 amino acid residue of adenovirus type 5. The proteins translated from the 13S and 12S mRNAs of adenovirus 7 wild type contain 261 and 230 amino acids respectively. Due to the high homology between adenoviruses type 3 and type 7 it would be reasonable to assume that this is also true of adenovirus type 3.

The E3 lesion could be cloned out as a HindIII-EcoR1 fragment of co-ordinates 78.8 m.u. - 87 m.u. By sequencing across the join of the deletion and beyond, it would be possible to predict if there was a reading frame which would code for a 19K + 15.2K fusion product.

D. Protein analysis of adenovirus 3var100

Comparison of the protein profile of adenovirus 3var100 may show if any proteins were altered relative to the wild type and could be carried out in conjunction with the sequencing strategy.

E. Isolation of revertants from the adenovirus variants

If the E1a lesion is a base pair change, then one should be able to isolate revertant progeny of adenovirus 3var100 and adenovirus 3hr102 on Hela cell monolayers.

Revertants of adenovirus 3var100 should behave like adenovirus 3dl101 in that they would be no longer host range for growth in Hela cells and would be able to suppress the DNA synthesis of adenovirus type 5.

Revertants of adenovirus 3hr102 should behave like adenovirus type 3 in that they would be no longer host range for growth in Hela cells and would be able to suppress adenovirus 3var100 DNA synthesis.

Sequencing of these revertants would be desirable to show that the reverted phenotype was the result of a reversion to wild type DNA sequence.

References

REFERENCES

Akusjärvi, G., and Pettersson, U., (1979). Sequence analysis of adenovirus DNA : Complete nucleotide sequence of the spliced 5' noncoding region of adenovirus 2 hexon messenger RNA. Cell 16 : 841-850.

Akusjärvi, G., Mathews, M. B., Anderson, P., Vennström, B., and Pettersson, U., (1980). Structure of genes for virus-associated RNA 1 and RNA 2 of adenovirus type 3. Proc. Natl. Acad. Sci. (USA). 77 : 2424-2428.

Akusjärvi, G., Zabielski, J., Perricaudet, M., and Pettersson, U., (1981). The sequence of the 3' non-coding region of the hexon mRNA discloses a viral adenovirus gene. Nucleic Acids Res. 9 : 1-17.

Alestrom, P., Stenlund, A., Li, P., Bellett, A., and Pettersson, U., (1982). A common sequence in the inverted terminal repetitions of human and avian adenoviruses. Gene 18 : 193-197.

Anderson, C.W., Baum, P. R., and Gesteland, R. F., (1973). Processing of adenovirus-2-induced proteins. J. Virol. 12 : 241-252.

Arrand, J. E., (1978). Mapping of adenovirus type 5 temperature-sensitive mutations by marker rescue in enhanced double DNA infections. J. Gen. Virol. 41 : 573-586.

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

Axelrod, N., (1978). Phosphoproteins of Adenovirus 2. Virology 87 : 366- 383.

Babich, A., and Nevins, J. R., (1981). The stability of early adenovirus mRNA is controlled by the viral 72Kd DNA-binding protein. Cell 26 : 371-379.

Babiss, L. E., Young, C. S. H., Fisher, P. B., and Ginsberg, H. S., (1983). Expression of adenovirus E1a and E1b gene products and the Esherichia coli XGPRT gene in KB cells. J. Virol. 46 : 454-465

Babiss, L. E., Fisher, P. B., and Ginsberg, H. S., (1984). Deletion and insertion mutations in early region 1a of type 5 adenovirus producing cold sensitive or defective phenotypes for transformation. J. Virol. 49 : 731-740.

Babiss, L. E., and Ginsberg, H. S., (1984). Adenovirus type 5 early region 1b gene product is required for efficient shut off of host protein synthesis. J. Virol. 50 : 202-212.

Baker, C. C., Herisse, J., Courtois, G., Gallibert, F., and Ziff, E., (1979). Messenger RNA for the Ad2 DNA binding protein : DNA sequences encoding the first leader and heterogeneity at the mRNA 5' end. Cell 18 : 569-580.

Baxendal, W., (1978). Egg drop syndrome 76'. Vet. Rec. 102 : 285-286

Bégin, M., and Weber, J., (1975). Genetic analysis of adenovirus type 2. 1. Isolation and genetic characterisation of temperature-sensitive mutants. J. Virol 15 : 1-7.

Berge, T. O., England, B., Mauris, C., (1955). Etiology of acute respiratory disease among service personnel at Fort Ord, California. Am. J. Hyg. 62 : 283-294.

Berget, S. M., Moore, C., and Sharp, P. A., (1977). Spliced segments at the 5' terminus of adenovirus 2 late mRNA. Proc. Natl. Acad. Sci. (U.S.A.) 74 : 3171-3175.

Berk, A. J., and Sharp, P.A., (1977). Sizing and mapping of early adenovirus mRNAs by gel electrophoresis of S1 endonuclease digested hybrids. Cell 12 : 721-732.

Berk, A. J., and Sharp, P. A., (1978). Structure of adenovirus 2 early mRNAs. Cell 14 : 695-711.

Berk, A. J., Lee, F., Harrison, T., Williams, J., and Sharp, P.A., (1979) Pre-early adenovirus 5 gene products regulate transcription and processing of early viral messenger RNAs. Cell 17 : 935-944.

Berkner, K., and Sharp., P., (1983). Generation of adenoviruses transfection of plasmids. Nucleic Acids Res. 11 : 6003-6020.

Bhargawa, P. M., and Shanmugam, G., (1971). Uptake of nonviral nucleic acid by mammalian cells. Prog. Nucl. Acid Res. 11 : 103-192.

Bhatti, A. R., and Weber, J., (1979). Protease of adenovirus type 2 : partial characterisation. Virology 96 : 478-485.

Binger, M. H., Flint, S. J., Rekosh, D., (1982). Expression of the gene encoding the adenovirus DNA terminal protein precursor in productively infected cells. J. Virol. 42 : 488-501.

Bos., J. C., Polder, L. J., Bernards, R., Schrier, P. I., van den Elsen, P. J., van der Eb., A. J., van Ormondt, H., (1981). The 2.2Kb E1b mRNA of human adenovirus 12 and adenovirus 5 codes for two tumour antigens starting at different AUG triplets. Cell 27 : 121-131.

Boursnell, M. E. G., and Mautner, V., (1981). In vitro construction of a recombinant of adenovirus Ad2 : Ad5. Gene 13 : 311-317.

Brackmann, K. H., Green, M., Wold, W. S. M., Cartas, M., Matsuo, T.J. and Hashimoto, S., (1980). Identification and peptide mapping of human adenovirus type 2 - induced early polypeptides isolated by two dimension gel electrophoresis. J. Biol. Chem. 255 : 6772.

Brandt, C. D., Kim, H. W., Yolken, R. H., Kapikian, A. Z., Arrobio, J. O., Rodriguez, W. J., Wyatt, R. G., Chanock, R. M., and Parrot, R. H., (1979). Comparative epidemiology of two rotavirus serotypes and other viral agents associated with pediatric gastroenteritis. Am. J. Epidemiol. 110 : 243-254.

Brown, D. T., Westphal, M., Burlingham, B. T., Winterhoff, U., and Doerfler, (1975). Structure and composition of the adenovirus

type 2 core. J. Virology. 16 : 366-387.

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

Bürki, F., Hinaidy, B., and Böckmann, J., (1979). Bovine adenoviruses. III Subclassification into two subgroups by means of immunodiffusion-tests. Microbiologica 2: 65 -

Burnett, J. P., and Harrington, J. A., (1968). Simian adenovirus SA7 DNA : Chemical, physical and biological studies. Proc. Natl. Acad. Sci. (U.S.A.) 60 : 1023-1029.

Busby, D. W. G., House, W., and MacDonald, J. R., (1964). In :"Virological Techniques", Churchill, London.

Byrd, P. J., Chia, W., Rigby, P. W. J., Gallimore, P. H., (1982). Cloning of DNA fragments from the left end of the adenovirus type 12 genome : transformation by cloned early region 1. J. Gen. Virol. 60 : 279-293.

Carlock, L. R., and Jones, N. C., (1981). Tranformation - defective mutants of adenovirus type 5 containing a single altered E1a mRNA species. J. Virol. 40 : 657-664.

Castro, B. C., Atchison, R. W., and Hammon, W. McD., (1967). Studies on the relationship between Adeno- Associated - Virus Type 1 (AAV-1) Virology 32 : 52-59.

Celma, M. L., Pan, J., and Weissman, S., (1977a) Studies of low molecular weight RNA from cells infected with adenovirus 2.1. The sequence at the 3' end of VA-RNA 1. J. Biol.Chem. 252 : 9032-9042.

Celma, M. L., Pan, J., and Weissman, S., (1977b). Studies of low molecular weight RNA from cells infected with adenovirus 2.II. Heterogeneity at the 5' end of VA-RNA 1. J. Biol. Chem. 252 : 9043-

9054.

Challberg, M. D., Desiderio, S. V., and Kelly, T. J. Jr., (1980). Adenovirus DNA replication *in vitro* : Characterisation of a protein covalently linked to nascent DNA strands. Proc. Natl. Acad. Sci. (U.S.A.). 77 : 5105-5109.

Challberg, M. D., and Kelly, T. J. Jr., (1981). Processing of the adenovirus terminal protein. J. Virol. 38 : 272-277.

Challberg, S. S., and Ketner, G., (1981). Deletion mutants of adenovirus 2 : Isolation and initial characterisation of virus carrying mutations near the right end of the viral genome. Virology 114 : 196-209.

Chardonnet, Y., and Dales, S., (1970). Early events in the interaction between Adenoviruses and Hela cells. II. Penetration of type 5 and intracellular release of the DNA genome. Virology 40 : 462-477.

Chen, T. R., (1977). In situ detection of mycoplasma contamination in cell cultures by Fluorescent Hoechst 33258 stain. Experimental Cell Research 104 : 255.

Chin, W. W., and Maizel, J. V., (1976). The polypeptides of adenovirus. VII Further studies of early polypeptides *in vivo* and localisation of E2 and E2A to the cell plasma membrane. Virology 7 : 518-530.

Chinnadurai, G., Chinnadurai, S., and Green, M., (1978). Enhanced infectivity of adenovirus DNA and a DNA-protein complex. J. Virol. 26 : 195-199.

Chinnadurai, G., Chinnadurai, S., and Brusca, J., (1979). Physical mapping of a large-plaque mutation of adenovirus type 2. J. Virol. 32 : 623-628.

Chinnadurai, G., (1983). Adenovirus $21p^+$ locus codes for a 19Kd tumour antigen that plays an essential role in cell transformation. Cell 33 : 759-766.

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

Chow, L., Lewis, J. B., and Broker, T., (1979b). RNA transcription and splicing at early and intermediate times after adenovirus - 2 infection. Cold Spring Harbor Symp. Quant. Biol. 44 : 401-414.

Chow, L. T., Roberts, J. M., Lewis, J. B., Broker, T. R., (1977). A map of cytoplasmic RNA transcripts from lytic adenovirus type 2 determined by electron microscopy of RNA-DNA hybrids. Cell 11 : 819-836.

Cladaras, C., and Wold, W., S., M., (1985). DNA sequence of early E3 transcription unit of adenovirus 5. Virology 140 : 28-43.

Clarke, A. J., (1973). Recombination deficient mutants of E. coli and other bacteria. Ann. Rev. Genetics 7 : 67.

Cleaver, J. E., (1974). Advances in Radiation Biology. eds. Lett, J. T., Adler, H., and Zelle, M. (Academic Press, New York) Vol 4 : 1-75.

Clewel, D. B., and Helinski, D. R., (1970). Properties of a supercoiled DNA-protein relaxation complex and strand specificity of the relaxation event. Biochem. 9 : 4428-4440.

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

Corden, J., Engelking, H. M., and Pearson, G. D., (1976). Chromatin-like organisation of the adenovirus chromosome. Proc. Natl. Acad. Sci. (U.S.A.) 73 : 401-404.

Cornick, G., Sigler, P. B., and Ginsberg, H. S., (1971). Characterisation of crystals of adenovirus type 5 hexon. J. Mol. Biol. 57 : 397-401

Cornick, G., Sigler, P. B., and Ginsberg, H. S., (1973). Mass of protein

in the asymmetric unit of hexon crystals - a new method. J. Mol. Biol. 73 : 533-537.

Dales, S., (1962). An electron microscopic study of the early association between two mammalian viruses and their hosts. J. Cell Biol. 13 : 309-

Dales, S., and Silverberg, H., (1968). Controlled double infection with unrelated animal viruses. Virology 34 :531-543.

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

Danna, K. J., Sack, G. H., Jr., and Nathans, D., (1973). A cleavage map of the SV40 genome. J. Mol. Biol. 78 : 363-376.

Darbyshire, J. H., Dawson, P. S., Lamont, P. H., Ostler, D. C., and Pereira, H. G., (1965). A new adenovirus serotype of bovine origin. J. Comp. Path. 75 : 327.

Das Gupta, C., and Radding, C. M., (1982). Polar branch migration promoted by recA protein : Effect of mismatched base pairs. Proc. Natl. Acad. Sci. (U.S.A.). 79 : 762-766.

de Jong, J. C., Wigand, R., Waddell, G., Keller, D., Muzerie, C. J., Wermenbol, A. G., and Schaap, G. J., (1981). Adenovirus 37 : identification and characterisation of a medically important new adenovirus type of subgroup D. J. Med. Virol. 7 : 105.

de Jong, J. C., Wigand, R., Kidd, A. H., Wadell, G., Kapsenberg, J. G., Muzerie, C. J., Wermenbol, A. G., Firtzlaff, R. G., (1983). Candidate adenovirus 40 and 41 : fastidious adenovirus from human infantile stool. J. Med. Virol. 11 :215-231.

Delsert, C., and D'Halliun, J-C., (1984). Genetic expression of human adenovirus in simian cells. Evidence for interserotypic inhibition of viral DNA synthesis. Virus Research 1 : 365.

Denhardt, D. T., (1966). A membrane filter technique for the detection of complementary DNA. Biochem Biophys. Res. Commun. 23 : 641-646.

Desiderio, S. V., and Kelly, T. J. (Jr.), (1981). The structure of the linkage between adenovirus DNA and the 55,000 dalton terminal protein. J. Mol. Biol. 145 : 319-337.

Devaux, C., Zulauf, M., Boulanger, P., and Jacrot, B., (1982). Molecular weight of adenovirus serotype 2 capsomers : A new characterisation. J. Mol. Biol. 156 : 927-939.

Devaux, C., Timmins, P. A., and Berthet-Colominas, C., (1983). Structural studies of adenovirus type 2 by neutron and X-ray scattering. J. Mol. Biol. 167 : 119-132.

D'Halluin , J-C., Milleville, M., Martin, G. R., and Boulanger, P., (1980). Morphogenesis of human adenovirus type 2 studied with fibre and penton base defective temperature-sensitive mutants. J. Virol. 33 : 88-99.

D'Halluin , J-C., Cousin, C., and Boulanger, P., (1982). Physical mapping of adenovirus type 2 temperature sensitive mutations by restriction endonuclease analysis of interserotypic recombinants. J. Virol. 41 : 401-413.

D'Halluin, J-C., Milleville, M., and Boulanger, P. A., (1983). Restriction maps of human adenovirus types 2, 5 and 3 for Bcl 1, Cla 1, Pvu 1 and Sph 1. Gene 21 : 165-169.

Dykema, R., and Dekker, B. M. M., (1979). The inverted terminal repetition of the DNA of the weakly oncogenic adenovirus type 7. Gene 8 : 7-15.

Dressler, D., and Potter, H., (1982). Molecular mechanisms in genetic recombination. Ann. Rev. Biochem. 51 : 727-761.

Dunn, A. R., and Hassell, J. A., (1977). A novel method to map transcripts : Evidence for homology between an adenovirus mRNA and discrete multiple regions of the viral genome. Cell 12 : 23-36.

Ellens, D., J., Sussenbach, J. S., and Jansz, H. S., (1974). Studies

on the mechanism of replication of adenovirus DNA. III. Electron microscopy of replicating DNA. Virology 61 : 427-442.

Engler, J. A., and Kilpatrick, B. A., (1981). Cloning and characterisation of class B adenoviruses Ad.3 and Ad.7. Gene 13 : 125-132.

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

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

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

Evans, R. M., Fraser, N., Ziff, E., Weber, J., Wilson, M., and Darnell, J. E., (1977). The initiation sites for RNA transcription in Ad.2 DNA. Cell 12 : 733-739.

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

Everitt, E., and Philipson, L., (1974). Structural proteins of adenoviruses. XI. Purification of three low molecular weight virion proteins of adenovirus type 2 and their sunthesis during productive infection. Virology 62 : 253-269.

Ezoe, H., and Mak, S., (1974). Comparative studies on functions of human adenovirus type 12 and its low oncogenic mutant virions. J. Virol. 14 : 733-739.

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

Ferguson, B., Jones, N., Richter, J., and Rosenberg, M., (1984). Adenovirus E1a gene product expressed at high levels in Escherichia coli is functional. Science 224 : 1343-1346.

Flewett, T. H., Bryden, A. S., Davies, H., and Morris, C. A., (1975). Epidemic viral enteritis in a long-stay children's ward. Lanet 1 : 4-5.

Flint, S. J., Werwerka-Lutz, Y., Levine, A. S., Sambrook, J., and Sharp, P. A., (1975). Adenovirus transcription. II. RNA sequences complementary to simian virus 40 and adenovirus 2 DNA in Ad.2^{+}ND$_{1}$ and Ad.2^{+}ND$_{3}$-infected cells. J. Virol. 16 : 662-673.

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

Flint, S. J., (1982). Expression of adenoviral genetic information in productively infected cells. Biochem. Biophys. Acta. 651 : 175-208.

Fowlkes, D. M., Lord, S. T., Linné, T., Pettersson, U., and Philipson, L., (1979). Interaction between the adenovirus DNA-binding protein and double stranded DNA. J. Mol. Biol. 132 : 163-180.

Franklin, R. M., Pettersson, U., Akervall, K., Strandberg, B., and Philipson, L., (1971). Structural proteins of adenoviruses. V. On the size and structure of the adenovirus type 2 hexon. J. Mol. Biol. 57 : 383-395.

Freeman, A. E., Black, P. H., Vanderpool, E. A., Henry, P. H., Austin, J. B., and Huebner, R. J., (1967). Transformation of primary rat

embryo cells of adenovirus type 2. Proc. Natl. Acad. Sci. (U.S.A.) 58 : 1205-1212.

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

Fujinaga, K., Sekikawa, K., Yamazaki, H., (1975). Method for determination of nucleotide sequence homology between viral genomes by DNA reassociation kinetics. J. Virol. 15 : 466-470.

Fukui, Y, Saito, I., Shiroki, K., and Shimojo, H., (1984). Isolation of transformation-defective replication non-defective, early region 1b mutants of adenovirus 12. J. Virol. 49 : 154-161.

Fütterer, J., and Winnaker, E-L., (1984). Adenovirus DNA replication. Current Topics in Microbiology and Immunology Vol. III : The Molecular Biology of Adenoviruses 3. (Ed. W. Doerfler) pp 41-64 Springer-Verlag, Berlin, Heidelberg.

Gallimore, P. H., Sharp, P. A., and Sambrook, J., (1974). Viral DNA in transformed cells. III. A study of the sequences of adenovirus 2 DNA in nine cell lines of transformed rat cells using specific fragments of the viral genome. J. Mol. Biol. 89 : 49-71.

Galos, R. S., Williams, J., Shenk, T., and Jones, N., (1980). Physical location of host range mutations of adenovirus type 5 : Deletion and marker rescue mapping. Virology 104 : 510-513.

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

Galos, R., and Williams, J. F., (1980). Summary of locations of ts mutations. Distributed at EMBO Workshop on adenoviruses. Peebles, Scotland. June 1980.

Gambke, C., and Deppert, W., (1983). Specific complex of the late nonstructural 100,000-dalton protein and newly synthesised hexon in adenovirus type-2 infected cells. Virology 124 : 1-12.

Garon, C. F., Berry, K. W., and Rose, J. A., (1972). A unique form of terminal redundancy in adenovirus DNA molecules. Proc. Natl. Acad. Sci. (U.S.A.). 69 : 2391-2395.

Garon, C. F., Berry, K. W., Hierholzer, J. C., and Rose, J. A., (1973). Mapping of base sequence heterologues between genomes of different adenovirus serotypes. Virology 54 : 414-426.

Garon, C. F., Parre, R. P., Padmanabhan, R., Roninson, I., Garrison, J. W., Rose, J. A., (1982). Structural characterisation of the adenovirus-18 inverted terminal repetition. Virology 121 : 230-239.

Gey, G. O., Coffman, W. D., Kubicek, M. T., (1952). Tissue culture of the proliferative capacity of cervical carcinoma and normal epithelium. Cancer Res. 12 : 264-265.

Gingeras, T. R., Sciaky, D., Gelinas, R. E., Bing-Dong, J., Yen, C. E., Kelly, M. M., Bullock, P. A., Parsons, B. L., O'Neill, K. E., and Roberts, R. J., (1982). Nucleotide sequences from adenovirus 2 genome. J. Biol. Chem. 257 : 13,375-13,491.

Ginsberg, H. S., Ensinger, M. J., Kauffman, R. S., Mayer, A. J., and Lundholm, U., (1974). Cell transformation : a study of regulation with types 5 and 12 adenovirus temperature-sensitive mutants. Cold Spring Harbor Symp. Quant. Biol. 39 : 419-426.

Girardi, A. S., Hilleman, M. R., and Zwickey, R. E., (1964). Tests in hamsters for the oncogenicity of ordinary viruses including adeno-virus type 7. Proc. Soc. Exp. Biol. Med. 115 : 1141-1150.

Glenn, G., and Ricciardi, R. P., (1937). An adenovirus type 5 E1a protein with a single amino acid substitution blocks wild type E1a transcription. Mol. and Cellular Biol. 7 : 1004-1011.

Gonda, D. K., and Radding, C. M. (1983). By searching processively RecA protein pairs DNA molecules that share a limited stretch of homology. Cell 34 : 647-654.

Graham, F. L., and van der Eb, A. J., (1973). A new technique for the assay of infectivity of human adenovirus DNA. Virology 52 : 456-467.

Graham, F. L., Abrahams, P. J., Mulder, G., Heijneker, H. L., Warnaar, S. O., De Vries, F. A. J., Fiers, W., and van der Eb., A. J., (1974). Studies on in vitro transformation by DNA and DNA fragments of human adenoviruses and simian virus 40. Cold Spring Harbor Symp. Quant. Biol. 39 : 637.

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

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

Green, M., Pina, M., Kemes, R. C., Wensick, P. C., MacHattie, L. A., Thomas, C. A., Jr., (1967). Adenovirus DNA. I. Molecular weight and conformation. Proc. Natl. Acad. Sci. (U.S.A.). 57 : 1302-1309.

Green, M., MacKay, J. K., Wold, W. S. M., Rigden, P., (1979a). Thirty-one human adenovirus serotypes (Ad1-Ad31) form five groups (A--E) upon DNA genome homologues. Virology 93 : 481-492.

Green, M., Wold, W. S. M., Brackmann, K., and Cartas, M. A., (1979b). Studies on Early Proteins and Transformation Proteins of Human Adenoviruses. Cold Spring Harbor Symp. Quant. Biol. 44 : 457-469.

Green, N. M., Wrigley, N. G., Russell, W. C., Martin, S. R., and McLachlan, A. D., (1983). Evidence for a repeating cross 3 sheet structure in the adenovirus fibre. EMBO J. 2 : 1357-1365.

Grodzicker, T., Williams, J., Sharp, P. A., and Sambrook, J., (1974). Physical mapping of temperature sensitive mutations of adenoviruses. Cold Spring Harbour Symp. Quant. Biol. 39 : 439-446.

Groff D. E., and Danniell, E., (1980). Isolation and characterisation of a viable deletion mutant of Adenovirus 3. Virology 106 : 191-194.

Gronenborn, B., and Messing, J., (1978). Methylation of single stranded DNA in vitro introduces new restriction endonuclease cleavage sites. Nature, 272 : 375-377.

Guilfoyle, R. A., Osheroff, W. P. and Rossini, M., (1985). Two functions encoded by adenovirus early region 1A are responsible for the activation and repression of the DNA-binding protein gene. EMBO. J. 4 : 707-713.

Gütter, M., and Franklin, R. M., (1974). Studies on the molecular weight of the adenovirus type 2 hexon and its subunit. J. Mol. Biol. 89 : 163-178.

Halbert, D. N., Cutt, J. R., And Shenk, T., (1985). Adenovirus early region 4 encodes functions required for efficient DNA replication, late gene expression and host cell shut-off. J. Virology 56 : 250-257.

Hanahan, D., (1983). Studies on transformation of E. coli with plasmids. J. Mol. Biol. 166 : 557-580.

Handa, H., Kingston, R. E., and Sharp, P. A., (1983). Inhibition of adenovirus early region. IV. Transcription in vitro by a purified viral DNA binding protein. Nature 302 : 545-547.

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

Harter, M. L., Shanmugam, G., Wold, W. S., and Green, M., (1976). Deletion of adenovirus type 2 induced Early polypeptides using

Cycloheximide Pretreatment to Enhance Viral Protein Synthesis. J. Virol. 19 : 232-242.

Harter, M. L., and Lewis, J. B., (1978). Adenovirus type 2 early proteins synthesised in vitro and in vivo. Identification in infected cells of the 38,000-50,000 molecular weight protein encoded by the left end of adenovirus type 2 genome. J. Virol. 26 : 736-749.

Hartley, J. W., and Rowe, W. P. (1960). A new mouse virus apparently related to the adenovirus group. Virology 11 : 645-647.

Hassell, J. A., and Weber, J., (1978). Genetic analysis of adenovirus type 2. VIII. Physical locations of temperature-sensitive mutations. J. Virol. 28 : 671-678.

Hay, R. T., Stow, N. D., and McDougall, I. M., (1984). Replication of adenovirus mini-chromosomes. J. Mol. Biol. 175 : 493-510.

Hay, R. T., (1985a). The origin of adenovirus DNA replications : Minimal DNA sequence requirement in vivo. EMBO J. 4 : 421-426.

Hay, R. T., (1985b). Origin of adenovirus DNA replication : Role of nuclear factor 1 binding site in vivo. J. Mol. Biol. 186 : 129-136.

Hay, R. T., and McDougall, I. M., (1986). Viable viruses with deletions in the left inverted terminal repeat define the adenovirus origin of DNA replication. J. Gen. Virol. 67 : 321-332.

Hierholzer, J. C., (1973). Further subgrouping of the human adenoviruses by differential hemagglutination. J. Infect. Dis. 128 : 541-550.

Hilleman, M. R., and Werner, J. H., (1954). Recovery of a new agent from patients with acute respiratory illness. Proc. Soc. Exp. Biol. Med. 85 : 183-188.

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

Huebner, R. J., Rowe, W. R., Ward, T. G., Parrott, R. H., and Bell, J. A.,

(1954). Adenoidal-pharyngeal-conjunctival agents : A newly recognized group of common viruses of the respiratory system. N. Engl. J. Med. 251 : 1077-1086.

Huebner, R. J., Rowe, W. P., and Lane, W. T., (1962). Oncogenic effects in hamsters of human adenoviruses type 12 and 18. Proc. Natl. Acad. Sci. (U.S.A.) 48 : 2051-2058.

Huebner, R. J., Rowe, W. P., Turner, H. C., and Lane, W. T., (1963). Specific adenovirus complement-fixing antigens in virus-free hamster and rat tumours. Proc. Natl. Acad. Sci. (U.S.A.) 50 : 379-389.

Huebner, R. J., Casey, M. J., Chanock, R. M., Schell, K., (1965). Tumours induced in hamsters by a strain of adenovirus type 3. Sharing of tumour antigens and "neoantigens" with those produced by adenovirus type 1 tumours. Proc. Natl. Acad. Sci. (U.S.A.) 54 : 381.

Huebner, R. J., (1967). Adenovirus-directed tumour and T-antigens in : Perspectives in Virology, Vol 5 (M. Pollard, ed.)., pp 147-166, Academic Press, New York.

Hull, R. N., Minner, J. R., and Smith, J. W., (1956). New viral agents recovered from tissue cultures of monkey kidney cells. Am. J. Hyg. 63 : 204-215.

Hurst, P. D., Fogel, F., and Mortimer, R. K., (1972). Conversion associated recombination in yeast. Proc. Natl. Acad. Sci. (U.S.A.) 69 : 101-105.

Ho. Y. S., Galos, R., and Williams, J., (1982). Isolation of type 5 adenovirus mutants with a cold sensitive host range phenotype : Genetic evidence of an adenovirus transformation maintainance function. Virology 122 : 109-124.

Holliday, R., (1964). A mechanism for gene conversion in fungi. Genetic Res. 5 : 282-304.

Holliday, R., (1968). In Replication and Recombination of Genetic Material, ed. W. Peacock, R. Brock, pp 157-74, Canberra, Australian Acad. Sci.

Holliday, R., (1974). Molecular Aspects of Genetic Exchange and Gene Conversion. Genetics, 78 : 273-85.

Holmes, D. S., and Quigley, M., (1981). A rapid boiling method for the preparation of bacterial plasmids. Anal. Biochem. 114 : 193.

Horwitz, M. S., Maizel, J. V., Scharff, M. D., (1970). Molecular weight of adenovirus type 2 hexon polypeptide. J. Virol 6 : 569-571.

Houweling, A., van den Elsen, P. J., and van der Eb., A. J., (1980). Partial transformation of primary rat cells by the leftmost 4.5% fragment of adenovirus 5 DNA. Virology 105 : 537-550.

Ikeda, J-E., Enomoto, T., and Hurwitz, J., (1982). Adenoviral protein-primed initiation of DNA chains in vitro. Proc. Natl. Acad. Sci. (U.S.A.) 79 : 2442-2446.

Ishibashi, M., and Maizel, J. V., (1974). The polypeptides of adenovirus. V. Young virions, structural intermediates between top components and mature virions. Virology 57 : 409-424.

Jeng, J. H., Wold, W.S.M., Sugawara, K., Gilead, Z., and Green, M., (1977). Adenovirus type 2 coded single-stranded DNA-binding protein : In vivo phosphorylation and modification. J. Virol, 22 : 402-411.

Jeng, Y. H., Wold, W. S. M., Sugawara, K., and Green, M., (1978). Evidence for an adenovirus type 2-coded early glycoprotein. J. Virol. 28 : 314-323.

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

Jones, N. C., and Shenk, T., (1979a). An adenovirus type 5 early gene function regulates expression of other early viral genes. Proc. Natl. Acad. Sci. (U.S.A.) 76 : 3665-3669.

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

Jörnvall, H., Pettersson, U., and Philipson, L., (1974). Structural studies of adenovirus type 2 hexon protein. Eur. J. Biochem. 48 : 179-192.

Jörnvall, H., Akusjärvi, G., Alström, P., von Bahr-Lindström, H., Pettersson, U., Apella, E., Fowler, A., Philipson, L., (1981). The adenovirus hexon protein : the primary structure of the polypeptide and its correlation with hexon gene. J. Biol. Chem. 256 : 6181-6186.

Kalter, S. S., Ablashi, D., Espana, C., Heberling, R. L., Hull, R. N., Lennette, E. H., Malherbe, H. H., McConnel, S., and Yohn, D., (1980). Simian virus nomenclature 1980. Intervirology, 13 : 317-330.

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

Kauffman, R. S. and Ginsberg, H. S., (1976). Characterisation of a temperature-sensitive hexon transport mutant of type 5 adenovirus. J. Virol. 19 : 643-658.

Kaufman, R. J., (1985). Identification of the components necessary for adenovirus translational control and their utilisation in cDNA expression vectors. Proc. Natl. Acad. Sci. (U.S.A.). 82 : 689-693.

Kawai, S., and Nishazawa, M., (1984). New procedure for DNA transfection with polycation and dimethyl sulphoxide. Mol. and Cellular Biol. 4 : 1172-1174.

Kawamura, H., Shimizu, F., and Tsubahara, H., (1964). Avian adenovirus : Its properties and serological classification. Natl. Inst. Anim. Health Q. 4 : 183-193.

Kitchingmann, G. R., Lai, S. P., and Westphal, H., (1977). Loop structures in hybrids of early RNA and the separated strands of adenovirus DNA. Proc. Natl. Acad. Sci. (U.S.A.) 74 : 4392-4395.

Kitchingmann, G. R., and Westphal, H., (1980). The structure of adenovirus 2 early nuclear and cytoplasmic RNAs. J. Mol. Biol. 137 : 23-48.

Klein, M., Earley, E., and Zellat, J., (1959). Isolation from cattle of a virus related to human adenovirus. Proc. Soc. Exp. Biol. Med. 102 : 1-4.

Klein, H., Maltzman, W., and Levine, A. J., (1979). Structure-function relationships of the adenovirus DNA-binding protein. J. Biol. Chem. 254 : 11051-11060.

Klessig, D. F., (1977). Two adenovirus mRNAs have a common 5' terminal leader sequence encoded at least 10Kb upstream from their main coding regions. Cell, 12 : 9-21.

Klessig, D. F., and Grodzicker, T., (1979). Mutations that allow human ad.2 and ad.5 to express late genes in monkey cells map in the viral gene encoding the 72K DNA-binding protein. Cell, 17 : 957-966.

Kruijer, W., van Schaik, F. M. A., and Sussenbach, J. S., (1980). Nucleotide sequence analysis of a region of adenovirus 5 DNA encoding a hitherto unidentified gene. Nucleic Acids Res. 8 : 6033-6042.

Kruijer, W., van Schaik, F. M. A., and Sussenbach, J. S., (1981). Structure and organisation of a gene coding for the DNA-binding protein of adenovirus type 5. Nucleic Acids Res. 9 : 4439-4457.

Kruijer. W., van Schaik, F. M. A., and Sussenbach, J. S., (1982). Nucleotide sequence of the gene encoding adenovirus type 2 DNA-binding protein. Nucleic Acids Res. 10 : 4493-4500.

Kvist, S., Ostberg, L., Persson, H., Philipson, L., and Pettersson, P. A., (1978). Molecular association between transplantation antigens and a Cell Surface antigen on an adenovirus transformed cell line. Proc. Natl. Acad. Sci. (U.S.A.). 75 : 5674-5678.

Lai, C-J., and Nathans, D., (1974). Mapping temperature-sensitive mutants of simian virus 40 : Rescue of mutants by fragments of viral DNA. Virology, 60 : 466-475.

Lai Fatt, R. B., and Mak, S., (1982). Mapping of an adenovirus function involved in the inhibition of DNA degradation. J. Virol. 42 : 969-977.

Larsson, V. M., Girardi, A. J., Hilleman, M. R., and Zwickey, R. E., (1965). Studies on the oncogenicity of adenovirus type 7 virus in hamsters. Proc. Soc. Exp. Biol. Med. 118 : 15-24.

Lasky, L. A., Lev, Z., Xin, J-H., Britten, R. J., and Davidson, E. H., (1980). Messenger RNA prevalence in sea urchin embryos measured with cloned cDNAs. Proc. Natl. Acad. Sci. (U.S.A.). 77 : 5317-5321.

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

Lebowitz, J., and Horwitz, M. S., (1975). Synthesis and assembly of adenovirus polypeptides. III. Reversible inhibition of hexon assembly in adenovirus type 5 temperature-sensitive mutants. Virology, 66 : 10-24.

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

Ledinko, N., (1974). Temperature-sensitive mutants of adenovirus type 12 defective in viral DNA synthesis. J. Virol. 14 : 457-468.

Leff, T., Elkaim, R., Godin, C. R., Jalinot, P., Sassone-Corsi, P.,

Perricaudet, C., Kedinger, C., and Chambon, P., (1984). Individual products of the adenovirus 12S and 13S E1a mRNAs stimulate viral EIIa and EIII expression at the transcriptional level. Proc. Natl. Acad. Sci. (U.S.A.), 81 : 4381-4385.

Leite, J. P. G., Niel, C., D'Halliun, J. C., (1986). Expression of the chloramphenicol acetyl transferase gene in human cells under the control of early adenovirus subgroup C promotors : effect of E1a gene products from other subgroups on gene expression. Gene 41 : 207-215.

Levine, A. J., and Ginsberg, H. S., (1967). Mechanism by which fibre antigen inhibits multiplication of type 5 adenovirus. J. Virol. 1 : 747-757.

Levinson, A., Levine, A. J., Anderson, S., Osborn, M., Rosenwirth, B., and Weber, K., (1976). The relationship between group C adenovirus tumour antigen and the adenovirus single-strand DNA-binding protein. Cell, 7 : 575-584.

Levinson, A. D., and Levine, A. J., (1977). The group C adenovirus tumour antigens : infected and transformed cells and a peptide map analysis. Cell 11 : 871-879.

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

Lewis, J. B., Anderson, C. W., Atkins, J. F., (1977). Further mapping of the late adenovirus genes by cell-free translation of RNA selected by hybridisation to specific DNA fragments. Cell 12 : 37-44.

Lichy, J. H., Horwitz, M. S., and Hurwitz, J., (1981). Formation of a covalent complex between the 80,000-dalton adenovirus terminal

protein and 5' dCMP in vitro. Proc. Natl. Acad. Sci. (U.S.A.) 78 : 2678-2682.

Lichy, J. H., Field, J., Horwitz, M. S., and Hurwitz, J. (1982). Separation of the adenoviral terminal protein precursor from its associated DNA polymerase : Role of both proteins in the initiation of adenovirus DNA replication. Proc. Natl. Acad. Sci. (U.S.A.) 79 : 5225-5229.

Liu, G. Q., Babiss, L. E., Volkert, F. C., Young, C. S. H., and Ginsberg, H. S., (1985). A thermolabile mutant of type 5 adenovirus has a substitution mutation in protein VIII. J. Virol. 53 : 920-925.

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

Luftig, R. B., and Weihing, R. R., (1975). Adenovirus binds to rat brain microtubules in vitro. J. Virol. 16 : 696-706.

Maizel, J. V., Jr., White, D. O., and Scharff, M. D., (1968). The polypeptides of adenovirus. II. Soluble proteins, cores, top components and the structure of the virion. Virology 36 : 126-136.

Mak, S., (1969). Transcription and replication of viral deoxyribonucleic acid in cells co-infected with adenovirus types 2 and 12. J. Virol. 4 : 651-656.

Martin, G. R., Warocquier, R., Cousin, C., D'Halliun, J-C., and Boulanger, P. A., (1978). Isolation and phenotypic characterisation of human adenovirus type 2 temperature-sensitive mutants. J. Gen. Virol. 41 : 303-314.

Mathews, M. B., (1975). Genes for VA RNA in adenovirus 2. Cell 6 : 223-230.

Matsuo, T., Hashimoto, S., Wold, W. S. M., Symington, J., Rankin, A., and Green, M., (1982). Identification of adenovirus 2 early region

4 polypeptides by *in vitro* translation and tryptic peptide map analysis. J. Virol. 41 : 334-339.

Mautner, V., and Boursnell, M. E. G., (1983). Recombination in adenovirus : DNA sequence analysis of crossover sites in intertypic recombinants. Virology 131 : 1-10.

Mautner, V., and MacKay, N., (1984). Recombination in adenovirus : Analysis of crossover sites in intertypic overlap recombinants. Virology 139 : 43-52.

Medical Research Council Working Party on Acute Respiratory Virus Infection, (1965). A Collaborative study of the etiology of acute respiratory infections in Britain (1961) - 4 : A report of the Medical Research Council Working Party on Acute Respiratory Virus Infections. Br. Med. J. 2 : 319-326.

Messing, J., (1979). A multipurpose cloning system based on the single-stranded DNA bacteriophage M13. Recombinant DNA Technical Bulletin 2 : 43-48.

Messing, J., and Vieira, J., (1982). A new pair of M13 vectors for selecting either DNA strand of double-digest restriction fragments. Gene 19 : 269-276.

McAllister, R. M., Nicolson, M. O., Lewis, A. M., MacPherson, I., Huebner, R. J., (1969a). Transformation of Rat Embryo Cells by Adenovirus type 1. J. Gen. Virol. 4 : 29-36.

McAllister, R. M., Nicolson, M. O., Reed, G., Kern, J., Gilden, R. V., and Huebner, R. J., (1969b). Transformation of rodent cells by adenovirus 19 and other group D adenoviruses. J. Natl. Cancer Inst. 43 : 917-922.

McBride, W. D., and Weiner, A., (1964). *In vitro* transformation of hamster kidney cells by human adenovirus type 12. Proc. Soc. Exp.

Biol. Med. 115 : 870-874.

McChesney, A. E., England, J. J., and Rich, L. J., (1973). Adenovirus infection in foals. J. Am. Vet. Med. Assoc. 162 : 545.

McChesney, A. E., England, J. J., Whiteman, C. E., Adcock, J. L., Rich, L. J., and Chow, T. L., (1974). Experimental transmission of equine adenovirus in Arabian and non-Arabian foals. Am. J. Vet. Res. 35 : 1015.

McDonough, J. S., and Rekosh, D. M., (1982). Differential complementation of adenovirus type 5 temperature-sensitive early mutants by adenovirus types 3 and 12. Virology 120 : 383-398.

McKinnon, R. D., Bacchetti, S., Graham, F. L., (1982). Tn5 mutagenesis of the transforming genes of human adenovirus type 5. Gene 19 : 33-42.

Miller, J. H., ed. (1972). Experiments in molecular genetics. Cold Spring Harbor Laboratory, Cold Spring Harbor, New York.

Miller, L. K., and Fried, M., (1976). Construction of the genetic map of the polyoma genome. J. Virol. 18 : 824-832.

Minekawa, T., Ishibashi, M., Yasue, H., and Takahashi, M., (1976). Characterisation 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.

Mirza, M. A., and Weber, J., (1982). Structure of adenovirus chromatin. Biochem. Biophys. Acta. 696 : 76-86.

Montell, C., Fisher, E. F., Caruthers, M. H., and Berk, A. J., (1982). Resolving the functions of overlapping viral genes by site-specific mutagens at a mRNA splice site. Nature 295 : 380-384.

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

Mulder, C., Arrand, J. R., Delius, H., and Pettersson, U., (1974a). Specific fragmentation of DNA of adenovirus serotypes 3, 5, 7 and 12 and aden-simian virus 40 hybrid virus Ad.2^+ ND_1 by restriction endonuclease R.EcoR1. J. Virol. 14 : 68-77.

Mulder, C., Arrand, J. R., Delius, H., Keller, W., Pettersson, U., Roberts, R. J., and Sharp, P. A., (1974b). Cleavage maps of DNA from adenovirus types 2 and 5 by restriction endonucleases EcoR1 and Hpa1. Cold Spring Harbor Symp. Quant. Biol. 39 :

Nermut, M. V., (1978). Structural elements in adenovirus cores. Studies by means of freeze-fractioning and ultra-thin sectioning. Arch. Virol. 57 : 323-337.

Nermut, M. V., (1979). Structural elements in adenovirus cores. Evidence for a core shell and linear structure in "relaxed cores". Arch. Virol. 62 : 101-116.

Nevins, J. R., (1981). Mechanism of activation of early viral transcription by the adenovirus E1a gene product. Cell 26 : 213-220.

Nicolas, J-C., Suarez, F., Levine, A. J., and Girard, M., (1981). Temperature-independent revertants of adenovirus H5ts125 and H5ts107 mutants in the DNA binding protein : Isolation of a new class of host-range temperature conditional revertants. Virology 108 :521-524.

Nicolson, M. V., and McAllister, R. M., (1972). Infectivity of human adenovirus 1 DNA. Virology 48 : 14-21.

Norrby, E., (1968). Biological significance of structural adenovirus components. Curr. Top. Microbiol. Immunol. 43 : 1-43.

Norrby, E., Marusyk, H., and Hammarskjöld, M-L., (1969). The relationship between soluble antigens and the virion of adenovirus type 3. V. Identification of antigen specificities available at the surface of virions. Virology 38 : 477-482.

Norrby, E., (1969a). The relationship between the soluble antigens and the virion of adenovirus type 3. IV. Immunological characteristics. Virology 37 : 565-576.

Norrby, E., (1969b). The structural and functional diversity of adenovirus capsid components. J. Gen. Virol. 5 :221-236.

Numazaki, Y., Shigeta, S., Kumasaka, T., Mizazawa, T., Yamanaka, M., Yano, N., Takai, S., and Ishida, N., (1965). Acute hemorrhagic cystitis in children : Isolation of adenovirus type 11. N. Engl. J. Med. 278 : 700-704.

Numazaki, Y., Kumasaka, T., Yano, N., Yamanaka, M., Mizazawa, T., Takai, S., and Isheda, N., (1973). Further study on acute hemorrhagic cystitis due to adenovirus type 11. N. Engl. J. Med. 289 : 344-347.

Ohe, K., and Weissman, S. M., (1970). Nucleotide sequence of an RNA from cells infected with adenovirus 2. Science 167 : 879-881.

Oosterom-Dragon, E. A., and Ginsberg, H. S., (1981). Characterisation of two temperature-sensitive mutants of type 5 adenovirus with mutations in the 100,000 dalton protein gene. J. Virol. 40 : 491-500.

Oosterom-Dragon, E. A., and Anderson, C. W., (1983). Polypeptide structure and encoding location of the adenovirus serotype 2 late non-structural 33K protein. J. Virol. 45 : 251-263.

Pan, J., Celma, M. L., and Weissman, S. M., (1977). Studies of low molecular weight RNA from cells infected with adenovirus 2. III. The sequence of the promotor for VA-RNA 1. J. Biol. Chem. 252 : 9047-9054.

Pereira, H. G., Allison, A. C., and Farthing, C., (1959). Study of adenovirus antigens by immunoelectrophoresis. Nature 183 : 895-896.

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

Pereira, M. S., Periera, H. G., and Clarke, S. K. R., (1965). Human adenovirus type 31. A new serotype with oncogenic properties. Lancet 1 : 21-23.

Pereira, H. G., Valentine, R. C., and Russell, W. C., (1968). Crystallisation of an adenovirus protein. Nature 219 : 946-947.

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

Persson, S., Signäs, C., and Philipson, L., (1979). Purification and characterisation of an early glycoprotein from adenovirus type 2 infected cells. J. Virol. 29 : 938-948.

Persson, H., Jörnvall, H., and Zabielsik, J., (1980a). Multiple mRNA species for the precursor to an adenovirus-encoded glycoprotein : Identification and structure of the signal sequence. Proc. Natl. Acad. Sci. (U.S.A.) 77 : 6349-6353.

Persson, H., Janssen, M., and Philipson, L., (1980b). Synthesis and genomic site for an adenovirus type 2 early glycoprotein. J. Mol. Biol. 136 : 375-394.

Petterson, U., Philipson, L., Höglund, S., (1968). Structural proteins of adenoviruses. II. Purification and characterisation of adenovirus type 2 fibre antigen. Virology 35 : 204-215.

Petterson, U., and Höglund, S., (1969). Structural proteins of adenoviruses. III. Purification and characterisation of adenovirus type 2 penton antigen. Virology 39 : 90-106.

Pettersson, U., and Sambrook, J., (1973). Amount of viral DNA in the

genome of cells transformed by adenovirus type 2. J. Mol. Biol. 73 : 125-130.

Philipson, L., Lonberg-Holm, K., and Pettersson, U., (1968). Virus Receptor Interaction in an Adenovirus System. J. Virol. 2 : 1064-1075.

Piña, M., and Green, M., (1965). Biochemical studies on adenovirus multiplication. IX. Chemical and base composition analysis of 28 human adenoviruses. Proc. Natl. Acad. Sci. (U.S.A.) 54 : 547-551.

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

Pincus, S., Robertson, W., and Rekosh, D., (1981). Characterisation of the effect of aphidicolin on adenovirus DNA replication : Evidence in support of a protein primer model of initiation. Nucleic Acid Res. 9 : 4919-4938.

Rabson, A. S., Kirschstein, R. L., and Paul, F. J., (1964). Tumours produced by adenovirus 12 in Mastomys in mice. J. Natl. Cancer Inst. 32 : 77-87.

Radding, C. M., (1982) Homologous pairing and strand exchange in genetic recombination. Ann. Rev. Genet. 16 : 405-437.

Rao, R. N., and Rogers, S., (1979). Plasmid pKC7 : A vector containing ten restriction endonuclease sites suitable for cloning DNA segments. Gene 7 : 79-82.

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

Revet, B., and Benichou, D., (1981). Electron microscopy of Ad 5 replicating molecules after in vivo photocrosslinking with trioxsalen. Virology 114 : 60-70.

Ricciardi, R. P., Jones, R. L., Cepko, C. L., Sharp, P. A., and Roberts, B. E., (1981). Expression of early adenovirus genes requires a viral encoded acidic polypeptide. Proc. Natl. Acad. Sci. (U.S.A.) 78 : 6121-6125.

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

Richmond, S. J., Caul, E. O., Dunn, S. M., Ashley, C. R., Clarke, S. K. R., Seymour, N. R., (1979). An outbreak of gastroenteritis in young children caused by adenovirus. Lancet 1 : 1178-1180.

Rigby, P. W. J., Dieckmann, M., Rhodes, C., and Berg, P., (1977). Labelling deoxyribonucleic acid to a high specific activity in vitro by nick translation with DNA polymerase 1. J. Mol. Biol. 113 : 237-251.

Robinson, A. J., Younghusband, H. B., and Bellett, A. J. D., (1973). A circular DNA-protein complex from adenoviruses. Virology 56 : 54-69.

Robinson, A. J. and Bellett, A. J. D., (1974). A circular DNA-protein complex from adenoviruses and its possible role in DNA replication. Cold Spring Harbor Symp. Quant. Biol. 39 : 523-539.

Robinson, A. J., and Bellett, A. J. D., (1975). Complementary strands of CELO virus DNA. J. Virol. 15 : 458-465.

Rosen, L., (1958). Hemagglutination by adenoviruses. Virology 5 : 574-577.

Rosen, L., (1960). A hemagglutination-inhibition technique for typing adenoviruses. Am. J. Hyg. 71 : 120-128.

Ross, S. R., Flint, S. J., Levine, A. J., (1980). Identification of the adenovirus early proteins and their genomic map positions. Virology

100 : 419-432

Rowe, W. P., Huebner, R. J., Gilmore, L. K., Parrott, R. H., and Ward, T. G., (1953). Isolation of a cytopathogenic agent from human adenoids undergoing spontaneous degradation in tissue culture. Proc. Soc. Exp. Biol. Med. 84 : 570-573

Rowe, W. P., Hartley, J. W., and Huebner, R. J., (1958). Serotype composition of the adenovirus group. Proc. Soc. Exp. Biol. Med. 97 : 465-470

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

Rubenstein, F. E., and Ginsberg, H. S., (1974). Transformation characteristics of temperature-sensitive mutants of type 12 adenovirus. Intervirology 3 : 170

Russell, W. C., Valentine, R. C., and Pereira, H. G., (1967). The effect of heat on the anatomy of the adenovirus. J. Gen. Virol. 1 : 509-522

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

Russell, W. C., Newman, C., and Williams, J. F., (1972). Characterisation of temperature-sensitive-mutants of adenovirus type 5-serology. J. Gen. Virol. 17 : 265-279

Russell, W. C., and Skehel, J. J., (1972). The polypeptides of adenovirus-infected cells. J. Gen. Virol. 15 : 45-57

Russell, W. C., Skehel, J. J., and Williams, J. F., (1974). Characterisation of temperature-sensitive mutants of adenovirus type 5 : Synthesis of polypeptides in infected cells. J. Gen. Virol. 24 : 247-259

Russell, W. C., and Blair, G. E., (1977). Polypeptide Phosphorylatin in Adenovirus-Infected Cells. J. Gen. Virol. 34 : 19-35

Russell, W. C., and Precious, B., (1982). Nucleic acid binding properties of adenovirus structural polypeptides. J. Gen. Virol. 63 : 69-79

Sarnow, P., Sullivan, C. A., and Levine, A. J., (1982a). A monoclonal antibody detecting the adenovirus type 5 E1b-58Kd tumour antigen : Characterisation of the E1b-58Kd tumour antigen in adenovirus-infected and transformed cells. Virology 120 : 510-517

Sarnow, P., Hearing, P., Anderson, C. W., Reich, N., and Levine, A. J., (1982b). Identification and characterisation of the immunologically conserved adenovirus early region E4-11K protein and its association with the nuclear matrix. J. Mol. Biol. 162 : 565-583

Sarnow, P., Hearing, P., Anderson, C. W., Halbert, D. N., Shenk, T., and Levine, A. J., (1984). Adenovirus early region 1B 58,000-dalton tumour antigen is physically associated with an early region 4 25,000-dalton protein in productively infected cells. J. Virol. 49 : 692-700.

Sato, K., and Hosokawa, K., (1981). The structure of adenovirus chromatin revealed by ultraviolet light-induced cross-linking. Biochem. Biophys. Res. Commun. 101 : 1318-1323

Schaap, G. J. P., de Jong, J. C., van Büsterveld, O. P., and Beekhuis, W. H., (1979). A new intermediate adenovirus causing conjunctivitis. Arch. Ophthalmol. 97 : 2336-2339

Schneider, R. J., Weinberger, C., and Shenk, T., (1984). Adenovirus VAI RNA facilitate the initiation of translation in virus-infected cells. Cell 37 : 291-307

Schoub, B. D., Koornhof, H. L., Lecatsas, G., Prozesky, O. W., Freiman, I., Hartman, E., and Kassel, H., (1975). Virus in acute summer gastro-

enteritis in black infants. Lancet 1 : 1093-1094

Schutzbank, T. E., (1980). Use of DNA-minus temperature-sensitive mutants H5ts1125 and H5ts149, to study DNA replication, transformation and transcription. PhD Thesis, Columbia University, New York.

Sharp, P. A., Sugden, B., and sambrook, J., (1973). Detection of two restriction endonuclease activities in Haemophilus parainfluenza using analytical agarose. Biochemistry 12 : 3055-3063

Sharp, P. A., Gallimore, P. H., and Flint, S. J., (1975). Mapping of adenovirus 2 RNA sequences in lytically infected cells and transformed cell lines. Cold Spring Harbor Symp. Quant. Biol. 39 : 457-474

Sharp, P. A., Moore, C., and Haverty, J. L., (1976). The infectivity of Adenovirus 5 DNA-protein complex. Virology 75 : 442-456

Shinagawa, M., and Padmanabhan, R., (1979). Nucleotide sequences at the inverted terminal replication of Ad 2 DNA. Biochem. Biophys. Res. Commun. 87 : 671-678

Shinagawa, M., and Padmanabhan, R., (1980). Comparative sequence analysis of the inverted terminal repetitions from different adenoviruses. Proc. Natl. Acad. Sci. (U.S.A.) 77 : 3831-3835

Shiroki, K., Irisawa, J., and Shimojo, H., (1972). Isolation and preliminary characterisation of temperature-sensitive mutants of adenovirus 12. Virology 49 : 1-11

Shiroki, K., Shimojo, H., Sawada, Y., Uemizu, Y., and Fujinaga, K., (1979). Incomplete transformation of rat cells by a small fragment of adenovirus 12 DNA. Virology 95 : 127-136

Shiroki, K., Maruyama, K., Saito, I., Fukui, Y., Yazaki, K., and Shimojo, H., (1982). Dependence of tumour-forming capacities of cells transformed by recombinants between adenovirus types 5 and 12 on expression of early region 1. J. Virol. 42 : 708-718

Signäs, C., Katze, M. G., Persson, H., and Philipson, L., (1982). The adenovirus glycoprotein binds heavy chains class 1 transplantation antigens from man and mouse. Nature 299 : 175-177

Signäs, C., Akusjärvi, G., and Petterson, U., (1987). Region E3 of human adenoviruses; differences between the oncogenic adenovirus-3 and the non-oncogenic adenovirus-2. Gene in press

Singer, B. S., Gold, L., Gauss, P., and Doherty, D. H., (1982). Determination of the amount of homology required for recombination in bacteriophage T_4. Cell 31 : 25-33

Smart, J. E., and Stillman, B. W., (1982). Adenovirus terminal protein precursor, partial amino acid sequence and the site of covalent linkage to virus DNA. J. Biol. Chemistry. 257 : 13499-13506

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

Solnick, D., and Anderson, M. A., (1982). Transformation-deficient adenovirus mutant defective in expression of region E1a but not region E1b. J. Virol. 42 : 106-113

Southern, E., (1975). Detection of specific sequences among DNA fragments separated by gel electrophoresis. J. Mol. Biol. 98 : 503-518

Spizizen, J., Reilly, B. F., and Evans, A. H., (1966). Microbial transformation and transfection. Annu. Rev. Microbiol. 20 : 371-400

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

Stillman, B. W., Lewis, J. B., Chow, L. T., Mathews, M. B., and Smart, J. E., (1981). Identification of the gene and mRNA for the adeno-

virus terminal protein precursor. Cell 23 : 497-508.

Stillman, B. W., Topp, W. C., and Engler, J. A., (1982). Conserved sequences at the origin of adenovirus DNA replication. J. of Virol. 44 : 530-537.

Stillman, B. W., White, E., and Grodzicker, T., (1984). Independent mutations in Ad2ts111 cause degradation of cellular DNA and defective viral DNA replication. J. Virol. 50 : 598-605.

Stinski, M. F., and Ginsberg, H. S., (1974). Antibody to a type 5 adenovirus hexon polypeptide : detection of nascent polypeptides in cytoplasm of infected KB cells. Internurology 4 : 226-236.

Stow, N. D., (1982). The infectivity of adenovirus genomes lacking DNA sequences from their left hand termini. Nucl. Acids Res. 10 : 5105-5119.

Straus, S., Wilson, T., Raskas, H., (1981). Transfection of KB cells by liposomes containing adenovirus type 2 DNA. J. Virol. 39 : 290-294.

Subramanian, T., Kuppuswamy, M., Mak, S., Chinnadurai, G., (1984a). Adenovirus Cyt^+ locus, which controls cell transformation and tumourgenicity is an allele of lp^+ locus which codes for a 19 Kilodalton tumour antigen. J. Virol. 52 : 336-343.

Subramanian, T., Kuppuswamy, M., Gysbers, J., Mak, S., and Chinnadurai, G., (1984b). 19-KDa tumour antigen coded by early region E1b of adenovirus 2 is required for efficient synthesis and for protection of viral DNA. J. Biol. Chem. 259 : 11777-11783.

Sugisaki, H., Sugimoto, K., Takanami, M., Shiroki, K., Saito, I., Shimojo, H. Sawada, Y., Uemizu, Y., Uesugi, S-I., and Fujinaga, K., (1980). Structure and gene organisation in the transforming HindIII-G fragment of Ad12. Cell 20 : 777-786.

Sussenbach, J. S., (1967). Early events in the infection process of

Structural properties of adenovirus DNAs. Biochem. Biophys. Acta. 182 : 530-541

van der Eb A. J., Mulder, C., Graham, F. L., and Houweling, A., (1977). Transformation with specific fragments of adenovirus DNAs. I. Isolation of specific fragments with transforming activity of adenovirus 2 and 5 DNA. Gene 2 : 115-132

van der Eb, A. J., and Houweling, A., (1977). Transformation with specific fragments of adenovirus DNAs. II. Analysis of the viral DNA sequences present in cells transformed with 7% fragment of adenovirus 5 DNA. Gene 2 : 133-146

van der Eb, A. J., van Ormondt, H., Schrier, P. I., Lupker, J. H., Jochenisen, H., van den Elsen, P. J., De Leys, R. J., Kratt, J., van Bevern, C. P., Dykema, R., De Wood, A., (1979). Structure and function of the transforming genes of human adenoviruses and SV40. Cold Spring Harbor Symp. Quant. Biol. 44 : 383-399

van Ormondt, H., Maat, J., and van Beveren, C. P., (1980). The nucleotide sequence of the transforming early region E1 of adenovirus type 5 DNA. Gene 11 : 299-399

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

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

van der Vliet, P. C., Landberg, J., and Jansz, H. S., (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

Vayda, M. E., Rogers, A. E., and Flint, S. J., (1983). The structure of

nucleoprotein cores released from adenoviruses. Nucleic Acids Res. 11 : 441-460

Vieira, J., and Messing, J., (1982). The pUC plasmids, an M13 mp7-derived system for insertion mutagenesis and sequencing with synthetic universal primers. Gene 19 : 259-268

Virtanen, A., and Pettersson, U., (1983). The molecular structure of the 9S mRNA from early region 1A of adenovirus serotype 2. J. Mol. Biol. 165 : 496-499

Volkert, F., and Young, C. S. H., (1983). The genetic analysis of recombination using adenovirus overlapping terminal DNA fragments. Virology 125 : 175-193

Wadell, G., and Norrby, E., (1969). Immunological and other biological characteristics of pentons of human adenovirus. J. Virol. 4 : 671-680

Weber, J., Bégin, M., and Khitto, G., (1975). Genetic analysis of adenovirus type 2. II. Preliminary phenotypic characterisation of temperature-sensitive mutants. J. Virol. 15 : 1049-1056

Weber, J., (1976). Genetic analysis of adenovirus type 2. III. Temperature sensitivity of processing of viral proteins. J. Virol. 17 : 462-471

Weber, J., Blanchard, J. M., Ginsberg, H., and Darnell, J. E., (1980). Polyadenylic acid addition and splicing events in early adenovirus mRNA formation. J. Virol. 33 : 286-291

White, E., Grodzicker, T., and Stillman, B., (1984). Mutations in the gene encoding the adenovirus early region 1b 19,000 molecular weight tumour antigen cause the degradation of chromosomal DNA. J. Virology 52 : 410-419

Werner, G., and zur Hausen, H., (1978). Deletions and insertions in

adenovirus type 12 DNA after viral replication in Vero cells. Virology 86 : 66-77

White, G. B. B., and Stancliffe, D., (1975). Viruses and gastroenteritis. Lancet 2 : 703-703

Whitton, J. L., Hundley, F., O'Donnell, B., and Desselberger, U., (1983). Silver staining of nucleic acids. Application in virus research and diagnostic virology. J. Virol. Methods 7 : 185-198

Wigand, R., Bartha, A., Dreizin, R. S., Esche, H., Ginsberg, H. S., Green, M., Hierholzer, J. C., Kalter, S. S., McFerran, J. B., Pettersson, U., Russell, W. C., and Wadell, G., (1982). Adenoviridae, second report. Intervirology 18 : 169-176

Wigler, M., Pellicer, A., Silverstein, S., and Axel, R., (1978). Biochemical transfer of single-copy eukaryotic genes using total cellular DNA as donor. Cell 14 : 725-731

Wilcox, W. C., and Ginsberg, H. S., (1963). Structures of type 5 adenovirus. I. Antigenic relationship of virus structural proteins to virus specific soluble antigens from infected cells. J. Exp. Med. 118 : 295-306

Wilcox, W. C., Ginsberg, H. S., and Andersson, T. F., (1963). Structure of type 5 adenovirus. II. Fine structure of virus subunits : Morphological relationship of structural subunits to virus specific soluble antigens from infected cells. J. Exp. Med. 118 : 307-314

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

Willcox, N., and Mautner, V., (1976). Antigenic determinants of adenovirus capsids. I. Measurement of antibody cross-reactivity. J. Immunol. 116 : 19-24

Williams, J. F., (1970). Enhancement of adenovirus plaque formation on Hela cells by magnesium chloride. J. Gen. Virol. 9 : 251-255

Williams, J. F., and Ustacelebi, S., (1971a). Complementation and Recombination with temperature-sensitive mutants of adenovirus type 5. J. Gen. Virol. 13 : 345-348

Williams, J. F., Gharpure, M., Ustacelebi, S., and McDonald, S., (1971b). Isolation of temperature-sensitive mutants of adenovirus type 5. J. Gen. Virol. 11 : 95-101

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

Williams, J., Grodzicker, T., Sharp, P., and Sambrook, J., (1975b). Adenovirus recombination : Physical mapping of crossover events. Cell 4 : 113-119

Williams, J., Ho., T. S., and Galos, R., (1981). Evidence for functional relatedness of products encoded by the transforming sequences of human Adenovirus types 5 and 12. Virology 110 : 208-212

Wolfson, J., and Dressler, D., (1972). Adenovirus DNA contains an inverted terminal repetition. Proc. Natl. Acad. Sci. (U.S.A.) 69 : 3054-3057

Wolgemuth, D. J., and Hsu, M-T., (1980). Visualization of genetic recombination intermediates of human adenovirus type 2 DNA from infected Hela cells. Nature 287 : 168-171

Yabe, Y., Samper, L., Bryan, E., Taylor, G., and Trentin, J. J., (1964). Oncogenic effect of human adenovirus type 12 in mice. Science 143 : 46-47

Yeh-Kai, L., Akusjärvi, G., Alestrŏm, P., Pettersson, U., Tremblay, M., and Weber, J., (1983). Genetic identification of an endoproteinase

encoded by the adenovirus genome. J. Mol. Biol. 167 : 217-222

Young, C. S. H., and Williams, J. F., (1975). Heat-stable variant of human adenovirus type 5 : Characterisation and use in Three-Factor Crosses. J. Virol. 15 :1168-1175

Young, C. S. H., and Silverstein, S. J., (1980). The Kinetics of adenovirus recombination in homotypic and heterotypic genetic crosses. Virology 101 : 503-515

Young, C. S. H., Shenk, T., and Ginsberg, H., (1984). The Genetic System. The Adenoviruses - (1984). Ed. Ginsberg, H. S., Plenum Press, New York.

Younghusband, H. B., and Bellet, A. J., (1971). Mature form of DNA from chicken embryo lethal orphan virus. J. Virol. 8 : 265-274

Zain, S., Sambrook, J., Roberts, R. J., Keller, W., Fried, M., and Dunn, A. R., (1979). Nucleotide sequence analysis of the leader segments in a cloned copy of adenovirus 2 fibre mRNA. Cell 16 : 851-861

Ziff, E. B., and Evans, R., (1978). Coincidence of the promotor and capped 5' terminus of RNA from the adenovirus 2 major late transcription unit. Cell 15 : 1463-1475

Zsák, L., and Kisary, J., (1984). Grouping of fowl adenoviruses based upon the restriction patterns of DNA generated by BamH1 and HindIII. Intervirology 22 : 110-114