MOLECULAR FATE OF ADENOVIRUS TYPE 5 DNA IN EUKARYOTIC CELLS

By

Martha M. Ruben de Campione, M.D.

A Thesis

Submitted to the School of Graduate Studies

in Partial Fulfillment of the Requirements

for the Degree

Doctor of Philosophy

McMaster University

December , 1983

MOLECULAR FATE OF ADENOVIRUS TYPE 5 DNA IN EUKARYOTIC CELLS

DOCTOR OF PHILOSPHY (1983)
(Medical Sciences)

MCMASTER UNIVERSITY
Hamilton, Ontario

TITLE: Molecular fate of adenovirus DNA in eukaryotic cells

AUTHOR: Martha M. Ruben de Campione, M.D.

SUPERVISOR: Dr. Frank L. Graham, Ph.D.

NUMBER OF PAGES: xviii, 211

ABSTRACT

Adenovirus type 5 is able to oncogenically transform cells growing in culture. These transformed cells show different degrees of transformation. The interest of my studies was to establish the presence and characterize the structure of virus DNA in adenovirus 5 transformed cell lines and to look for a possible correlation between the integrated viral sequences and the phenotype of the cells. For this purpose, cell lines transformed by wild type (virions or DNA) and by host range mutants (virions) were analyzed for their viral DNA content and for their transformed phenotype.

More than the left 8% was always present in virion transformed cells. Cells transformed by host range mutants of complementation Group I generally contained a larger fraction of the genome than did their counterparts transformed by wild type virus. In some host range transformed cells, virtually the entire viral DNA molecule was found colinearly integrated.

In the case of the cells transformed by wild type DNA or virions, it was not possible to correlate any particular phenotype with a specific integration pattern. The same pattern was found in the tumorigenic derivatives of a non oncogenic cell line (293) as in the parental line. Studies with cells transformed by Group I host range mutants showed that partial

or complete integration of viral DNA into transformed cells was always associated with the production of some viral proteins and with the induction of a partially transformed, non oncogenic phenotype.

An interesting finding was the limited number of inserts in cell lines isolated from uncloned populations within a small number of passages after transformation. This suggested the possibility of a limited number of sites available for transformation or alternatively, that a few cells rapidly overgrew the rest. In an attempt to answer this question and to obtain more information on the integration process, DNA was extracted from semipermissive rat cells at different times shortly after infection. Adenovirus 5 wild type and host range mutants from Groups I and II were used in individual experiments. The extracted DNA was analyzed for the presence and state of viral DNA. New forms of intracellular viral DNA, which might be intermediates for integration, were found. A fragment having the size of both ends of the viral DNA joined together, and which hybridizes with both ends when these are used separately as probes, was detected in Southern blots after digestion of rat cell DNA with different restriction enzymes. This structure was detected earlier after infection with host range 1 (Group I), than after infection with host range 6 (Group II) or with wild type, and was found also in Hela cells and in two rat cell lines after infection with host

range 1 or wild type. Studies followed to determine the nature of this new arranged viral DNA provided evidence for covalent head to tail joining and for the formation of circular Ad5 DNA molecules.

Acknowledgements

I wish to express my thanks to my supervisor, Dr. F.L. Graham, for his aid and advise during the course of these studies, and for his patience and guidance when I was writing this manuscript.

I also would like to acknowledge the advise from the other members of my supervisory committee: Dr. P.E. Branton, Dr. S. Mak and Dr. W.C. Leung.

Thanks is also due to Dr. S. Bacchetti, for many helpful discussions and for her help with the nick translations, to Mr. R. Mackinnon for providing the plasmid DNA used in these studies, and to Dr. D.T. Rowe for the antisera required for the immunoprecipitations.

I wish to express my gratitude to Dr. D. Palmer and Miss S. Howley who gave me invaluable help in overcoming the difficulties of the English language.

The technical assistance of Mr. J. Rudy and Mrs. J. Ng, and the excellent work done by Miss E. McNally in typing this manuscript are greatly appreciated.

I wish to specially thank my husband for his encouragement to pursue these studies, and for his continued support criticism, and advise.

This thesis is dedicated with affection to my parents, Helmut Ruben and Aurora Barretto de Ruben; to my sisters, Cristina, Loreley and Beatriz; as well as to my husband, Jose and to my son Nicolas (who, upon his birth, put an end to the experimental work leading to this thesis).

Table of Contents

List of Tables

List of Figures

Chapter IV cont'd:

Appendix 2:

Appendix 3:

List of Abbreviations

Ad 2, 5, 12	adenovirus type 2, 5, 12
Ara C	cytosine arabinose
ARD	acute respiratory disease
ATP	adenosine triphosphate
bp	base pairs
BRK	baby rat kidney
BSA	bovine serum albumin
Ci	curie
CPE	cytopathic effect
cpm	counts per minute
DBP	DNA binding protein
dCMP	deoxycytidine monophosphate
dATP, dCTP, dGTP, dTTP	deoxy nucleoside triphosphate
dl	deletion mutant
DNA	deoxyribonucleic acid
DNAse	deoxyribonuclease
E1 - E4	early regions 1 - 4 of adenovirus DNA
EDTA	ethylendiamine-tetraacetate
EKC	epidemic kerato cojunctivitis
EL	early-late promoter
et al.	and coworkers
EtBr	ethidium bromide
FBS	fetal bovine serum

List of Abbreviations (Cont'd)

Fig.	figure
HEK	human embryonic kidney
hr	host range mutant
HS	horse serum
kb	kilobasepairs
l strand	strand of adenovirus DNA transcribed in leftward orientation
Ll - 5	late region l - 5 of adenovirus DNA
M	molar
MEM	minimal essential medium
MOI	multiplicity of infection
mRNA	messenger ribonucleic acid
MW	molecular weight
ME	mercaptoethanol
N	normal
ND	not done
NCS	newborn calf serum
OD	optical density
PAGE	polyacryamide gel electrophoresis
PBS	phosphate-buffered saline
PCF	pharingo conjunctivitis febril
PEG	polyethylene glycol
PFU	plaque forming unit

List of Abbreviations (Cont'd)

PI	post infection
pTP	precursor of the terminal protein
PVP	polyvinylpyrrolidone
r strand	strand of adenovirus DNA transcribed in the rightward orientation
RNA	ribonucleic acid
RNAse	ribonuclease
rpm	revolutions per minute
SDS	sodium dodecyl sulphate
SSC	saline sodium citrate
SV40	simian virus 40
TCA	trichloroacetic acid
^{3}H TdR	tritiated thymidine
TP	terminal protein
ts	temperature sensitive mutant
UV	ultraviolet radiation
wt	wild type virus

CHAPTER I

INTRODUCTION

INTRODUCTION

The Adenovirus Family

Adenoviruses constitute a family of DNA viruses originally isolated from the respiratory tract of humans and other animals (Green et al., 1980; Persson and Philipson, 1982; Tooze, 1980; Fenner, 1974). This family has been subdivided into two genera: mastadenovirus and aviadenovirus according to whether their isolation was from mammalian or avian hosts. There are 35 serotypes of human adenovirus recognized (31 universally accepted; Green et al., 1980) which can be subdivided into five groups according to DNA sequence homology (Green et al., 1979). About 80% DNA homology exists within each group but only 10-20% homology between DNAs corresponding to different groups. Members of each group also share similarities in their pathologic and epidemiologic as well as other properties (see Table I).

The most extensively studied serotypes are the closely related adenoviruses type 2 and 5 (Ad2, Ad5), both from group C. For this reason and because Ad5 was the serotype used in all my studies, the information given in this introduction will refer mainly to serotypes 2 and 5. It is, however, true that adenoviruses represent a very homogeneous family so that observations in Ad2 and Ad5 frequently apply to other human serotypes.

TABLE I

PROPERTIES OF HUMAN ADENOVIRAL DNA HOMOLOGY GROUPS A THROUGH E

Group	Type[a]	DNA homology[b] (%)	Incidence of tumors in newborn hamsters	Cell transformation	Transforming region homology[b] (%)	Pathogenicity & epidemiology
A	12,18,31	48-69/8-20	high: tumors in most animals in 4 months	+	35-71/2-7	associated with upper respiratory infections & diarrhea; isolated from stools of children
B	3,7,11,14, 16,21	89-94/9-20	weak: tumors in few animals in 4-18 months	+	85-99/2-12	common (3,7); moderate to severe infections; ARD[c] epidemics among military recruits (7, also 3,14,21); bad colds & severe illness (7); summer PCF[d] (3,7); pharyngitis (3,7); febrile pneumonia (3,7,14); acute hemorrhagic cystitis (11, occasionally 21); diarrhea (16, 21)
C	1,2,5,6	99-100/10-16	nil	+	98-100/1-15	most common (2,1); mild to severe infections of upper respiratory tract (especially nasopharynx) of infants & children; latent infections of lymphoid tissue; readily isolated from throat and anal swabs of children

TABLE I (cont'd)

D	8,9,10,13, 15,17,19,20, 22,23,24,25, 26,27,28,29, 30	94-99/4-17	nil; 9 induces mammary fibro-adenomas in rats	+	n.d.	8 & 19 commonly cause epidemic keratoconjunct-ivitis (EKC)[e]; isolated from stool
E	4	4-23 with other types	nil	?	n.d.	ARD[c] epidemics in military recruits; cause of EKC[e] severe PCF[d], and pneumonia

[a] Types 32-35 have undetermined homology groups.

[b] Percentage within group/percentage with other types; n.d. indicates not determined.

[c] ARD: acute respiratory disease

[d] PCF: pharingo conjunctivitis febril

[e] EKC: epidemic kerato conjunctivitis

from Green et al. (1980).

All the viruses belonging to this family have a characteristic icosahedral shape and contain at least 9 polypeptides with sizes between 7,500 and 120,000 daltons (Philipson et al., 1975). Inside the virus the DNA is associated with two core proteins and with a terminal protein covalently attached to the DNA; this structure is surrounded by a capsid composed of 252 capsomers: 240 hexons and 12 pentons (so called because of the number of neighbouring capsomers; Tooze, 1980). Each penton is formed by a base within the body of the capsid and the fiber, an antenna-like projection (Tooze, 1980; Valentine and Pereira, 1965).

The DNA inside the capsid is linear, double stranded, with a molecular weight (MW) of about 23 X 10^6 (35 kilobasepairs; van der Eb et al., 1979; Green, 1970; Persson and Philipson, 1982) which could code for as many as 50-60 polypeptides. This viral DNA contains inverted terminal repeats of about 100 basepairs (bp) yielding identical ends (Garon et al., 1972; Wolfson and Dressler, 1972; Steenbergh et al., 1977), whose significance is still obscure; in addition, a terminal protein of MW 55,000 is covalently linked to both 5' ends. Pure adenovirus DNA is infectious, but the specific infectivity on a molar basis is at least 10^6 times lower than that of the virus (Graham and van der Eb, 1973). However, if the terminal protein is not removed, the infectivity of the DNA can be enhanced several fold (Sharp et al., 1976).

Adenovirus Gene Expression in Infected Cells.

The adenovirus system is considered a very useful tool for the study of gene expression in mammalian cells and both productive

infection and cell transformation have been used for this purpose.

I. Productive infection.

The productive infection of permissive cells has been traditionally divided into early and late phases (Tooze, 1980). The early phase includes the events which precede the synthesis of adenovirus DNA, and recently this phase has been further subdivided into immediate early, pre-early and delayed early periods according to the sensitivity of gene expression to protein inhibitors and the time of appearance of gene products in the infected cells (Lewis and Mathews, 1980). An intermediate phase, required for the onset of late transcription and synthesis of virus proteins, has recently been identified after the early phase (Lewis and Mathews, 1980). This intermediate stage is independent of viral DNA replication.

The late phase is initiated after the onset of DNA replication (6-8 hours P.I.) and leads to a drastic change in viral gene expression (see below). Inhibitors of protein synthesis can prolong artificially the early phase, and inhibitors of DNA synthesis prolong both early and intermediate gene expression (Eggerding and Raskas, 1978).

A) Early phase

Uncoated DNA is found in the nucleus of the cell within 1 to 3 hours P.I. and transcription of early mRNA starts at this time (Lonberg and Philipson, 1969). The parental DNA acquires a nucleosome-like structure which has been related either to rearrangement of core proteins or to the acquisition of cellular

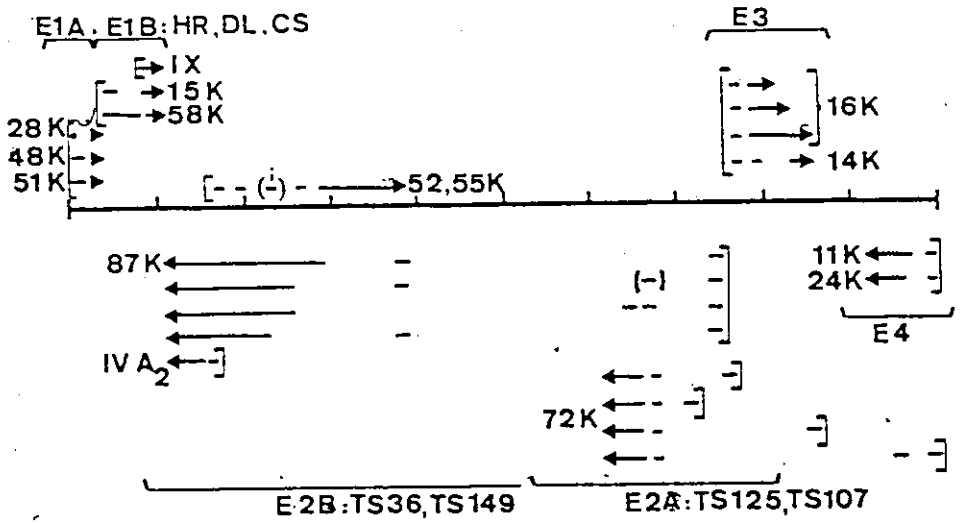
histones (Sergeant et al., 1979; Tate and Philipson, 1979). It is believed that this structure serves as a template for early transcription and for DNA replication (Persson and Philipson, 1982). Depending on the multiplicity of infection, between 5% and 18% of the total polysomal mRNA labeled at early times, P.I. hybridizes to viral DNA (Lindberg et al., 1972). This processed mRNA is complementary to about 40% of the viral genome (Pettersson et al., 1976). At least 19 distinct early mRNAs have been recovered and mapped (Berk et al., 1979; Chow et al., 1979; Kitchingman and Westphal, 1980) and around 20 viral coded early polypeptides have been identified (Lewis et al., 1976; Harter and Lewis, 1978; Lewis and Mathews, 1980).

i) Early regions

Several procedures have been used to map and quantitate early transcription, and four to five early regions have been described. These regions are numbered, from left to right, E1 to E4 (early region 1 to early region 4, Lewis et al., 1976; Berk and Sharp, 1977a; Chow et al., 1977a; Lewis and Mathews, 1980). Six early promoters have been identified (see Fig. 1) with a tentative order of expression: E1A, EL, E3, E4, E1B, E2A (Nevins et al., 1979; Nevins, 1981); these promoters are differentially expressed during the viral cycle.

Region E1 is divided into two independent transcription blocks E1A and E1B, controlled by different promoters (Sehgal et al., 1979; Wilson et al., 1979) and at least 3 different mRNAs with overlapping sequences have been mapped to each of these regions

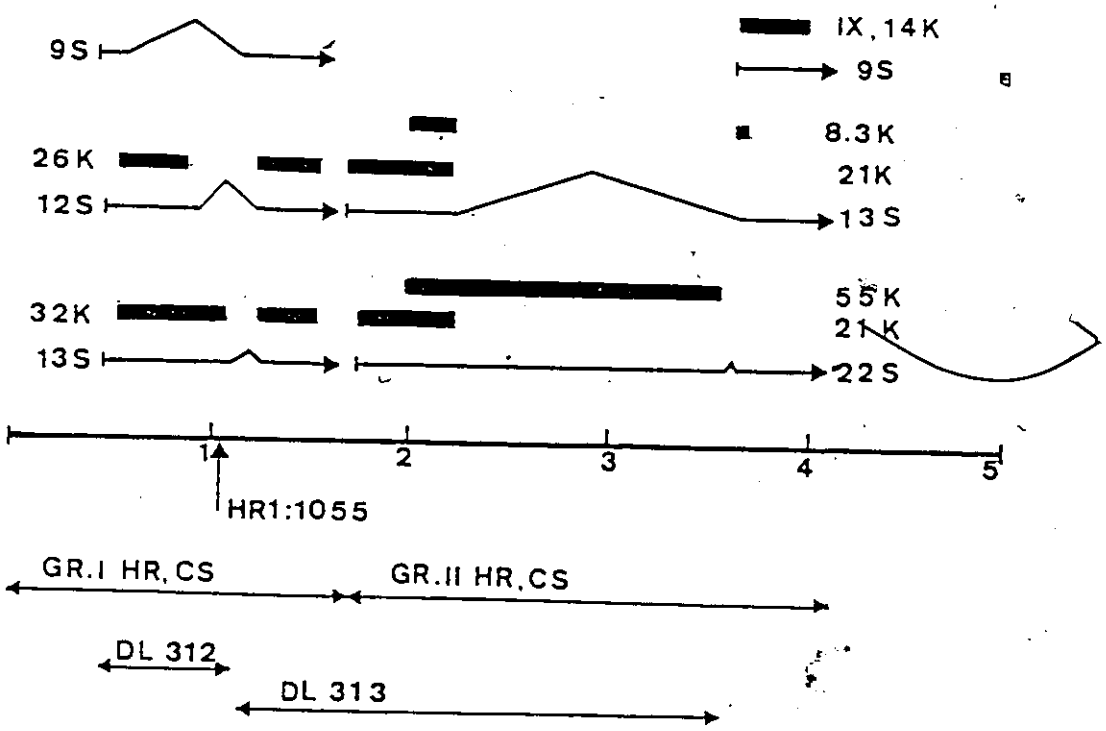
Figure 1: A genomic map of Ad2-coded early proteins and their mRNAs: The arrows in the figure represent early and intermediate mRNAs, both can be expressed in the absence of viral DNA replication (see text). Arrowheads show the 3' end, and tentative promoter sites are indicated with brackets. Each protein encoded in a specific early viral mRNA is indicated beside its mRNA. Proteins whose mRNA structure has not yet been elucidated are not shown. The map position of the mutants mentioned in Table III is shown beside the corresponding early region. HR: host range mutants; DL: deletion mutants; CS: cold sensitive mutants; TS: temperature sensitive mutants. Modified from Persson and Philipson (1982).


E1A, E1B: HR, DL, CS
E3
IX
15K
58K
28K
48K
51K
52,55K
16K
14K
87K
IV A2
(-)
11K
24K
E4
72K
E2B: TS36, TS149
E2A: TS125, TS107

(Berk and Sharp, 1977b; Spector et al., 1978; Chow et al., 1979; Perricaudet et al., 1979, 1980) (see Fig. 2). Region E1A is the first to be transcribed, around 1 hour P.I., and its maximal transcription is maintained for at least 6 hours. The two overlapping early mRNAs transcribed from this region, 12S and 13S, share identical 5' and 3' termini and differ by 138 nucleotides due to the different sized introns removed by RNA splicing (Perricaudet et al., 1979; Baker and Ziff, 1981; see Fig. 2). Both are transported to the cytoplasm where they accumulate at a constant rate throughout the early phase (Spector et al., 1978). A third E1A mRNA of 9S, giving a 28,000 dalton polypeptide in in vitro translation, is detected only after initiation of DNA replication and it accumulates during the late phase of infection (Spector et al., 1980). Two early mRNAs have been identified for region E1B, a 22S mRNA and a 13S mRNA (Wilson and Darnell, 1981); a third transcription unit from which polypeptide IX is derived, is also located in this region. This latter promoter is considered intermediate or quasi late and it is not activated in transformed cells (Pettersson and Mathews, 1977; Persson et al., 1978; Spector et al., 1978).

In region E2 of the DNA, the transcription pattern changes during the switch from early to intermediate times. At early times, a promoter mapped at 75 map units is active (Berk and Sharp, 1977a); from this transcription, one or two mRNAs are generated which code for a DNA binding protein (DBP) required for DNA replication. At intermediate times the DBP promoter is located at

Figure 2. Map of regions E1A and E1B and their transcription products. The DNA sequence is marked off in kilobases from the left end of the genome. Above this, horizontal lines represent exons of the E1A and E1B mRNAs, and the arrowheads indicate the 3' ends. Boxes above the axons indicate translated regions and the theoretical molecular weights for the proteins which can be translated from the open reading frames. Mutants mapped in E1A or E1B are indicated below the adenovirus map. Modified from Gaynor et al. (1982), Halbert and Raskas (1982), Montell et al. (1982), Graham (1984).


9S
IX, 14K
9S
8.3K
26K
21K
12S
13S
55K
32K
21K
13S
22S
1
2
3
4
5
HR1:1055
GR.I HR.CS
GR.II HR.CS
DL 312
DL 313

72 map units (Chow et al., 1979) and there is also some promoter activity at 86 and 99 map units (Chow et al., 1979). This shift in promoter activity requires synthesis of virus coded proteins. At intermediate times, the promoter located at position 75 generates a transcript which terminates at or after position 11 on the left of the DNA (Stillman et al., 1981). This unit has been called E2B and at least 3 polypeptides are generated from this transcript (see Fig. 1). The terminal protein (Stillman et al., 1981) and a virus coded polymerase (Stillman et al., 1982; Lichy et al., 1982) belong to these E2B coded proteins. An additional promoter on the l strand at 16 map units is also turned on at intermediate and late times (Chow et al., 1977a). The mRNA controlled by this promoter codes for a protein known as IVa2 (Lewis and Mathews, 1980) which appears to be involved in the maturation of precursor capsids into virions (Persson et al., 1979). Early region 2 is the last early region to be turned on and its transcription rate reaches a maximum at 7 hours P.I. followed by a decline (Nevins et al., 1979).

The E3 region is transcribed rightward into a set of 6-8 different mRNAs, all from the same promoter at 76 map units and at least 3 different polypeptides have been assigned to this region (Persson et al., 1980b; Ross et al., 1980a). One of these proteins is the 16,000 dalton precursor of the 19,000 dalton membrane-bound glycoprotein; it is translated from 3 different mRNAs which vary in the site of addition of poly A (Persson et al., 1980b; Herisse et al., 1980). Part of region E3, located between coordinates 83.5 and 85, is

not required for growth in human cells since deletion mutants which lack this region multiply normally (Persson et al., 1980a).

Region E4 is transcribed from the l strand and encodes multiple mRNAs, all with the same poly A addition site at 91.3 map units (Berk and Sharp, 1977b; Chow et al., 1979). Several polypeptides have been identified by in vitro translation, but their in vivo function is still unknown (Matsuo et al., 1982; Downey et al., 1983). The transcription of regions E3 and E4 reaches a maximum rate at 3 hours P.I. and then declines over the next 6 hours (Nevins et al., 1979).

In addition to these regions, the major late promoter located at 16.3 map units on the r strand of the genome is also expressed at early times (EL promoter, Shaw and Ziff, 1980; Akusjarvi and Persson, 1981a). The mRNA transcribed from it contains the same tripartite leader used at late times, but only one poly A addition site, mapped at 38.5 map units, has been identified. This mRNA is heterogeneous and sometimes contains an extra leader fragment (i) mapped at 22.0 - 23.5 map units (Akusjarvi and Persson, 1981a; Falvey and Ziff, 1983).

Early gene expression is controlled at different levels: E1A products seem to govern the accumulation of mRNA from other regions (Berk et al., 1979a; Persson et al., 1981a,b) and other viral products like the 72,000 dalton DNA binding protein, seem to act at the transcriptional level (Nevins and Winkler, 1980; Persson et al., 1981b). A brief discussion of these control mechanisms

is presented in the next section.

ii) Early proteins

At least 19 early viral coded proteins have been identified and mapped by studies carried out using in vitro translation of selected mRNAs or with the cell proteins labelled in vivo and then immuno precipitated with antisera raised against early antigens (Lewis et al., 1976; Harter and Lewis, 1978; Persson et al., 1979b; Halbert et al., 1979; Esche et al., 1980; Lewis and Mathews, 1980). The large number of related transcripts derived from each of the four early regions complicate the efforts to sequence mRNAs and to identify the proteins they encode (Chow et al., 1979; Wilson et al., 1979; Kitchingman and Westphal, 1980). Figures 1 and 2 show the locations of the mRNA corresponding to the early regions and the tentative molecular weights (MW) of the early proteins which are, or might be, encoded by these messengers. Some of these products will be discussed in more detail in the following sections of the introduction.

The role of early proteins in the control of early promoters has been studied with the aid of protein synthesis inhibitors and of virus mutants, but the picture is not yet clear. The protein encoded by the E1A - 13S mRNA is required for transcription of viral early regions other than E1A (Berk et al., 1979; Jones and Shenk, 1979b; Nevins, 1981; Montell et al., 1982) at early times after infection. Protein inhibitors added before infection, depress accumulation of mRNA from regions 2, 3 and 4, three to four times

with respect to untreated controls (Persson et al., 1981a). Similar results were obtained with E1A mutants defective for the synthesis of 13S mRNA; E1A 12S mRNA is not required for the normal control of transcription of other early regions (Berk et al., 1979; Nevins, 1981; Montell et al., 1982; Ricciardi et al., 1981; Jones and Shenk, 1979a; Ross et al., 1980b; Shenk et al., 1979).

In addition, if the inhibitor is added 1-2 hours after infection, there is an increase in the accumulation of early mRNA (Lewis and Mathews, 1980); the normal decline of mRNA synthesis from transcription units 3 and 4 does not occur in the absence of protein synthesis or after infection with ts 125 at nonpermissive temperature. Ts 125 is temperature sensitive for the production of 72,000 dalton DNA binding protein and this protein seems to be involved in the control of the stability of early mRNAs and, therefore, in their cytoplasmic abundance (Carter and Blanton, 1978a,b; Nevins and Winkler, 1980; Babich and Nevins, 1981).

B) Late phase

i) The early to late phase switch

At intermediate times during the lytic cycle, the major change appears to be a shift in promoter recognition by the cellular polymerase II and at least 7 distinct new mRNAs appear (Chow et al., 1979; Stillman et al., 1981). The major event leading to the early to late transition is the replication of viral DNA (Tooze, 1980; Thomas and Mathews, 1980), which is required for almost all the structural proteins. Only structural polypeptides IX and IVa2 can

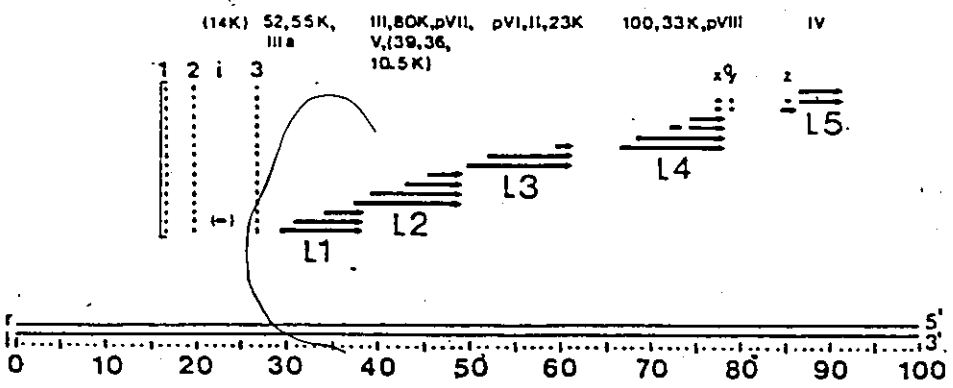
be synthesized in the absence of DNA replication (Esche et al., 1980; Persson et al., 1978), though their rate of transcription increases considerably after DNA replication. Therefore, both are classified as intermediate proteins.

ii) Late phase

During the late phase of the productive infection almost all the information in the adenovirus genome is transcribed. The transcription pattern is markedly different from the pattern observed at early and intermediate times (see Fig. 1 and 3) (Sharp et al., 1974b; Philipson et al., 1975; Pettersson et al., 1976; Tooze, 1980) and at least a 30-fold increase in transcription is observed (Shaw and Ziff, 1980). At least 22 late mRNAs and 12-14 proteins have been identified and mapped. Several early mRNAs are also transcribed during the late phase of the cycle (Ginsberg, 1979).

The major late promoter is located at 16.3 map units (Lewis et al., 1977) and some late transcripts extend toward the right end of the genome, beyond coordinate 92 (Fraser et al., 1979a). However, only 20% of the transcripts extend all the way, due to inefficient elongation of the chains (Fraser et al., 1979b). The mRNAs clustering along the long transcript have been classified into 5 classes (L1 - L5, see Fig. 3) of 3' coterminal mRNA species located between coordinates 30 and 92 (Chow et al., 1977b; Nevins and Darnell, 1978a; Ziff and Fraser, 1978). Each of these classes is translated into more than one protein (Fraser and Ziff, 1978; Nevins and Darnell, 1978a; Ziff and Fraser, 1978) but each mRNA species codes for a single protein only,

Figure 3. A genomic map of Ad5-coded late proteins and their mRNAs. All late mRNAs originate from the major promoter at coordinate 16.3 and contain tripartite leader segments derived from coordinates 16.4, 19.6 and 26.6 joined to the body of each mRNA. A fraction of all late mRNA also contains a fourth leader segment, the i leader, and a 14K protein has tentatively been located within the i leader itself. The relative order of the polypeptides (parentheses) has not been unequivocally determined. Modified from Persson and Philipson (1982).


(14K) 52,55K, IIIa
III,80K,pVII, V,(39,36, 10.5K)
pVI,II,23K
100,33K,pVIII
IV
1 2 i 3
x
y
z
L1
L2
L3
L4
L5
(-)
5'
3'
0 10 20 30 40 50 60 70 80 90 100

even when it contains sequences specific for more than one protein. As mentioned above, transcripts of the major late promoter containing the tripartite leader are also found in the cytoplasm of infected cells at early times but they terminate prematurely and belong only to the L1 family.

Poly A addition and splicing are the critical steps which determine the kind of protein translated. This selection must be done in a coordinate manner, but the mechanisms of control are still unknown. Cleavage at the 3' terminus and poly A addition seem to precede splicing (Nevins and Darnell, 1978b; Akusjarvi et al., 1981b). Some differences observed in the abundances of late mRNAs might also be regulated at the level of mRNA stability as in the case of some early transcripts. However, late mRNA has a relatively long half life compared with early transcripts, suggesting that regulation of late mRNA concentrations may be at some other level.

The proteins synthesized predominantly after the onset of viral DNA replication are considered late proteins. These proteins are mainly structural proteins and represent almost all the proteins synthesized by the cell 16-18 h after infection (Philipson et al., 1975). Figure 3 shows the position of the different classes of late mRNA and the tentative molecular weights for the proteins translated from these mRNAs.

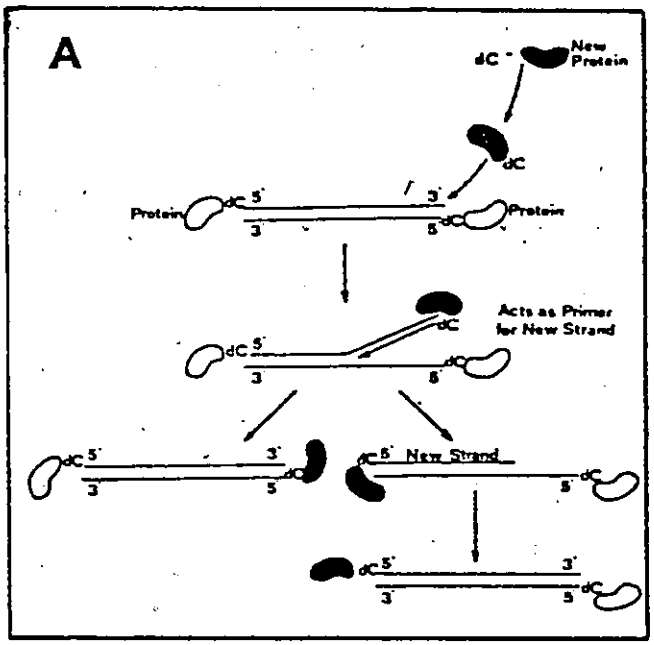
C) Adenovirus DNA replication

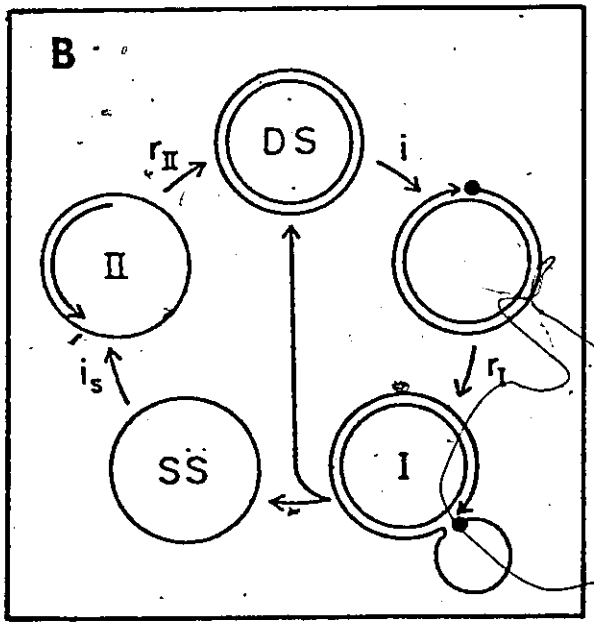
The synthesis of viral DNA begins 6 to 8 hours after infection of human cells with adenovirus 2 or 5 and reaches a maximum 6 to 10

hours later (Tooze, 1980). DNA synthesis is initiated at either end of the duplex and proceeds by strand displacement (Lechner and Kelly, 1977; Sussenbach and Kuijk, 1977, 1978) producing branched and unbranched linear molecules (see Fig. 4). Recent in vitro experiments have demonstrated that initiation of replication is primed at the ends of the molecule by a virus encoded protein with a mass of 80-87,000 daltons (Rekosh et al., 1977; Challberg et al., 1980, 1982; Tamanoi and Stillman, 1982). The protein is subsequently cleaved into the 55,000 dalton terminal protein found in DNA isolated from mature virus (Challberg et al., 1980; Enomoto et al., 1981; Lichy et al., 1981). The 80-87,000 dalton precursor of the terminal protein (pTP) has been found on the 5' ends of nascent DNA strands replicated in vitro and also as a component of the DNA-protein complex isolated from virions of the protease deficient Ad2 mutant ts1 (Challberg et al., 1980; Stillman et al., 1981). This precursor copurifies with a DNA polymerase activity. The purest fraction which is still active for in vitro replication contains viral DNA plus 2 viral coded polypeptides: an 80-87,000 dalton polypeptide which has been identified as the precursor for the terminal protein and another polypeptide of 140,000 dalton with DNA polymerase activity (Enomoto et al., 1981; Lichy et al., 1982; Stillman et al., 1982). This last protein appears to be inactive when prepared from cells infected with Ad5 ts 149 at non-permissive temperatures (Stillman et al., 1982). Cellular factors are required as well (see below).

Viral DNA treated with pronase does not support replication in a cell free system using infected cell extracts (Challberg and Kelly, 1979).

Figure 4. Replication models for adenovirus DNA. The model shown in (A) has been proposed by Rekosh et al. (1977). In this model the terminal protein (◖) covalently linked to the 5' ends of the viral DNA acts as a primer for the new strand. A free protein molecule becomes covalently attached to a deoxycytidine (dCTP) residue which in turn provides the 3' OH terminus required for initiation of DNA replication. Model (B) was proposed by Pearson et al. (1982). In this case circular molecules are formed before replication, and the terminal protein (●) introduces a nick in the DNA at the origin of replication (i) and remains covalently attached to the 5' phosphate. The 3' hydroxyl (arrowhead) acts as the primer for displacement synthesis (rI). The displaced strand is locked into the replication fork in the type I molecule. After termination of displacement synthesis initiation (is) of complementary synthesis (rII) requires a cellular priming event and results in the formation of Type II molecules.


A
dC
New Protein
dC
Protein
dC
5'
3'
3'
5'
dC
Protein
dC
Acts as Primer for New Strand
dC
5'
3'
dC
5'
3'
dC
5'
New Strand
5'
dC
dC
5'
3'
3'
5'
dC


B
DS
r_{II}
i
II
i_s
SS
I
r_I

If this inactive DNA is further treated with piperidine, its activity is restored (Tamanoi and Stillman, 1982), presumably because the removal of the residual peptide by piperidine allows the protein-DNA complex to be formed again. In addition, when a plasmid containing an origin of viral DNA replication was used as a template and added to infected Hela cells extracts, the pTP-dCMP complex was formed provided that the plasmid was linearized and the origin was located at the end of the molecule (Tamanoi and Stillman, 1982). Furthermore, initiation of DNA replication occurred whether the sequence was in double stranded or single stranded form. With both templates, viral or plasmid DNA, initiation occurs at the deoxycytidine residue at the 5' end of the adenovirus-protein complex. Pearson et al. (1982) also reported high efficiency of recognition of the viral end by the pTP in circular plasmids containing cloned terminal sequences of adenovirus DNA (Wasylyk et al., 1980). A specific nick was introduced into replicating molecules at the adenovirus origin and these molecules replicated as rolling circles with displaced, single stranded tails. The sequences important for controlling recognition and replication of the cloned termini of Ad5 DNA seem to be located at least 10 ± 5 bp within the terminal repeat unit, perhaps including the highly conserved sequence found in all adenovirus serotypes between nucleotides 9 - through 22 (Pearson et al., 1982; Tolun et al., 1979). Figure 4 summarizes the models proposed for adenovirus replication by Tamanoi and Stillman (1982) and other groups (Fig. 4A), and by Pearson et al. (1982) (Fig. 4B). In the first situation (Fig. 4A), the function of the pTP in replication is to form

a complex with the first nucleotide in the nascent chain and serve as a primer for DNA synthesis. The primary initiation event in adenovirus DNA replication would be the formation of this ester linkage between the α phosphoril group of dCMP and the β OH of a serine residue in the 80,000 dalton terminal protein precursor (Desiderio and Kelly, 1981; Tamanoi and Stillman, 1982; Challberg et al., 1982). Some kind of association, mediated by interactions with specific terminal nucleotide sequences might be formed between the 80,000 dalton pTP and the terminus of the linear parental DNA. As a result, the dCMP residue attached to the 80,000 dalton pTP could be positioned in such a way that it could serve as a primer for subsequent chain elongation (Challberg et al., 1980; 1982). The essential features of the model proposed by Pearson et al. (1982) (Fig. 4B) are that the viral DNA replicates as a covalently closed circular molecule; and the terminal protein, which binds tightly at a specific recognition sequence, catalyzes the breakage and reunion of a single polydeoxynucleotide chain at a fixed distance from the recognition site. This cleavage results in the constant attachment of the terminal protein to the 5' phosphate and the 3' hydroxyl now acts as a primer for replication. The circular molecules which might be predicted by this model had not been observed in vivo until recently (see chapter V of this thesis).

Another virus encoded protein known to be required for DNA replication is the single stranded DNA binding protein (DBP). Experiments using Ad5 mutant ts 125, which is temperature sensitive for the 72,000 dalton DBP, have shown that this protein is required for DNA elongation but not for the in vitro formation of the first nucleotide linkage

between the 80,000 pTP and the dCTP group (Van der Vliet and Sussenbach, 1975; Van der Vliet et al., 1977; Kaplan et al., 1979; Enomoto et al., 1981; Challberg et al., 1982).

Recently, Nagata et al. (1982) reported the requirement of a cellular factor, called "nuclear factor I" for adenovirus replication. It is a 47,000 dalton protein, isolated from nuclear extracts of uninfected Hela cells. In the presence of DBP there is a requirement of factor I for pTP-dCMP complex formation. No polymerase activity either α, β, or γ has been detected in association with factor I.

II. Cell Transformation

A. Transformation: lysogeny vs. transformation of eukaryotic cells

Certain bacteriophage genomes, such as the lambda phage genome can be covalently integrated into the host bacterial chromosome. This integration leads to lysogeny, a condition which affects the fate of both the phage and the host bacterium: the integrated phage does not replicate independently; the host cell, on the other hand, becomes resistant to superinfection by the same phage strain and can continue to grow and replicate (Hershey, 1970).

Since the discovery of DNA tumor viruses, investigators have attempted to obtain experimental evidence for an analogy between lysogenic conversion of bacteria by bacteriophage and animal cell transformation by viruses. After inoculation with a tumor virus, the infected cells do not always undergo lysis and under certain circumstances they continue growing and become significantly altered. Often, the most prominent changes are an increased growth rate, loss

of contact inhibition, and the ability to replicate indefinitely in serial subcultures (Pastan et al., 1978; Wittelsberger et al., 1981). Changes in cell morphology and antigenicity, anchorage independence and tumorigenicity are other properties of some transformed cells. Many attempts have been made to relate the different transformed phenotypes to the expression of one or more viral functions and also to find a relationship between in vitro phenotypes and tumorigenicity. Several different approaches have been used to detect the presence and expression of viral genes in transformed cells. The demonstration of the presence of viral DNA sequences within the host genome requires the use of sensitive RNA-DNA or DNA-DNA hybridization analysis. Before such techniques were available, the detection of virus specific antigens in virus infected cells using antisera from animals bearing tumors induced by the same virus, suggested the presence and expression of viral genes in transformed cells (Huebner et al., 1963; Habel, 1965; Rapp et al., 1964). Convincing evidence for the continued presence of viral DNA in SV_{40} transformed cells was first obtained in a series of virus rescue experiments (Gerber, 1966; Koprowski et al., 1967; Boyd and Butel, 1972).

The use of filter hybridization (Westphal and Dulbecco, 1968) and of the kinetics of reassociation in solution (Britten and Kohne, 1968; Wetmur and Davidson, 1968; Gelb et al., 1971) of labelled viral DNA in the presence of cold transformed cell DNA, gave quantitative evidence for the presence of viral DNA sequences in transformed cells (Green et al., 1970; Pettersson and Sambrook, 1973). More recently,

the blotting procedure described by Southern (1975) allowed more detailed studies of the presence and state of viral sequences in transformed cells and of the cell-viral DNA junctions.

B) Transformation by adenovirus

The human adenoviruses were among the first DNA viruses shown to be able to induce tumors in animals or to transform cells in culture (Tooze, 1980). Indeed, they were the first human viruses demonstrated to be oncogenic in laboratory animals. As shown in Table I, only adenovirus from groups A and B are able to induce tumors in animals, group A being strongly oncogenic and group B only weakly oncogenic. Nevertheless, viruses of groups A, B, C and D can transform cells in culture; only adenovirus 4, from group E, has never been shown to transform cells or to induce tumors in animals (Green et al., 1980, see Table I).

Hamster cells are partially permissive for replication of adenovirus 2 or 5 but nonpermissive for replication of adenovirus 12; rat cells, on the other hand, are semipermissive for the replication of all three serotypes (Tooze, 1980). For that reason, both are commonly used to study cell transformation by these viruses. A wide variety of transformed phenotypes, similar to that observed in other kinds of transformed cells, can be induced in either cell type after adenovirus infection. For example, adenovirus transformed cells are less susceptible to contact inhibition of movement and growth than the untransformed cells. In addition, adenovirus induced transformants can grow in low calcium medium and some transformants

can grow in suspension or in medium with low concentration of serum (Tooze, 1980). Adenovirus transformed cells contain subgroup-specific antigens and some adenovirus transformed cells are tumorigenic when transplanted into animals.

All the adenovirus-transformed cells reported up to date contain and express at least part of region E1, but other regions of the viral DNA can also be present and transcribed in the cells. Sharp et al. (1974) first demonstrated that the reassociation of some EcoR1 fragments of Ad2 DNA was accelerated when mixed with DNA extracted from Ad2 transformed cells. Gallimore et al. (1974) quantitated the viral DNA sequences present in 9 transformed rat cells. The authors consistently found sequences located at the left-hand end of the viral genome, with varying amounts of other DNA segments also present in some cells. It was concluded that none of the Ad2 transformed cell lines studied contained a complete set of viral DNA sequences and that as little as 14 percent of the viral genome was sufficient to maintain cells in the transformed state. In addition to that, transfection studies using different restriction fragments mapped the transforming region in the left most 8% of the genome for Ad12 (group A; Shiroki et al., 1977; Mackey et al., 1979; Mak et al., 1979), Ad7 (Group B; Sekikawa et al., 1978) and Ad5 (group C; Graham et al., 1974).

Houweling et al. (1980) and Van der Eb et al. (1979) reported partial transformation of rat cells by the HpaIE fragment of Ad5 (4.5% of the extreme left); these transformed cells have an unlimited

life span and are aneuploid, but they grow slower than other adenovirus transformed cells, are unable to reach high saturation density and have an elongated, fibroblast-like morphology (fully transformed cells transformed by adenovirus have an epithelial-like morphology and form multilayers in culture dishes).

All these studies indicate that transformation is basically a function of early region 1 (E1), but they raised the question of the role played by E1 encoded gene products in transformation by adenoviruses. To answer this question, the phenotypes of adenovirus mutants in region E1 have been analyzed in different laboratories (see Table II).

C. Mutants in the transforming regions (Table II, Fig. 1 and 2)

i) Host range (hr) mutants.

A number of host range mutants of Ad5 have been isolated on the basis of their preferential growth in an adenovirus transformed human cell line (293 cells) which contains and expresses the left end (E1 region) of the adenovirus DNA (Graham et al., 1977). These mutants are transformation defective (Graham et al., 1978) and, on the basis of complementation analysis and marker rescue studies, they have been subdivided into two complementation groups: I and II, mapping in E1A and E1B, respectively (Harrison et al., 1977; Frost and Williams, 1978; Galos et al., 1979). Group I mutants are unable to transform rat embryo or rat embryo brain cells but can induce a partial or semi-abortive transformation of primary baby rat kidney cells (Graham et al., 1978). Group II hr mutants, on the other hand, fail to transform any of these cell types in assays using virus (Graham et al., 1978). Nevertheless,

TABLE II

Ad5 MUTANTS AFFECTING TRANSFORMATION *

	MAP POSITION		MUTANT
1) Host range mutants	Group I	E1A	hr1, 2, 3, 4, 5
	Group II	E1B	hr6, 7, 50, 51
2) Deletion mutants	Group I	E1A	dl 311, dl 312
	Group II	E1B	dl 313, dl 314
3) Temperature sensitive mutants		E2A	ts 107, ts 125
		E2B	ts 36, ts 149
4) Cold sensitive mutants	Group I	E1A	cs11, cs12
	Group II	E1B	cs13

*See references in the text.

they are able to induce a fully transformed phenotype in experiments using DNA (Rowe and Graham, 1983).

The DNA belonging to the E1A region of hr1 (a group I hr mutant) has been recently sequenced (Ricciardi et al., 1981) between positions 917 and 1112, and shown to contain a single bp deletion at nucleotide 1055. The sequences containing this deletion are removed by splicing from the 12S mRNA, giving a polypeptide which runs in polyacrylamide gels as having a molecular weight of 48,000 daltons (same as in wt infections). On the other hand, the frameshift produced in the 13S mRNA generates a premature stop codon at nucleotides 1085-1088 and the polypeptide obtained migrates in polyacrylamide gels with a molecular weight of 28,000 daltons instead of the 51,000 polypeptide produced in wt infections. This truncated polypeptide seems to be responsible for the changes in the phenotype exhibited by hr1 mutants.

ii) Deletion mutants (dl)

Several deletion mutants in E1 have been isolated which grow to wt levels in 293 cells but will not replicate in Hela cells (Jones and Shenk, 1979a). Dl 312 and dl 313 are the most commonly used and best characterized dl mutants. Essentially all of region E1A is deleted in dl 312, a mutant which is transformation negative, while dl 313, which has all region E1B deleted, is able to partially transform 3Y1 cells (Kimura et al., 1975) at high multiplicity of infection (moi) (Shiroki et al., 1981; Mak and Mak, 1983).

iii) Temperature sensitive mutants (ts)

None of the ts mutants isolated to date map in the transforming region E1. Nevertheless, 2 groups of ts mutants have a pattern of

transformation different from wt. virus. These mutants are: 1) the transformation defective mutants ts 36 and ts 149, both mapping in region E2B (Galos et al., 1979; Stillman et al., 1982) and E2A mutants ts 125 and ts 107 which transform with enhanced efficiency (Ginsberg et al., 1974). Group N ts mutants (ts 36 and ts 149) fail to transform rat embryo cells at the non-permissive temperature (Williams et al., 1974, 1979), and temperature shift experiments indicated that the affected gene product is required for initiation but not for maintenance of transformation. The gene product affected in these mutants seems to be the viral coded DNA polymerase (see section IC), the role of this enzyme in transformation is unknown. A similar example of a viral protein required for replication and transformation has been reported for SV40 T-antigen (Rigby, 1979). The supertransforming capacity of ts 125 and ts 107 at non-permissive temperature may be related to the lack of an active 72,000 dalton DBP. This protein has been involved in the rapid turnover of early viral mRNA, including E1A and E1B mRNAs (Babich and Nevins, 1981; see section IA). The absence of 72,000 dalton activity may result in the accumulation of E1 mRNA and hyperproduction of transforming proteins. Other hypotheses have been proposed to explain the higher transforming efficiency of ts 125 and ts 107. Ginsberg et al. (1974), for example, proposed that the 72,000 dalton protein acts as an excision protein, and that the lack of 72,000 dalton activity allows a higher number of viral sequences to remain integrated and a higher number of cells to become transformed.

iv) Cold sensitive mutants (hr^{cs})

Host range mutants of Ad5 which possess a cold sensitive host range phenotype have been recently isolated (Ho et al., 1982). The authors showed, in addition, that some of the previously isolated host range mutants (i.e. hr1 and hr2) were also cold sensitive. Cold sensitive mutants grow well in 293 cells at 32.5° and 38.5°C but show a cold (32.5°) sensitive phenotype in Hela cells. They map in the E1A (group I) and E1B (group II) regions and are also cold sensitive for transformation. Group I cs mutants transform rat embryo cells at 38.5°C as efficently as wild type virus but they do not transform, or transform poorly at 32.5°C. Temperature shift experiments showed that upon shiftdown to 32.5°C after 5 days grown at 38.5°C, transformation was almost completely abolished. On the other hand, transformation at 32.5°C followed by shift to 38.5°C gave a number of transformants similar to experiments done and kept at 38.5°C. From these experiments the authors concluded that E1A function is required for maintenance but not for initiation of transformation. Group II cs mutants fail to transform or transform very poorly at both temperatures (32.5°C and 38.5°C). Transformants could be obtained only at 38.5°C and showed a cold-sensitive phenotype. Therefore, it seems that E1B gene function is required for both initiation and maintenance of transformation.

Purpose of the Study

As previously indicated in this introduction, several approaches have been used by others to gain an insight into the mechanism of cell transformation by adenovirus. These studies have revealed that all the analyzed adenovirus-transformed cells contain and express at least part of region E1, but other regions of the viral DNA can also be present and transcribed in the cells. This raises the possibility that other regions may also be involved in the transformation process.

The studies presented in this thesis were undertaken to find out whether the transformed phenotype was dependent on the pattern of integration of the viral DNA and to obtain information about the mechanism by which this DNA becomes integrated into the host chromosome. The phenotypes of cell lines transformed either by Ad5 or by host range mutants were analyzed and correlated with the presence of viral sequences. The results reported in Chapters III and IV confirm that at least part of region E1 is always present in the transformed cells, but no connection was found between the pattern of integration and the phenotypes of the transformed cells. These phenotypes are, however, different if the E1 sequences integrated in the cells belong to wt virions or to hr mutants.

In some hr transformed cells, virtually the entire viral DNA molecule was found colinearly integrated in the cell chromosome. This finding suggests a function for both viral ends in the process of cell transformation by adenovirus. Observations done by other groups, which reported the presence of both viral DNA ends joined

together in some wt transformed cells, give further support to this hypothesis. To test this idea I looked for intermediate DNA forms of the integration process and for precursors for integration in permissive and semipermissive cells immediately after infection. Covalently closed circular forms of Ad5 DNA were discovered (see Chapter V), a finding which opens new possibilities for the understanding of adenovirus integration.

CHAPTER II

MATERIALS AND METHODS

MATERIALS AND METHODS

1) Cells and Cultures

a) Cell lines: The human cell line Hela, used for propagation and assay of adenovirus 5 (Ad5) was originally obtained from Dr. J. Williams. Rat cells 3Y1 (Kimura et al., 1975) were provided by Dr. S. Mak and the rat line 1074-9, a spontaneously transformed primary baby rat kidney cell line, was developed in our laboratory (Graham, personal communication). The transformed human line 293 (Graham et al., 1977) was derived from primary cultures of human embryonic kidney cells (HEK) transfected with sheared Ad5 DNA using the calcium technique (Graham and Van der Eb, 1973). Some cell lines derived from 293 cells were used in the experiments reported in Chapter III: 1) 293-NM3 is a tumorigenic derivative of 293 cells obtained after 3 successive passages through nude mice (Graham, unpublished), 2) 293-SC4 is a subclone of 293-NM3 (Graham, unpublished) and 3) A9 and A11 are two human 293-chinese hamster hybrids provided by Dr. L. Aiello (Aiello et al., personal communication). Several Ad5 transformed rat cell lines were also used: 512C8 (Lassam et al., 1979) was transformed with the HindIII G fragment representing the left end 8% of the viral DNA (Tooze, 1980); 637C3 and 822C2 (Lassam et al., 1979) were transformed by infection with wild type (wt) virions while 637-1 and 637-4 were transformed by Group I host range (hr) mutants hr3 and hr1, respectively (Graham et al., 1977). The cell line 149-637-4 corresponds to 637-4 cells infected with Group II host range mutant hr6, and

637-4' are 637-4 cells obtained from the same experiment, which were treated in a similar way as the infected cultures but didn't receive any virus.

Hela and 3Y1 cells were grown in α minimal essential medium (αMEM, Flow laboratories) supplemented with 5% newborn calf serum. 293 cells were maintained in Joklik's modified MEM (Flow laboratories supplemented with 10% heat-inactivated horse serum (HS, Gibco). Rat transformed lines were grown in Joklik's medium plus 10% fetal bovine serum (FBS) or 10% newborn calf serum (NCS). 100 units of penicilin per ml, 100 µg of streptomycin per ml (Gibco) and 1.2 mg/ml of L-glutamine were added to all the cultures. The cells were grown at 37°C in an atmosphere of 5% CO_2 and 95% air.

b) Primary cultures: Monolayers of primary baby rat kidney (BRK) cells were prepared from kidneys of one week old Wistar rats and seeded in αMEM supplemented with 10% FBS (Graham et al., 1978). The cultures were fed with the same medium until they reached 80% confluency at which point they were used in transformation assays.

2) Virus Strains and Viral Infections

The wild type strain of Ad5 was the one regularly used in our laboratory (Williams, 1970). Host range mutants hr1, hr3, hr5 and hr6 (Harrison et al., 1977) were also obtained from our laboratory stocks. Virus infections were usually done for 1 hour with virions diluted in αMEM, (1 ml of the appropriate dilution per 60 mm dish) and then incubated further in αMEM plus 5% horse serum or as required for each experiment.

For the transformation assays, 1-2 day old subconfluent cultures of primary BRK cells (approximately 10^6 cells/60 mm dish)

were infected with wild type virus or host range mutant virus at a multiplicity of infection (moi) of 0.01-2 PFU/cell. Host range mutants hr1, hr3 and hr5 from Group I and hr6 from Group II were used. After 1 hour absorption at 37°C, the cultures were refed with αMEM plus 10% FBS and three days later they were switched to Joklik's modified MEM (with low Ca^{++}) plus 5% horse serum. This medium was changed 2 or 3 times per week. Dishes in which colonies could be detected were transferred as mass cultures at different times after infection (see Chapter IV). Once established, some of the lines were subcloned by plating 20-50 cells/60 mm dish and picking no more than one clone from each dish.

For the study of new forms of viral DNA early after infection, subconfluent Lux dishes (150 mm) with primary BRK or cell lines 3Y1, 1074-9 or Hela were infected with hr1, hr6 or wt Ad5 at a moi of 1 PFU/cell. The dishes were then incubated in αMEM plus 10% FBS for different periods of time (see Chapter V) and harvested as indicated for DNA extraction (see below).

3) Purification of Ad5 Virions

Wild type Ad5 virions were purified from suspension cultures of infected KB cells harvested 48 hr after infection. The infected cells were pelleted at 2,000 rpm in a MSE centrifuge and resuspended in 100 mM Tris pH 7.5. The suspension was then sonicated on ice for several 15 sec bursts in a Biosonik II Sonicator, followed by centrifugation. An equal volume of Freon 20 was added to the supernatant and the solution was homogenized on ice in a Sorvall omnimixer. The Freon was centrifuged, and extracted

two times with 100 mM Tris pH 7.5. The pooled supernatants were loaded in a SW25 tube containing a 5 ml cushion of CsCl with a density of 1.435 gm/ml and centrifuged in a Beckman centrifuge for 30-45 min at 25,000 r.p.m. After centrifugation the virus band was on the top of the cushion. Some CsCl from the bottom plus the aqueous phase were removed and discarded; the final density of the virus band was adjusted to 1.35 gm/ml and again centrifuged for 20 hr at 35,000 r.p.m. in a SW50 rotor. The virus band was then collected and dialized against 10 mM Tris pH 7.5, 1 mM EDTA for DNA extraction.

4) Transfection Assays with Transformed Cell DNA

Total cellular DNA was extracted from 637-4 cells (see below) and 15, 20, 25 and 30 µg of DNA were inoculated on 293 cells growing in αMEM plus 5% HS using the calcium technique (Graham and Van der Eb, 1973; Graham et al., 1980). Briefly, cell DNA was diluted in 10 mM Tris pH 7.0, 1 mM EDTA and mixed with 2 x $CaCl_2$ to give a final concentration of 125 mM. This solution was added slowly (1 to 1), with bubbling, to Hepes buffer pH 7.1 (2 x concentrated = 280 mM NaCl, 50 mM Hepes, 1.5 mM Na_2HPO_4) and kept 20 min-30 min at room temperature to form a precipitate. Finally, 0.5 ml of the solution plus the precipitate were inoculated in 60 mm dishes. The medium was not removed or changed until the next day and the cultures were incubated at 37° C in a CO_2 oven.

293 cells transfected with 637-4 DNA were followed daily for about 1 month looking for CPE. Weekly changes of media were made

during this period and 1 ml of medium was removed before the media change and inoculated into fresh 293 cells. Some dishes were frozen and thawed 3 times and the lysates were inoculated into fresh 293. Other dishes were used for in situ hybridization.

5) Growth and Plating Efficiency Studies

Cell saturation densities and doubling times were measured in cultures growing in Joklik's medium with 10% FBS. Cells were seeded in 60 mm dishes at 2×10^5 cells/dish; each day, over a 10 day period, a set of cultures was trypsinized and the cells counted in a Neubauer chamber. The medium was changed once in the middle of the experiment (at about 5 days). The doubling time of the cells was calculated from the exponential part of the growth curve and the saturation density, expressed as number of cells per 60 mm dish, from the plateau level reached in each case. The efficiency of plating in soft agar was determined according to Macpherson and Montagnier (1964), by plating 10^3-10^5 cells in 2 ml medium containing 0.25% Difco Noble Agar onto a basal layer of 0.5% agar. Colonies were counted after 6-8 weeks with the aid of a low power microscope.

6) Tumorigenicity in Nude Mice

Confluent cultures were trypsinized and the cells resuspended in PBS with 2% horse serum. The cells were counted, centrifuged and resuspended in PBS without serum, at a concentration of 0.8 to 2×10^8 cells/ml, and 0.1 ml of the cell suspension was

inoculated subcutaneously into 6-8 week old congenitally athymic nude mice (Pantelouris, 1968).

7) Cell Fusion

Mixed populations (1:1) of 293 and 637-4 cells were seeded at about 25% confluency and left overnight to attach and grow. These mixed monolayers were then treated with 50% PEG 6.000 in Joklik medium (w/v) for 1 minute, followed by washes in two progressively diluted solutions of PEG (1/3 and 1/8) and finally growth in Joklik plus 10% FBS (Pontecorvo et al., 1977). The cultures were followed daily for a period of 6 to 8 weeks, with weekly changes of media. One milliliter of medium was removed on occasions and inoculated on fresh 293 cells. Some dishes were frozen and thawed 3 times and the lysates were inoculated on fresh 293.

8) DNA Extraction

Confluent monolayers of transformed cells were rinsed twice with phosphate buffer saline lacking Ca^{++} and Mg^{++} (PBS^{--}) and the cells were lysed in 2-3 ml of 10 mM Tris-HCl pH 7.5, 10 mM NaCl, 1 mM EDTA, 0.4% SDS plus 0.5 mg/ml of predigested Pronase B (Calbiochem-Boehring Corp.). After digestion of cell proteins at 37°C overnight and extraction with Tris-buffer saturated phenol and chloroform:isoamyl alcohol (24:1), the lysate was treated with 10 µg/ml of RNase A (Sigma) for 30 min at 37°C and again extracted with chloroform:isoamyl alcohol. Following dialysis against 10 mM Tris-HCl pH 7.5, 10 mM NaCl, 1 mM EDTA, the DNA was precipitated

with ethanol and redissolved in 20 mM Tris HCl pH 7.5, 1 mM EDTA. The DNA extraction from purified virions was carried out similarly except that digestion with Pronase was for 2 hr and RNase treatment was omitted. DNA from recombinant plasmids containing the cloned fragments HindIII F, G or I, XhoI C or the PvuII leftmost 1.2% fragment of Ad5 DNA was kindly provided by Mr. R. McKinnon (McMaster University).

For the experiments described in Chapter V, covalently closed circular DNA was partially purified following a modification of the procedure developed by Birnboim and Doly (1979) for purification of plasmid DNA from bacteria. Specifically, the cells were washed with PBS and lysed for 10 min at room temperature by addition of alkaline SDS (1 ml of 50 mM glucose, 25 mM Tris, 10 mM EDTA plus 2 ml of freshly prepared 1% SDS, 0.2 N NaOH) and 3 N sodium acetate pH 4.8 was added to a final concentration of 1 M for 1 hour at $4^{o}C$. Following centrifugation, the DNA in the supernatant was precipitated with ethanol and resuspended in 20 mM Tris pH 7.5, 1 mM EDTA. CsCl and ethidium bromide were then added to a final density of 1.60 g/cc and a concentration of 200 µg/ml, respectively. The gradients were centrifuged to equilibrium at 35,000 rpm, $15^{o}C$ for 65 hours in a Beckman 50 Ti rotor and were then fractionated by collecting 10 drop fractions from the bottom of the tube. Aliquots (20 µl) from each fraction were counted in a liquid scintillation counter. A second aliquot from most of the fractions was used to measure the

refractive index in a Bausch & Lomb refractometer from which the density along the gradient was calculated. The fractions selected were pooled, extracted with isoamyl alcohol to remove the ethidium bromide and dialized in 20 mM Tris pH 7.5, 1 mM EDTA buffer.

9) Gel Electrophoresis and Southern Blotting of Cell DNA

DNA from control, infected or transformed cells was digested with different restriction endonucleases (see text) following the manufacturer's instructions (New England Biolabs, Bethesda Research Labs, Boehringer Mannheim). For transformed cells, usually 30 µg of DNA were digested and analyzed, for infected cells variable amounts were used as described in Chapter V. The DNAs were then electrophoresed on 0.5 or 0.7% agarose horizontal slab gels (16 x 26 x 1 cm) for about 36 hr at 1 V/cm in Tegtmeyer's buffer (36 mM Tris, 30 mM NaH_2PO_4, 1 M EDTA, pH 7.5; Tegtmeyer and Macasaet, 1972) with 1 µg/ml of ethidium bromide. One or 3 genome equivalents of Ad5 DNA per cell, mixed with 20-30 µg of carrier DNA and digested in the same way as test DNA, were also electrophoresed as a marker. Direct visualization of the DNAs was obtained by UV illumination, and pictures were taken using type 57 Polaroid film (Polaroid Corp., Oak Brook III) and a red filter. Transfer of the DNA from the gels to nitrocellulose filters (Schleicher and Schwell, BA 85 R597) was done by the Southern technique (Southern, 1975) as modified by Ketner and Kelly (1976): The DNAs were denatured in situ with 1 N KOH for 20' in a shaking

bath. The same volume of 1 M Tris pH 7.5 plus 1 N HCl was added after this time and shaken for 2 hours with control of the pH which should be ∿ 7. The gels were then transferred to a 6 x SSC bath and shaken 20 min. Water soaked nitrocellulose filters were added at this time and the gel plus the nitrocellulose filter and 6-7 pieces of Whatman paper Nr. 1 and 3 were placed on a piece of Saran Wrap and maintained for more than 4 hours with constant changes of the Whatman filters. When the gel was less than 1 mm thin, it was removed and discarded and the nitrocellulose paper was washed with ice cold 6 x SSC for 20 min at 4° C. After that the filter was dried and baked in a vacuum oven at 80° C for two hours. At that point the filter could be stored or used right away for hybridization.

10) In Vivo Labeling of Cells

a) Labeling of the DNA: Primary BRK cells were infected with Ad5 hr1 mutant at a moi of 1 Pfu/cell. ^{3}H TdR (.5 µCi/ml, New England Nuclear 20 Ci/mmol) was added at both 24 hr and 48 hr after infection and the cultures were harvested at 72 hr PI for DNA extraction.

b) Labeling of proteins: The growth medium was removed from subconfluent monolayers of transformed cells (150 mm Lux dishes) and substituted for 5 ml per dish of methionine free medium containing 10 µCi/ml of [^{35}S] methionine (New England Nuclear, specific activity ∿ 1,000 Ci/mmol). After a labeling period of 8 to 12 hours, the cells were scraped with a rubber policeman

into the medium and pelleted for 10 min at 4,000 rpm in a MSE centrifuge. The pellets were used for preparation of cell-free extracts and immunoprecipitation (see below).

11) In Vitro Labeling of DNA

Adenovirus 5 DNA or viral DNA fragments were labeled in vitro with [α^{32}P] dCTP (New England Nuclear, prepared in tricine buffer, specific activity ~ 700 Ci/mmol) by nick translation.

Most of the nick translation were done following the procedure described by Maniatis et al. (1975) with some changes: 0.5 - 1.0 µg of DNA was incubated in 50 mM Tris pH 7.9, 5 mM $MgCl_2$, 10 mM mercapto ethanol, 15 µM of each dTTP, dATP, dGTP, 100 µCi of [α^{32}P] dCTP plus 10 ng/ml of activated DNAse and 10 units of Polymerase I. The DNAse (pancreatic DNAse, Sigma) was initially dissolved in 0.1 M NaCl at 1 mg/ml and stored in 10-20 µl aliquots at -20° C. Before use it was diluted 1/10 in activation buffer (10 mM Tris pH 7.5, 5 mM $MgCl_2$, 1 mg/ml BSA, 0.15 M NaCl) and incubated 2 hr at 4° C. The activated DNAse was further diluted 1/1000 in activation buffer (final concentration 100 ng/ml) before adding to the reaction, which was equilibrated at room temperature or 37° C before adding the enzymes. The DNAse was added first and incubated for 1 min at room temperature followed by the addition of 10 units of DNA Polymerase I (Boehringer-Mannheim, 1000 U/200 µl). The final volume used was 100 µl which were incubated at 14° C for 2-3 hr.

The procedure described by Rigby et al. (1977) was used for some experiments, the main difference with the procedure described above was that phosphate buffer pH 7.4 was used, instead of Tris buffer pH 7.9. Specific activities of 0.8 - $1.8 \cdot 10^8$ cpm/µg of DNA were obtained with both procedures. On occasion, the reaction was monitored by removing 0.5 µl which were mixed with 500 µl of ice-cold calf thymus or salmon sperm DNA (100 µg/ml in H_2O). 100 µl were spotted and dried on a glass fiber filter to obtain the total input of cpm and 10-50% ice cold TCA was added to the remaining 400 µl. This tube was kept on ice for 5-10 min and then filtered onto a glass fiber filter and washed with TCA and ethanol. Both filters were dried and counted in toluene in a Scintillation counter. The cpm of the 2nd filter versus the cpm in the first filter, corrected by the volumes used, represent the percentage of incorporation of ^{32}P into the nick translated DNA.

The reaction was stopped by adding 10 µl of 0.25 M EDTA and the labeled DNA was separated from unicorporated label on Sephadex G50 (7 ml column on a 10 ml plastic pipette equilibrated with 10 mM Tris pH 7.5, 10 mM of NaCl, 2 mM of EDTA and 0.1% SDS). Two peaks of counts could be eluted from the column: the first peak represented the incorporated cpm and the 2nd peak was the free nucleotides.

12) Hybridization and Autoradiography

Two procedures were followed for DNA-DNA hybridization and similar results were obtained in both. In some experiments the hybridization was performed overnight at 60° C in Denhardt's solution II (Botchan et al., 1976): 0.02% Ficoll (MW: 400.000), 0.02% polyvinylpyrrolidone (PVP, MW: 360.000), 0.02% bovine serum albumin (BSA), 0.5% SDS and 1 mM EDTA in 6 x SSC. The filters were previously preincubated in Denhardt's solution I which only contains 0.02% Ficoll, PVP and BSA in 6 x SSC (Denhardt, 1966).

In the 2nd protocol (Wahl et al., 1979) hybridization was done at 42° C in the presence of formamide. The filters were preincubated first for 2 hr in 6 x SSC or in Denhardt's I solution at 42° C and then overnight in 50% formamide, 5 x SSC, 5 x Denhardt's solution, 100-200 µg/ml denatured salmon sperm DNA and 50 mM sodium phosphate pH 6.8. The hybridization solution contained 50% formamide, 5 x SSC, 1 x Denhardt's, 10% dextran sulfate and 2 mM sodium phosphate. Both hybridizations were done overnight at the respective temperatures (65° C or 42° C) and with $1\text{-}2 \cdot 10^6$ cpm/ml of labeled probe mixed with the hybridization solution and boiled for 5 min. The final volume used was 15-20 ml.

Filters hybridized following protocol 1 were washed at 65° C with 2 x SSC (several changes). Filters hybridized according to the 2nd protocol were washed initially at 42° C with 2 x SSC plus 0.2% SDS, then with 0.2 x SSC plus 0.2% SDS at 50° C and finally

with 2 x SSC with or without SDS at 65°C. For each different wash about 3 changes of the same buffer were made over a 1-2 hour period.

The filters were then dried and exposed to Kodak X-Omat R films XR1 or XR5, in some cases using intensifying screens at -70°C.

13) Densitometric Studies of the Autoradiographs

The appropriate bands in the autoradiographs were scanned with a Joyce Loebl MK III C5 microdensitometer and the relative radioactivity was estimated from the peak areas. To measure these surfaces a MOP-3 planimeter (Carl Zeiss Inc) was used and 4-5 readings were done for each peak.

Some readings were done using a Beckman DV-8 slab gel scanning system, which gives the peaks and the areas below the peaks simultaneously.

14) Plaque Hybridization Procedure (Perez-Villarreal and Berg, 1977)

a) Cell and plaque imprints on nitrocellulose: 60 mm dishes containing 293 cells fused with 637-4, or 293 cells inoculated with DNA extracted from 637-4 were drained and the moist monolayer was covered by dry nitrocellulose filters and pressed with a rubber policeman. The filters were then removed and placed for one minute on Whatman paper saturated with 0.5 N NaOH. 1.5 M NaCl. This step was followed by drying by suction under vacuum and the NaOH treatment plus suction was repeated 3 times. Finally,

the filter was soaked in 1 M Tris pH 7.4 and in 2 X SSC and fixed for 2 hours at 80°C in a vacuum oven.

b) Hybridization of plaque imprints: The prehybridization of the filters was done in Denhardt's solution I (Denhardt, 1966) for 4 hr at 66°C and the incubation with the labeled probe was done for 20 hr at 66°C in 6 X SSC, 100 µg of salmon sperm (SS) and 12 to $15 \cdot 10^3$ cpm/ml ($\sim 10^5$ cpm total) of labeled Ad5 DNA. The filters were then washed several times in 2 X SSC plus 0.1 M NaH_2PO_4 pH 6.5 and once with 2 X SSC and exposed to Kodak X Omat R films XR1.

15) Immunoprecipitation and SDS-Polyacrylamide Gel Electrophoresis (PAGE)

Cultures of transformed cells at ~ 80% confluency were labeled for 8-12 hr with 10 µCi/ml of [^{35}S] Methionine in αMEM lacking methionine. To detect Ad5 antigens two sera were used: Megaserum, provided by Mr. D.T. Rowe (McMaster University), and P antiserum, supplied by Dr. W.C. Russell (National Institute for Medical Research, Mill Hill, London). Megaserum is pooled serum from hamsters bearing tumors as a result of injection of several different lines of hamster cells transformed by various fragments of Ad5 DNA. This antiserum precipitates E1A and E1B proteins from virus infected KB cells (D.T. Rowe et al., unpublished). P antiserum was obtained from rabbits injected with extracts of Ad5 infected Ara C-treated RK_3 cells (Russell et al., 1967). It is directed mainly against the 72,000 dalton single-stranded DNA binding protein, but it is also active against other adenovirus early polypeptides (Lassam et al., 1979;

Saborio and Oberg, 1976). The protein A-sepharose technique was used (Lassam et al., 1979): labeled cells were washed with cold PBS and resuspended in RIPA buffer (1% vol/vol Triton X-100, 1% Deoxycholate, 0.2% SDS wt/vol 150 mM NaCl, 50 mM Tris pH 7.4). The cell lysate was sonicated several times for 15 sec on ice and then centrifuged at 4.000 rpm in a MSE centriguge. The supernatant was shaken overnight at 4° C and then mixed with 20 µl of the appropriate antiserum and 20-50 µl of Protein A-Sepharose beads (Pharmacia Fine Chemicals, Inc.), 10% suspension in RIPA buffer. The Sepharose beads were pelleted by centrifugation in an Eppendorf microcentrifuge, washed 3 times with ice cold 20 mM Tris buffer pH 7.5 containing 0.25 M lithium chloride and 0.1% ME and finally resuspended in the electrophoresis sample buffer (0.625 M Tris, pH 6.8, 2% [wt/vol] SDS, 0.1 M dithiothreitol, 10% [vol/vol] glycerol, 0.02% bromophenol blue) and boiled 10 min.

For the electrophoresis, a vertical slab gel apparatus 180 mm long and 1 mm thick was used for 16 hours at 70 volts. The running gel contained 18% acrylamide and the stacking gel 4.5% acrylamide with a ratio of acrylamide:bisacrylamide of 30:0.2. After electrophoresis, the slab gels were fixed in methanol:acetic acid:water (1:1.4:20) and desicated under vacuum. Finally, the dry gels were autoradiographed on Kodak X-Omat film XR1 or XR5.

CHAPTER III

INTEGRATION OF VIRAL DNA IN CELLS TRANSFORMED BY WILD TYPE ADENOVIRUS 5

I. Introduction

A number of laboratories have shown that the left 8-12% of the adeno genome is necessary and sufficient for the induction of a complete transformed phenotype in rodent cells (see general introduction). When I started the studies reported in this thesis, my interest was to analyze the patterns of integration of Ad5 sequences in the host cell chromosome, and also to determine whether any correlation existed between patterns of integration and transformed cell phenotypes.

With this purpose I analyzed the state of viral sequences in cell lines transformed by 1) host range (hr) mutants with mutations in the transforming genes (see chapter IV) and 2) by wild type (wt) Ad5. I was particularly interested in analyzing cell line 293 and certain cell lines derived from these cells. 293 is a human cell line transformed by sheared DNA extracted from wt virions (Graham et al., 1977); this line is very widely used because it contains and expresses the E1 region of the Ad5 genome (Graham et al., 1977; Aiello et al., 1979) and therefore, it allows the growth of mutants which are defective in functions encoded by this region. I was also interested in these cells because of the availability in the laboratory of non-tumorigenic or very weakly tumorigenic cells (all the passages of the 293 cells) and of tumorigenic derivatives of 293 cells. These tumorigenic lines resulted from successive passages of 293

cells in nude mice (see below). Cell hybrids of the 293 cells to hamster cells were also analyzed. These hybrids retain the same transformed phenotype as the parental 293 cells.

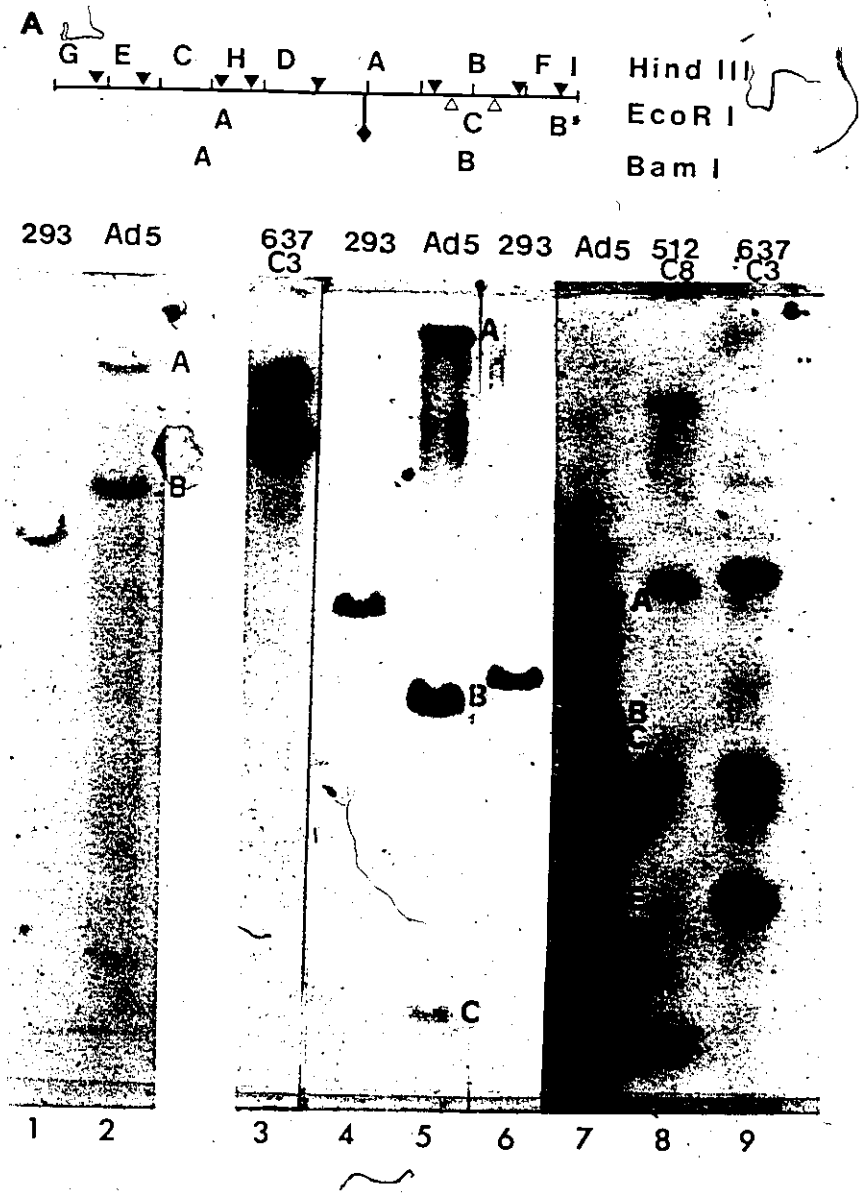
The experiments reported in this chapter were carried out, in part, to set up the techniques required to characterize integration patterns of viral DNA in transformed cells. The patterns obtained were very simple and stable. In addition to that, the results obtained with the 293 cells showed that changes in tumorigenicity of the cells were not associated with changes in the integration pattern.

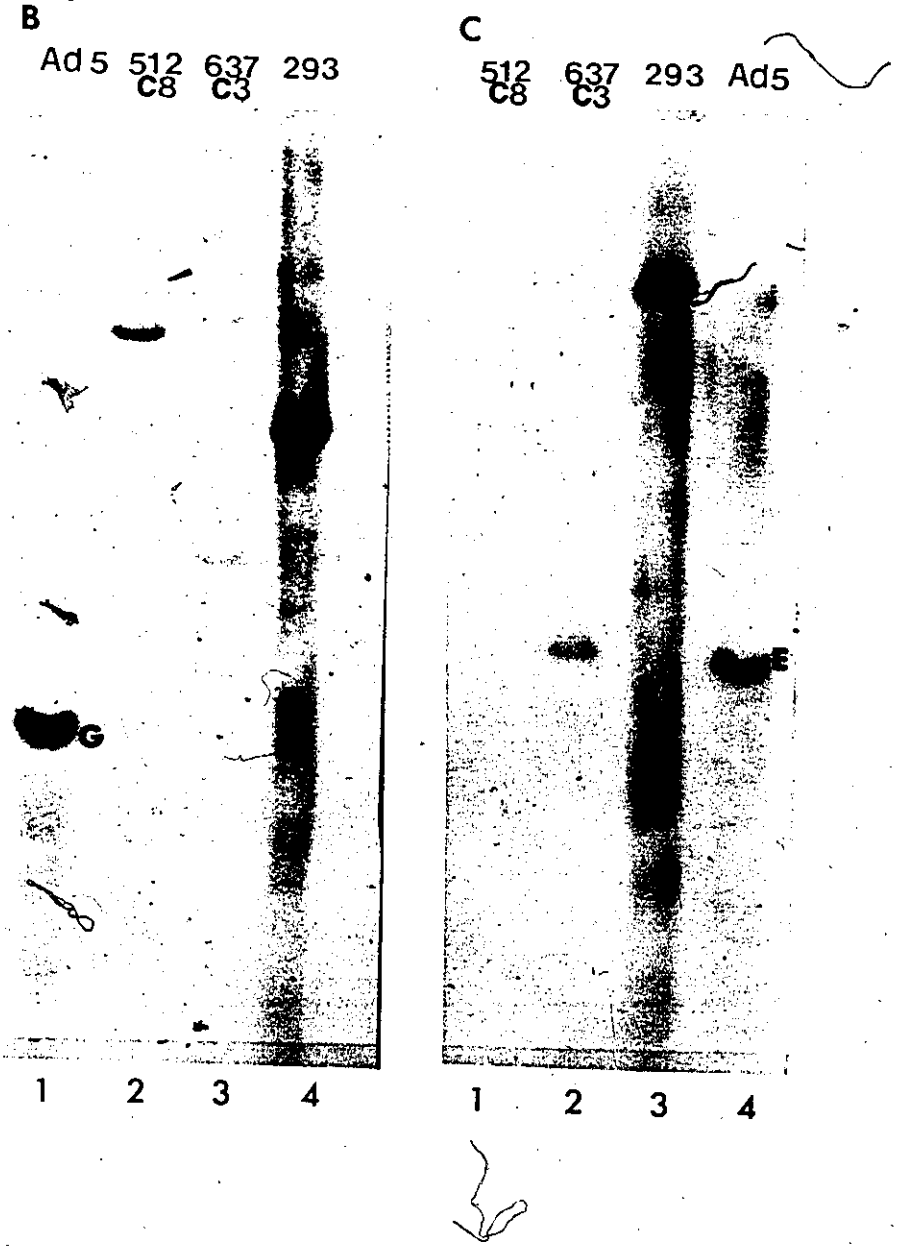
IV. Results

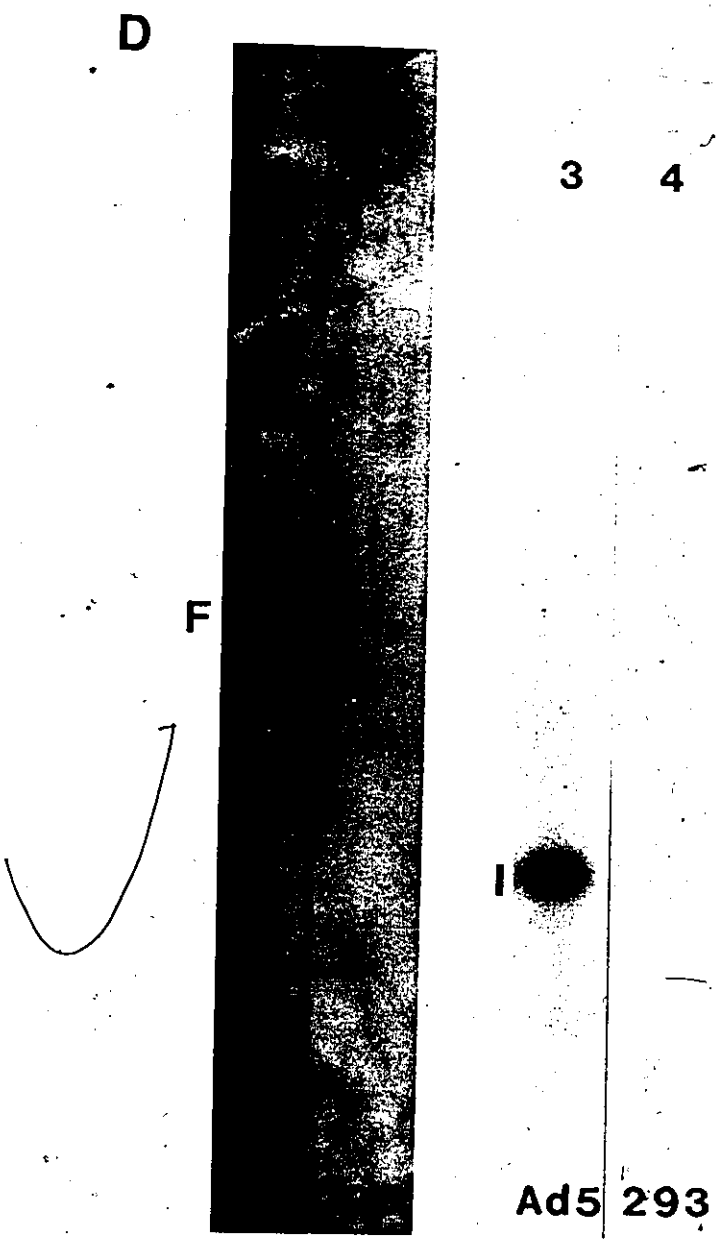
1) Ad5 DNA Integration in wt Transformed Cells

DNA extracted from 293 cells was analyzed by Southern blotting (see Materials and Methods). The nitrocellulose filters were probed with labeled total Ad5 DNA or with labeled restriction fragments (Hind III G or E) extracted from gels (Girvitz et al., 1980). The results obtained are shown in Fig. 5. According to these results, only one site of integration is present in the transformed 293 cells (Fig. 5A tracks 1 and 4) and the integrated fragment hybridizes with Hind III fragments G and E (Fig. 5B track 4 and 5C track 3) but not with Hind III fragments F and I (Fig. 5D tracks 2 and 4). As shown in figure 5, G and E represent the left end of the viral genome and F and I are both located at the extreme right of the viral DNA; therefore, we can conclude that the 293 cells grown and passaged in our laboratory contain only the left end of the viral DNA and that no sequences of the right end can be detected, either free or joined to the left end. This result disagrees with one published report (Aiello et al., 1979) but is consistent with the fact that neither protein nor mRNA from right end has been detected, and confirms results obtained in at least one other laboratory (D. Spector, personal communication).

Figure 5. Analysis of Ad5 DNA sequences present in wt transformed cells. DNAs extracted from transformed cells were digested with different restriction enzymes and analyzed by the Southern blotting procedure followed by hybridization with ^{32}P-labeled Ad5 DNA (panel A), HindIII G fragment (panel B) HindIII E fragment (panel C), HindIII F or HindIII I fragments (panel D). Restriction enzymes BamHI (tracks 1 and 2), EcoRI (tracks 3-5) or HindIII (tracks 6-9) were used in panel A, and HindIII in panels B, C and D. The cleavage map of Ad5 DNA with BamHI (◆), EcoRI (△) or HindIII (▼) is shown above panel A. The cell DNAs were as follows: Panel A) 293, channels 1, 4 and 6; 637C3, channels 3 and 9; 512C8, channel 8., Panel B) 512C8, channel 2; 637C3, channel 3; 293, channel 4. Panel C) 512C8, channel 1; 637C3, channel 2; 293, channel 3. Panel D) 293, channels 2 and 4. Marker Ad5 DNA was run in tracks 2, 5 and 7 of panel A in tracks 1 and 4 of panels B and C, respectively and in tracks 1 and 3 of panel D.


A
G E C H D A B F I
Hind III
A
A
C
B*
EcoR I
A
B
Bam I
293 Ad5
637 293 Ad5 293 Ad5 512 637
C3
C8 C3
A
B
A
B
C
1 2 3 4 5 6 7 8 9


B
Ad 5
512
C8
637
C3
293
G
1
2
3
4
C
512
C8
637
C3
293
Ad5
1
2
3
4


D
3
4
F
I
Ad5 293

Tracks 3, 8 and 9 in Fig. 5A contain two wild type transformed rat cell lines: 512C8 cells, transformed by Hind III G fragment extracted from agarose gels (Fig. 5A track 8; FLG personal communication) and 637C3 cells, transformed by virions (Fig. 5A tracks 3 and 9; Graham et al., 1978). As shown in Fig. 5, cell line 512C8 has two fragments hybridizing with the G probe after digestion of the cell DNA with Hind III; this can be interpreted as two sites of integration, both containing at least part of the Hind III G fragment (Fig. 5B track 2). The percentage of G fragment present in each site and their transcriptional activity are not known at this moment. At least one of the integrated G fragments must be transcribed because Ad5 coded proteins have been detected in 512C8 cells (Lassam et al., 1979). As expected, fragment E is not present in these cells (Fig. 5C track 1).

Cell line 637C3 has also two sites of integration and at least one of them contains more than the left 17% of the viral DNA. It can be seen in figure 5B track 3 and 5C track 2, that two of the fragments detected after hybridization with total Ad5 (Fig. 5A track 9) represent the junction of fragment G to cell DNA, and one of them is the entire E fragment. A tentative diagram for the integration pattern of Ad5 DNA in this cell line is shown in Fig. 12 (see Chapter IV).

2) Integration Patterns of Ad5 DNA in Tumorigenic Derivatives of 293 Cells

293 cells are very weakly oncogenic (Gallimore et al., 1977; Graham, unpublished) and are able to induce tumors only in

5 days old nude mice or in x rays-irradiated adults (300 rads whole body; Gallimore et al., 1977) after long latent periods (30 days or more). The tumor cells, however, can be transplanted to adult nude mice in which they produce tumors in a period of 10-12 days (Gallimore et al., 1977). Two tumorigenic derivatives of 293 cells were used for these experiments: 293-N3, obtained after 3 successive passages of 293 cells through nude mice and 293-SC4, a subclone isolated from 293-N3 cells (see Materials and Methods). I analyzed these 2 lines using the procedure described by Southern and compared the patterns obtained with these cells to the patterns of the parental 293 cells (see Fig. 6). No differences could be detected between the parental line and the tumorigenic derivatives. A similar result was obtained after the analysis of two 293-hamster hybrid lines (not shown). I conclude from these experiments that the integration of Ad5 DNA in the human line is very stable, and that changes in tumorigenicity are not necessarily associated with changes in the integration pattern of viral sequences.

In addition to that, cell lines 637C3 and 512C8 containing the entire E1 region (637C3) or only part of it (512C8) are both highly tumorigenic for nude mice (see Chapter IV, Table V). Therefore, the entire E1 region does not seem to be required for rat transformed cells to induce tumors (see below), whereas on the other hand, the presence of the entire E1 region is not always associated with tumorigenicity since 293 cells are only very weakly tumorigenic.

Figure 6. Patterns of integration of Ad5 DNA in 293 cells and in two tumorigenic lines derived from this human cells line. The DNAs were digested with restriction enzymes XbaI (panel A) and HindIII (panel B). Total Ad5 DNA labeled with ^{32}P dCTP was used as a probe with both filters. For both panels track 1 contains marker Ad5 DNA and the cell lines are from left to right: 293 (track 2), 293-N3 (track 3) and 293-SC4 (track 4). The cleavage map of Ad5 with both enzymes is shown below the figure.


A
B
1 2 3 4
1 2 3 4
G E C H D A B F I
Hind III
E B A D C
Xba I

III. Discussion

Several wt Ad5 transformed cells were analyzed for their viral DNA content. Some of these cells were tumorigenic for nude mice, and it was considered of interest to look for a relation between integrated sequences and tumorigenicity and also to find if any change in the integrated sequences could be related to changes in tumorigenicity.

Using Southern's blotting procedure, I found a very simple pattern of integration of Ad5 DNA in the virus or DNA transformed cells I analyzed. Only one (293, Fig. 5A tracks 1 and 4) or two (512C8, Fig. 5A track 8 and 637C3, Fig. 5A track 3and 5B track 2) sites of integration were detected and the integrated sequences seemed to be very stable.

Changes in tumorigenicity may be associated with a selection in the first host of cells which are able to survive in the new environment (Shin et al., 1975), or to a decreased rejection by the second host, due to changes in the antigenicity of the cells during the initial passage (Gallimore et al., 1977; 1979; McDougall et al., 1974; Gallimore and Paraskeva, 1979). The observed changes in tumorigenicity of 293 cells were however, not related to detectable changes in the integrated sequences (see Fig. 6).

It should be mentioned that group C adenovirus transformed cells are only weakly oncogenic in rodents, and newborn animals, immunosuppressed rats or nude mice should usually be used to detect tumorigenicity (Tooze, 1980). Nude mice are functionally devoid of T cell-dependent immunity (Pantelouris, 1968; Povlsen et al., 1973; Freedman and Shin, 1974) but in some cases their rejection of tumor cells can be highly efficient (Kiessling et al., 1976; Smets, 1980). Alternative mechanisms involve antibody dependent cell-mediated cytolysis, activated macrophages or naturally occurring killer cells. One or more of these mechanisms may recognize and destroy 293 cells when injected into adult nude mice.

As mentioned above, differences in antigenicity or in viability of the cultured tumor cells have been proposed by different laboratories to explain their success or failure in producing tumors in nude mice with transplanted cells. My results neither favor nor reject either hypothesis, but allow the conclusion that the presence and expression of the entire Ad5 E1 region is not sufficient for the human cell line 293 to induce tumors in nude mice and that no qualitative changes in the integrated sequences are required to increase tumorigenicity.

An alternative explanation for the increase in tumorigenicity may be a change in the number of copies of integrated viral sequences. At least for cell line 293-SC4, this seems

an unlikely possibility since no obvious change in the viral DNA content is detected in Figure 6.

The rat cells studied, on the other hand, confirm that cells containing only part (7.5%) of the E1 region can be as tumorigenic as cells with the entire E1 region (see Table V). The number of cell lines studied is not large enough to derive any general conclusion but the results presented in this chapter agree with previous publications (Graham et al., 1974; Van der Eb et al., 1977; Lassam et al., 1979). In the following chapter the results of new transformation experiments will be presented. Cell lines transformed by wt and hr mutant virus (mutants in region E1) are analyzed in order to obtain more information first, on the role played in transformation by region E1, and second, on the relation between integrated sequences and the phenotype displayed by the transformed cell.

CHAPTER IV

INTEGRATION AND EXPRESSION OF VIRAL DNA IN CELLS TRANSFORMED BY HOST RANGE MUTANTS OF ADENOVIRUS 5

I. Introduction

Only the E1 region of Ad5 can be clearly implicated in cell transformation and if any viral products are required for maintenance of the transformed phenotype, these products must be coded within region E1. This region can be subdivided into two transcriptional units: E1A and E1B and, as mentioned above, the transformation defective group I and group II host range mutants have been mapped in these regions (Frost and Williams, 1978; Graham et al., 1978). Group II mutants fail to transform any of the cell types used to date in assays using virions whereas group I mutants, on the other hand, induce an abnormal or semi-abortive transformation of baby rat kidney cells (Graham et al., 1978). It has been shown that lines of cells transformed by group I mutants can be established and cultured indefinitely even though the hr I mutants are defective in E1. Preliminary studies on their properties indicated that, though immortal, they failed to express a fully transformed phenotype (Graham et al., 1978).

In the studies reported in this chapter, I established several additional lines of cells transformed by group I mutants and characterized these and previously established lines in terms of extent and structure of integrated viral DNA, expression of

viral specific proteins, a variety of growth parameters and tumorigenicity. The results are consistent with the hypothesis that group I hr mutants are defective in one or more functions required for the induction and maintenance of a fully transformed phenotype. In addition, the structure of integrated viral DNA sequences in several hr mutant transformed cells appears to be atypical. Most of the results reported in this chapter have been published (Ruben et al., 1982).

II. Results

1) Isolation of Cell Lines Transformed by Host Range Mutant Virus

Although group I hr mutants are considered defective for transformation, a few group I transformed lines were isolated in previous experiments (Graham et al., 1978). These cell lines exhibit an abnormal, partially transformed phenotype. I attempted new transformation experiments to obtain additional information on the transformation process by group I mutants and to establish more transformed lines to be characterized in terms of their phenotype and the structure of integrated viral sequences.

Subconfluent monolayers of primary BRK cells were infected with wild type (wt) Ad5, with group I hr mutants, 1, 3 or 5, or with group II hr mutant 6 at a multiplicity of infection (moi) of 0.01-2 pfu/cell. Three days after infection, the cultures were switched to low calcium medium (Joklik's) plus 5% horse serum to select against untransformed rat cells. Extensive cytopathic effect (cpe) was observed by 2-3 days post-infection in cultures infected with wt virus and, to a lesser degree, in group II mutant infected cultures; no cpe was observed following infection with group I mutant virus. Transformed colonies were detected 10-14 days after infection in cultures inoculated with either wt or group I hr mutant virus, whereas no transformed foci

developed in cultures infected with group II mutant virus, as reported previously (Graham et al., 1978). In agreement with results published by Graham et al. (1978), the number of foci in cultures receiving group I mutant virus was higher than in cultures infected with wt virus. Attempts to establish cell lines from isolated transformed clones in the mutant infected cultures were unsuccessful; however, polyclonal transformed lines could be established by passaging entire cultures containing 20 or more colonies. Transformed cultures were trypsinized at various times ranging from 2 to 11 weeks post infection and replated to 25 cm^2 plastic bottles in Joklik's medium plus 5% horse serum. From a total of 48 attempts, 21 cultures continued to grow after the 2nd or 3rd passage and indefinitely thereafter. The protocol for the isolation of the various lines is presented in Table III; hr virus transformed lines 637-1, 637-3, 637-4 and 809-1 and wt transformed lines 637-C3 and 822-C2 (both clonal isolates) were derived from previous experiments (Graham et al., 1978).

As shown in Fig. 7, the hr mutant transformed lines have a fibroblastic morphology (panel B), different from the epithelioid morphology of the wt transformants (panel A). Small groups of cells with epithelial morphology could be observed in some hr

TABLE III

CELL LINES TRANSFORMED BY GROUP I hr MUTANT AND w.t. VIRUS

	Cell line	Transformed by	Transformed at MOI[a] of:	Time of 1st subculturing[b]	DNA extracted at passage no.[d]
Expt. 1	637-4	hr1	$5\ 10^{-2}$	19	13, 41
	809-1	hr1	2	28	8
	637-1	hr3	1	19	11
	637-3	hr3	$2\ 10^{-1}$	19	15
	637C3	wt	$5\ 10^{-1}$	19[c]	32
	822C2	wt	2	21[c]	12
Expt. 2	1H1	hr1	10^{-1}	32	4-5
	1H2	hr1	10^{-1}	34	5, 11-13
	3H1	hr3	1	13	5-6, 9-11
	3H2	hr3	1	34	4-5
	5H1	hr5	1	13	6-8, 9-10
	5H2	hr5	1	19	6, 8-10
	5H3	hr5	1	34	5, 8-11
	5H4	hr5	10^{-1}	77	10-12
	W86	wt	1	47	11-13
EXPT. 3	1H3	hr1	1	15	5-7
	3H3	hr3	1	15	9-16
	5H5	hr5	1	15	7-12

Primary baby rat kidney cells were transformed by wt virus or by hr mutants, 1, 3 or 5 as described in the text, and at various times post infection polyclonal populations of transformed cells were trypsinized. Listed in the table are all the established cell lines which are studied in the following sections.

[a]Multiplicity of infection (PFU/cell) at which the rat cells were infected.

[b]Number of days post infection at which the first passage of the entire culture was done, or

[c]at which two wt clones were isolated.

[d]Number of passages at which the cell DNA was extracted and studied.

(Ruben et al., 1982).

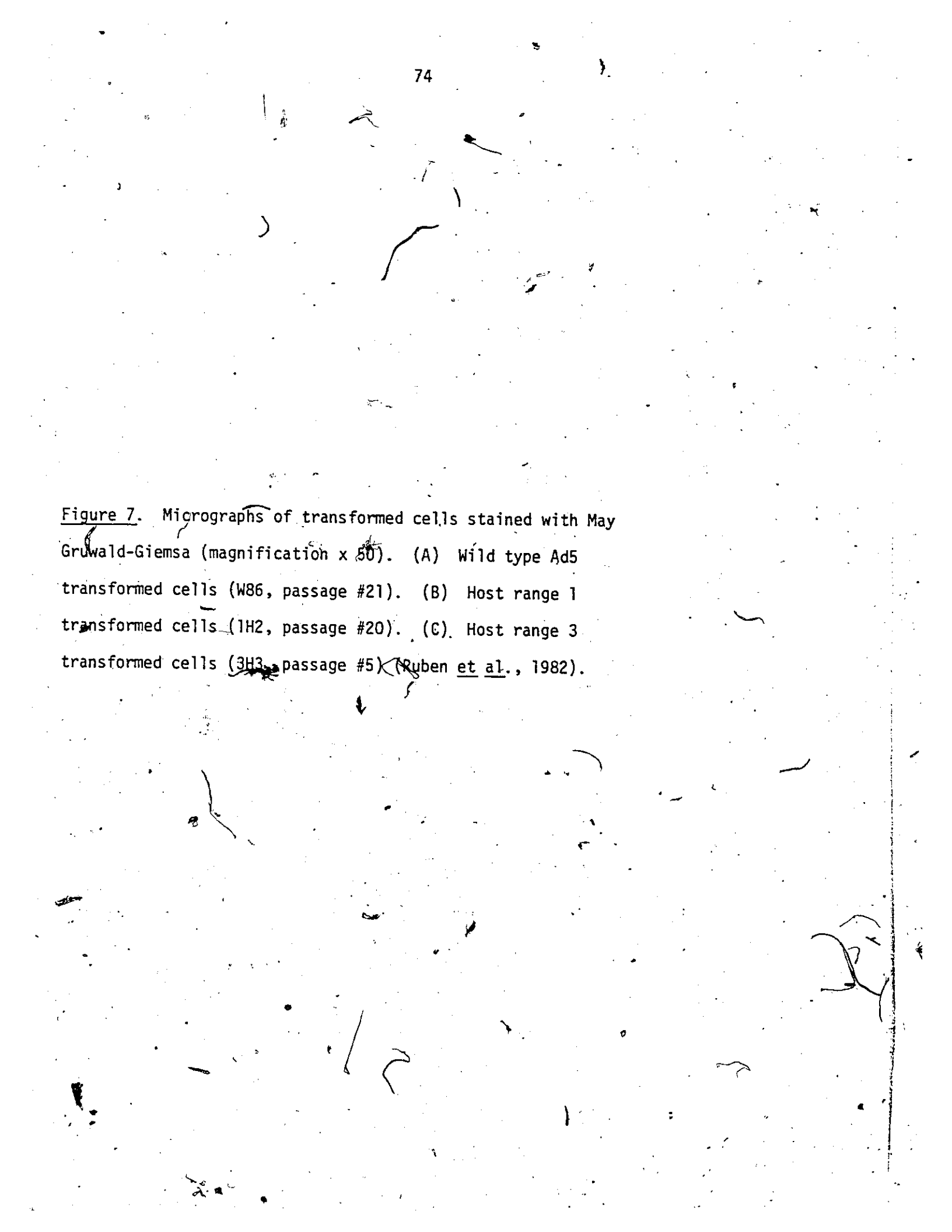
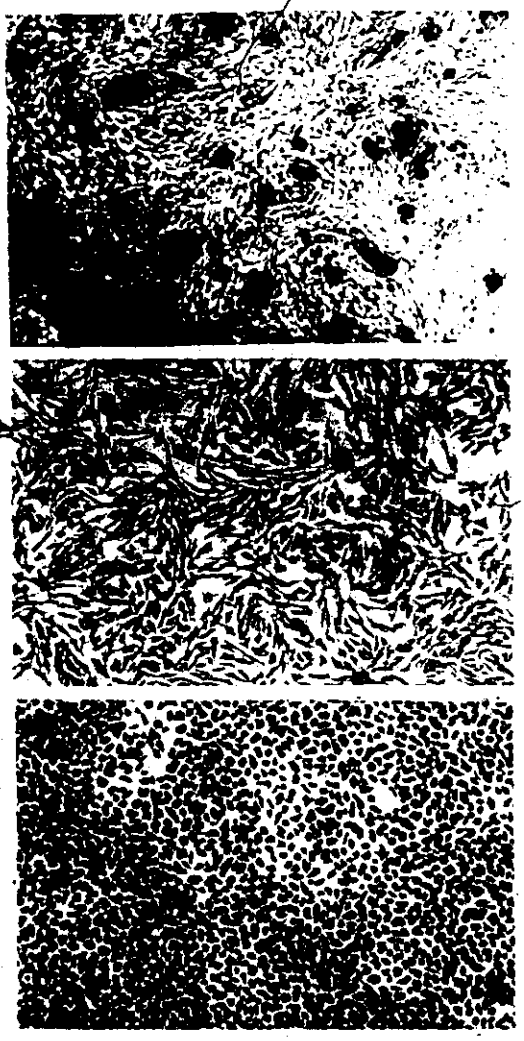


Figure 7. Micrographs of transformed cells stained with May Grüwald-Giemsa (magnification x 50). (A) Wild type Ad5 transformed cells (W86, passage #21). (B) Host range 1 transformed cells (1H2, passage #20). (C) Host range 3 transformed cells (3H3, passage #5) (Ruben et al., 1982).



transformed cultures at early passages (Fig. 7, panel C) but disappeared after 8-12 passages. Clonal isolates could be obtained from the fibroblastic cells after 8-9 passages in culture. Attempts to clone the epithelial cells from hr transformed cultures were usually unsuccessful, but a few clones could be isolated and studied (see below).

2) Growth Characteristics and Tumorigenicity of Transformed Cells

Transformed lines, as yet uncloned, were initially characterized on the basis of several growth parameters. Both mutant and wt transformants were capable of growth in low calcium ion medium and have so far been passaged over 50 times in culture. Initially, mutant transformed lines grew slower than wt transformants but after 6-8 passages in culture, both types of transformants had similar doubling times and saturation densities (see Table IV). Cells transformed by hr mutants, however, did not form colonies in soft agar whereas wt transformed cells usually plated with an efficiency of 10-15%. Several transformed lines were injected into 6-8 week old nude mice at concentrations ranging from 10^5 to 10^7 cells per animal. As can be seen from the data in Table V, hr transformed cell lines were much less tumorigenic than lines transformed by wt Ad5. In fact only one hr transformant, 637-1, induced any tumors in nude mice, and in this case the latent period (16 weeks) was much longer than the latent period for most wt transformed lines and only two of four animals developed tumors.

TABLE IV

GROWTH OF HOST RANGE TRANSFORMED CELLS IN JOKLIK MEDIUM SUPPLEMENTED WITH 10% FBS

Cell line	Transformed by	Doubling time (h) Expt. 1	Doubling time (h) Expt. 2	Saturation density (10^5cells/cm^2) Exp. 1	Saturation density (10^5cells/cm^2) Expt. 2
637C3 p13[a] and p18	wt	21:7	18	7.3	11
W86 p32	wt	33.2		9.3	
637-4 p42 and p46	hr1	22.6	20	13.1	10.0
1H2 p12 and p33	hr1	28	33	9.2	8.0
1H3 p20	hr1	29		7.4	
5H2 p25 and p32	hr5	29	24.5	8.5	10.1
5H5 p23	hr5	33.8		11.5	

Cell lines transformed by wt Ad5 and by hr virus were analyzed for different growth parameters.

[a]Passage number at which the cell growth was analyzed.

TABLE V

TUMORIGENICITY OF Ad5 TRANSFORMED CELL LINES IN ADULT ATHYMIC NUDE MICE

Cell line	Transformed by	No. cells inoculated	Expt. 1	Expt. 2	Expt. 3
637C3	wt	10^7	5/5[a] (3)[b]	4/4 (3)	4/4 (1)
		10^6	4/4 (3)		
		10^5	3/4 (3)		
424C1	wt	10^7	4/4 (2)	4/4 (7)	4/4 (2)
	(sheared DNA)	10^6	3/3 (7)		
642C1	wt	10^7	4/4 (2)		
822C2	wt	10^7	0/4 (13)[c]	0/4 (25)	
W86	wt	10^7	1/4 (4)[c]		
1H2	hr1	10^7	0/4 (8)[c]	0/4 (5)[c]	
1H3	hr1	10^7	0/4 (9)[c]		
637-4	hr1	10^7	0/4 (26)	0/4 (16)	
3H3	hr3	10^7	0/4 (10)		
637-1	hr3	10^7	2/4 (16)	0/4 (14)	
5H2	hr5	10^7	0/4 (8)[c]		

Transformed rat cells growing in culture were harvested, injected subcutaneously into nude mice and scored for tumorigenicity as described in Materials and Methods.

[a]No. animals with tumor/no. animals inoculated.

[b]Latent period in weeks or time at which the experiment was terminated when all animals were negative.

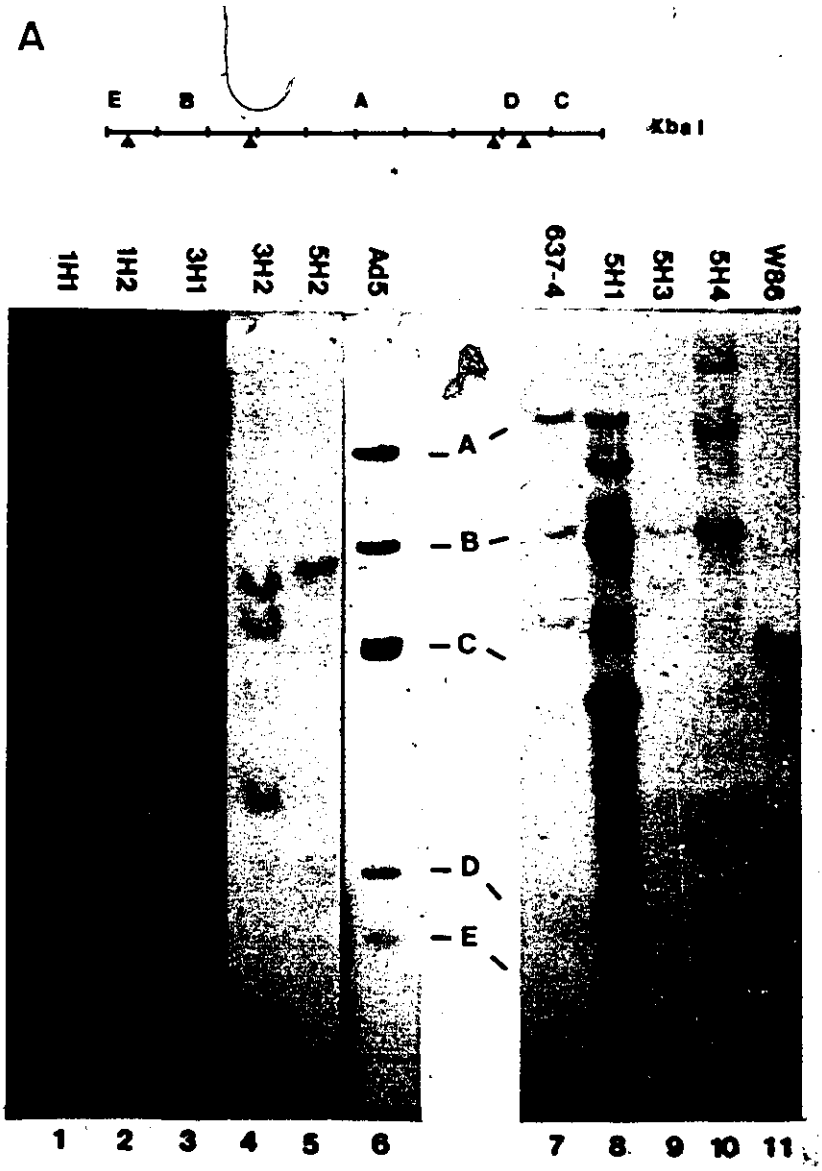
[c]At this time some animals died without any tumor and it was not possible to continue the experiment.

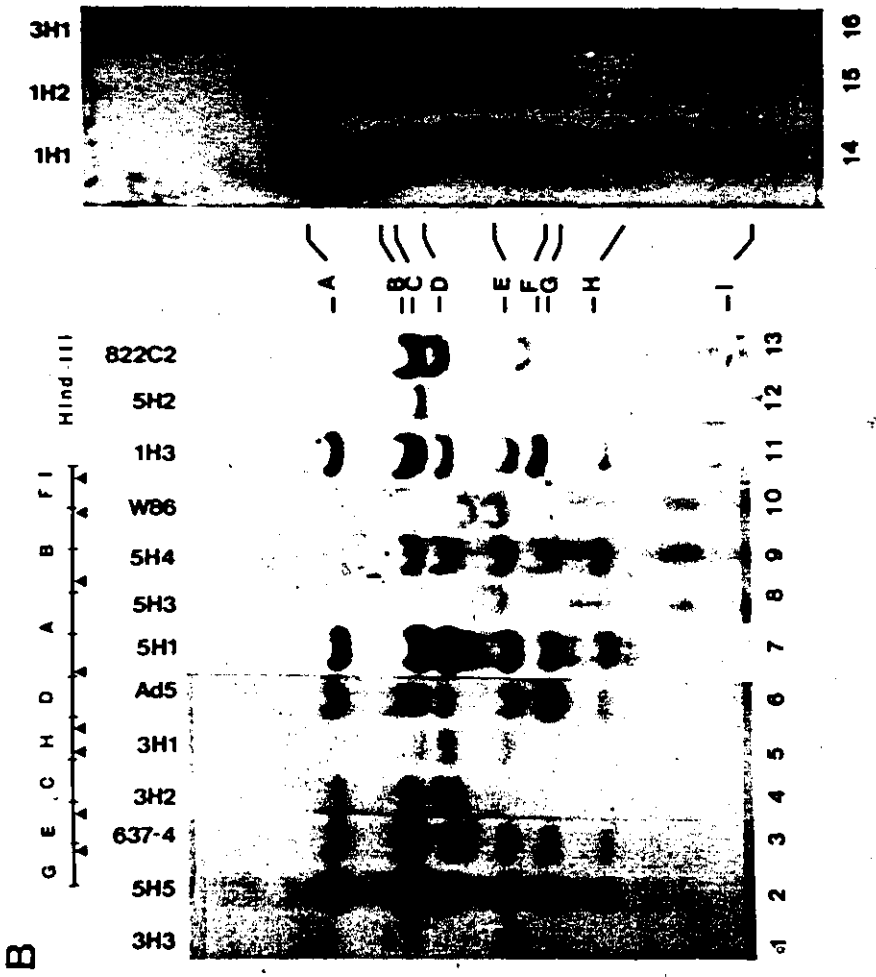
(Ruben et al., 1982)

3) Viral DNA Sequences in Transformed Cells

As described in the previous section, wt and hr transformed cells exhibit different phenotypes which could conceivably result from differences in integrated viral sequences and/or differences in the expression of viral genes. In order to explore these possibilities, the presence and state of viral DNA in hr transformed lines was studied and compared to that in wt transformants. DNA from transformed cells was digested with Xba I (Fig. 8A) or Hind III (Fig. 8B) and the digestion products were separated by electrophoresis, transferred to nitrocellulose filters and hybridized with ^{32}P-labeled Ad5 DNA. The cleavage maps of the viral DNA with either enzyme are shown above each panel of Fig. 8 and the migration patterns of the respective cleavage products of viral DNA markers are illustrated in track 6 of panel A (Xba I) and track 6 of panel B (Hind III). Comparison of these patterns to those obtained by electrophoresis of restricted DNA from transformed cells indicated that host range transformed lines 1H2, 637-4, 5H1 and 5H5 (Fig. 8A, tracks 2, 7, 8, and Fig. 8B, tracks 15, 3, 7 and 2) contain all of the internal fragments of Ad5 DNA (i.e., Xba I fragments A, B, D and Hind III fragments A, B, C, D, E, F, H.). Although the terminal fragments of viral DNA (Xba I C, E or Hind III G, I) could not be directly identified in the transformed cell DNA, all of the above lines contained two or more new fragments migrating slower than the DNA termini. The new fragments most likely represent

Figure 8. Analysis of Ad5 DNA sequences present in hr and wt transformed cells. DNAs extracted from transformed cells were digested with XbaI (A) or HindIII (B) and analyzed by Southern's blotting procedure followed by hybridization using ^{32}P Ad5 DNA as probe. The cell DNAs electrophoresed were as follows: Panel A (XbaI):1H1 (channel 1); 1H2 (channel 2); 3H1 (channel 3); 3H2 (channel 4); 5H2 (channel 5); 637-4 (channel 7); 5H1 (channel 8); 5H3 (channel 9); 5H4 (channel 10); and W86 (channel 11). XbaI digested Ad5 DNA was run in channel 6 as a marker. Panel B (HindIII): 3H3 (channel 1); 5H5 (channel 2); 637-4 (channel 3); 3H2 (channel 4); 3H1 (channel 5); 5H1 (channel 7); 5H3 (channel 8); 5H4 (channel 9); W86 (channel 10); 1H3 (channel 11); 5H2 (channel 12); 822C2 (channel 13); 1H1 (channel 14); 1H2 (channel 15) and 3H1 (channel 16). HindIII digested Ad5 DNA was run in channel 6 as a marker. (Ruben et al., 1982).


A
E
B
A
D
C
XbaI
1H1
1H2
3H1
3H2
5H2
Ad5
637-4
5H1
5H3
5H4
W86
A
B
C
D
E
1
2
3
4
5
6
7
8
9
10
11

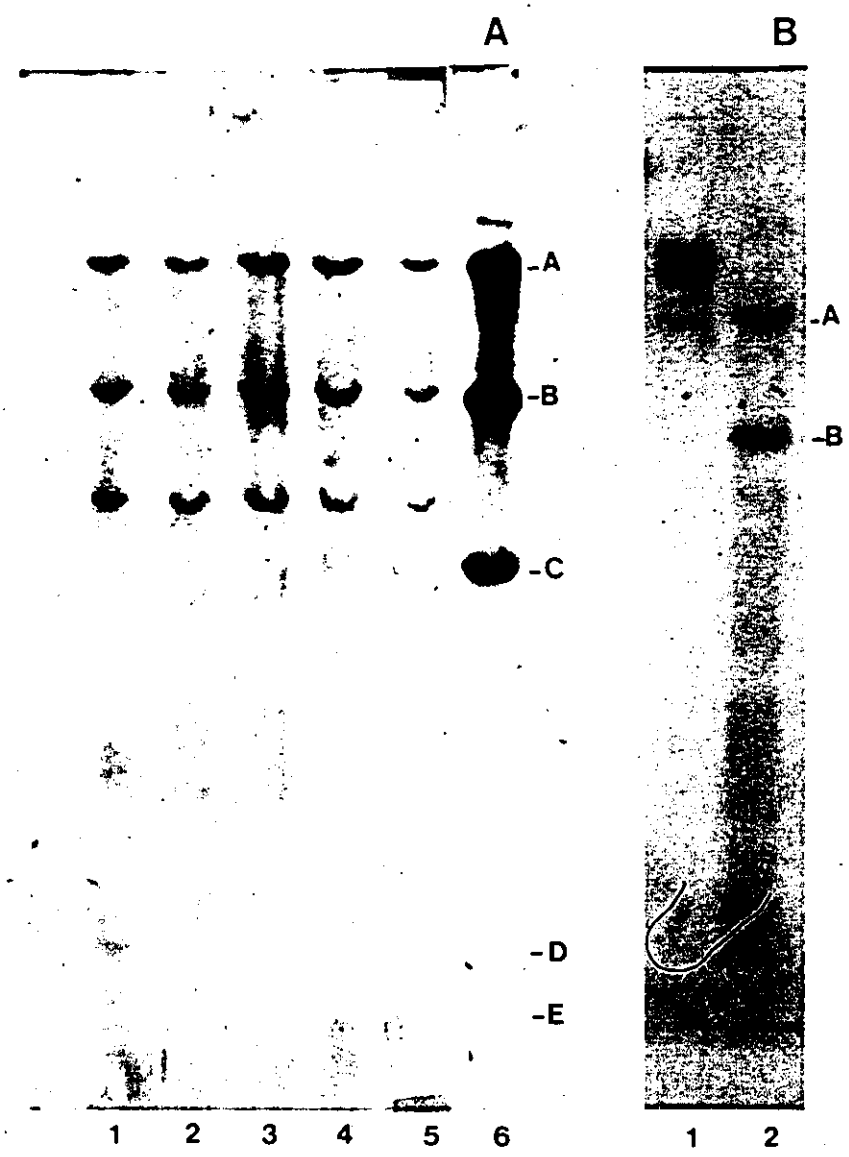

3H1
1H2
1H1
16
15
14
A
B
C
D
E
F
G
H
I
Hind III
822C2
5H2
1H3
W86
5H4
5H3
5H1
Ad5
3H1
3H2
637-4
5H5
3H3
G E C H D A B F I
13
12
11
10
9
8
7
6
5
4
3
2
1
B

joint fragments between viral and host DNA sequences; if so the patterns described above are consistent with the hypothesis that lines 1H2, 637-4, 5H1 and 5H5 contain all of the viral genome colinearly integrated.

As already mentioned, these hr transformed lines were not clonal isolates and therefore it seemed possible that the apparent integration of the entire genome could be due to a mixed population of cells carrying different regions of the viral DNA.

This hypothesis, however, could be discounted when the DNA content of subclonal lines isolated from one hr transformed line, 637-4, was analyzed. As shown in Fig. 9, panel A, for DNA restricted with XbaI, all of the subclones derived from line 637-4 (tracks 2-5) exhibited the same integration pattern of Ad5 DNA as the parental line (track 1); this pattern moreover, did not vary over a period of several months in culture. The results, which are in agreement with the hypothesis that the entire viral genome is colinearly integrated in the transformed cells were further confirmed by analysis of cell DNA restricted with BamH1, which cleaves Ad5 DNA at a single site (at 59.5%) generating only two fragments. As shown in Fig. 9B, track 1, DNA from line 637-4 contains only two fragments complementary to the labeled probe, both of which migrate slower than the BamH1 A and B fragments of viral DNA (track 2), as expected if the viral genome is integrated at a single site into host DNA. Subclones from other cell lines

Figure 9. Integration patterns of Ad5 DNA in the hr transformed cell line 637-4 and its subclones. Panel A: Cell DNA extracted at passage 13 from line 637-4 (channel 1) or from 4 subclones isolated at passage 13 (channels 2-5) was digested with XbaI and hybridized to ^{32}P Ad5 DNA as described in the text. Ad5 DNA marker was electrophoresed in channel 6. Panel B: BamHI digested 637-4, Channel 1; and BamHI digested Ad5 DNA, channel 2. (Ruben et al., 1982).

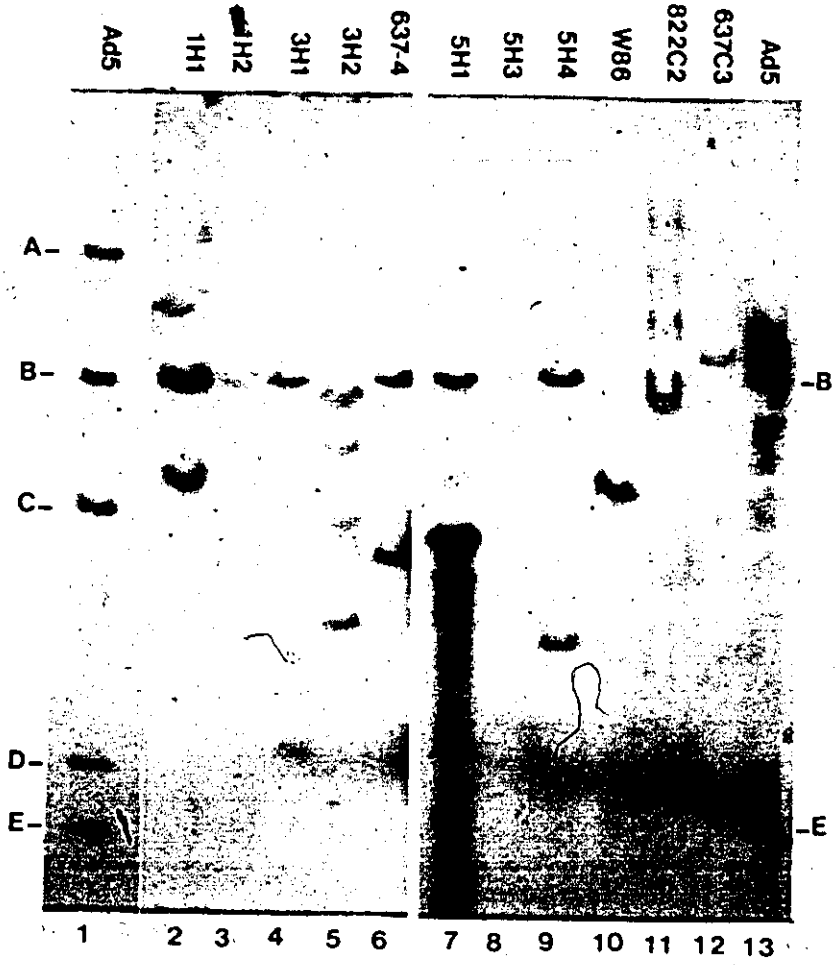

A
B
-A
-B
-C
-D
-E
1
2
3
4
5
6
-A
-B
1
2

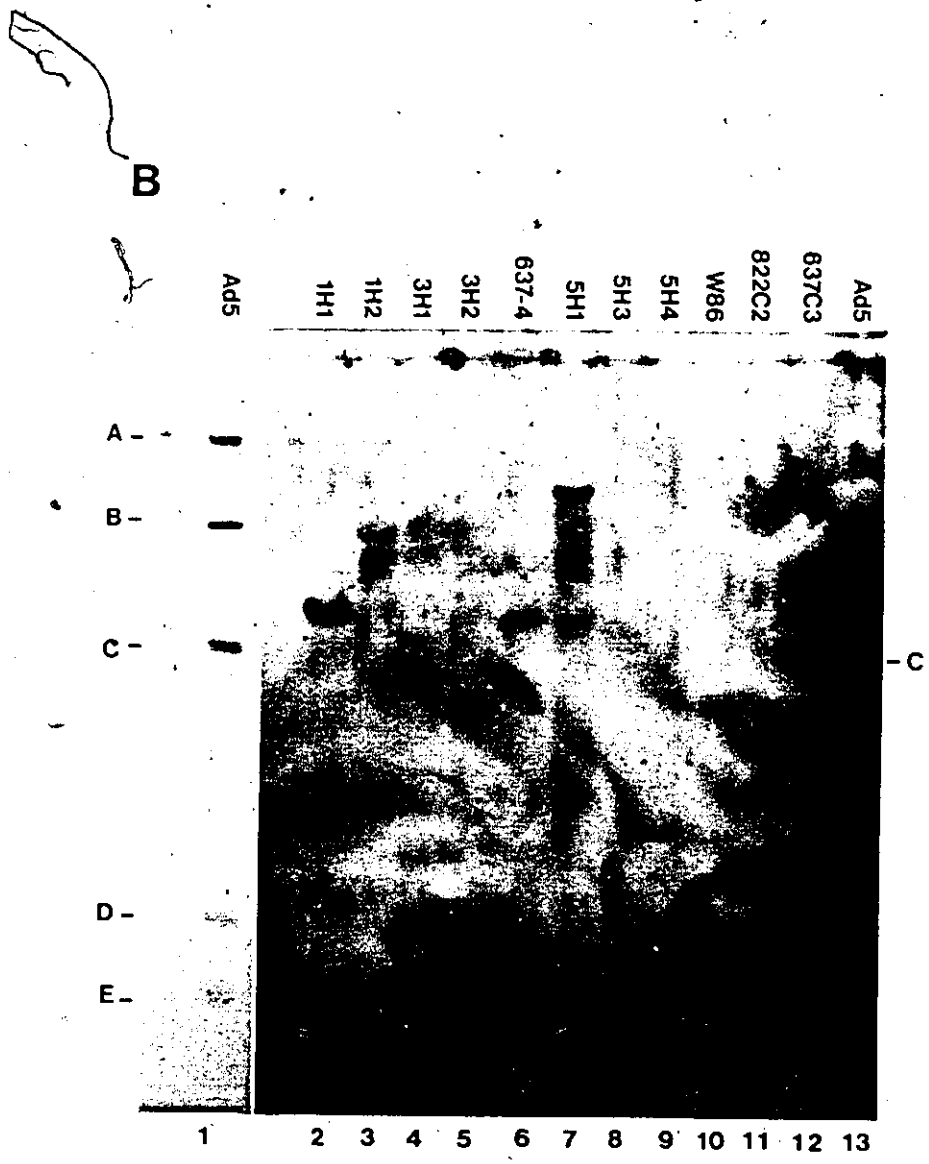
have not been analyzed by Southern blotting. However, in most cases the lines contain a single integration site for viral DNA and therefore are probably monoclonal. In cases where more than one site is found (see Fig. 12) it is possible that the lines contain a mixed population of two or more cell types (see below).

The results of hybridization with probe made from total Ad5 DNA indicated that the hr transformed lines contained widely differing amounts of viral DNA. Based on the presence of fragments comigrating with internal fragments of viral DNA, upon digestion with two restriction endonucleases, and assuming that all transformed cells must contain at least the left-end portion of the viral genome (Gallimore et al., 1974; Sambrook et al., 1975; Visser et al., 1979), the extent of viral DNA in the cells was estimated at between less than 17% up to as much as the entire genome. On the other hand, all of the wt transformed cell lines which were analyzed contain only a portion of the viral DNA amounting to less than the leftmost 28% of the genome (see Fig. 8A, track 11 and 8B, tracks 10 and 13). In order to obtain more precise data on the viral DNA sequences present in each transformed line, particularly with respect to sequences near the viral termini, additional studies were performed using as labeled probes the Xho I C (0-15.5%) or Hind III F (89.1-97.1%) fragments of Ad5 DNA cloned in pBR322. Figure 10A shows the results obtained when the Xho I C probe was hybridized to cell DNA (tracks 2-12) or viral DNA (track 13) restricted with Xba I. In the

Figure 10. Analysis of Ad5 DNA present in hr and wt transformed cell lines. Filters prepared as described in the previous figures were hybridized with ^{32}P labeled plasmid DNA containing the XhoI C fragment (panel A) or the HindIII F fragment (panel B) of Ad5 DNA. In both panels the cell and virus marker DNA was digested with XbaI. Both panels illustrate from left to right DNA from lines: 1H1 (channel 2); 1H2 (channel 3); 3H1 (channel 4); 3H2 (channel 5); 637-4 (channel 6); 5H1 (channel 7); 5H3 (channel 8); 5H4 (channel 9); W86 (channel 10); 822C2 (channel 11); 637C3 (channel 12). Channel 1 contains ^{14}C in vivo labeled-Ad5 DNA and channel 13 contains cold Ad5 DNA hybridized with the same ^{32}P probe as the cell DNAs. (Ruben et al., 1982).

A

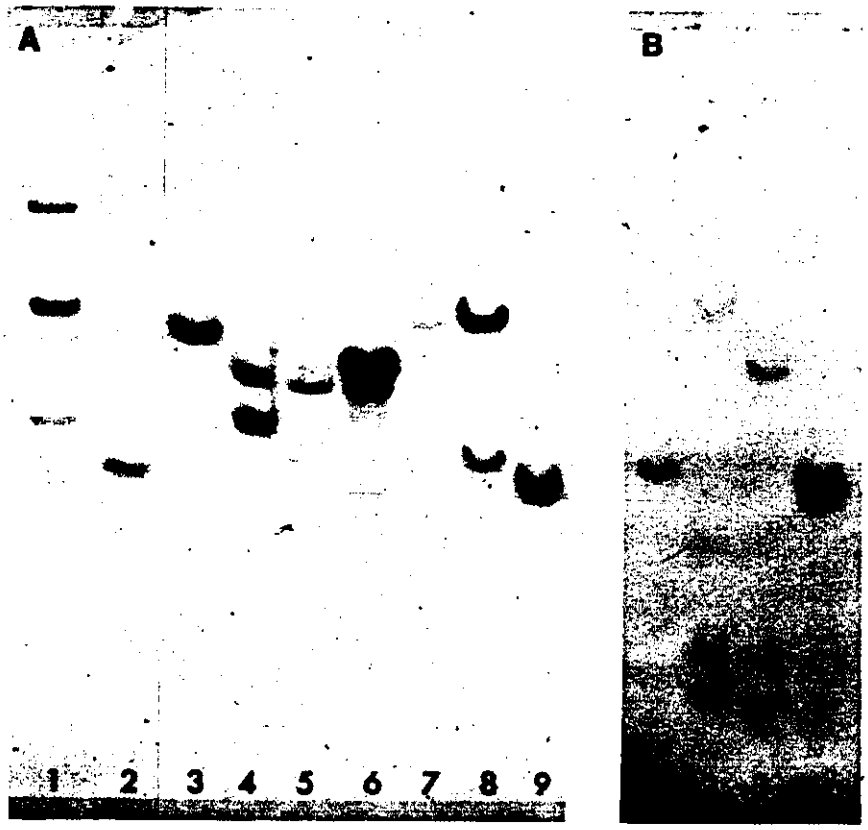

Ad5
1H1
1H2
3H1
3H2
637-4
5H1
5H3
5H4
W86
822C2
637C3
Ad5
A–
B–
C–
D–
E–
–B
–E
1 2 3 4 5 6 7 8 9 10 11 12 13


B
Ad5
1H1
1H2
3H1
3H2
637-4
5H1
5H3
5H4
W86
822C2
837C3
Ad5
A
B
C
D
E
C
1
2
3
4
5
6
7
8
9
10
11
12
13

case of Ad5 DNA, as expected, the probe hybridized to the XbaI E (0-3.8%) and B (3.8-28.8%) fragments. Two or more hybridization fragments were also detected in the transformed cell DNAs. Some of the lines (1H1, 1H2, 3H1, 637-4, 5H3, 5H4, tracks 2-4 and 6, 8, 9) contained a fragment comigrating with XbaI B and an additional fragment slower than XbaI E, which was assumed to be the viral left end-host junction fragment. Other lines did not contain any fragments of native size but rather what was assumed to be XbaI E-host DNA junction and a subset of XbaI B, again in the form of a cell-virus DNA joint (lines 3H2, W86, 822C2, 637C3, tracks 5 and 10-12).

Finally, some lines (3H2 and 5H1, Fig. 10A tracks 5 and 7) contained more than two fragments hybridizing to the Xho I C probe which suggests more than one site of integration for the viral DNA. Similar conclusions were derived from the results obtained with cell DNA digested with Hind III and hybridized with the Hind III G probe (Fig. 11A). Additional experiments using the cloned extreme left-end 1.2% of Ad5 DNA (Pvu II left end fragment 454 bp) to probe DNA from lines 637-4, 1H2 and 1H3 showed a single hybridization band with the same mobility as one hybridizing to the Xho I C probe (Fig. 11B). This result allows us to conclude that in these lines the virus-host junction on the left end of Ad5 DNA deletes less than 454 bp of the viral sequences.

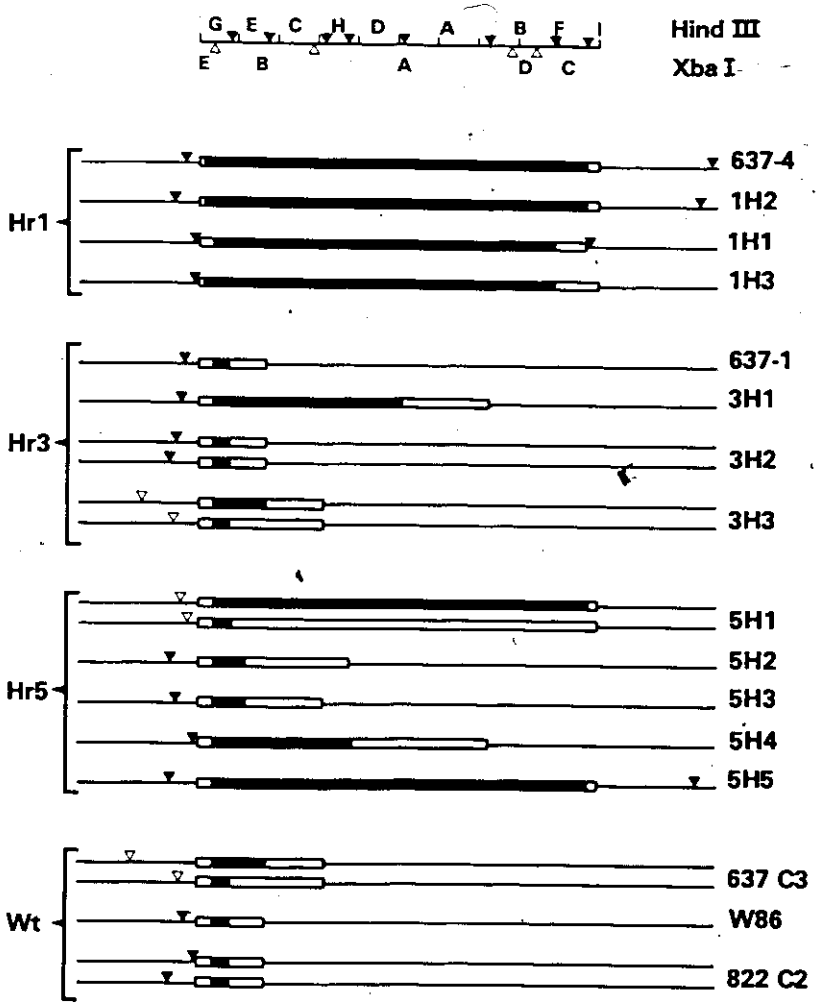
Figure 11. Analysis of left end sequences present in hr and wt transformed cells. For both panels the enzyme used was HindIII and ^{32}P labeled plasmid DNAs containing the HindIII G fragment (7.9%) (panel A) or the extreme left end fragment after digestion with PvuII enzyme (1.2%) (panel B) were used as probes. From left to right the cell DNAs are: Panel A: 1H3 (channel 2; 5H5 (channel 3); 149-637-4 (channel 4); 637-4 (channel 5); 5H1 (channel 6); 5H2 (channel 7); 822C2 (channel 8). HindIII digested wt Ad5 DNA was run in channel 9 as a marker. Panel B: 1H3 (channel 1); 1H2 (channel 2) and 637-4 (channel 3); Ad5 DNA extracted from hr1 virions (channel 4). Track 1 in panel A contains ^{14}C in vivo labeled-Ad5 DNA digested with HindIII.


A
B
1 2 3 4 5 6 7 8 9

In contrast to the results obtained probing with the cloned left end of viral DNA (Xho I C), the right end of the viral genome was absent from several lines (Fig. 10B). Hybridization with the cloned Hind III F fragment (89.1-97.1%) gave positive results only for lines which, from the results shown in Figs. 8 and 9, appeared to contain the entire genome. In these cases (Fig. 10B, tracks 2, 3, 6 and 7) one band, migrating slower than the marker XbaI C fragment (84.3-100%, track 13) could be detected.

The results presented in Figs. 8-11 were used to construct the diagram in Fig. 12 showing the most probable integration pattern of Ad5 DNA in 16 different transformed lines. The fact that all the hr transformed cell lines were isolated as polyclonal populations leaves open the possibility that some of the results used to construct this diagram are due to mixed populations of cells carrying different fragments. However, the simplicity of the patterns obtained with different enzymes (XbaI, Hind III, Kpn I, BamHI) make this improbable except possibly in the case of cell lines 3H2, 3H3 and 5H1 which have two apparent sites of integration. This can be interpreted as two sites per cell or as the mixture of two or more populations of cells. Two clones were isolated from cell line 3H3, one of them has epithelial morphology and the other is fibroblastic. Southern analysis of DNA extracted from these cloned populations showed the same pattern of integration in both sublines as was seen in the

Figure 12. Schematic representation of the integration patterns of Ad5 DNA in transformed rat cells. Only the most likely model is presented. _____ cell DNA; ▼ HindIII restriction sites in the cell DNA; ■ virus DNA sequences present in the cells; □ virus DNA fragments for which the fraction present in the cells is uncertain. Lines 3H2, 3H3, 5H1, 637C3 and 822C2 have two distinct inserts of viral DNA (c.f. Figure 10). Because of the complexity of the pattern obtained for cell lines 3H3, 5H1 and 637C3 it is not possible to say which left end junction corresponds to which insert and the resulting uncertainty in the host cell DNA cleavage site is indicated in the figure by open triangles ▽ . The integration pattern after digestion with KpnI and BamHI (not shown) is consistent with the scheme illustrated above and does not reveal the existence of new integrated fragments. The right end host DNA cleavage sites, when known, are included for cells which appear to contain the entire genome. (Ruben et al., 1982).

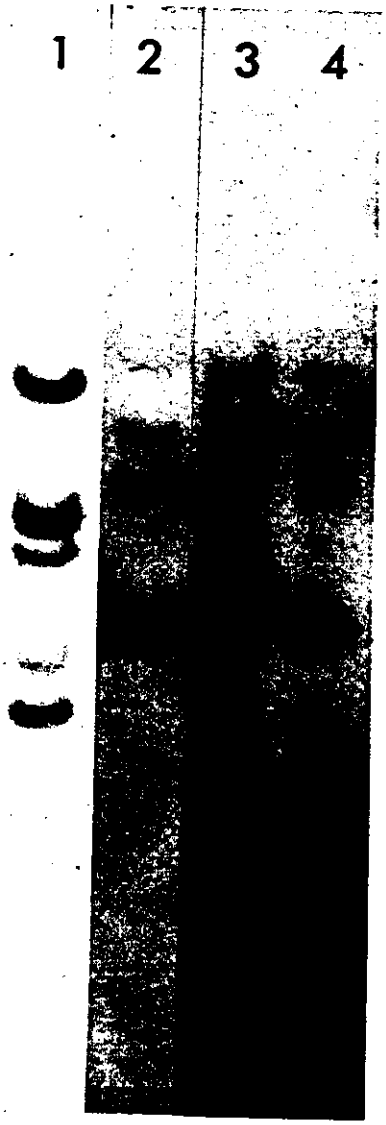

G E C H D A B F I
E B A D C
Hind III
Xba I
Hr1
637-4
1H2
1H1
1H3
Hr3
637-1
3H1
3H2
3H3
Hr5
5H1
5H2
5H3
5H4
5H5
Wt
637 C3
W86
822 C2

parental cells (Fig. 13 and some data not shown). This lends support to the hypothesis that cell lines presented in Fig. 12 correspond to clonal populations of cells which have spontaneously overgrown the original mixed population.

4) Virus Rescue Experiments

The inability to rescue infectious virus from cells transformed by adenoviruses of group C has been attributed to the fact that these cells usually contain only a fraction of the viral DNA (Tooze, 1980; Sambrook, 1975). Some of the hr transformed lines described here, on the other hand, appear to contain the entire genome and as such are suitable candidates for virus rescue attempts. For these experiments two procedures successfully used in the papovavirus system, were followed using line 637-4. In one case, high molecular weight DNA from the hr-transformed cells was used to infect 293 cells (Boyd and Butel, 1972) using the calcium technique (Graham and Van der Eb, 1973); in the second case, hr-transformed and 293 cells were fused with the aid of polyethylene glycol (Pontecorvo et al., 1977). The 293 cells were chosen as recipients for the transformed cell DNA or as partner in the fusion experiments since they are fully permissive for hr mutant virus (Harrison et al., 1977). With either procedure, however, no infectious virus could be rescued: No cpe could be detected after absorption of fresh 293 cells with either supernatant or cell lysate from the fused or transfected cultures (see Materials and Methods). "In situ blots" (Perez-Villarreal

Figure 13. Ad5 DNA sequences in hr transformed line 3H3 and in two subclones with epithelial or fibroblastic morphology. The enzyme used to digest the cell DNAs was HindIII and the filter was hybridized against ^{32}P labeled total Ad5 DNA. Track 4, cell line 3H3; track 2; F1-3H3; track 3, E1-3H3; and track 1 Ad5 DNA. Cell line 3H3 contains mixed populations of epithelial and fibroblastic cells (see text). F1-3H3 has fibroblastic morphology whereas E1-3H3 cells are epithelial.

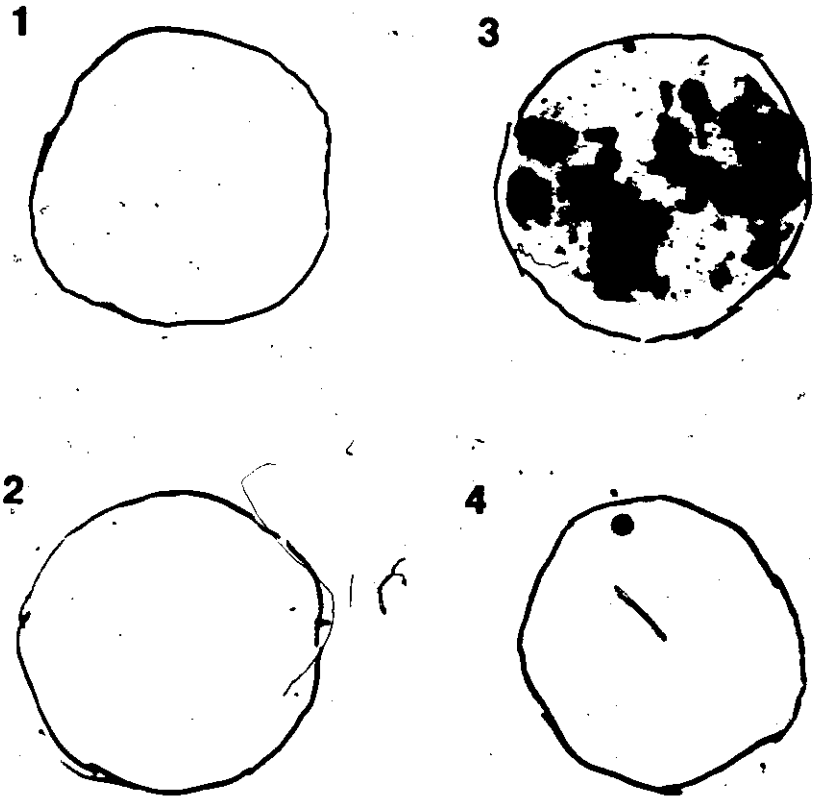

1
2
3
4

and Berg, 1977) of 293 cells transfected with 637-4 DNA were also negative (see Fig. 14). In this type of experiment the transfected dishes were harvested 15 days post transfection and analyzed according to the procedure of Perez-Villarreal and Berg (1977), using ^{32}P labeled Ad5 DNA as a probe. The results of the autoradiographs are shown in Fig. 14. One radioactive spot can be observed in dish 4. This spot was interpretated as an artifact because no infectious virus was rescued from that dish and the same result could not be repeated in further plaque hybridizations. However, the posibility of DNA replication without virus production in this particular spot can not be discarded. Several reasons can account for the failure in rescuing the integrated viral DNA: first, the integrated sequences may not extend all the way to the extreme ends of the viral DNA; second, excision of viral DNA may not occur or products required for viral DNA replication may not be produced after fusion or transfection; and finally, the original transformation may have been caused by virus particles with an additional defect besides the hr mutantion. This last hypothesis could also explain the lack of rescue of infectious virus from dish 4, if the spot that we observed there was not an artifact.

5) Expression of Viral Proteins in hr Transformed Cells

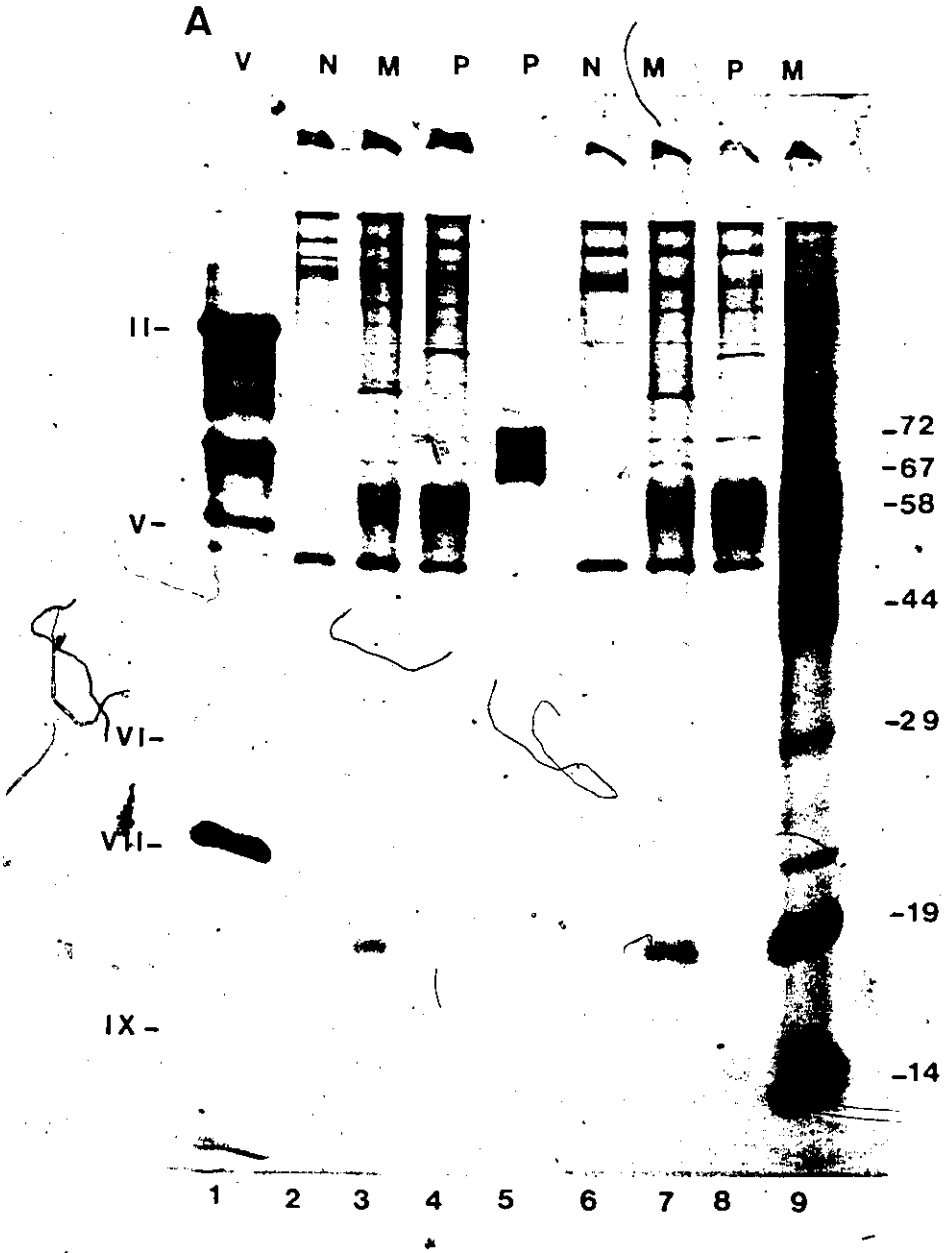
A number of workers have reported that deletion mutants and hr mutants mapping in E1A (Jones and Shenk, 1979a) are unable

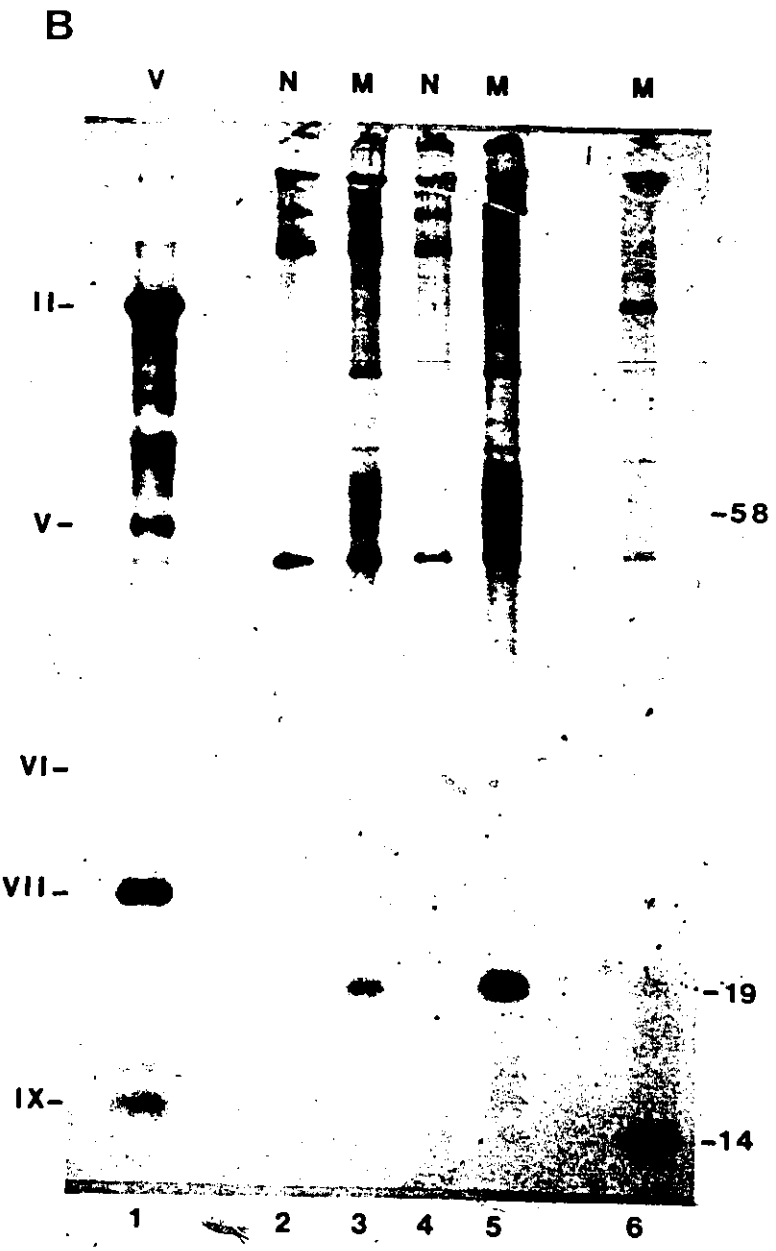
Figure 14. Plaque hybridization of 293 cells inoculated with Ad5 virions (3) or 293 cells transfected with 637-4 DNA (2-4). Semi-confluent dishes of 293 cells were treated as described in the text. Filter 1, Mock infected 293; filter 3, 293 inoculated with 20 PFU of Ad5 virions and filters 2 and 4, 293 inoculated with 15 (2) and 25 (4) µg of 637-4 DNA.


1
3
2
4

to transcribe other early regions, including E1B, in nonpermissive Hela or KB cells (Berk et al., 1979b; Berk and Sharp, 1977; Jones and Shenk, 1979b). We considered it of interest therefore to examine the expression of early viral proteins in hr transformed cells. Extracts of cells, labeled with [^{35}S] methionine, were immunoprecipitated with either Megaserum or P antiserum (see Materials and Methods) and the reaction products electrophoresed on polyacrylamide gels. The results of these experiments are shown in the autoradiograms of Fig. 15. P antiserum (Fig. 15A) was used to detect the expression of the 72,000 dalton and the related 67,000 dalton protein in two transformed lines containing the E2 region of Adenovirus DNA (i.e. 637-4 and 1H2). As can be seen from the results in tracks 4 and 8, none of these transformed lines express either the 72,000 or the 67,000 dalton proteins. However, they express a polypeptide which migrates in the region of 58,000 daltons as a broad band. This protein was also detected in immunoprecipitates using Megaserum which is specific for E1 proteins. (Fig. 15A, tracks 3 and 7 for lines 1H2 and 637-4, and Fig. 15B, tracks 3 and 5 for lines 5H2 and 637-C3). In addition to the 58,000 dalton protein, a polypeptide of molecular weight 19,000 was also detected in every cell line when immunoprecipitation was carried out using Megaserum. These two polypeptides are thought to be coded by sequences in E1B (Ross et al., 1980b; D.T. Rowe et al., unpublished).

Figure 15. Autoradiographs of SDS-polyacrylamide gels after electrophoresis of immunoprecipitated Ad5 early polypeptides. Panel A: ^{35}S-methionine labeled cell extracts from transformed cell lines 1H2 (channels 2-4); 637-4 (channels 6-8) and from virus infected KB cells pulse-labeled for 2 hours (8-10 hr) after infection (channels 5 and 9). Panel B: extracts from cell lines 5H2 (channels 2 and 3); 637C3 (channels 4 and 5) and from virus infected KB cells pulse-labeled for 2 hours (3-5 hr) after infection (channel 6); N: non-immuno hamster serum; M: hamster Megaserum; P: rabbit P antiserum; V: ^{35}S-methionine-labeled Ad5 virus marker. (Ruben et al., 1982).


A
V
N
M
P
P
N
M
P
M
II–
V–
VI–
VII–
IX–
–72
–67
–58
–44
–29
–19
–14
1
2
3
4
5
6
7
8
9


B
V
N
M
N
M
M
II–
V–
VI–
VII–
IX–
–58
–19
–14
1
2
3
4
5
6

Both P antiserum and Megaserum are capable of precipitating E1A proteins from infected cells (44,000 dalton and 29,000 dalton proteins, see Fig. 15 track 9) but as shown in Fig. 15, precipitation of these proteins from transformed cells is very inefficient, a finding consistently observed (Lassam et al., 1979a, b; D.T. Rowe et al., unpublished). For this reason, I cannot reach any conclusion about the expression of E1A proteins in hr transformed cells.

III. Discussion

As mentioned in the general introduction, group 1 hr mutants of Ad5 map in the E1A region of the viral genome and are defective for transformation (Frost and Williams, 1978; Graham et al., 1978; Harrison et al., 1977). These mutants are unable to transform rat embryo and rat embryo brain cells, but as shown previously (Graham et al., 1978), and in the present studies, they appear to induce a semi-abortive or abnormal transformation of baby rat kidney cells. In this latter case, transformed cell lines could not be established from individual foci of transformed cells but could only be derived (with about 30-50% success rate) by passaging entire polyclonal cultures. Once polyclonal lines had survived two or three passages it was possible to subculture them indefinitely as well as to establish sublcones. No transformed foci were observed in mock-infected cultures and 4 out of 4 attempts failed to establish continuous lines from control cultures in low calcium medium. Thus, although the group I mutants are defective in one or more E1A functions, they are nevertheless still capable of immortalizing cells under certain conditions.

That lines established in this way are not the result of rare cells transformed by revertants is suggested by their aberrant properties. Although after 6-8 passages in culture, the mutant transformed lines achieved the same growth rates and acquired the ability to reach the same saturation densities as wt transformants, they differ from wt transformed cells in their morphology and in their being unable to form colonies in soft agar. In addition, the mutant transformed cells are either nontumorigenic or very weakly tumorigenic in nude mice, in contrast to the majority of wt transformed cells. The hr mutants of group I are known to map in E1A (Frost and Williams, 1978) and presumably affect one or more E1A functions. Thus, these phenotypic differences between mutant and wt transformed cells are presumably related to differences in the expression of E1A functions. In the present studies with hr mutant transformed rat cells I was unable to detect E1A coded proteins either in mutant or wt transformed cells. Nor did I detect synthesis of the 72,000 dalton polypeptide coded by E2 in cells which contained the entire viral genome. This polypeptide has been reported to be present in Ad2 wt transformed hamster cells (Levinson and Levine, 1977; Ross et al., 1980b) but not in Ad2 wt transformed rat cells (Wold and Green, 1979). I did, however, detect the presence of the E1B polypeptides of molecular

weight 58,000 and 19,000 (the latter formerly denoted 15,000 (Lassam et al., 1979a, b) at levels comparable to those seen in wt transformed cells (cf. Fig. 15B). Thus, E1B proteins alone are evidently not sufficient for the complete transformation of rat cells. The explanation for the aberrant phenotype of hr transformed cells may be related to a defect in the expression of another early region, presumably E1A, suggesting that one or more E1A functions may be involved in maintenance of some of the phenotypic properties of Ad5 transformed cells.

One of the most interesting properties of the hr transformants was the structure of integrated viral sequences. Most lines contained a good deal more of the viral genome than was seen in wt transformed lines and many of the mutant transformed lines appeared to contain virtually the entire viral genome colinearly integrated into host sequences. In several lines the integrated sequences extended beyond the Hind III cleavage site at 97.1 map units (i.e. within 2.9% of the right terminus) and to within less than about 1.2% of the left terminus. Whether or not the integrated viral sequences in these cells extend to the extreme ends of the viral genome will be clear once joint fragments are cloned and sequenced.

One rather surprising observation was that even when all DNA was extracted and analyzed at the earliest possible times after establishing mutant transformed lines (passage 4-6) the integration patterns were simple and remained stable after

further subculture. Thus, although the lines were initially polyclonal, having been established from cultures usually containing 20-30 foci, it appears likely that by the time of the first DNA extraction only a single cell type predominated. In all the cell lines analyzed in the present study, the viral DNA sequences were integrated into different sites in the host DNA.

Transformation of rodent cells by Ad2 and Ad5 generally results in lines containing only part of the viral genome (Gallimore et al., 1974; Sambrook et al., 1975; Visser et al., 1979). Although on occasion a larger proportion of the viral DNA is present (Gallimore et al., 1974; Sambrook et al., 1975; Visser et al., 1979), colinear integration of the entire genome has been observed only in exceptional situations. Dorsch-Hasler et al., (1980) have recently described the integration patterns in rat cells transformed at nonpermissive or semipermissive temperature by Ad5 mutants ts 107 and ts 125. These mutants are DNA negative under restrictive conditions as a result of defects in the 72,000 dalton DNA binding protein (Van der Vliet et al., 1975) and transform rat cells with higher efficiency than does wt virus (Ginsberg et al., 1975; Mayer and Ginsberg, 1977). Many of the ts 107 and ts 125 transformed lines characterized by Dorsch-Hasler et al. (1980) appeared to contain all of the viral genome colinearly integrated like the cells characterized in the studies reported in this chapter.

There are at least two other examples of adenovirus transformed cell lines which contain the entire viral genome: these are rodent lines transformed by Ad12 (or lines established from Ad12 induced tumors) (Ibelgaufs et al., 1980; Stabel et al., 1980) and a particularly nonpermissive rat line transformed by wt Ad5 (Fisher et al., 1982).

All of these examples have several features in common: They involve relatively stringent nonpermissive conditions under which functions needed for DNA replication are suppressed and they often involve enhanced transformation efficiencies. A reasonable explanation for the findings reported here and those of others mentioned above is that during transformation of cells by adenoviruses, usually, or perhaps always, the entire viral genome is initially integrated intact but the expression of viral functions other than those required for transformation (late genes, 72,000 protein), or the replication of viral DNA, has a cytotoxic effect. In semipermissive cells infected by wt virus, presumably only those cells which retain a defective genome, as a result of deletion or rearrangement of viral DNA sequences, are able to survive. When a mutation prevents the expression of putative cytotoxic functions (eg. hr group I; ts 107, ts 125) or when the cells are totally nonpermissive to the virus, then survival of transformed cells containing the entire intact viral genome becomes possible.

Consistent with this hypothesis, is the extensive cpe seen in rat kidney cultures infected with wt Ad5 but not with the group I mutants. In addition, the efficiency of transformation of rat kidney cells by wt Ad5 drops rapidly at multiplicities of infection higher than 1 (Graham et al., 1978) (presumably as a consequence of cell killing), whereas the efficiency of transformation by hr mutants continues to increase.

If colinear integration of the entire viral genome into host DNA is a common mechanism by which integration initially occurs during transformation by virions, the study of the integrated sequences early after infection may allow us to detect covalently linked entire viral DNA in wild type virus infected rat cells as well. With this purpose I infected primary BRK with wt, hr1 and hr6 virions and extracted and analyzed total cell DNA at early times after infection. The results of these studies are reported in the next chapter.

CHAPTER V

HEAD TO TAIL JOINING AND CIRCULARIZATION OF ADENOVIRUS 5 DNA DURING INFECTION OF PERMISSIVE AND SEMIPERMISSIVE CELLS

I. Introduction

Although cells transformed by wt Ad2 or Ad5 virions usually contain only part of the viral genome, the results presented in chapter IV show that colinear integration of the entire genome is a frequent event in cells transformed by group I hr mutants. This kind of integration has also been found in Ad12 transformed rat cells and in two Ad5 non permissive systems (see Chapter IV). The hypothesis suggested in the previous chapter is that colinear integration of the entire genome may be a general event for adenovirus transformation, an event which cannot be detected regularly because of the expression of viral functions lethal for the recipient cell, and that only under certain conditions, it becomes possible to find the entire adeno genome persisting in the transformed cell (see Chapter IV).

In addition, several wild type transformed cell lines contain the left and right ends of viral DNA linked together, an observation which also suggested an involvement of both ends in the integration process, possibly through the formation of circular intermediates (Visser et al., 1981).

The studies reported in this chapter were carried out to determine the structure of intracellular viral DNA sequences at early times during transformation and to examine the

earliest patterns of integration detectable before any selection procedure was used and before transformed lines were established. During the first 5 days after infection, I was unable to obtain any evidence for integration. I could, however, find new forms of rearranged viral DNA which may be involved in the integration process.

Part of the results presented in this chapter have been published in Nature (Ruben et al., 1983). These results have also been reported at the 1982 Tumor Virus Meeting in Cold Spring Harbor (Ruben et al., 1982b)

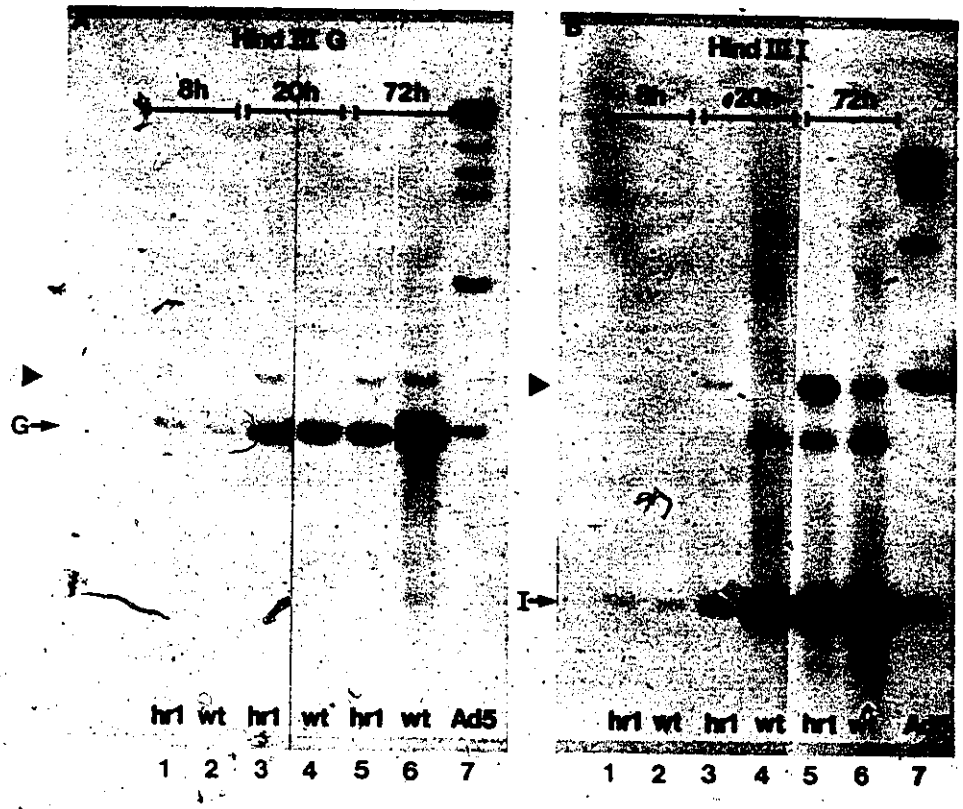
II. Results

1) Fate of Viral DNA in Primary Baby Rat Kidney Cells Early After Infection

In an attempt to study the transformation event at early times after infection, total DNA was extracted from BRK cells at 8 hours, 20 hours, 3 days and 5 days post infection with hr 1 (group I), hr 6 (group II) or wt Ad5 (see Materials and Methods). The DNAs were then digested with different restriction enzymes, electrophoresed on agarose, transferred to nitrocellulose filters and hybridized to cloned Ad5 HindIII G or HindIII I fragments. Both fragments represent the two ends of the adenovirus genome (see Fig. 16); they were selected for these experiments because fragment G is always present in transformed cells and it and fragment I should appear with a larger size if total and colinear integration occur (see Chapter IV).

Figure 16 shows the autoradiographs obtained after digestion of the extracted DNAs with HindIII and hybridization of the filters with fragments G (Fig. 16A) or I (Fig. 16B) cloned in pBR322. A partial HindIII digest of viral DNA was run as a size marker in track 7 of both panels. At the times studied, all the infected cells contained HindIII fragment G (A) and HindIII fragment I (B) with sizes comparable to the marker. As expected, there was some cross hybridization between G and I due to the terminal repeats (Steenbergh et al., 1977). In addition, some channels contained a new band which was of

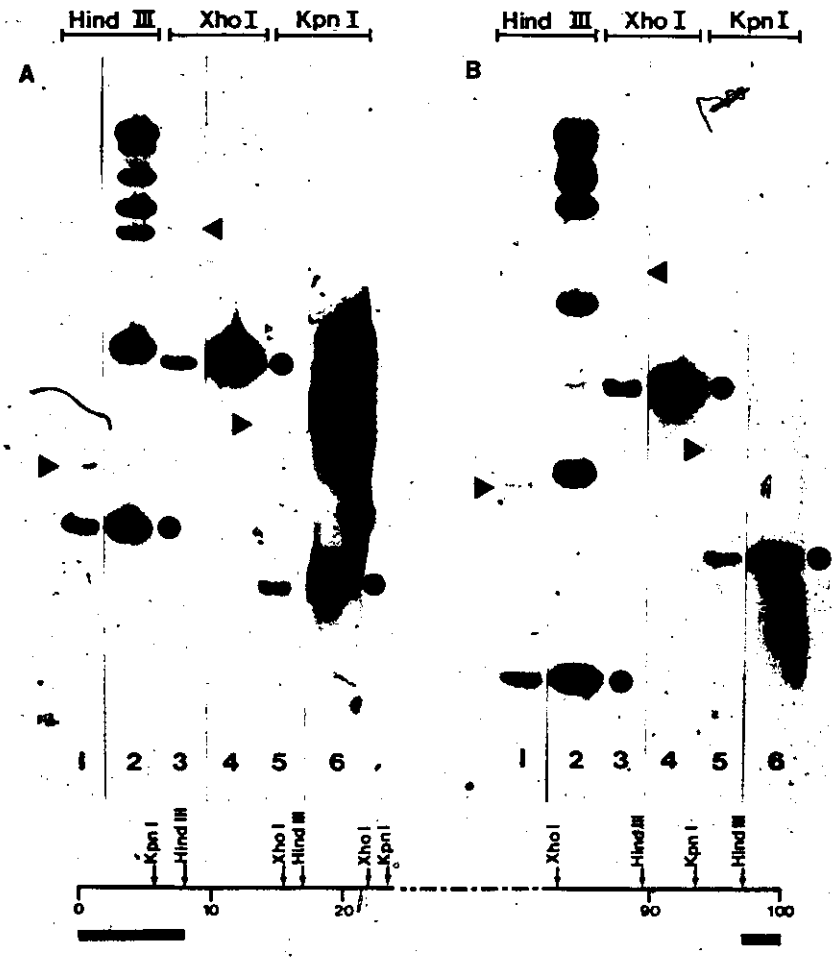
Figure 16. Analysis of the state of viral DNA in BRK cells early after infection with wt Ad5 or with hr1 mutant. Total DNA was extracted from BRK cells at 8h (tracks 1 and 2), 20h (tracks 3 and 4) and 72h (tracks 5 and 6) after infection with hr1 or wt virus. The cell DNAs were then digested with HindIII, electrophoresed and transferred to nitrocellulose filters as described. The filters were hybridized with ^{32}P-labeled plasmid DNA containing the HindIII fragment G (A) or I (B). A partial HindIII digest of Ad5 DNA extracted from virions was run in track 7 as a size marker. ▶ joint fragment.


Hind III G
8h
20h
72h
G
hr1 wt hr1 wt hr1 wt Ad5
1 2 3 4 5 6 7
Hind III I
8h
20h
72h
I
hr1 wt hr1 wt hr1 wt
1 2 3 4 5 6 7

particular interest because it had the size of both ends of the DNA cut with HindIII and joined together (G + I = 3.8 kb). and because it hybridized with both ends when used as labeled probes in separate blot hybridizations (Figs. 16A and 16B). This joint fragment appeared earlier in hr 1 infected cells, where it could be detected at 8, 20, 72 and 120 hours after infection (tracks 1, 3 and 5 of Figs. 16A and 16B for 8, 20 and 72 hours P.I., and Fig. 20 track 2 for 120 hr P.I.). In cells infected with hr6 (from complementation group II) or with wt, this new band could be first detected after 3 to 5 days P.I. (tracks 2, 4 and 6, Fig. 16A and 16B for wt and Fig. 20, tracks 3 and 4 for hr6 and wt 5 days P.I.).

As shown in Fig. 16, a new band with the size and hybridization properties of a head to tail joint appeared clearly at 8 hr P.I. only in cells infected with hr1. This experiment was repeated using different enzymes. Total DNA extracted from BRK cells at 8 hr after infection with hr1, hr6, or wt was digested with HindIII, KpnI or XhoI and analyzed by Southern blotting and hybridization as before. The results are shown in Figures 17A and B. A partial HindIII digest of marker DNA was run in channel 2 as a size marker and tracks 4 and 6 contained complete digests of marker DNA with XhoI (track 4) and KpnI (track 6). The results shown in tracks 1, 3 and 5 confirmed the presence in group I

Figure 17. Detection of head to tail joining of Ad5 DNA molecules in primary BRK cells infected with hr1 mutant virus. Subconfluent cell monolayers were infected with hr1 (group I) mutant Ad5 at a multiplicity of 1 pfu/cell. Eight hours after infection, the monolayers were rinsed with PBS and the DNA extracted as described in Materials and Methods. 10 µg of each DNA preparation were digested with restriction endonucleases HindIII, XhoI or KpnI and electrophoresed on 0.7% agarose gels. The nitrocellulose filters obtained after Southern blotting of the gels were hybridized with labeled HindIII G (panel A) and I (panel B) fragments as in Figure 16. In each panel, channels 1, 3 and 5 contain DNA extracted from infected cells and channels 2, 4 and 6 contain marker Ad5 DNA mixed with DNA from uninfected BRK cells prior to digestion. The HindIII digest in both channels 2 is a partial digest which serves as size marker. The restriction map of Ad5 DNA at the bottom of the figure shows the relevant restriction endonuclease sites with the left and right ends of the genome. The map location of the HindIII G and I fragments used as probes is indicated by solid bars. Free viral DNA termini in the infected cell DNA are identified by closed circles. Joint fragments, discussed in the text, are indicated by arrows. The hybridization band above the joint fragment in panel A, channel 5 results from hybridization between HindIII G (0-8 mp) and KpnI A (5.8-23.-mp). (Ruben et al., 1983).

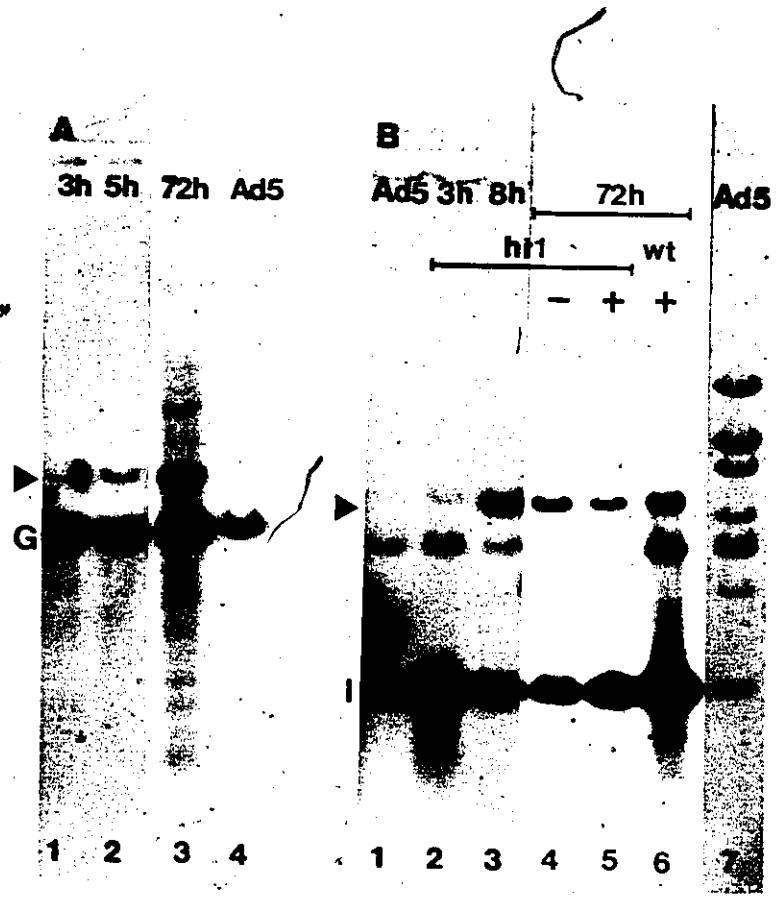

Hind III
Xho I
Kpn I
A
B
1
2
3
4
5
6
Kpn I
Hind III
Xho I
Hind III
Xho I
Kpn I
Xho I
Hind III
Kpn I
Hind III
0
10
20
90
100

infected cells of new bands with sizes expected for a head to tail joint after digestion with each of the above enzymes and the new bands hybridized with either end when terminal fragments were used as probes (Fig. 17A and B). Cells infected with hr6 or wt did not contain any new band at that time (8 h) (not shown). DNAs digested with KpnI showed two bands of hybridization in addition to the joint fragment; these bands corresponded to KpnI fragments H and A which hybridized to the HindIII G probe (see below, Fig. 17).

Further experiments were done to determine if the junction fragment appeared in hrl infected cells at times earlier than 8 h post infection, when DNA replication has not yet commenced. DNA was extracted from hrl infected BRK at 3 and 5 hr post infection and analyzed as before. As seen in Figure 18A, tracks 1 and 2, and Figure 18B, track 2, the new band is already present even at these early times, although in much lesser amounts. Since this is well before DNA replication, these results indicate that, at least in this system, the incoming molecules must form head to tail joints before initiation of viral DNA synthesis. Joint fragments were never detected in DNA extracted from purified virions, whether wt or hrl (see below).

Estimates of the amount of joint fragment (j) in the infected cells in relation to the amount of total viral DNA

Figure 18. Joint formation in primary BRK cells infected with hrl or wt. The formation of joint fragment was investigated after 3 hours (track 1, panel A and track 2, panel B), 5 hours (track 2, panel A), 8 hours (track 3, panel B) and 72 hours (track 3, panel A and tracks 4, 5 and 6, panel B) post infection. All the DNAs were digested with HindIII and the nitrocellulose filters were hybridized with ^{32}P labeled HindIII G (A) and I (B) fragments clonned in PBR 322. The DNAs run in tracks 5 and 6 (+) were redigested with Pronase (see text) after their digestion with HindIII. Ad5 DNA treated in the same way as the cell DNAs was run in tracks 4 in panel A and 1 in panel B. Track 7 in panel B contains ^{14}C Ad5 DNA, labeled in vivo. ▶ Joint fragment.


A
3h 5h 72h Ad5
G
1 2 3 4
B
Ad5 3h 8h 72h Ad5
hr1 wt
– + +
I
1 2 3 4 5 6 7

detected with the probe (j+e, e = amount of terminal fragment hybridizing with the probe) were done by microdensitometry. Table VI was constructed with the fraction $\frac{j}{j+e}$ calculated from microdensitometric tracings of different autoradiographs. The procedures used to do the quantitations are described in appendix 2.

In hr1 infected BRK cells, there was an increase in the relative amount of joint fragment very early after infection. This fraction increased rapidly up to 8 hrs post infection (see Fig. 19), then remained relatively constant up to 120 hr. No evidence of head to tail joints was seen before 72 hr P.I. with hr6 or wt virus; by that time the background usually found late after infection was very high and the quantitation of the bands was not possible.

From the series of experiments described above, it is possible to conclude that a head to tail joint fragment appears after infection of BRK with Ad5 virions. This structure appears very early (3 hr) in hr1 (complementation Group I) infected cells but is also present 3 days post infection with hr6 (complementation Group II) or wt.

2) Presence of Head to Tail Junctions in Permissive Hela Cells as Well as in Lines of Semipermissive Rat Cells

Head to tail junctions consistently appear in different extractions of primary BRK DNA after infection with wt or hr1 mutant. It was considered of interest to investigate if the

TABLE VI

FRACTION OF VIRAL DNA JOINED HEAD TO TAIL IN hr1 INFECTED CELLS

Time Post infection	Cells			
	BRK	Hela	3Y1	1074-9
3 hr	0.01 (1)[a]	ND[b]	ND	ND
5 hr	0.08 (1)	ND	ND	ND
8 hr	0.15 (4)	0.08 (1)	ND	ND
20 hr	0.11 (3)	0.09 (2)	ND	ND
72 hr	0.13 (3)	0.04 (2)	0.18 (2)	0.12 (2)
120 hr	0.09 (2)	ND	ND	ND

The amount of viral DNA present as head to tail joints was determined by scanning autoradiographs using a Joyce-Loebl scanning spectrophotometer and is expressed as the ratio of label hybridizing with the joint fragment to the sum of label hybridizing with the joint plus free terminal fragments.

[a]Number of experments from which the values were estimated. The mean value was calculated when more than one data was available.

[b]ND not done

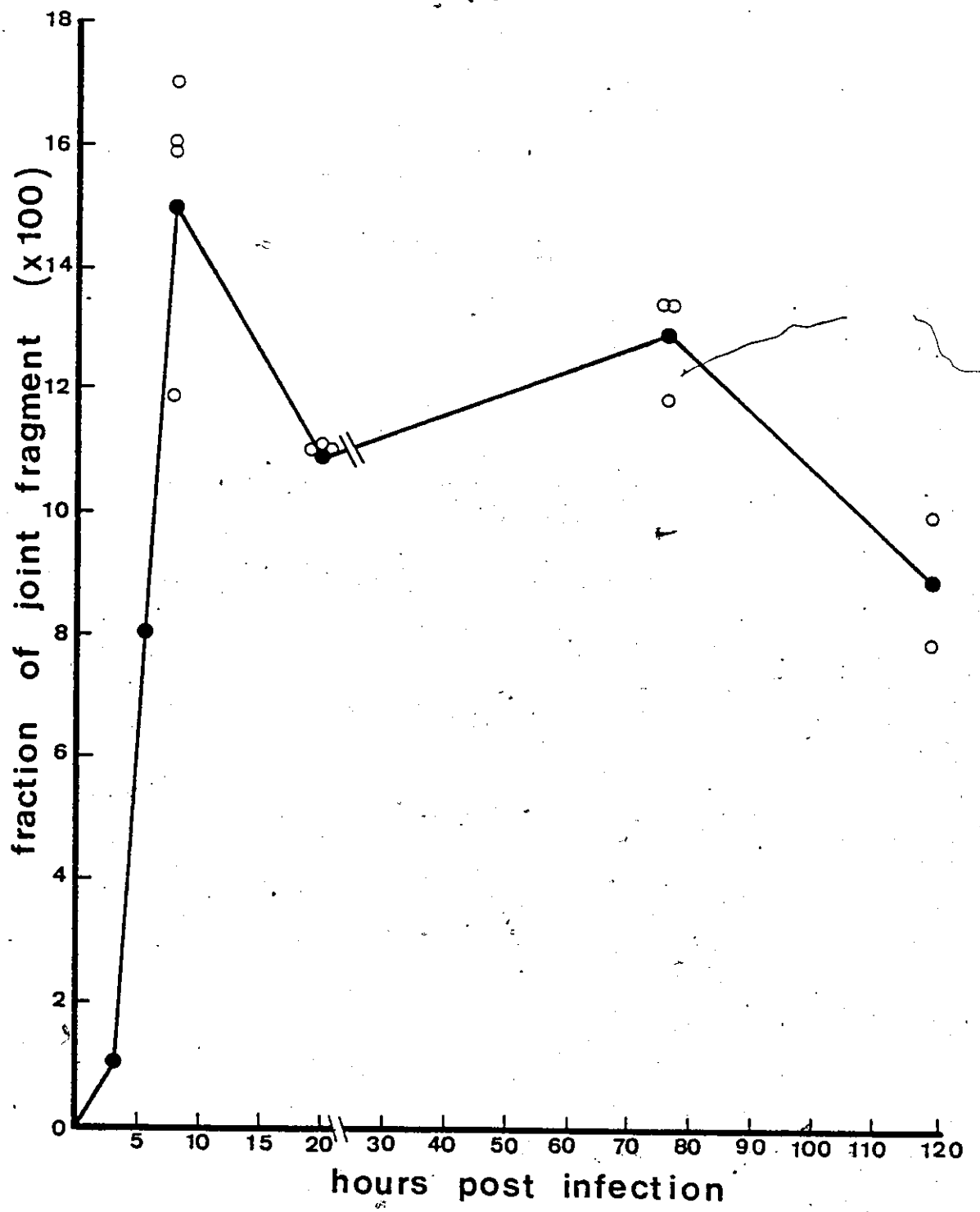
(Ruben et al., 1983)

Figure 19. Time course for the formation of head to tail joints in BRK cells infected with hr1.

The values tabulated in Table VI were used to construct this graph.

○ Actual values obtained by microdensitometry

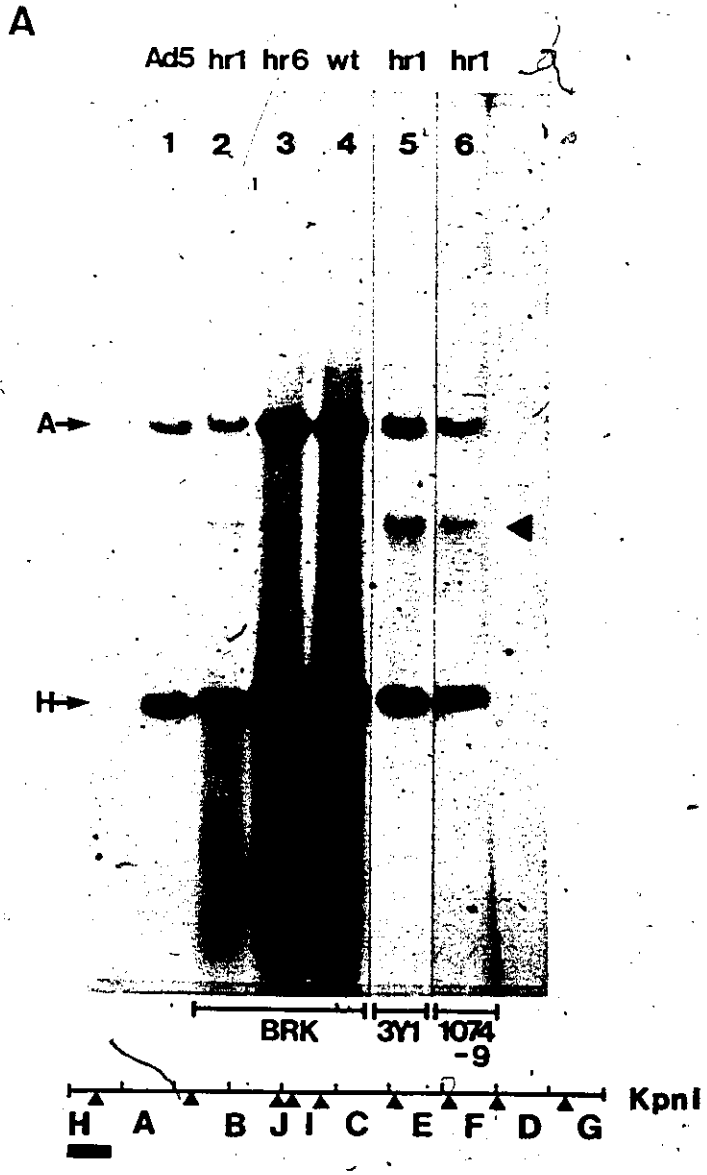
● Mean values from different experiments

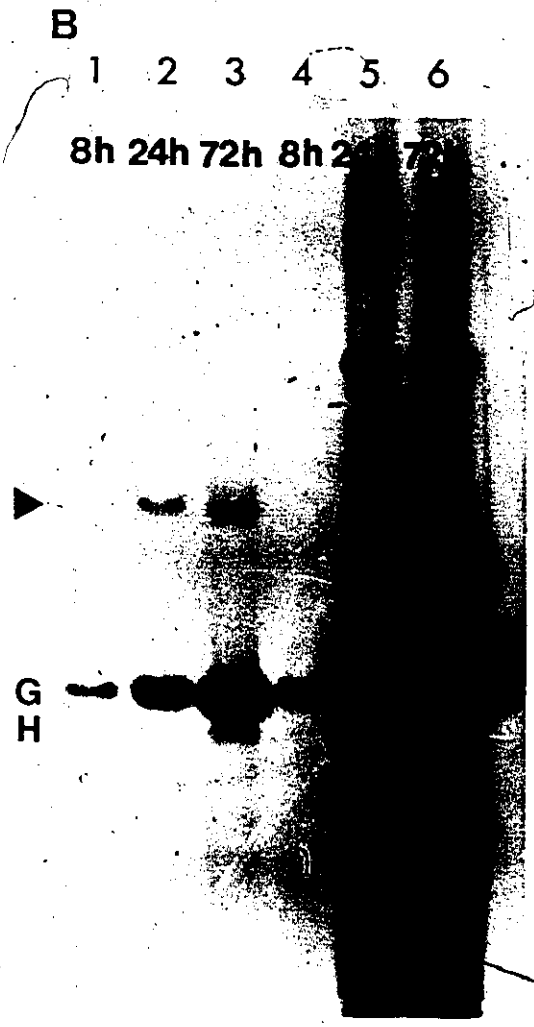

fraction of joint fragment (x100)
18
16
14
12
10
8
6
4
2
0
5
10
15
20
30
40
50
60
70
80
90
100
110
120
hours post infection

formation of these joints was only a property of primary rat cells or if they also appeared in continuous cell lines: semipermissive rat lines or permissive human lines. For this reason two rat cell lines were studied: 3Y1 (Kimura et al., 1975) and 1074-9 (a spontaneously transformed BRK cell line, Graham, unpublished) as well as human Hela cells. As shown in Figure 20A, tracks 5 and 6, a single band migrating in the same position as the head to tail junction seen earlier (track 2) was found in both rat cell lines after digestion with KpnI. The DNAs were extracted 3 days P.I. with hr1, and the filters were hybridized with labeled HindIII G fragment. The same fragment also appears in Hela cells after infection with hr1 or wt as can be seen in Fig. 20B, after digestion of the cell DNA with KpnI and hybridization of the filter with labeled HindIII I fragment. This head to tail joint seems to appear earlier after infection with hr1 than after infection with wt, in Hela cells as well as in BRK cells (see Figs. 16 and 20B). The extra bands observed in tracks 5 and 6 of Figure 20B may correspond to partial digests of the DNA and to some extent of cross hybridization.

From the experiments shown in Figures 16 and 20A and B it is possible to conclude that the joint formation is not affected by the mutation carried by hr1 (complementation Group I) and hr6 (complementation Group II), although hr1 follows different kinetics: in BRK as well as in Hela cells, head to tail joints appear earlier after infection with hr1 than after infection with

Figure 20. Joint formation in primary BRK cells or in established cell lines infected with wt virus or hr mutants of Ad5. The DNAs were extracted and treated as described in the previous figures. The results shown in this figure were obtained after digestion with KpnI and hybridization with HindIII G (panel A) or HindIII I (panel B) fragment of Ad5 DNA. Panel A: Joint fragments (▶) appear in BRK cells infected with hr1 (Group I), hr6 (Group II) and wt (channels 2, 3,4, respectively) 120 hours after infection. Channels 5 and 6 contain DNA extracted from established rat cell lines (3Y1 and 1074-9, respectively) 72 hours after infection with hr1. Marker Ad5 DNA digested with KpnI is shown in channel 1. In all channels the slowest migrating band results from the hybridization of the HindIII G probe to the KpnI A fragment of Ad5 DNA as shown in the Kpn restrication map below the figure. The map location of the HindIII G fragment is indicated by a solid bar. (Ruben et al., 1983). Panel B: Hela cells infected with hr1 (tracks 1-3) or wt virus (tracks 4-6). HindIII I fragment of Ad5 DNA was used as labeled probe.


A
Ad5 hr1 hr6 wt hr1 hr1
1 2 3 4 5 6
A
H
BRK
3Y1
1074
-9
H A B J I C E F D G
KpnI


B
1 2 3 4 5 6
8h 24h 72h 8h
G
H

wt. Therefore, the time of detection of joints seems to be dependent on the infecting virus but not on the permissiveness of the infected cells: Hela cells are fully permissive for wt virus and only partially permissive for hr1 DNA replication (see appendix 3) whether BRK cells are semipermissive for both virus.

3) Circle Formation in BRK Cells Infected With hr1.

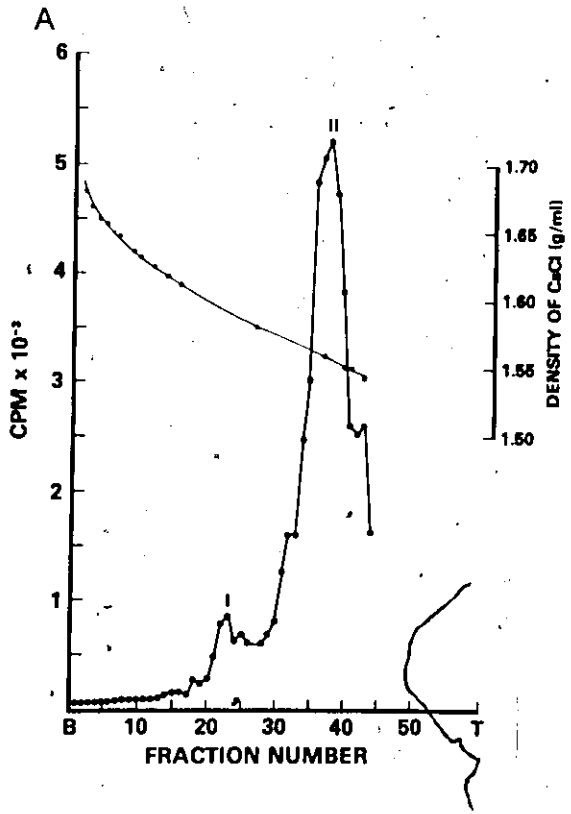
Head to tail joints could result from circular molecules, head to tail concatemers or other kinds of rearranged molecules. The junction between both ends could be covalent, as a result of hybridization between the bases of the inverted terminal repeats or they could be attached by a linker consisting of RNA or protein. The presence of a protein joining both ends of Ad2 DNA, and the formation of circles or concatemers by this DNA was reported several years ago by Robinson et al. (Robinson et al., 1973; Robinson and Bellet, 1974). After extraction of DNA from virions without the use of SDS and Pronase, they found several kinds of molecules: electron microscopy of samples at various stages of preparation of DNA-protein complexes showed that initially linear DNA was released from the virus particles, but some of this DNA was subsequently circularized or formed oligomers. The authors found left to right, left to left or right to right end junctions which disappeared after treatment with SDS, Pronase or alkali.

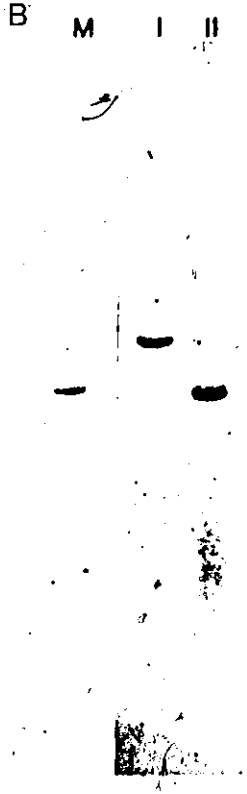
The procedure I used for DNA extraction from infected cells included pronase, SDS, phenol and chloroform:isoamylalcohol (see Materials and Methods), therefore a head to tail joining as a result of a DNA-protein interaction is unlikely. In addition, I redigested overnight, some HindIII cut DNA with 0.5 mg/ml of pronase, and no change was observed in the pattern seen in Southern blots (Fig. 18B, tracks 5 and 6). As mentioned in the previous sections, left to right junctions were never detected in DNA extracted from virions: either hr1 or wt. All the Southern blots shown in this thesis contain as a marker; DNA extracted from virions by a procedure similar to the procedure used to extract cell DNA (see Materials and Methods), and in addition to that I used three different preparations of wt DNA and one of hr1 DNA (Fig. 11B, track 4) extracted from purified virions. Furthermore, no left to left or right to right end junctions were detected in infected cell extracts. Therefore, from all these considerations, it is possible to rule out a protein as a linker between both ends and I conclude that the molecules described in this chapter are different from the ones detected by Robinson et al. (1973) in DNA extracted from virions. The experiments outlined below also indicate that head to tail joining of the ends of Ad5 were not the result of transient base pairing between the inverted repeated sequences at either end of the DNA molecule, since joint structures were resistant to treatment with alkali. Because of this resistance, and of the

resistance to RNAse used for DNA extraction, it is also possible to rule out the possibility of an RNA linker.

As already mentioned, head to tail joining of Ad5 DNA molecules could result from covalent circularization of the DNA or from formation of concatemers. To detect the presence of any covalently closed circles, primary rat cells were infected with hr1 at an moi of 1 PFU/cell and labeled with 0.5 μCi/ml of [^{3}H] thymidine at 24 hr and again at 48 hr after infection (in vivo labeling of the DNA was done to help to monitor the formation of circular molecules). At seventy-two hours post infection the cells were harvested and the DNA was extracted using a modification of the procedure developed by Birnboim and Doly (1979) for purification of circular plasmid DNA from bacteria. This procedure involved cell lysis in alkali followed by precipitation of denatured DNA and proteins after neutralization with acetate buffer (see Materials and Methods). The supernatant from this extraction, which retains renaturated covalently closed circles, was banded in CsCl-ethidium bromide equilibrium gradients which are routinely used to separate covalently closed, supercoiled circles from linear DNA molecules. The principle behind this procedure is that supercoiled circles bind less ethidium bromide than nicked circles or linear molecules, and as a result, supercoiled molecules band at higher densities. After 72 hr, fractions were collected from the bottom of the tubes (see Materials and Methods). The plotting of the counts, presented in Figure 21A,

Figure 21. Characterization of Ad5 DNA from infected BRK cells by equilibrium sedimentation. Panel A: Primary BRK cells were infected with Ad5 hr1 mutant at a multiplicity of 1 pfu/cell. ^{3}HTdR (0.5 µCi/ml) was added at both 24 and 48 hours after infection, the cultures were harvested at 72 hours and covalently closed circular DNA was partially purified following a modification of the procedure developed by Birnboim and Doly (1979) for purification of plasmid DNA from bacteria (see Materials and Methods). The DNA extracted by this procedure was then centrifuged to equilibrium in a CsCl-EtBr gradient (see text) and the radioactivity profile obtained after counting aliquots from the fractions is shown in this figure. A second aliquot was used to measure the refractive index and calculate the density along the gradient. Panel B: DNA recovered from peaks I and II of the gradients was digested with HindIII and analyzed by Southern blot hybridization as described before, using HindIII G fragment as labeled probe. A complete HindIII digest of Ad5 DNA purified from virions was used as marker (M). (Ruben et al., 1983).


A
6
5
4
3
2
1
CPM x 10⁻³
II
I
1.70
1.65
1.60
1.55
1.50
DENSITY OF CsCl (g/ml)
B
10
20
30
40
50
T
FRACTION NUMBER


B
M
I
II

revealed the presence of two distinct peaks of radioactivity. The fractions corresponding to each of the peaks were pooled and extracted, and the DNAs were then digested with restriction endonucleases and analyzed as in previous experiments by Southern blot hybridization using plasmids containing the left or right termini of Ad5 DNA as radioactive probes. The results obtained after digestion of peak I and peak II with HindIII and subsequent hybridization with nick translated HindIII G fragment, are shown in Figure 21B. Peak II, which has a density of 1.56 g/cc (Fig. 21A), contains the terminal G fragment as well as a small fraction of the head to tail joint (Fig. 21B, track 2). Peak I, on the other hand, is highly enriched for joint fragments and contains only traces of contaminating fragment G (Figure 21B, track 3). Peak I has a density of 1.60 g/cc (Fig. 21A) which corresponds to the density observed for covalently closed super-coiled molecules such as SV-40 and polyoma (Tooze, 1980). This fact, together with the presence of both ends of the adenogenome joined together instead of the individual fragments, are strong support for the hypothesis that circular molecules are produced in BRK cells after infection with hr1 and that at least part of the joint fragments seen in the previous experiments are due to the presence of covalently closed circles.

Peak II of the gradient, with a density of 1.56, contains some joint fragment as well as a band which comigrates with fragment G (Fig. 21B, track 2). It is likely that this material

represents the sum of some nicked circles plus some linear molecules which reassociate after the alkaline denaturation.

III. Discussion

The original goal of the studies pursued in this chapter was to detect early forms of integration in Ad5 infected BRK cells as well as viral forms involved in the integration process. With this purpose, I extracted and analyzed total cell DNA at various times post infection, relatively early during the transformation process. Host range mutants from complementation Groups I or II (hr1 or hr6) as well as wt virions were used for the infections. With the protocols used in this chapter, I was unable to detect integration with any of the virus used. Nevertheless, integration can not be discarded because of the limitations of the procedure used, which would have allowed me to detect cell to virus DNA junctions only if integration has occurred into a limited number of sites. However, these protocols turned out to be ideal for the detection of new intracellular forms of viral DNA which had gone undetected in a large number of previous studies (Doerfler et al., 1972, 1973; Burger and Doerfler, 1974; Fanning and Doerfler, 1977; Tyndall et al., 1978; Schick et al., 1976; Pearson, 1975; Groneberg et al., 1975).

The results presented in this chapter describe the formation of head to tail joints in Ad5 DNA molecules. The formation of these joints was independent of the type of cell used: a) semi-permissive: primary BRK cells or established rat cell lines 3Y1

and 1074-9 or b) permissive: Hela cells. The results presented in this chapter also indicated that the mutations carried by hr mutants hr1 (from complementation Group I) and hr6 (from complementation Group II) do not affect the formation of head to tail joints. Only the time of appearance of this fragment was different in hr1 infected cells, in which joint fragments could be detected as early as 3 hours after infection, while cells infected by hr6 or wt virus exhibit the joint fragment at times later than 24 hr.

From the procedure used to extract total cellular DNA plus the results of Birnboim's extraction used to detect circles, it seems likely that both ends are covalently linked. The simplest structure which can account for the observed joint fragments would result from flush end joining of left and right termini. However, although bands corresponding to joint fragments are reasonably well defined, their size cannot be determined with precision greater than 100-200 bp. Consequently, the fine structure of head to tail joints is at present obscure. Attempts to clone the joint fragments (FLG, personal communication) have consistently resulted in plasmids containing Ad5 inserts with deletion of all or most of the terminal repeated sequences probably because these sequences, when joined together, generate a structure (a 200 bp palindrome) thought to be lethal for plasmids (Lilly, 1981).

As proposed in section 3 of this chapter, the observed joint fragments could derive from circular molecules, head to tail concatemers or other kinds of rearranged molecules. I was able to show the presence of circles after Birnboim extraction of cellular DNA at 3 days after infection with hr1, followed by centrifugation in a CsCl-EtBr gradient (see section 3). Compared with the amount of joint fragment obtained with the regular procedure, the efficiency of the Birnboim extraction was very low (see appendix 2b). From my results I cannot calculate the proportion of head to tail joint due to circles since I have no estimate of the loss of circular molecules (due to formation of single strand nicks or to other causes) incurred during the purification procedure. However, the results of Figures 21A and 21B establish that at least a portion of the hr1 DNA in infected BRK cells must be in the form of covalently closed circles (see appendix 2b). It seems likely that such circles also exist under the other experimental conditions in which head to tail joining was detected.

Other laboratories have investigated early forms of integration and the fate of viral DNA shortly after infection with adenovirus 2, 5 and 12(Doerfler et al., 1972, 1973; Tyndall et al., 1978). None of them described new forms of rearranged DNA similar to the structures presented in this chapter. Nevertheless, forms of viral DNA which migrate in

propidium iodide-CsCl gradients at densities higher than the density of viral DNA or which migrate in sucrose gradients faster than viral DNA were described. These molecules could be similar to the structures described in this chapter. The authors couldn't find circles or concatemers and the structures they found instead, were described as DNA-RNA-protein complexes or as viral DNA linked to cellular DNA. Considerable differences in the approaches used to purify and to analyze the DNAs extracted from infected cells as well as the use of hrl in my experiments, may be the explanation for the differences observed between previously reported results and the results reported in this chapter.

The significance of head to tail joining is still not clear. Circles and concatemers of viral DNA could be precursors for DNA replication or could be involved in integration. It is now well established that the terminal protein linked to adenovirus DNA, plays a role in viral DNA replication, possibly acting as a primer for initiation of DNA synthesis (Rekosh et al., 1977; Challberg and Kelly, 1979; Tamanoi and Stillman, 1982). However, none of the studies carried out to date eliminate the possibility that the terminal protein, or its 80,000 dalton precursor is also a nicking closing enzyme which could generate head to tail joining or that the terminal protein functions in DNA replication as an origin specific topoisomerase (Pearson et al., 1981). Alternatively, a cellular enzyme may be involved in covalent joining of viral

DNA termini and the terminal protein might act to bring the ends together, a property it is known to possess (Rekosh et al., 1977). Detection of the joint fragment as early as 3 and 5 hrs post infection suggests that incoming molecules may be circularized before the onset of viral DNA replication. However, the absence of joint fragments in DNA extracted from purified virions suggests that covalently closed circular viral DNA is never packaged as such, even when as much as 10% of the intracellular viral DNA is joined head-to tail. The results shown in this chapter, in which the efficiency of head to tail formation is higher in the less permissive systems (ie. hrl infected cells), do not support a connection between replication and circle formation but neither do they rule it out.

On the other hand, my results suggest a possible relation between integration and circle formation; conditions which most efficiently generate head to tail joining (ie. infection of BRK cells with group I hr mutants), also result in exceptionally high transformation frequencies (Graham et al., 1978) and often give rise to transformants containing the entire viral genome covalently integrated into cellular DNA (see Chapter IV). In addition, linked terminal fragments have been detected in Ad2 and Ad5 transformed cell lines and it has been suggested that circularization may precede integration (Vardimon and Doerfler, 1981; Visser et al., 1981).

Circular molecules have been proposed as precursors for the integration of proviral DNA from several RNA tumorvirus: avian sarcoma virus (Guntaka et al., 1976), murine leukemia virus (Shoemaker et al., 1980) and mammary tumor virus (Shank et al., 1978). Proviral circular DNA has been isolated from the nucleous of avian cells, where it apparently originates from linear DNA molecules (Shank and Varmus, 1978). In FV-1 restricted systems, circle formation is strongly inhibited as well as the subsequent integration of proviral DNA (Jolicoeur and Rassart, 1980; Tang et al., 1980). Formation of linear DNA molecules, on the other hand, decreases only slightly in these systems, strongly suggesting that circles and not linear molecules are those directly involved in integration. A similar relation between circle formation and integration was suggested by studies using ethidium bromide or aphidicolin (Guntaka et al., 1975; Chinski and Soeiro, 1982; Hsu and Taylor, 1982). The presence of ethidium bromide supresses the accumulation of closed circular viral DNA, presumably by intercalation into DNA duplexes, but leaves unaltered the formation of linear molecules (Guntaka et al., 1975). The subsequent integration of proviral DNA is strongly inhibited. Aphidicolin, is a strong inhibitor of eucariotic DNA polymerase α (Ikegami et al., 1975) but has no effect on reverse transcriptase-mediated viral DNA synthesis (Chinski and Soeiro, 1982). However, in the presence of the drug circular DNA fails to accumulate and viral DNA integration does not occur (Chinski and Soeiro, 1982; Hsu and Taylor, 1982).

Proviral circular DNA molecules have been cloned and the junction fragment has been sequenced (Shoemaker et al., 1980, 1981; Shank et al., 1978; Majors and Varmus, 1981) showing blunt end ligation of both ends or deletion of one terminal repeat, as if they arose by homologous recombination of the LTR ends of linear DNA (Shank et al., 1978; Yoshimura and Weinberg, 1979). The exact mechanism of provirus integration is unknown, but circular precursors may explain two characteristics of this process: 1) Four to six bases of host DNA become reiterated on both sides of the viral insertion (Dhar et al., 1980; Shoemaker et al., 1980, 1981; Hughes et al., 1978; Shimotohno and Temin, 1980) and 2) usually two bases at the ends of both LTR are lost after colinear integration of the entire proviral DNA (Shoemaker et al., 1980, 1981). Shoemaker et al. (1980, 1981) have proposed models of integration based on this information and after cloning and sequencing several free circular forms as well as some integrated proviral DNA.

Circular viral DNA molecules have also been detected in Epstein-Barr transformed cells (Lindhal et al., 1976), and circular copia molecules have been detected in Drosophila cells (Flavell and Horowitz, 1981). Both are additional examples in eucaryotes of systems in which DNA sequences may be inserting into (or excising from) cellular DNA using a possible circular intermediate.

In order to elucidate the role, if any, of head to tail joining of a[illegible] molecules in DNA replication and transformation,

it will be useful to examine the effect of agents which have been found to block circularization of retrovirus genomes. In addition, sequencing of Ad5 joint fragments as well as of the cell-virus joint in hr group I transformed cell lines might indicate whether the mechanism of adenovirus DNA insertion into host DNA shares any similarity with retrovirus DNA integration.

CHAPTER VI

CONCLUSIONS

CONCLUSIONS

The studies presented in this thesis were undertaken in an attempt to gain more information on the mechanism of cell transformation by adenovirus. The blotting procedure described by Southern (1975) was used to characterize the presence and state of viral DNA sequences in cells infected and transformed by Ad5. As pointed out earlier, only the leftmost 8-12% (E1 region) is required for cell transformation. Some of the transformed cell lines analyzed by other workers contain greater amounts of the viral genome, but colinear integration of the entire genome is a very rare event seen only under particular conditions.

In the studies presented in Chapters III and IV, I characterized the structure of the integrated viral DNA in tumorigenic and non tumorigenic cells transformed by wt Ad5 virions or by group I hr mutants and also looked for a possible correlation between the structure of integrated sequences and the transformed phenotype. My results confirm what has been previously reported with respect to the minimal sequences required to transform cells with Ad5: all the virus transformed lines which I analyzed contain at least the entire region E1. Only cell line 512C8, a cell line transformed by the HindIII G fragment of the viral DNA lacks part of region E1B, as expected;

however, it shows a fully transformed, tumorigenic phenotype (see Chapter III). Human cells 293, on the other hand, are very weakly or non tumorigenic, although they contain the entire region E1 integrated into the cell chromosome. In addition, some tumorigenic cell lines derived from the 293 showed no difference in the integrated sequences.

These results are consistent with the hypotheses that: 1. Not all of E1 is needed for oncogenic transformation and 2. integration of E1 is not sufficient for oncogenicity, hypotheses which have been suggested on the basis of more extensive studies on a variety of transformed cell lines (Rowe and Graham, personal communication).

In addition, cells transformed by Group I hr mutants (mutated in region E1A) show always a partially transformed phenotype, independent of the amount of viral sequences integrated. These cells have a very particular pattern of integration: most of the hr1 transformed cells and some hr5 transformed cells contain virtually the entire viral DNA colinearly integrated (see Chapter IV). The hypothesis proposed at the end of Chapter IV to explain this finding and some similar findings by other groups, was that colinear integration of the entire genome may be a common event in adenovirus transformation but the expression of other functions toxic to the recipient cells may be lethal to them, allowing

the survival only of cells which retain deleted, rearranged or mutated viral DNA. When this thesis was in preparation, Fisher et al. (1982) published additional characterization of cells transformed by hr1. Host range 1 is also a cold sensitive mutant (Ho et al., 1982) and cells transformed by this virus exhibit a cold sensitive phenotype similar to the phenotype described by Ho et al. (1982) for cold sensitive mutants mapped in region E1A (see general Introduction). In addition to that, one of the hr1 transformed cell lines described by Fisher et al. (1983) has virtually all the adeno genome colinearly integrated, similar to some of the cells reported in Chapter IV.

In Chapter V, I analyzed the early intracellular events after infection with Ad5, in an attempt to detect early forms of integration or viral DNA structures involved in the integration process. The results reported in this chapter document the finding of circular forms of viral DNA which may be involved in the replication and/or integration of viral DNA. As discussed in Chapter V, these circles may be precursors for integration of adenovirus in transformed cells and could explain the finding of colinear integration of the entire viral DNA and of head to tail junctions in some transformed cells.

The finding of circular forms of adenovirus DNA, presented in this thesis, bring a new dimension to the molecular

biology of adenovirus replication and transformation of mammalian cells. These forms have been investigated by different groups who wanted to propose a circular intermediate for adenovirus replication. In addition to that, the finding of circular viral DNA gives support to hypotheses which propose a circular intermediate for viral DNA integration into the cell chromosome as a general mechanism for cell transformation by viruses.

APPENDICES

Appendix I

Retransformation of 637-4 Cells by hr6:

As shown in Chapter IV, cell line 637-4 was obtained after several passages of a multiclonal population of hr1-transformed cells. This cell line exhibits a partially transformed phenotype (see Chapter IV) and contains the entire viral genome colinearly integrated into cellular DNA. To study a possible complementation between the resident genome (mutated in region E1A) and Group II mutants (mutated in region E1B), the following experiment was done (FLG, unpublished):

Subconfluent dishes of 637-4 were inoculated with hr6 (complementation Group II) at an moi between 0.1 and 1 PFU/cell and incubated with medium changes but without subculturing for about 3 months, to select for cells capable of growing at high cell densities. Mock infected cultures were treated in the same way. One hr6 infected cell line named 149-637-4, and one mock infected culture (637-4'), were established and further characterized. The mock infected cells, like the parental cell line, did not induce tumors in nude mice. On the contrary, cells infected with hr6 were tumorigenic (see Table VII) when tested several passages (5-6 passages) after infection.

I was interested in determining whether tumorigenicity, which indicates a fully transformed phenotype, could be due to new viral DNA inserts, which might indicate integration of hr6

TABLE VII

TUMORIGENICITY OF hr AND wt TRANSFORMED CELLS IN ADULT ATHYMIC NUDE MICE

Cell line	Transformed by	No. cells inoculated	Expt. 1	Expt. 2	Expt. 3
637C3	wt	10^7	5/5[a](3)[b]	4/4 (3)	4/4 (1)
		10^6	4/4 (3)		
		10^5	3/4 (3)		
512C8	HindIII G fragment (wt)	10^7	4/4 (9)		
		10^6	4/4 (11)		
		10^5	4/4 (18)		
637-4	hr1	10^7	0/4 (26)	0/4 (16)	
637-4'	hr1 + mock	10^7	0/4 (14)	0/4 (8)[c]	0/4 (18)
149-637-4	hr1 + hr6	10^7	2/2 (8)	3/3 (5)	4/4 (3)
		10^6	3/3 (8)	3/3 (7)	4/4 (3)
348	NM[d] inoculated with 149 cells	10^7	3/3 (11)		
		10^6	4/4 (11)		

Transformed cells growing in culture were harvested, injected subcutaneously into nude mice and scored for tumorigenicity as described in Materials and Methods.

[a]No. animals with tumor/no. animals inoculated

[b]Latent period in weeks or time at which the experiment was terminated when all animals were negative.

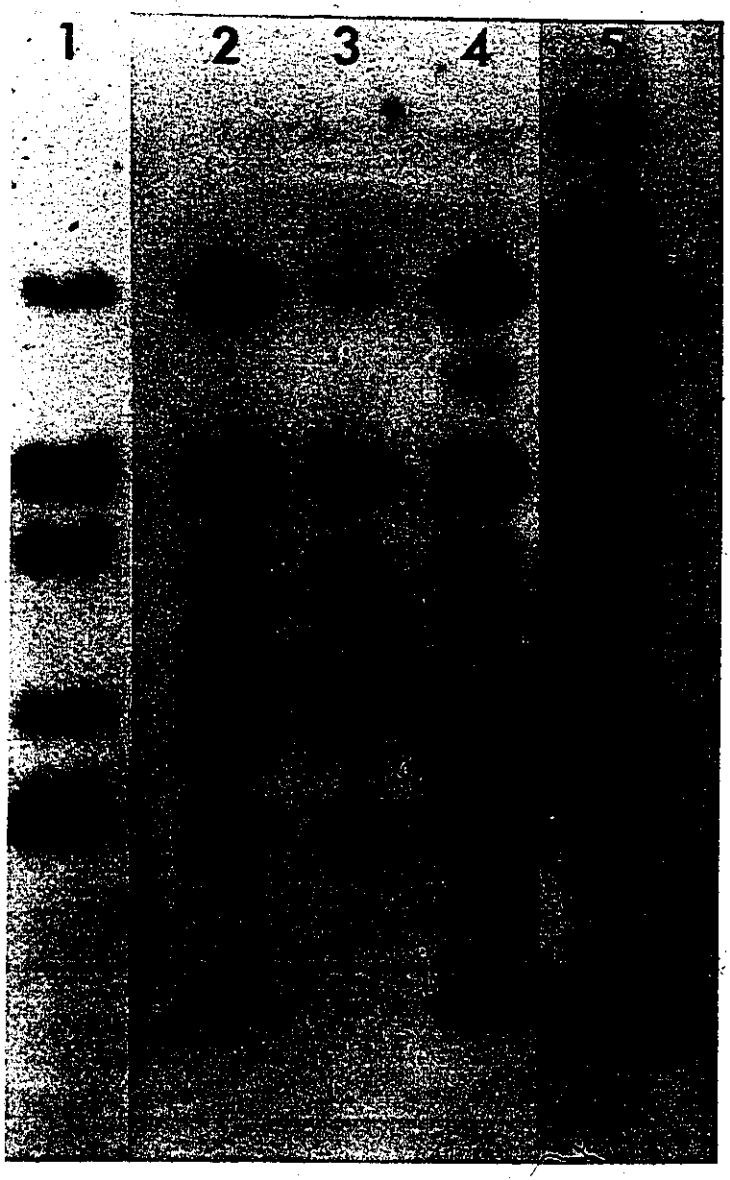
[c]At this time some animals died without any tumor and it was not possible to continue the experiment.

[d]Cells obtained from a tumor developed in a nude mouse inoculated with 149 cells.

DNA. For that reason I analyzed the tumorigenic cell line with the Southern procedure hybridizing with labeled total Ad5 DNA or cloned HindIII G fragment. Cell lines 637-4 and 348, a line obtained from a tumor which developed in a nude mouse inoculated with 149-637-4 cells, were analyzed as well. As can be seen from Figure 22, a new band appears in the 149 cells and in the tumor cell line. Further studies confirmed that this new band hybridized with the HindIII G probe, which represents the left end (see Fig. 11, track 4). Different explanations can be given for this finding. The simplest and most attractive explanation is that a second integration of the transforming region (in this case belonging to hr6) provides the functions lacking in Group I transformed cells. In addition, the resident genome may have complemented the defect shown by Group II mutants which are unable to initiate transformation in experiments using virus (Graham et al., 1978).

No apparent changes can be observed in the integrated viral sequences previously described for hr1 transformed 637-4 cells (see Fig. 11 and 22). The fragment which hybridizes with the G probe remains unchanged as well as all the internal fragments (Fig. 11, tracks 4 and 5; Fig. 22, tracks 2 and 4). This fact suggests a new site of integration and not a rearrangement of the resident genome.

Figure 22. Pattern of integration of Ad5 DNA after retransformation of 637-4 cells with hr6 (see text). Cell DNAs were digested with HindIII and the filters were hybridized with total Ad5 DNA labeled in vitro with [α ^{32}P]dCTP as described. The cell DNAs analyzed were as follows: track 2, 637-4; track 3, 637-4'; track 4, 149-637-4; and track 5, 348 cells. Track 1 contains HindIII digested Ad5 DNA, run as a marker.


1
2
3
4
5

To confirm the hypothesis proposed in this appendix, it may be useful to clone and sequence both integrated E1 regions to find if they correspond to both groups of hr mutants. In addition to that, if we want to propose a complementation between both groups, we have to demonstrate also that new viral E1A proteins are expressed after transformation with hr6.

Other hypotheses which can explain the findings reported here are: 1) the reversion of the defect exhibited by the integrated hr1 mutant followed by duplication and reinsertion of the E1 region into the host DNA or 2) the expression or repression of cellular genes produced by the new insertion or rearrangement. It would be of interest to sequence the integrated E1 regions and to study in more detail the viral proteins expressed in hr1 transformed cells and in the retransformed derivatives as well as to study more retransformed cell lines to get a more coherent picture which would confirm or exclude all the above proposed hypothesis.

Appendix 2

Estimation of the amount of viral DNA sequences in infected or transformed cells

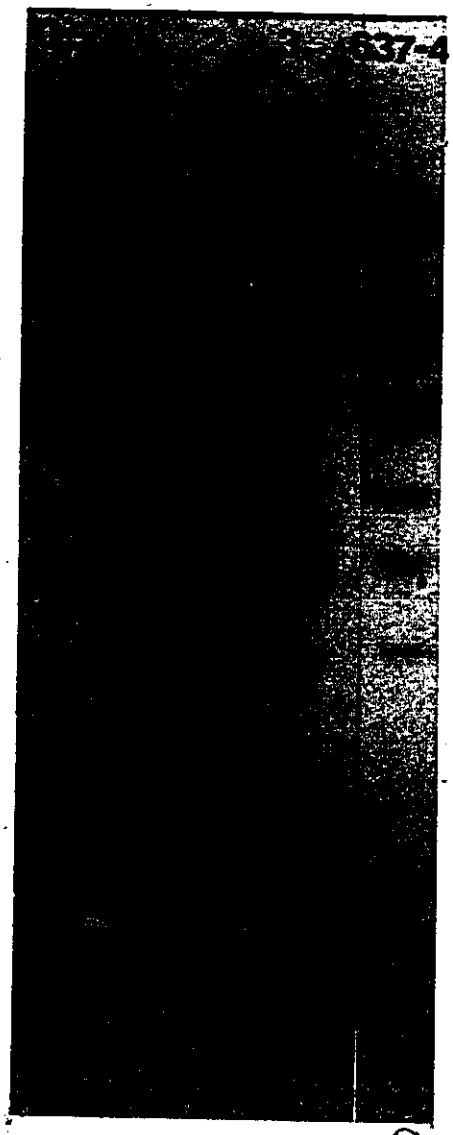
a) Estimation of the amount of DNA present in 637-4 cells:

As shown in Chapter IV, virtually the entire viral DNA is present colinearly integrated in most hrl transformed cells. To further characterize the presence of viral DNA sequences in transformed cells I wanted to determine the number of viral copies per cell and the molar amount of each of the internal restriction fragments. For this purpose, I made densitometric measurements of autoradiographs obtained with one of the transformed cell lines (637-4).

Known amounts of cold viral DNA mixed with 30 µg of uninfected rat cell DNA and digested with HindIII were used as marker DNA. After electrophoresis in agarose the DNA was transferred to nitrocellulose filters and hybridized with total ^{32}P labeled Ad5 DNA (Fig. 23A). Special care was taken to avoid saturation of the silver grains in the autoradiographs. The different tracks were scanned with a Joyce Loebl densitometer and the areas below the curves (Fig. 23B) were measured with a computerized MOP-3 digitizer (Carl Zeiss Inc., BDR). Table VIII and Figure 24 show the results of such readings. For each fragment an approximate linear relation was found between the densitometric readings and the number of genomic equivalents in the marker DNA (see Figure 24). Linear regression analysis

Figure 23. Quantiation of the viral DNA present in 637-4 cells. Panel A: Known amounts of cold Ad5 DNA (0.5, 1, 2 and 3 µg) extracted from virions were mixed with 30 µg of uninfected rat cell DNA, digested with HindIII, electrophoresed in 0.7% agarose (tracks 1-4) and hybridized to ^{32}P Ad5 DNA as described. 30 µg of 637-4 cell DNA was run in track 5. The autoradiograph obtained was scanned with a Joyce-Loebl densitometer (see Materials and Methods) and a sample of the scans is shown in panel B. The values obtained for the areas below the peaks (in relative units) were used to construct Tables VIII and IX and Figure 24.

A



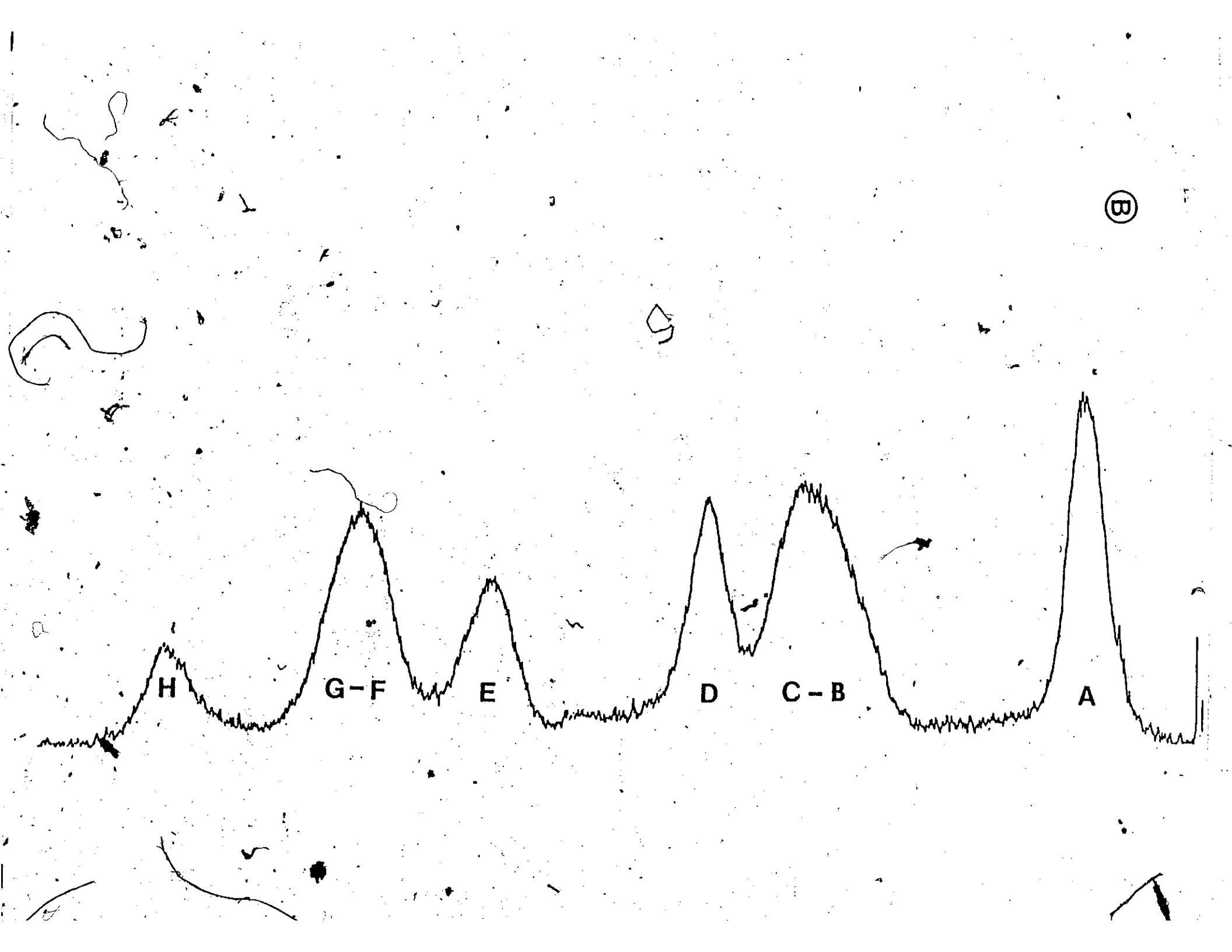

B
H
G–F
E
D
C–B
A

TABLE VIII

OPTICAL DENSITIES (IN RELATIVE UNITS) OBTAINED FOR FRAGMENTS DETECTED IN BLOTS OF DIFFERENT AMOUNTS OF Ad5 DNA DIGESTED WITH HINDIII

No. of copies per cell genomic equivalent	OD (relative units)[a]					
	A (5.22)[c]	B+C[b] (3.75)(3.34)	D (2.94)	E (2.09)	F+G[b] (1.84)(1.82)	H (1.33)
0.5	0.29[d]	0.37	0.16	-	-	-
1	0.77	0.98	0.36	0.35	0.83	-
2	1.29	1.96	0.79	0.80	1.43	0.44
3	1.83	2.68	1.24	1.09	2.02	0.60

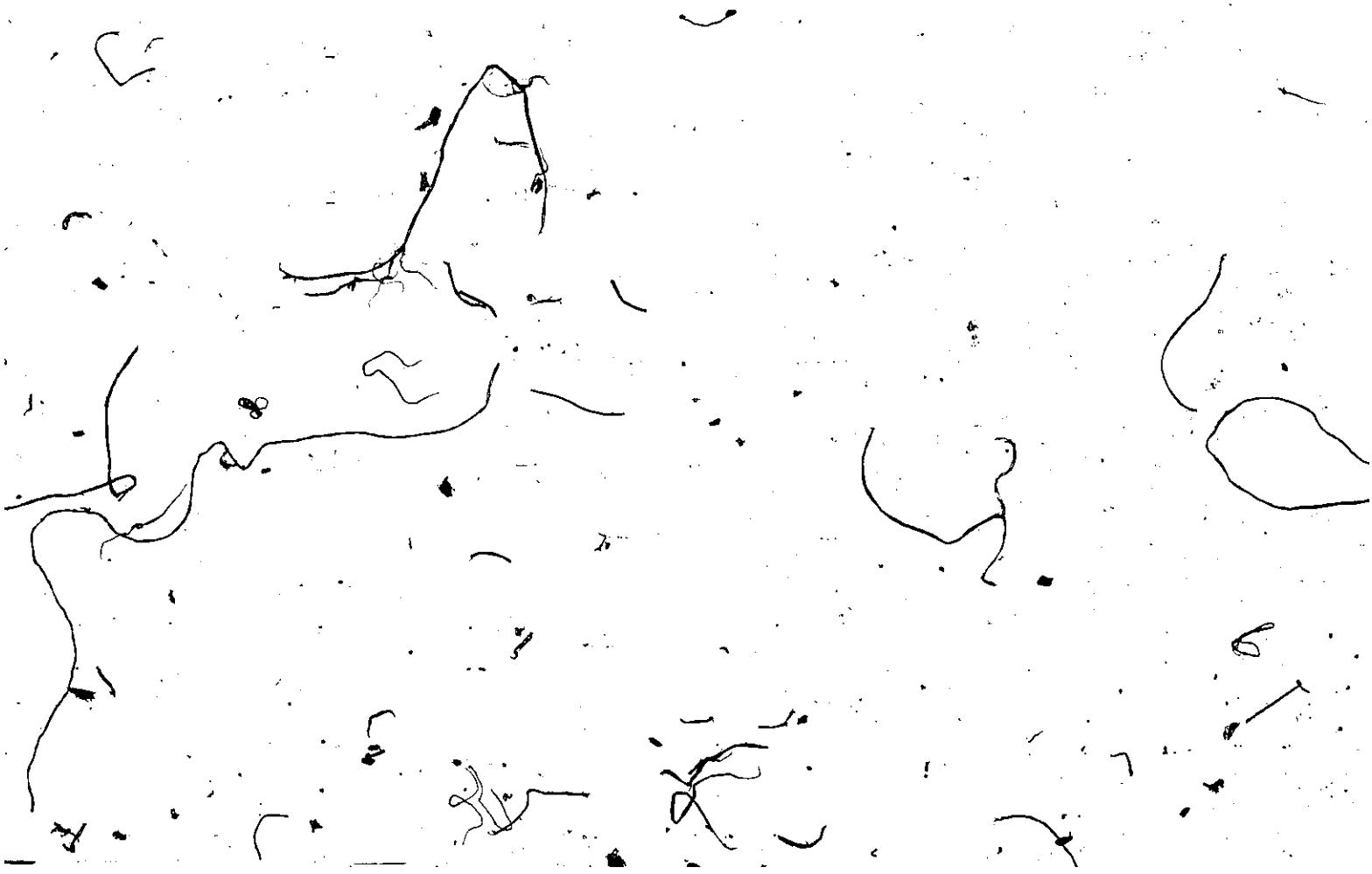
[a]The light density of the bands present in the autoradiograph of Figure 23 was measured as described in the text and the units used were the relative units given by the MOP-3.

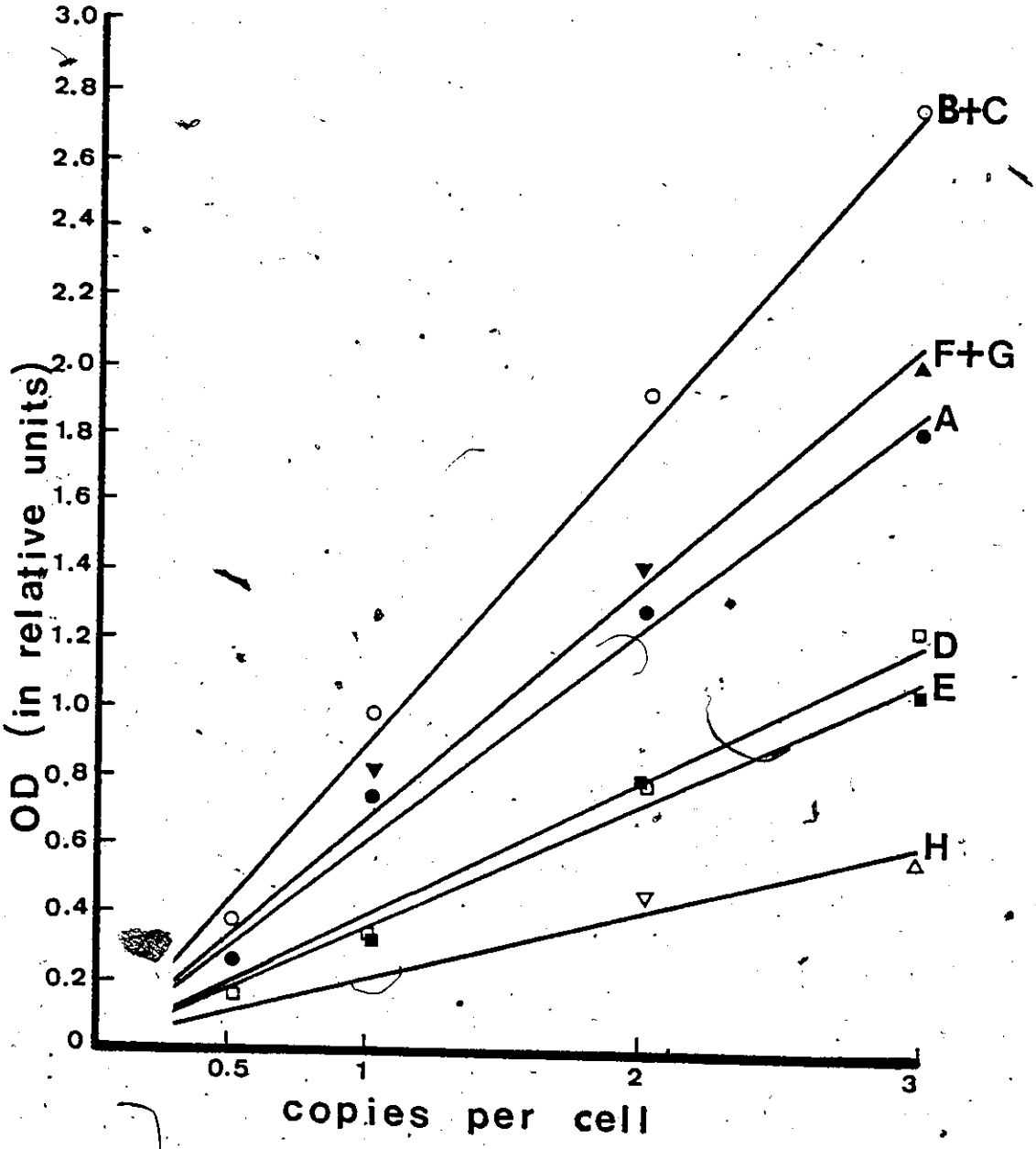
[b]Fragments B+C and F+G were not resolved in the scanning and were measured as a single peak.

[c]The numbers between brackets represent the molecular weights of Ad5 HindIII DNA fragments (x 10^6 daltons).

[d]The values in the table represent the average of four readings.

Figure 24. Densities measured for different amounts of HindIII digested Ad5 DNA. The values shown in Table VIII were used to construct this figure. O.D. values are given in relative units (see Table VIII).




3.0
2.8
2.6
2.4
2.2
2.0
1.8
1.6
1.4
1.2
1.0
0.8
0.6
0.4
0.2
0
OD (in relative units)
0.5
1
2
3
copies per cell
B+C
F+G
A
D
E
H

were used to obtain the straight lines giving the least squares fit to the points obtained for each fragment with the marker DNA (Dixon and Massey, 1957).

Thirty micrograms of DNA from the transformed cells 637-4 were processed in the same way as the marker DNA (Fig. 23A); the densities measured for each of the internal fragments of the viral DNA present in these cells are shown in Table IX and were used to calculate the number of copies per cell for each of these fragments, which was interpolated using the regression lines (Table IX). Both ends of the viral DNA, which are present in these cells joined to cellular DNA, were not measured in this experiment (see Chapter IV).

All the internal fragments, with the exception of HindIII H, appear to be present in equimolar amounts. The amount estimated for fragment H is, however, smaller than the estimations done for the other fragments. This result can be explained by the fact that fragment H is a small fragment and a fraction of it may be lost after transfer to nitrocellulose filters (Wahl et al., 1979). In addition to that, only two points (corresponding to 2 and 3 copies per cell) could be obtained to calculate the slope in Figure 24 because it was not possible to obtain measurable scans for fragment H when 0.5 or 1 copies of Ad5 DNA per cell were used. One unexpected finding was that the values obtained for all the internal fragments were smaller than 1. This may indicate that

TABLE IX

NUMBER OF COPIES OF Ad5 HINDIII DNA FRAGMENTS PRESENT IN 637-4 CELLS

Fragment	Relative OD units[a]	No. of copies/cell[b]
A	0.44	0.70
B+C	0.80 < 0.42[c] 0.38	< 0.86 0.87
D	0.34	0.84
E	0.25	0.67
F	0.21	0.60
G	-[d]	-
H	0.08	0.39

[a] The light density of the bands present in the autoradiograph of Figure 23 was measured as described in the text and the units used were the relative units given by the MOP-3.

[b] These values were obtained by interpolation in the linear graphs obtained with marker DNA (Fig. 24).

[c] B+C run together. To get an estimation of the density corresponding to each fragment, the total density obtained was divided according to the size of each fragment.

[d] Fragment G was present in 637-4 cells joined to cellular DNA (see Chapter IV) and was not used for these studies.

the value used to estimate the amount of DNA in rat cells (3.6×10^{12} daltons/diploid cell, Lehninger, 1975) is an underestimate for 637-4 cells, which are probably aneuploid. Alternatively, it may indicate heterogeneity in the cell population, although this is unlikely in view of the results shown in Chapter IV.

b) Estimation of the amount of joint fragment present in adenovirus infected cells:

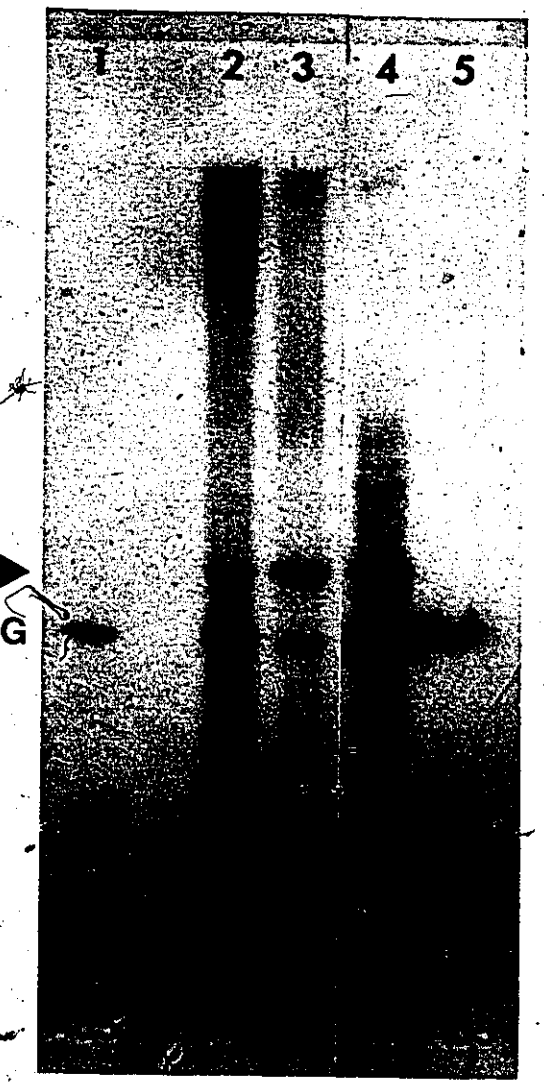
The same procedure used in Section a) to measure the number of copies of Ad5 DNA in 637-4 cells, was followed to quantitate joint fragments formed in Ad5 infected cells (see Chapter V). As shown in Chapter V, a fraction of the molecules which give head to tail joints after digestion with restriction enzymes, corresponded to circular DNA. I was interested to calculate the amount of circular molecules in relation to other kinds of molecules which also produce head to tail joints when digested with restriction enzymes. With this purpose, I compared the amount of joint fragment obtained after selection for circular DNA molecules, with the total amount of head to tail joints present in the cells.

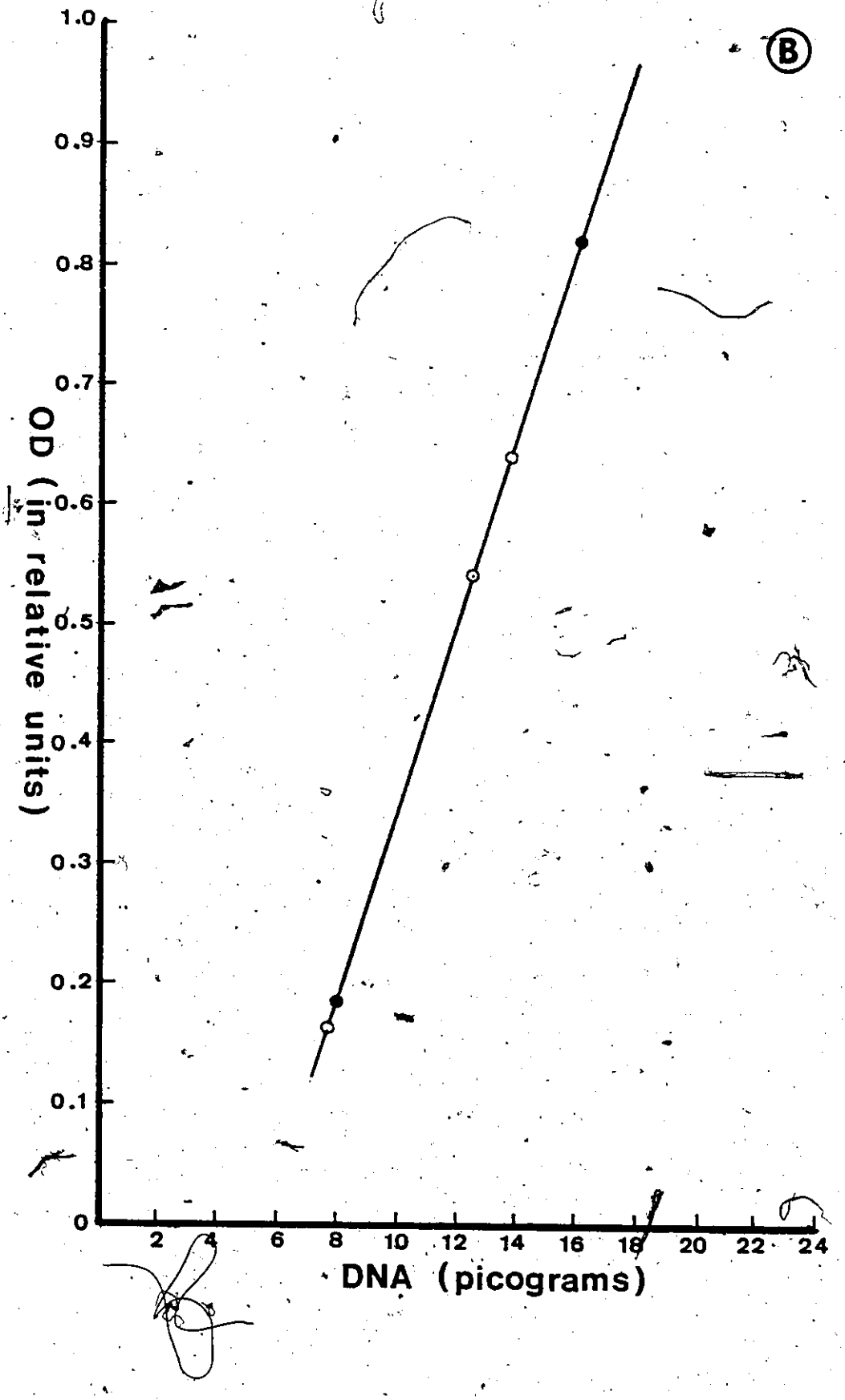
Figure 25A shows the results obtained after Southern blotting and hybridization with labeled HindIII G fragment, of a HindIII digest of different DNAs: Tracks 1 and 5 contain 0.1 ng and 0.2 ng, respectively, of marker Ad5 DNA extracted from virions. Tracks 2-4 contain DNA from BRK cells infected

Figure 25. Amount of joint fragment after selection for circular molecules. Panel A: DNA collected from peaks I(3) or II(2) of the gradient was digested with HindIII and coelectrophoresed with 0.1 ng(1) and 0.2 ng(5) of HindIII digested marker DNA. Ten micrograms of infected rat cell DNA(IC), digested with HindIII were run in track 4. The nitrocellulose filter was hybridized with ^{32}P labeled HindIII G fragment and the autoradiographs obtained were measured for their relative amount of joint fragment (see text). Panel B: The values presented in Table X were used to calculate the relative amount of joint fragment in band I and band II of the gradient, as shown in this figure. OD values are given in relative units (see Table X).

● G fragment

○ joint fragment


A
1
2
3
4
5
G


B
1.0
0.9
0.8
0.7
0.6
0.5
0.4
0.3
0.2
0.1
0
OD (in relative units)
2
4
6
8
10
12
14
16
18
20
22
24
DNA (picograms)

with hrl virions at an moi of 1, and extracted following the usual procedure used to extract cell DNA (track 4) or using the modified Birnboim extraction followed by CsCl-ethidium bromide gradient (tracks 2 and 3; see Chapter V). Five times more cells were used for the gradient, (ie. 2 large Lux dishes, 150 mm, $\sim 10^7$ cells/dish were used for total DNA extraction and 10 dishes for selection of circular molecules).

The values obtained after microdensitometry of the autoradiograph are shown in Table X. The amount of DNA running as a joint fragment in the gel was interpolated in Figure 25B. This figure was constructed with the values obtained for known amounts of marker G fragment. The size of the joint fragment after digestion with HindIII allows the assumption that it will transfer to nitrocellulose at the same efficiency of the G fragment. Therefore, I used the amounts obtained for the G fragment to estimate the amount of joint fragment. The values shown in column 4 of Table X were used to calculate total amount of head to tail joints per cell, or joint fragment per cell banding with circles, as follows (see column 5):

$$\frac{\text{amount of joint fragment in track "N"} \times \text{fraction of sample used in the gel}}{\text{Number of dishes extracted} \times \text{cells per dish}} =$$

$$\text{total amount of joint fragment per cell} = \frac{\text{track 4} \times 200}{2 \cdot 10^7} =$$

$$1.38 \cdot 10^{-4} \text{ picograms/cell}$$

TABLE X

DENSITOMETRIC ESTIMATIONS OF THE AMOUNT OF JOINT FRAGMENT PRESENT AFTER SELECTION FOR CIRCULAR MOLECULES

Track no.	DNA run on gel	OD (relative units)[a]	DNA amount (pg)	Joint fragment[b] pg/cell
1	Ad5	0.18	8[c]	-
2	I[d]	0.54	12.5	$5 \cdot 10^{-7}$
3	II[d]	0.16	7.6	$3.04 \cdot 10^{-7}$
4	total DNA[e]	0.64	13.8	$1.38 \cdot 10^{-4}$
5	Ad5	0.82	16	-

[a]Relative units given by the MOP-3.

[b]Values estimated as explained in the text. Each dish was estimated as containing 10^7 cells and five times more cells were used for the gradient (see text).

[c]Known amount of marker DNA (Ad5) was digested with HindIII and loaded in the gel. The values for tracks 2, 3 and 4 were interpolated in Figure 25B.

[d]Fraction I and II of the CsCl-ethidium bromide gradient (see text and Figure 21); ¼ of the total amount of DNA in fraction I or II, respectively, was digested and loaded in the gel.

[e]Total infected rat cell DNA extracted following the usual procedure, (see text) 1/200 of the DNA obtained was digested and loaded in the gel.

joint fragment extracted with circular

$$\text{DNA} = \frac{(\text{track 2} + \text{track 3})\ 4}{10 \times 10^7} = 8.04 \cdot 10^{-7} \text{ picograms/cell}$$

The amount of joint fragment in fraction II (track 3) could be due to linear concatemers or to nicked circles (see Chapter V). For the calculation done in this appendix, I assumed that this fraction corresponded to nicked circles exclusively.

From the data shown in Table X it is possible to conclude that the efficiency of the Birnboim extraction followed by the gradient (ie. amount of circular molecules resistant to alkaline extraction which banded as form I in the CsCl-ethidium bromide gradient) was very low in comparison to the amount of joint fragment obtained using the regular procedure used for all the other extractions (which does not discriminate between circular, linear or concatemeric molecules). As mentioned in Chapter V, the differences between both kinds of extractions may correspond to a low percentage of circular molecules, <1/100 compared to other forms (ie. linear concatemers), or to circular monomers or concatemers nicked and lost during the Birnboim extraction. Adenovirus 5 circles, even the monomers, would be large in size, which would make them more sensitive to extraction and increase the possibility of losing these molecules by single nicks.

Appendix 3

DNA replication of hrl mutants in semipermissive primary rat cells

The results reported in Chapter V show the formation of circular molecules of Ad5 DNA in cells infected with this virus. Head to tail joints, which may be a sign for circle formation (see Chapter V) appear in hrl (Group I hr mutant) as well as in wt infected cells (see Chapter V). Another interesting finding from the same experiments was that hrl virions were able to replicate their DNA in semipermissive rat cells almost as well as wt virions. That observation is reported in this appendix.

Group I hr mutants are considered negative for DNA replication in human cells, permissive for wt. (Lassam et al., 1979; Rowe and Graham, 1981). Nevertheless, Southern-blot analysis can detect a low level of replication in Hela cells (a human cell line), much smaller than the levels detected for wt virions (see below). When the same procedure was used with semipermissive rat cells infected with hr or wt virions, the level of DNA replication was similar for both viruses (see Fig. 26 and Table XI).

For the experiments shown in Figure 26, total DNA was extracted at different times after infection of BRK cells with hrl or wt, digested with HindIII and analyzed by the Southern technique. The labeled probe used for the hybridization was the right end of the DNA, HindIII I fragment. Panel A shows

Figure 26. Ad5-DNA-replication in infected BRK cells. Panel A: Southern blot analysis of hrl (tracks 1, 3, 5) or wt (tracks 2, 4, 6) infected cells at different times after infection. Infected cell DNAs were digested with HindIII and analyzed as described previously. ^{32}P labeled HindIII I fragment was used for the hybridizations. Tracks 1 and 2 contain 10 µg of infected cell DNA, tracks 3 and 4 contain 5 µg and tracks 5 and 6, 2.5 µg of infected BRK. Track 7 corresponds to a partial HindIII digest of 20 µg of uninfected rat DNA plus 1 ng of Ad5 DNA extracted from virions. ▶ Joint fragment (see Chapter V). Panel B: Photograph of the ethidium bromide stained gel used for panel A.

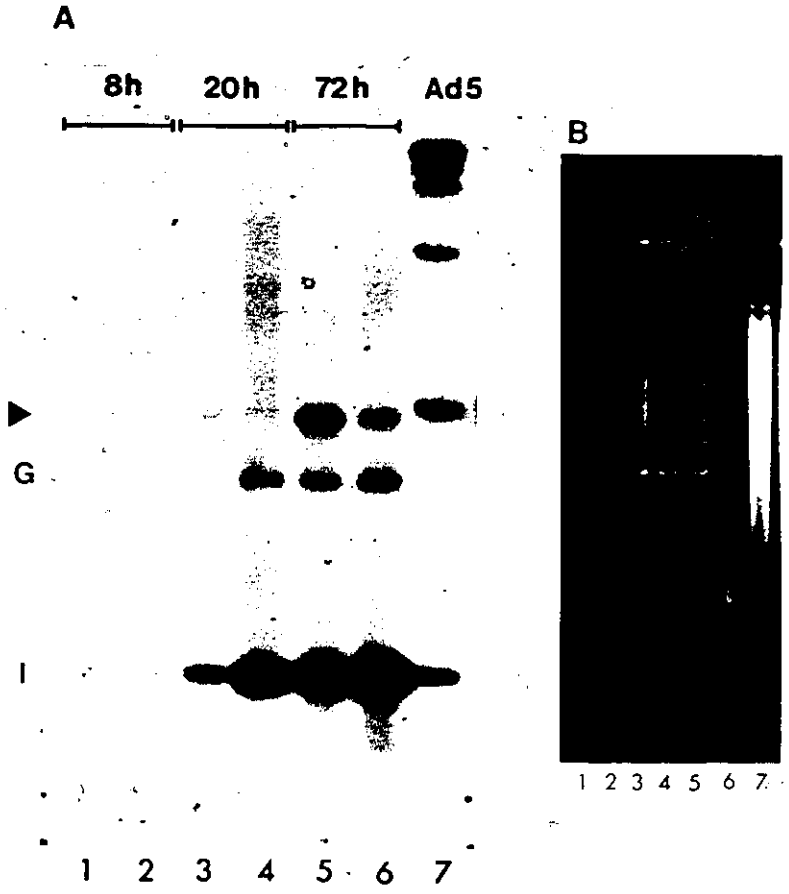

A
8h
20h
72h
Ad5
G
I
1 2 3 4 5 6 7
B
1 2 3 4 5 6 7

TABLE XI

Ad5 DNA REPLICATION IN PRIMARY BRK CELLS OR IN HELA CELLS

Time post infection	BRK						Hela					
	hrl			wt			hrl			wt		
	ug[a]	OD[b]	OD/10 ug	ug[a]	OD[b]	OD/10 ug	ug[a]	OD[b]	OD/10 ug	ug[a]	OD[b]	OD/10 ug
8 h	10	0.5	0.5	10	0.3	0.3	20	0.2	0.1	20	0.2	0.1
20 h	5	2.1	4.2	5	8.0[c]	16.0[c]	20	0.8	0.4	2	6.0[c]	30.0[c]
72 h	2.5	9.0[c]	36.0[c]	2.5	10.0[c]	40.0[c]	20	5.0	2.5	2	10.0[c]	50.0[c]

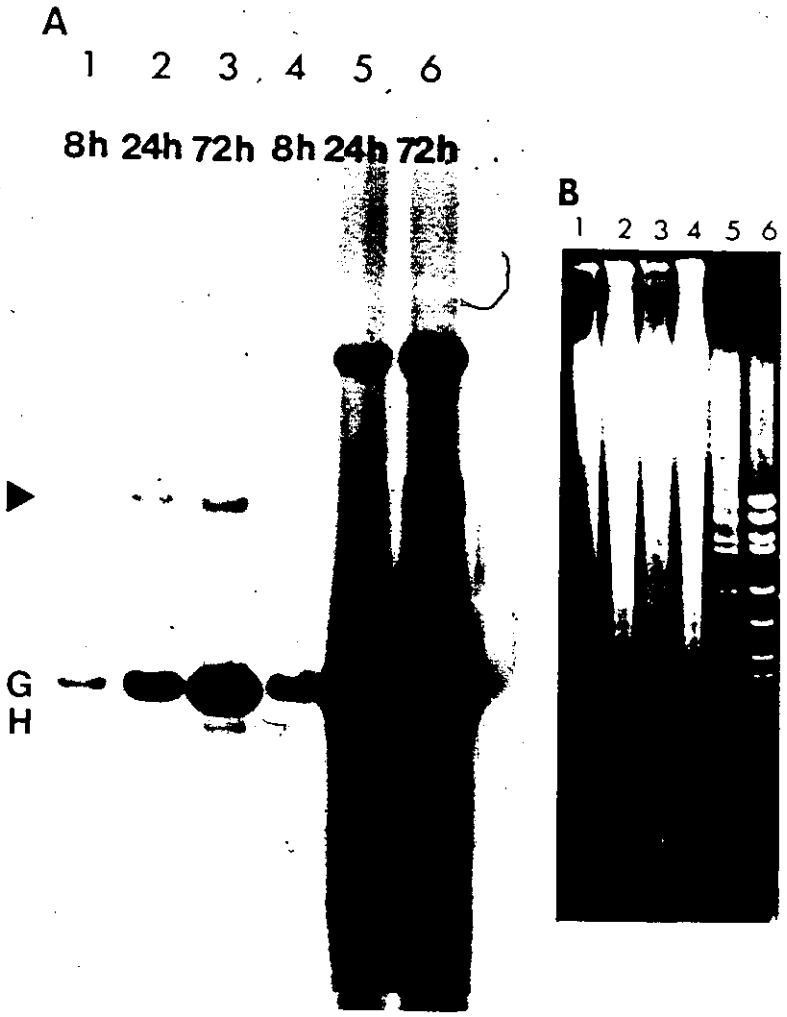
Autoradiographs similar to the ones shown in Figures 26 and 27 were scanned in a DV-8 Beckman spectrophotometer. Bands representing the right end of the viral DNA (ie. HindIII I, Fig. 26 or KpnI G, Fig. 27) were scanned, and the optical densities (in relative units) were used to construct this table (see Fig. 28).

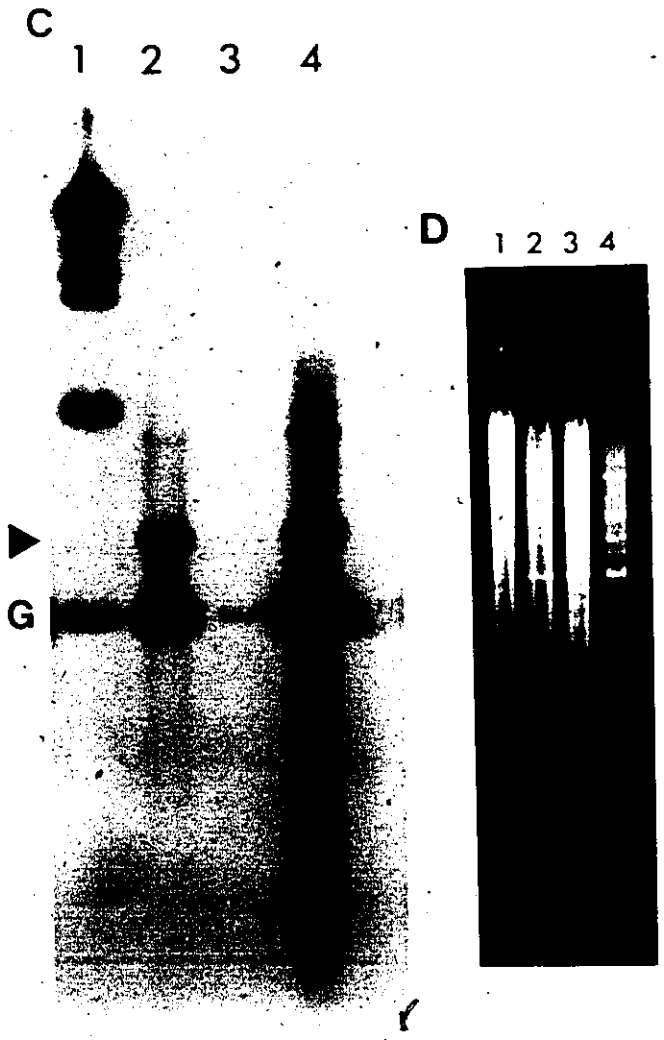
[a]ug of infected cell DNA loaded in each well.

[b]relative density of the bands measured with a densitometer.

[c]Saturation of the silver grains was reached and therefore the actual value could be higher. The values in the table are the values given by the DV-8 Beckman spectrophotometer.

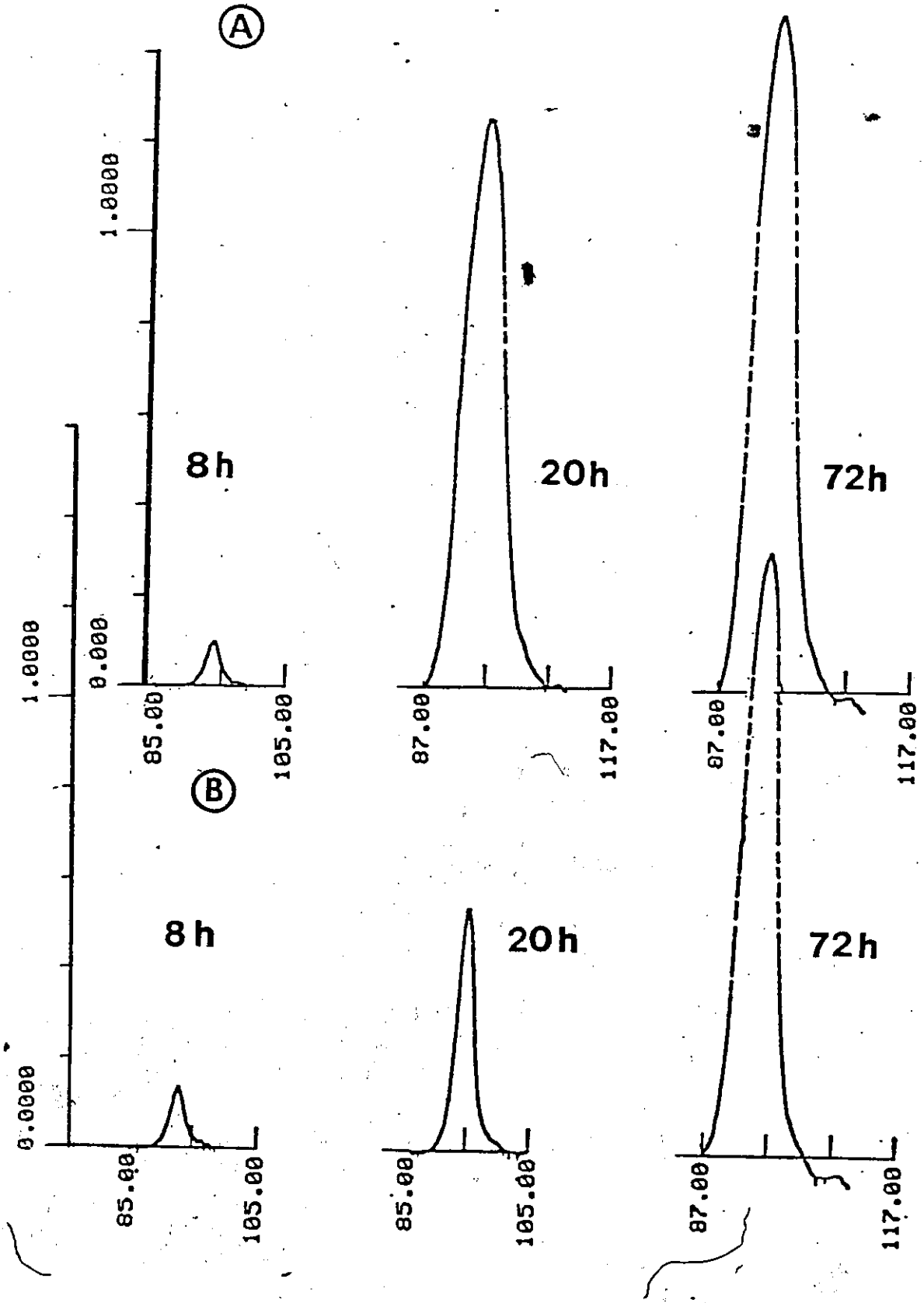
Figure 27. Ad5-DNA replication in continuous cell lines of permissive and semipermissive cells. Panel A: Autoradiograph of KpnI digested and HindIII I hybridized Hela cell DNA. The cells were previously infected with hr1 (tracks 1, 2, 3) or wt virus (tracks 4, 5, 6). Tracks 1-4 contain 20 µg of infected cell DNA. Tracks 5 and 6 contain 2 µg of wt infected cell DNA. Panel C: HindIII digest of DNA (8 µg) extracted from hr1 infected 1074-9 (track 2), 3Y1 (track 3) or BRK (track 4) hybridized with HindIII G probe. Panels B and D: ethidium bromide stained gels from which the nitrocellulose filters used in panel A and panel C, respectively, were obtained. Track 1 in panels C and D contains a partial HindIII digest of marker Ad5 DNA mixed with 20 µg of uninfected rat cell DNA. ▶ Joint fragment (see Chapter V).

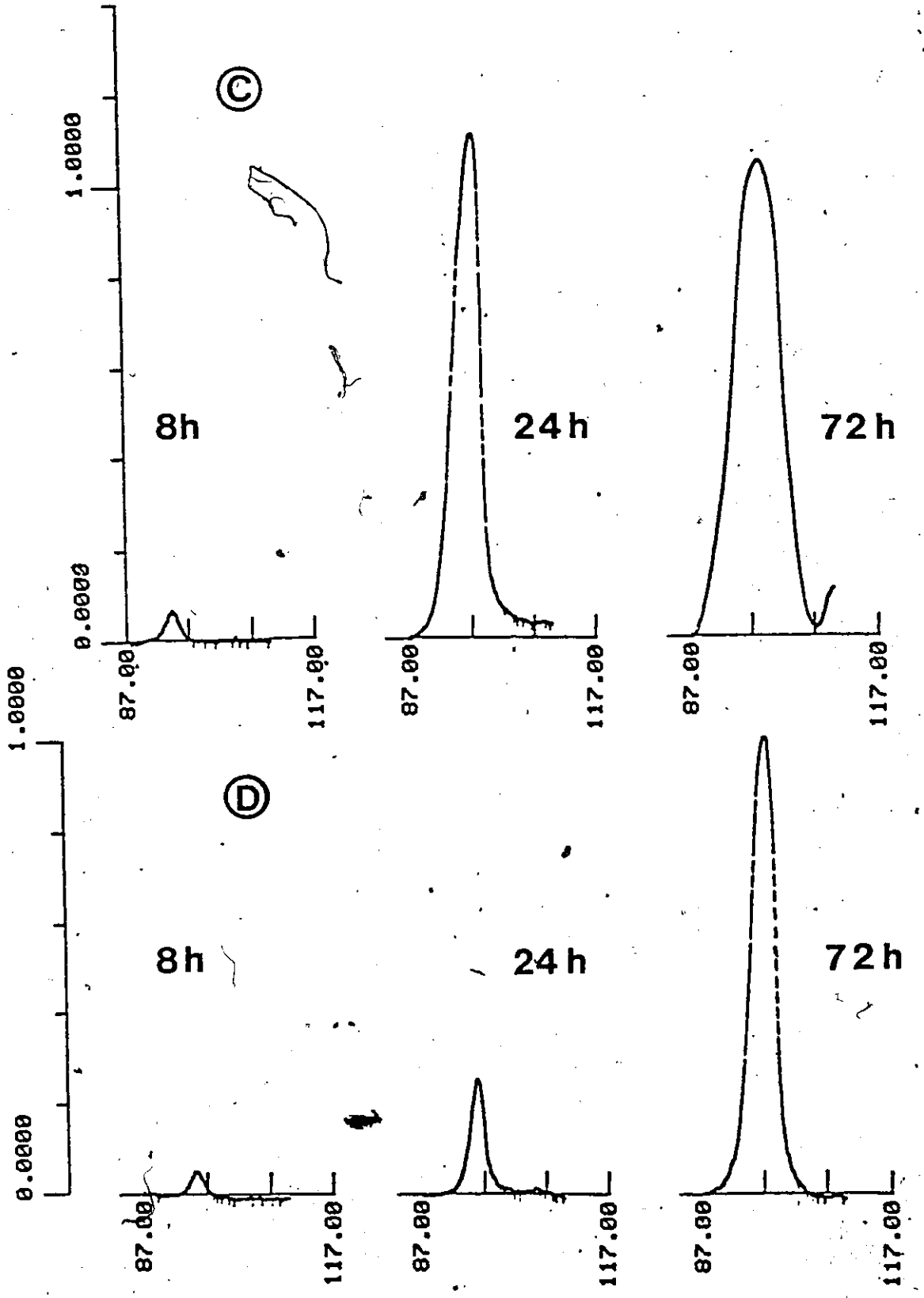

A
1 2 3 4 5 6
8h 24h 72h 8h 24h 72h
G
H
B
1 2 3 4 5 6


C
1 2 3 4
G
D
1 2 3 4

the autoradiograph and panel B shows the ethidium bromide stained gel, from which the DNA was transferred to nitrocellulose. The band migrating faster in the Southern-blot, represents the right end, HindIII fragment I of Ad5 DNA. This band is present in all the channels, and its intensity increases with the time post infection both with hr1 and wt (8, 20 or 72 hours). Note that the total amount of DNA loaded in the gels was decreased in tracks corresponding to longer times post infection (see legend of Figure 26). Table XI contains the values of the I band after scanning of the autoradiographs (see Fig. 28A and B) and corrected for the different amounts of DNA loaded in each track. A clear increase in the amount of fragment I, indicating viral DNA replication, can be observed in hr1 as well as in wt infected BRK. The same phenomena can be observed in the photographs of ethidium bromide stained gels (Fig. 26, panel B), where there is an increase of all bands corresponding to Ad5 DNA fragments 72 hours after infection with hr1 and wt (Fig. 26B, tracks 5 and 6). In addition to the viral bands, an extra band corresponding to repetitive rat sequences can be observed in the ethidium bromide stained gel (see Fig. 26B). The two extra bands seen in the autoradiographs (Fig. 26A) correspond to left to right end cross-hybridization and to head to tail joints (see Chapter V).

Figure 28. Ad5 DNA replication in eukaryotic cells. The bands representing the right end fragment of the viral DNA (see text) in the autoradiographs of Figure 26 and Figure 27A were scaned in a DV-8 Beckman spectrophotometer. Panels A and B: scans obtained for fragment HindIII I in the autoradiograph of Figure 26 (BRK cells infected with wt, A, or hrl, B). Panels C and D: scans obtained for fragment Kpn G in the autoradiograph of Figure 27A (Hela cells infected with wt, C or hrl, D). The values obtained for the areas below the curves were used to construct Table XI.


A
1.0000
0.000
8h
85.00
105.00
20h
87.00
117.00
72h
87.00
117.00
B
1.0000
0.0000
8h
85.00
105.00
20h
85.00
105.00
72h
87.00
117.00


©
1.0000
0.0000
8h
24h
72h
87.00
117.00
D
1.0000
0.0000
8h
24h
72h
87.00
117.00

As shown in Figure 27, the situation was different after infection of Hela cells with hrl or wt virus. The ethidium bromide stained gels did not show any sign of hrl DNA replication (no viral bands could be detected in hrl infected Hela cells, see Fig. 27B). Nevertheless, some hrl replication could be observed in the autoradiograph (Fig. 27A, tracks 1, 2 and 3). After digestion of cell DNA with KpnI and hybridization of the filter with labeled HindIII I, there was an increase in fragment G (right end fragment after digestion with KpnI, see Fig. 20) parallel to the time post infection at which the cell DNA was extracted. This increase is much higher in wt infected Hela cells as can be observed in Figure 27A, tracks 4, 5 and 6, as well as in the ethidium bromide stained gel (Figure 27B, tracks 4, 5 and 6). Table XI shows the results obtained after scanning of the KpnI G band in the autoradiograph (see Fig. 28C and D), corrected to the same amount of cell DNA. The extra bands observed in the autoradiograph correspond to the joint fragment, to left to right cross hybridization (see Chapter V) and to some partial digests. The additional bands observed mainly in tracks 1, 2, 3 and 4 of the ethidium bromide stained gel, are repetitive sequences detected with KpnI in human DNA.

Figure 27C and D show the results of similar studies done on established rat cell lines 3Y1 and 1074-9 (see Materials and Methods) 3 days after infection with hrl. The DNAs extracted from the infected cells were digested with HindIII and the nitrocellulose filter was hybridized to labeled HindIII G fragment (panel C). The ethidium bromide stained gel is shown in Figure 27D. Only track 4 in panel D, which contains hrl infected BRK cells, exhibits signs of hrl replication (given by the intensity of the viral DNA bands). The same result can be observed in the autoradiograph (panel C), where no replication is detected in 3Y1 cells (track 3) and only some replication in 1074-9, but to a smaller extent than in primary BRK cells (track 4).

From the experiments shown above, it is possible to conclude that hrl is able to replicate its DNA in semipermissive primary rat cells to levels similar to wt virus (Fig. 26). In contrast to this, in Hela cells which are fully permissive for wt, hrl replication is very inefficient (Lassam et al., 1979; Rowe and Graham, 1981) and can be detected only with very sensitive procedures, like Southern blotting and hybridization with labeled fragments (Fig. 27A). Some replication of hrl DNA could also be detected with this procedure in spontaneously transformed BRK after infection with hrl (cell line 1074-9, see Fig. 27C).

The differences in hrl DNA replication among different cells (BRK, Hela, 3Y1) may be related to host proteins overcoming the hrl defect. We know that Group I hr mutants are defective for DNA replication in permissive humans cells. We do not know the replication step at which the virus DNA replication is blocked and how it can be bypassed in BRK cells. The small amount of hrl DNA replication in Hela cells detected in the Southern blots can be related to leakiness of hrl in these cells. The level of replication observed in primary BRK, however, can not be explained in the same way, unless leakiness of the mutant is increased in these cells.

References

Aiello, L., R. Guilfoyle, K. Huebner and R. Weinmann (1979). Adenovirus 5 DNA sequences present and RNA sequences transcribed in transformed human embryo kidney cells. (HEK-Ad5 or 293). Virol. 94: 460-469.

Akusjarvi, G. and H. Persson (1981a). Controls of RNA splicing and termination in the major late adenovirus transcription unit. Nature 292: 420-426.

Akusjarvi, G., J. Zabielski, M. Perricaudet and U. Pettersson (1981b). The sequence of the 3' non-coding region of the hexon mRNA discloses a novel adenovirus gene. Nucl. Acids Res. 9: 1-17.

Babich, A. and J. Nevins (1981). The stability of early adenovirus mRNA is controlled by the viral 72 kd DNA-binding protein. Cell 26: 371-379.

Babiss, L.E., H.S. Ginsberg and P.B. Fisher (1983). Cold-sensitive expression of transformation by a host range mutant of type 5 adenovirus. Proc. Natl. Acad. Sci. U.S.A. 80: 1352-1356.

Baker, C.C. and E.B. Ziff (1981). Promoters and heterogeneous 5' termini of the messeger RNAs of adenovirus serotype 2. J. Molec. Biol. 149: 189-221.

Berget, A.M. and P.A. Sharp (1979). Structure of late adenovirus 2 heterogeneous nuclear RNA. J. Mol. Biol. 129: 547-565.

Berk, A.J. and P.A. Sharp (1977a). Ultraviolet mapping of the adenovirus 2 early promoters. Cell 12: 45-55.

Berk, A.J. and P.A. Sharp (1977b). Structure of adenovirus 2 early mRNAs. Cell 14: 695-711.

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

Berk, A.J., F. Lee, T. Harrison, J. Williams and P.A. Sharp (1979b). Phenotypes of adenovirus 5 host-range mutants for early mRNA synthesis. Cold Spring Harbor Symposium. Quant. Biol. 44: 429-436.

Birnboim, H.C. and J. Doly (1979). A rapid alkaline extraction procedure for screening recombinant plasmid DNA. Nucl. Acid Res. 7: 1513-1523.

Botchan, M., W. Topp and J. Sambrook (1976). The arrangement of simian virus 40 sequences in the DNA of transformed cells. Cell 9: 269-287.

Boyd, V.A. and J.S. Butel (1972). Demonstration of infectious deoxyribonucleic acid in transformed cells. I Recovery of simian virus 40 from yielder and non yielder transformed cells. J. Virol. 10: 399-409.

Britten, R.J. and D.E. Kohne (1968). Repeated sequences in DNA. Science 161: 529-540.

Burger, H. and W. Doerfler (1974). Intracellular forms of adenovirus DNA. III Integration of the DNA of adenovirus type 2 into host DNA in productively infected cells. J. Virol. 13: 975-992.

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

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

Challberg, M.D. and T.J. Kelly, Jr. (1979). Adenovirus DNA replication in vitro. Proc. Natl. Acad. Sci. U.S.A. 76: 655-659.

Challberg, M.D., S.V. Desiderio and T.J. Kelly, Jr. (1980). Adenovirus DNA replication in vitro: characterization of a protein covalently linked to nascent DNA strands. Proc. Natl. Acad. Sci. U.S.A. 77: 5105-5109.

Challberg, M.D., J.M. Ostrove and T.J. Kelly, Jr. (1982). Initiation of adenovirus DNA replication: Detection of covalent complexes between nucleotide and the 80-kilodalton terminal protein. J. Virol. 41: 265-270.

Chinsky, J. and R. Soeiro (1982). Studies with aphidicolin, on the Fv-1 host restriction of Friend Murine Leukemia Virus. J. Virol. 43: 182-190.

Chow, L.T., J.M. Roberts, J.B. Lewis and T.R. Broker (1977a). A map of cytoplasmic RNA transcripts from lytic adenovirus type 2, determined by electron microscopy of RNA:DNA hybrids. Cell 11: 819-836.

Chow, L.T., R.E. Gelinas, T.R. Broker and R.S. Roberts (1977b). An amazing sequence arrangement at the 5' ends of adenovirus 2 messenger RNA. Cell 12: 1-8.

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

Cohen, J.C., P.R. Shank, V.L. Morris, R. Cardiff and H.E. Varmus (1979). Integration of the DNA of mouse mammary tumor virus in virus-infected normal and neoplastic tissue of the mouse. Cell 16: 333-345.

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 T.J. Kelly, Jr. (1981). The structure of the linkage between adenovirus DNA and the 55.000 dalton terminal protein. J. Mol. Biol. 145: 319-337

Dhar, R., W.L. McClements, L.W. Enquist and G.F. Vande Woude (1980). Terminally repeated sequence (TRS) of integrated Moloney sarcoma provirus: nucleotide sequence of TRS and its host and viral junctions. Proc. Natl. Acad. Sci. U.S.A. 77: 3937-3941.

Dixon, W.J. and F.J. Massey, Jr. (1957). Introduction To Statistical Analysis, 2nd ed. McGraw-Hill Book Co. Inc., New York.

Doerfler, W., V. Lundholm and M. Hirsch-Kauffmann (1972). Intracellular forms of adenovirus deoxyribonucleic acid. I Evidence for a deoxyribonucleic acid-protein complex in baby hamster kidney cells infected with adenovirus type 12. J. Virol. 9: 297-308.

Doerfler, W., V. Lundholm, V. Rensing and L. Philipson (1973). Intracellular forms of adenovirus DNA. II Isolation in dye-buoyant density gradients of a DNA-RNA complex from KB cells infected with adenovirus type 2. J. Virol. 12: 793-807.

Dorsch-Hasler, K., P. Fisher, B. Weinstein and H. Ginsberg (1980). Patterns of viral DNA integration in cells transformed by wild type or DNA-binding protein mutants of adenovirus type 5 and effect of chemical carcinogens on integration. J. Virol. 34: 305-314.

Downey, J.F., D.T. Rowe, S. Bacchetti, F.L. Graham and S.T. Bayley (1983). Mapping of a 14.000-dalton antigen to early region 4 of the human adenovirus 5 genome. J. Virol. 45: 514-523.

Eggerding, F. and H.J. Raskas (1978). Effect of protein synthesis inhibitors on viral mRNAs synthesized early in adenovirus type 2 infection. J. Virol. 25: 453-458.

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

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

Esche, H. (1982). Viral gene products in adenovirus type 2-transformed hamster cells. J. Virol. 41: 1076-1082.

Falvey, E. and E. Ziff (1983). Sequence arrangement and protein coding capacity of the adenovirus type 2 "i" leader. J. Virol. 45: 185-191.

Fanning, E. and W. Doerfler (1977). Intracellular forms of adenovirus DNA: VI Quantitation and characterization of the four size-classes of adenovirus type 2 in productively infected cells. Virol. 81: 433-448.

Fenner, E., B.R. McAuslan, C.A. Mims and J. Sambrook, eds. (1974). In. The Biology of Animal Viruses, Academic Press, New York.

Fisher, P.B., L.E. Babiss, I.B. Weinstein and H.S. Ginsberg (1982). Analysis of type 5 adenovirus transformation with a cloned rat embryo cell line (CREF). Proc. Natl. Acad. Sci. U.S.A. 79: 3527-3531.

Flavell, A.J. and D. Ish-Horowicz (1981). Extrachromosomal circular copies of the eukaryotic transposable element "copia" in cultured Drosophila cells. Nature (London) 292: 591-595.

Fraser, N. and E. Ziff (1978). RNA structures near poly (A) of adenovirus-2 late messenger RNAs. J. Mol. Biol. 124: 27-51.

Fraser, N.W., J.R. Nevins, E. Ziff and J.E. Darnell (1979a). The major late adenovirus type-2 transcription unit: termination is downstream from the last poly (A) site. J. Mol. Biol. 129: 643-656.

Fraser, N. P. Segal and J.E. Darnell (1979b). Multiple discrete sites for premature RNA chain termination late in Ad2 infection: enhancement by 5, 6 dichloro 1βD-ribofuranosylbenzimidazole. Proc. Natl. Acad. Sci. U.S.A. 76: 2571-2575.

Freedman, V.H. and S. Shin (1974). Cellular tumorigenicity in nude mice: correlation with cell growth in semisolid medium. Cell 3: 355-359.

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

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

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

Gallimore, P.H., J.K. McDougal and L.B. Chen (1977). In vitro traits of adenovirus-transformed cell lines and their relevance to tumorigenicity in nude mice. Cell 10: 669-678.

Gallimore, P.H. and C. Paraskeva (1979). A study to determine the reasons for differences in the tumorigenicity of rat cell lines transformed by adenovirus 2 and adenovirus 12. Cold Spring Harbor Symposium. Quant. Biol. Med. 44: 703-713.

Gallimore, P.H., J.K. McDougall and L.B. Chen (1979). Malignant behaviour of three adenovirus-2-transformed brain cell lines and their methyl cellulose-selected sub-clones. Int. J. Cancer 24: 477-484.

Garon, C.F., K.W. Berry and J.A. Rose (1972). A unique form of terminal redundancy in adenovirus DNA molecules. Proc. Natl. Acad. Sci. U.S.A. 69: 2391-2395.

Gaynor, R., A. Tsukamoto, C. Montell and A. Berk (1982). Enhanced expression of adenovirus transforming proteins. J. Virol. 44: 276-285.

Gelb, L.D., D. Kohne and M.A. Martin (1971). Quantitation of SV40 sequences in African Green monkey, mouse and virus-transformed cell genomes. J. Mol. Biol. 57: 129-145.

Gerber, P. (1966). Studies on the transfer of sub-viral infectivity from SV40 induced hamster tumor cells to indicator cells. Virol. 28: 501-509.

Ginsberg, H.S., J.J. Ensinger, R.S. Kauffman, A.J. Mayer and V. Lundholm (1974). Cell transformation: a study of regulation with types 5 and 12 adenovirus temperature-sensitive mutants. Cold Spring Harbor Symposium. Quant. Biol. 39: 419-426.

Ginsberg, H.S. (1979). Adenovirus structural proteins. Comp. Virol. 13: 409-457.

Girvitz, S.C., S. Bacchetti, A.T. Rainbow and F.L. Graham (1980). A rapid and efficient procedure for the purification of DNA from agarose gels. Anal. Biochem. 106: 492-496.

Graham, F.L. and A.J. Van der Eb (1973). A new technique for the assay of infectivity of human adenovirus DNA. Virol. 52: 456-467.

Graham, F.L., A.J. Van der Eb and H. Heijneker (1974). Size and location of the transforming region in human adenovirus type 5 DNA. Nature 251: 687-691.

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

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

Graham, F.L., S. Bacchetti and R. McKinnon (1980). Transformation of mammalian cells with DNA using the calcium technique. Introduction of Macromolecules Into Viable Mammalian Cells. Alan R. Liss, Inc., New York.

Graham, F.L. (1984). Transformation by and oncogenicity of human adenoviruses. In The Adenoviruses. Comprehensive Virology. H. Ginsberg and C.H.F. Young, Eds. Plenum Press, N.Y. (in press).

Green, M. (1970). Oncogenic viruses. Ann. Rev. Biochem. 39: 701-756.

Green, M., J.T. Parsons, M. Pina, K. Fujinaga, H. Caffier and I. Landgraf-Lewis (1970). Transcription of adenovirus genes in productively infected and transformed cells. Cold Spring Harbor Symposium. Quant. Biol. Med. 35: 803-818.

Green, M., J.K. Mackey, W.S.M. Wold (1979). Thirty-one human adenovirus serotypes (Ad1-31) from five groups (A-E) based upon DNA genome homologies. Virol. 93: 481-492.

Green, M., W.S.M. Wold, K.H. Brookmann, M.A. Cartas, P.R. Sanders, K. Olson, T.K. Lee, L. Young, T. Matsuo and Q. Kapoor (1980). In. Viruses in Naturally Occurring Cancers, Essex, Todaro, Zur Hausen, eds. Cold Spring Harbor, New York.

Groneberg, J., D. Brown and W. Doerfler (1975). Uptake and fate of the DNA of adenovirus type 2 in KB cells. Virol. 64: 115-131.

Guntaka, R.J., B.W.J. Mahy, J.M. Bishop and H.E. Varmus (1975). Ethidium bromide inhibits appearance of closed circular viral DNA and integration of virus-specific DNA in duck cells infected by avian sarcoma virus. Nature 253: 507-511.

Guntaka, R.V., O.C. Richards, P.R. Shank, H.J. Kung, N. Davidson, E. Fritsch, J.M. Bishop and H.E. Varmus (1976). Covalently closed circular DNA of avian sarcoma virus: purification from nuclei of infected Quail tumor cells and measurement by electron microscopy and gel electrophoresis. J. Mol. Biol. 106: 337-357.

Habel, K. (1965). Specific complement-fixing antigens in polyoma tumors and transformed cells. Virol. 25: 55-61.

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

Halbert, D. and H. Raskas (1982). Tryptic and chymotryptic methionine peptide analysis of the in vitro translation products specified by the transforming region of adenovirus type 2. Virol. 116: 406-418.

Harrison, T., Graham, F. and J. Williams (1977). Host-range mutants of adenovirus type 5 defectives for growth in Hela cells. Virol. 77: 319-329.

Harter, M.L. and J.B. Lewis (1978). Adenovirus type 2 early proteins synthesized in vitro and in vivo: identification in infected cells of the 38.000 to 50.000 molecular weight protein encoded by the left end of the adenovirus type 2 genome. J. Virol. 26: 736-749.

Herisse, J., G. Courtoris and F. Galibert (1980). Nucleotide sequence of the Eco RI-D fragment of the adenovirus 2 genome. Nucleic Acids Res. 8: 2173-2191.

Hershey, A.D. (1970). In. Bacterioplage Lambda. Cold Spring Harbor Press, New York.

Ho, I.S., R. Galos and J. Williams (1982). Isolation of type 5 adenovirus mutants with a cold-sensitive host range phenotype: genetic evidence of an adenovirus transformation maintenance function. Virol. 122: 109-124.

Houweling, A., P.J. Van der Elsen and A.J. Van der Eb (1980). Partial transformation of primary rat cells by the left most 4.5% fragment of adenovirus 5 DNA. Virol. 105: 537-550.

Hsu, T.W. and J.M. Taylor (1982). Effect of aphidicolin on avian sarcoma virus replication. J. Virol. 44: 493-498.

Huebner, R.J., W.P. Rowe, H.C. Turner and W.T. Lane (1963). Specific adenovirus complement-fixing antigens in virus-free hamster and rat tumors. Proc. Natl. Acad. Sci. U.S.A. 50: 379-389.

Hughes, S.H., P.R. Shank, D.H. Spector, H.J. Kung, M. Bishop, H.E. Varmus, P.K. Vogt and M.L. Breitman (1978). Proviruses of avian sarcoma viruses are terminally redundant, co-extensive with unintegrated linear DNA and integrated at many sites. Cell 15: 1397-1410.

Ibelgaufts, H., W. Doefler, K.H. Scheidtmann and W. Wechsler (1980). Adenovirus type 12-induced rat tumor cells of neuroepithelial origin: persistence and expression of the viral genome. J. Virol. 33: 423-437.

Ikegami, S., T. Tuguchi, M. Ohashi, M. Oguro, H. Nagano and G. Mano (1978). Aphidicolin prevents mitotic cell division by interfering with the activity of DNA polymerase α. Nature (London) 275: 458-460.

Ikeda, J.E., T. Enomoto and J. Hurwitz (1982). Adenoviral protein-primed initiation of DNA chains in vitro. Proc. Natl. Acad. Sci. U.S.A. 79: 2442-2446.

Jolicoeur, P. and E. Rassart (1980). Effect of Fv1 gene product on synthesis of linear and supercoiled viral DNA in cells infected with murine leukemia virus. J. Virol. 33: 183-195.

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

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

Kaplan, L.M., H. Ariga, J. Hurwitz and M.S. Horwitz (1979). Complementation of the temperature-sensitive defect in H5 ts 125 adenovirus DNA replication in vitro. Proc. Natl. Acad. Sci. U.S.A. 76: 5534-5538.

Katze, M.G., H. Persson and L. Philipson (1981). Control of adenovirus early gene expression: a post transcriptional control mediated by region E1A products. Mol. Cell Biol. 1: 807-813.

Ketner, G. and T. Kelly (1976). Integrated simian virus 40 sequences in transformed cell DNA: analysis using restriction endonucleases. Proc. Natl. Acad. Sci. U.S.A. 73: 1102-1106.

Kiessling, R., G. Petranyi, G. Klein and H. Wigzell (1976). Non-T-cell resistance against a mouse Moloney limphoma. Int. J. Cancer 17: 275-281.

Kimura, G., A. Itagaki and J. Summers (1975). Rat cell 3Y1 and its virogenic polyoma and SV40 transformed derivatives. Int. J. Cancer 15: 694-706.

Kitchingman, G.R. and H. Westphal (1980). The structure of adenovirus 2 early nuclear and cytoplasmic RNAs. J. Mol. Biol. 137: 23-78.

Koprowski, H., F.C. Jensen and Z.S. Steplewski (1967). Activation of production of infectious tumor virus SV40 in heterokaryon cultures. Proc. Natl. Acad. Sci. U.S.A. 58: 127-133.

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

Lassam, N.J., S.T. Bayley and F.L. Graham (1979b). Transforming proteins of human adenovirus 5: studies with infected and transformed cells. Cold Spring Harbor Symposium. Quant. Biol. 44: 477-491.

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

Lehninger, A.L. (1975). Biochemistry, 2nd ed., Worth Pub. Inc.

Levinson, A.D. and A.J. Levine (1977). The group C adenovirus tumor antigens: identification in infected and transformed cells and a peptide map analysis. Cell 11: 871-879.

Lewis, J.B., J.F. Atkins, P.R. Baum, R. Solem, R.B. Gesteland and C.W. Anderson (1976). Location and identification of the genes for adenovirus type 2 early polypeptides. Cell 17: 141-151.

Lewis, J.B., C.W. Anderson and J.F. Atkins (1977). Further mapping of late adenovirus genes by cell-free translation of RNA selected by hybridization to specific DNA fragments. Cell 12: 37-44.

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

Lichy, J.H., M.S. Horwitz and J. Hurwitz (1981). Formation of a covalent complex between the 80.000 dalton adenovirus terminal protein and 5' dCMP in vitro. Proc. Natl. Acad. Sci. U.S.A. 78: 2678-2682.

Lichy, J., J. Field, M. Horwitz and J. Hurwitz (1982). Separation of the adenovirus terminal 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.

Lilly, D.M.J. (1981). In vivo consequences of plasmid topology. Nature 292: 380-382.

Lindberg, V., T. Persson and L. Phillipson (1972). Isolation and characterization of adenovirus mRNA in productive infection. J. Virol. 10: 909-919.

Lindhal, T., A. Adams, G. Bjursell, G. Bornkamm, C. Kaschka-Dierich and V. Jehn. Covalently closed circular duplex DNA of Epstein Barr Virus in a human lymphoid cell line. J. Molec. Biol. 102: 511-530.

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

Mackey, J.K., W.S.M. Wold, P. Rigden and M. Green (1979). Transforming regions of group A, B. and C adenoviruses: DNA homology studies with twenty-nine human adenovirus serotypes. J. Virol. 29: 1056-1064.

Macpherson, I. and L. Montagnier (1964). Agar suspension culture for the selective assay of cells transformed by polyoma virus. Virol. 23: 291-294.

Mak, S., I. Mak, J.R. Smiley and F.L. Graham (1979). Tumorigenicity and viral gene expression in rat cells transformed by Ad 12 virions or by the EcoRiC fragment of Ad 12 DNA. Virology 98: 456-460.

Mak, I. and S. Mak (1983). Transformation of rat cells by cyt mutants of adenovirus Type 12 and mutants of adenovirus Type 5. J. Virol. 45: 1107-1117.

Majors, J.E. and H.E. Varmus (1981). Nucleotide sequences at host-proviral junctions for mouse mammary tumor virus. Nature (London) 289: 253-258.

Maniatis, T., A. Jeffrey and D. Kleid (1975). Nucleotide sequence of the rightward operator of phage λ. Proc. Natl. Acad. Sci. U.S.A. 72: 1184-1188.

Mathews, M.B. and V. Pettersson (1978). The low molecular weight RNA of adenovirus 2-infected cells. J. Mol. Biol. 119: 293-328.

Matsuo, T., S. Hashimoto, W.M. Wold, J. Symington, A. Rankin and M. Green (1982). Identification of adenovirus 2 early region 4 polypeptides by in vitro translation and tryptic peptide map analysis. J. Virol. 41: 334-339.

Mayer, A.J. and H.S. Ginsberg (1977). Persistence of type 5 adenovirus DNA in cells transformed by a temperature-sensitive mutant, H5 ts 125. Proc. Natl. Acad. Sci. U.S.A. 74: 785-788.

McAllister, R.M., M.O. Nicholson, A.M. Lewis, JR., I. MacPherson and R.J. Huebuer (1969). Transformation of rat embryo cells by adenovirus type 1. J. Gen. Virol. 4: 29-36.

McDougall, J.C., A.R. Dunn and P.H. Gallimore (1974). Recent studies on the characteristics of adenovirus-infected and transformed cells. Cold Spring Harbor Sympsoium. Quant. Biol. Med. 39: 591-600.

Montell, C., E.F. Fisher, M.H. Caruthers and A.J. Berk (1982). Resolving the functions of overlapping viral genes by site-specific mutagenesis at a mRNA splice site. Nature (London) 295: 380-384.

Nagata, K., R.A. Guggenheimer, T. Enomoto, J.H. Lichy and J. Hurwitz (1982). Adenovirus DNA replication in vitro: identification of a host factor that stimulates the synthesis of the preterminal protein-dCMP complex. Proc. Natl. Acad. Sci. U.S.A. 79: 6438-6442.

Nevins, J.R. and J.E. Darnell (1978a). Groups of adenovirus type 2 mRNAs derived from a large primary transcript: probably nuclear origin and possible common 3' ends. J. Virol. 25: 811-823.

Nevins, J.R. and J.E. Darnell (1978b). Steps in the processing of Ad2 mRNA: poly $(A)^+$ nuclear sequences are conserved and poly (A) addition precedes splicing. Cell 15: 1477-1493.

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

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

Nevins, J.R. (1981). Mechanism of activation of early viral transcription by adenovirus E1A gene product. Cell 26: 213-220.

Pantelouris, E.M. (1968). Absence of thymus in a mouse mutant. Nature (London) 217: 370-371.

Pastan, I. and M. Willingham (1978). Cellular transformation and the "morphologic phenotype" of transformed cells. Nature 274: 645-649.

Pearson, G.D. (1975). Intermediate in adenovirus type 5 replication. J. Virol. 16: 17-26.

Pearson, G.D., K.C. Chow, J.L. Corden and J.A. Harpst (1981). Replication directed by a cloned adenovirus origin. ICN-UCLA Symp. Molec. Cell. Biol. 22: 581-595.

Pearson, G.D., K.C. Chow, R.E. Enns, K.G. Ahern, J.L. Corden and J.A. Harpst (1982). In vitro replication directed by a cloned adenovirus origin. J. Biol. Chem. (Submitted for publication).

Perez Villarreal, L. and P. Berg (1977). Hybridization in situ of SV40 plaques: detection of recombirant SV40 virus carrying specific sequences of nonviral DNA. Science 196: 183-185.

Perricaudet, M., G. Akusjarvi, A. Virtanen and V. Pettersson (1979). Structure of two spliced mRNAs from the transforming region of human sub-group C adenovirus. Nature 281: 694-696.

Perricaudet, M., J.P. LeMoullee and V. Pettersson (1980). The predicted structure of two adenovirus T-antigens. Proc. Natl. Acad. Sci. U.S.A. 77: 3778-3782.

Persson, H. V. Pettersson and M.B. Mathews (1978). Synthesis of a structural adenovirus polypeptide in the absence of viral DNA replication. Virol. 90: 67-79.

Persson, H., B. Mathison, L. Philipson and V. Pettersson (1979a). A maturation protein in adenovirus morphogenesis. Virol. 93: 198-208.

Persson, H., M. Perricaudet, A. Tolun, L. Philipson and V. Pettersson (1979b). Purification of RNA/DNA hybrids by exclusion chromatography. J. Biol. Chem. 254: 7999-8003.

Persson, H., M. Jansson and L. Philipson (1980a). Synthesis of and genomic site for an adenovirus type 2 early glycoprotein. J. Mol. Biol. 136: 375-394.

Persson, H., H. Jornvall and J. Zabielski (1980b). 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., H.S. Monstein, G. Akusjarvi and L. Phillipson (1981a). Adenovirus early gene products may control viral mRNA accumulation and translation in vivo. Cell 23: 485-496.

Persson, H., M. Katze and L. Philipson (1981b). Control of adenovirus early gene expression: accumulation of viral mRNA after infection of transformed cells. J. Virol. 40: 358-366.

Persson, H. and L. Philipson (1982). Regulation of adenovirus gene expression. Current Topics in Microbiol. & Immunol. 97: 157-203.

Pettersson, V. and J. Sambrook (1973). Amount of viral DNA in the genome of cells transformed by adenovirus type 2. J. Mol. Biol. 73: 125-130.

Pettersson, V., C. Tibbetts and L. Philipson (1976). Hybridization maps of early and late mRNA sequences of the adenovirus type 2 genome. J. Mol. Biol. 101: 479-502.

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

Philipson, L., V. Pettersson and V. Lindberg (1975). Molecular biology of adenoviruses. Virol. Monographs 14: 1-115.

Pontecorvo, G., P.N. Riddle and A. Hales (1977). Time and mode of fusion of human fibroblasts treated with polyethylene glycol (PEG). Nature 265: 257-258.

Povlsen, C.O., P.J. Fialkow, E. Klein, G. Klein, J. Rigaard and F. Wiener (1973). Growth and antigenic properties of a biopsy-derived Burkitt's lymphoma in tymus-less (Nude) mice. Int. J. Cancer 11: 30-39.

Rapp, F., T. Kitahara, J. Butel and J.L. Melnick (1964). Synthesis of SV40 tumor antigen during replication of simian papovavirus (SV40). Proc. Natl. Acad. Sci. U.S.A. 52: 1138-1142.

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

Ricciardi, R.P., R.L. Jones, C.L. Cepko, P.A. Sharp and B.E. Roberts (1981). Expression of early adenovirus genes requires a viral encoded acidic polypeptide (1981). Proc. Natl. Acad. Sci. U.S.A. 78: 6121-6125.

Rigby, P., M. Dieckmann, C. Rhodes and P. Berg (1977). Labeling deoxyribonucleic acid to high specific activity in vitro by nick translation with DNA polymerase I. J. Mol. Biol. 113: 237-251.

Rigby, P. (1979). The transforming genes of SV40 and polyoma. Nature 282: 781-784.

Robinson, A.J., H.B. Younghusband and A.J.D. Bellett (1973). A circular DNA-protein complex from adenoviruses. Virol. 56: 54-69.

Robinson, A.J. and A.J.D. Bellet (1974). A circular DNA-protein complex from adenoviruses and its possible role in DNA replication. Cold Spring Harbor Symposium. Quant. Biol. 39: 523-531.

Ross, S.R., S.J. Flint and A.J. Levine (1980a). The genome map position of the adenovirus type 2 glicoprotein. Virol. 99: 427-430.

Ross, S.R., A.J. Levine, R.S. Galos, J. Williams and T. Shenk (1980b). Early viral proteins in Hela cells infected with adenovirus type 5 host range mutants. Virol. 103: 457-492.

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

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

Rowe, D. and F.L. Graham (1983). Transformation of rodent cells by DNA extracted from transformation-defective adenovirus mutants. J. of Virol. 46: 1039-1044.

Ruben, M., S. Bacchetti and F.L. Graham (1982a). Integration and expression of viral DNA in cells transformed by host range mutants of adenovirus type 5. J. Virol. 41: 674-685.

Ruben, M., S. Bacchetti and F.L. Graham (1982b). Evidence for head to tail joining of human adenovirus type 5 DNA molecules. Cold Spring Harbor Symposium, Abstract 8.

Ruben, M., S. Bacchetti and F. Graham (1983). Covalently closed circles of adenovirus 5 DNA. Nature 301: 172-174.

Russell, W.C., K. Hayashi, P.J. Sanderson and H.G. Pereira (1967). Adenovirus antigens - A study of their properties and sequential development in infection. J. Gen. Virol. 1: 495-507.

Saborio, J.L. and B. Oberg (1976). In vivo and in vitro synthesis of adenovirus type 2 early proteins. J. Virol. 17: 865-875.

Sambrook, J., M. Botchan, P. Gallimore, B. Ozanne, V. Petterson, J. Williams and P.A. Sharp (1975). Viral DNA sequences in cells transformed by simian virus 40, adenovirus type 2 and adenovirus type 5. Cold Spring Harbor Symposium. Quant. Biol. 39: 615-632.

Schick, J., K. Baczko, E. Fanning, J. Groneberg, H. Burger and W. Doefler (1976). Intracellular forms of adenovirus DNA: IV Integrated form of adenovirus DNA appears early in productive infection. Proc. Natl. Acad. Sci. U.S.A. 73: 1043-1047.

Sehgal, P.B., N.W. Fraser and J.E. Darnell (1979). Early Ad2 transcription units: only promoter-proximal RNA continues to be made in the presence of DRB. Virol. 94: 185-191.

Sekikawa, K., K. Shiroki, H. Shimojo, S. Ojima and K. Fujinaga (1978). Transformation of a rat cell line by an adenovirus 7 DNA fragment. Virol. 88: 1-7.

Sergeant, A., M.A. Tigges and H.J. Raskas (1979) Nucleosome-like structural subunits of intracellular parental adenovirus type 2 DNA. J. Virol. 29: 888-898.

Shank, P.R. and H.E. Varmus (1978). Virus-specific DNA in the cytoplasm of avian sarcoma virus-infected cells is a precursor to covalently closed circular viral DNA in the nucleus. J. Virol. 25: 104-114.

Shank, P.R., J.C. Cohen, H.E. Varmus, K.R. Yamamoto and G.M. Ringold (1978). Mapping of linear and circular forms of mouse mammary tumor virus DNA with restriction endonucleases: Evidence for a large specific deletion occurring at high frequency during circularization. Proc. Natl. Acad. Sci. U.S.A. 75:2112-2116.

Shank, P.R., S.H. Hughes, H.-J. Kung, J.E. Majors, N. Quintrel, R.V. Gumtaka, J.M. Bishop and H.E. Varmus (1978). Mapping unintegrated avian sarcoma virus DNA: termini of linear DNA bear 300 nucleotides present once or twice in two species of circular DNA. Cell 15: 1383-1395.

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

Sharp, P.A., P.H. Gallimore and S.J. Flint (1974b). Mapping of adenovirus 2 RNA sequences in lytically infected cells and transformed cell lines. Cold Spring Harbor. Quant. Biol. 39: 457-474.

Sharp, P., C. Moore and J.L. Haverty (1976). The infectivity of adenovirus 5 DNA-protein complex. Virol. 75: 442-456.

Shaw, A.R. and E.B. Ziff (1980). Transcripts from the adenovirus 2 major late promoter yield a single family of coterminal mRNAs during early infection and five families at late times. Cell 22: 905-916.

Shenk, T., N. Jones, W. Colby and D. Fowlkes (1979). Functional analysis of adenovirus type 5 host range deletion mutants defective for transformation of rat embryo cells. Cold Spring Harbor Symposium. Quant. Biol. 44: 367-375.

Shimotohno, K. and H.M. Temin (1980). No apparent nucleotide sequence specificity in cellular DNA juxtaposed to retrovirus proviruses. Proc. Natl. Acad. Sci. U.S.A. 77: 7357-7361.

Shin, S.I., V.H. Freedman, R. Risser and R. Pollack (1975). Tumorigenicity of virus-transformed cells in nude mice is correlated specifically with anchorage independent growth in vitro. Proc. Natl. Acad. Sci. U.S.A. 72: 4435-4439.

Shiroki, K., H. Hamada, H. Shimojo, S. Yano, S. Ojima and K. Fujinaga (1977). Establishment and characterization of rat cells transformed by restriction endonuclease fragments of adenovirus 12 DNA. Virol. 82: 462-471.

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

Shoemaker, C.H., S. Goff, E. Gilboa, M. Paskind, S.W. Mitra and D. Baltimore (1980). Structure of a cloned circular Moloney murine leukemia virus DNA molecule containing an inverted segment: implications for retrovirus integration. Proc. Natl. Acad. Sci. U.S.A. 77: 3932-3936.

Shoemaker, C.H., J. Hoffmann, S.P. Goff and D. Baltimore (1981). Intramolecular integration within Moloney murine leukemia virus DNA. J. Virol. 40: 164-172.

Smets, L.A. (1980). Cell transformation as a model for tumor induction and neoplastic growth. Biochem. Biophys. Acta 605: 93-111.

Soderlund, H., V. Pettersson, B. Vennstrom, B. Philipson and M.B. Mathews (1976). A new species of virus-coded low molecular weight RNA from cells infected with adenovirus type 2. Cell 7: 585-593.

Southern, E.M. (1975). Detection of specific sequences among DNA fragments separated by gel electrophoresis. J. Mol. Biol. 98: 503-517.

Spector, D.N., M. McGrogan and H.J. Raskas (1978). Regulation of the appearance of cytoplasmic RNAs from region 1 of the adenovirus genome. J. Mol. Biol. 126: 395-414.

Spector, D.J., L.D. Crossland, D.N. Halbert and H.J. Raskas (1980). A 28K polypeptide is the translation product of 9S RNA encoded by region E1A of adenovirus 2. Virol. 102: 218-221.

Stabel, S., W. Doerfler and R.R. Friis (1980). Integration sites of adenovirus type 12 DNA in transformed hamster cells and hamster tumor cells. J. Virol. 36: 22-40.

Steenbergh, P.H., J. Maat, H. van Ormondt and J.S. Sussenbach (1977). The nucleotide sequence at the termini of adenovirus type 5 DNA. Nucleic Acid Res. 4: 4371-4389.

Steffen, D. and R.A. Weinberg (1978). The integrated genome of murine leukemia virus. Cell 15: 1003-1010.

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

Stillman, B., F. Tamanoi and M. Mathews (1982). Purification of an adenovirus-coded DNA-polymerase that is required for initiation of DNA replication. Cell 31: 613-623.

Stringer, J.R. (1982). Mutant of simian virus 40 large T-antigen that is defective for viral DNA synthesis, but competent for transformation of cultured rat cells. J. Virol. 42: 854-864.

Sussenbach, J.S. and M.G. Kuijk (1977). Studies on the mechanism of replication of termini. Virol. 77: 149-157.

Sussenbach, J.S. and M.G. Kuijk (1978). The mechanism of replication of adenovirus DNA. IV Localization of the origins of the displacement synthesis. Virol. 84: 509-517.

Sutter, D., M. Westphal and W. Doerfler (1978). Patterns of integration of viral DNA sequences in the genomes of adenovirus type 12 - transformed hamster cells. Cell 14: 569-585.

Tamanoi, F. and B. Stillman (1982). Function of adenovirus terminal protein in the initiation of DNA replication. Proc. Natl. Acad. Sci. U.S.A. 79: 2221-2225.

Tate, V. and L. Philipson (1979). Parental adenovirus DNA accumulation in nucleosome-like structures in infected cells. Nucleic Acid Res. 6: 2769-2785.

Tegtmeyer, P. and F. Macasaet (1972). Simian virus 40 deoxyribonucleic acid synthesis: analysis by gel electrophoresis. J. Virol. 10: 599-604.

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

Tolun, A., P. Alestrom and V. Pettersson (1979). Sequence of inverted terminal repeats from different adenovirus: demonstration of conserved sequences and homology between SA7 termini and SV40 DNA. Cell 17: 705-713.

Tooze, J., ed. (1980). In. DNA Tumor Viruses. Molecular Biology of Tumor Viruses. Part 2 (2nd ed.). Cold Spring Harbor Laboratory, New York.

Tyndall, C.L., H.B. Younghusband and A.J.D. Bellett (1978). Some adenovirus DNA is associated with the DNA of permissive cells during productive or restricted growth. J. Virol. 25: 1-10.

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

Van der Eb, A.J., L.W. van Kesteren and E.F. van Bruggen (1969). Structural properties of adenovirus DNAs. Biochim. Biophys. Acta 182: 530-541.

Van der Eb, A.J., C. Mulder, F.L. Graham and A. Howeling (1977). Transformation with specific fragments of adenovirus DNAs: Isolation of specific fragments with transforming activity of adenovirus 2 and 5 DNA. Gene 2: 115-132.

Van der Eb, A.J. and F.L. Graham (1979). Assay of transforming activity of tumor virus DNA. Methods in Enzymol. 65: 826-839.

Van der Eb, A.J., H. Van Ormondt, P.I. Schrier, H.J. Lupker, H. Jockemsen, P.J. Van der Elsen, R.J. DeLeys, J. Maat, C.P. Van Beveren, R. Dijkema and A. DeWaard (1979). Structure and function of the transforming genes of human adenovirus and SV40. Cold Spring Harbor Symposium. Quant. Biol. 44: 383-399.

Van der Putten, H., E. Terwidt and A. Berns (1979). The integration sites of endogenous and exogenous Moloney murine leukemia virus. Cell 18: 109-116.

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

Van der Vliet, P.C., A.J. Levine, M.J. Ensinger and H.S. Ginsberg (1975). Thermolabile DNA binding proteins from cells infected with a temperature-sensitive mutant of adenovirus defective in viral DNA synthesis. J. Virol. 15: 348-354.

Van der Vliet, P.C., J. Landberg and H.S. Janz (1977). Evidence for the function of the adenovirus DNA binding protein in initiation of DNA synthesis as well as in elongation of nascent DNA chains. Virol. 80: 98-110.

Van Ormondt, H., J. Maat, A. de Waard and A.J. Van der Eb (1978). The nucleotide sequence of the transforming HpaI-E fragment of adenovirus type 5 DNA. Gene 4: 309-328.

Vardimon, L. and Doerfler, W. (1981). Patterns of integration of viral DNA in adenovirus type 2 transformed hamster cells. J. Molec. Biol. 147: 227-246.

Visser, L., M.W. van Maarschalkerweerd, T.H. Rozijn, A.D.C. Wassenhaar, A.M.C.B. Reems and J.S. Sussenbach (1979). Viral DNA sequences in adenovirus-transformed cells. Cold Spring Harbor Symposium. Quant. Biol. 44: 541-550.

Visser, L., A.D.C. Wassenhaar, M.W. van Maarschalekerweerd and T.H. Rozijn (1981). Arrangement of integrated viral DNA sequences in cells transformed by adenovirus types 2 and 5. J. Virol. 39: 684-693.

Visser, L., A.C. Reemst. A.D.M. Van Mansfeld and T.H. Rozijn (1982). Nucleotide sequence analysis of the linked left and right hand terminal regions of adenovirus type 5 DNA present in the transformed rat cell line 5RK20. Nucleic Acid Res. 10: 2189-2198.

Wahl, G.M., M. Stern and G.R. Stark (1979). Efficient transfer of large DNA fragments from agarose gels to diazobenzyloxymethyl-paper and rapid hybridization by using dextran sulfate. Proc. Natl. Acad. Sci. U.S.A. 76: 3683-3687.

Wasylyk, B., C. Kedinger, J. Cordon, U. Brison and P. Chambon (1980). Specific in vitro initiation of transcription on conalbumin and ovalbumin genes and comparison with adenovirus 2 early and late genes. Nature 285: 367-373.

Weil, P.A., D. Sohnse, J. Segal and R.G. Roeder (1979b). Selective and accurate initiation of transcription at the Ad2 major late promoter in a soluble system dependent on purified RNA polymerase II and DNA. Cell 18: 469-484.

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

Weinman, R., T.G. Brendler, H.J. Raskas and R.G. Roeder (1976). Low molecular weight viral RNAs transcribed by RNA polymerase III during Ad2 infection. Cell 7: 557-566.

Westphal, H. and R. Dulbecco (1968). Viral DNA in polyoma- and SV40-transformed cell lines. Proc. Natl. Acad. Sci. U.S.A. 59: 1158-1165.

Wetmur, J.G. and N. Davidson (1968). Kinetics of renaturation of DNA. J. Mol. Biol. 31: 349-370.

Williams, J.F. (1970). Enhancement of adenovirus plaque formations on Hela cells by magnesium chloride. J. Gen. Virol. 9: 251-256.

Williams, J.F., H. Young and P. Austin (1974). Genetic analysis of adenovirus type 5 in permissive and non permissive cells. Cold Spring Harbor Symposium. Quant. Biol. 39: 427-437.

Williams, J., R. Galos, M. Binger and J. Flint (1979). Location of additional early regions within the left quarter of the adenovirus genome. Cold Spring Harbor Symposium. Quant. Biol. 44: 353-365.

Wilson, M.C., J.R. Nevins, J.M. Blanchard, M.S. Ginsberg and J.E. Darnell, Jr. (1979). The metabolism of mRNA from the transforming region of adenovirus type 2. Cold Spring Harbor Symposium. Quant. Biol. 44: 447-455.

Wilson, M.C. and J.E. Darnell, Jr. (1981). Control of mRNA concentration by differential cytoplasm half life: adenovirus mRNAs from transcription units 1A and 1B. J. Mol. Biol. 148: 231-251.

Wittelsberger, S., K. Kleene and S. Penman (1981). Progressive loss of shape-responsive metabolic controls in cells with increasingly transformed phenotype. Cell 24: 859-866.

Wold, W. and M. Green (1979). Adenovirus type 2 early polypeptides immunoprecipitated by antisera to cell lines of adenovirus-transformed rat cells. J. Virol. 30: 297-310.

Wolfson, J. and D. Dressler (1972). Adenovirus 2 DNA contains an inverted terminal repetition. Proc. Natl. Acad. Sci. U.S.A. 69: 3054-3057.

Yong, W.K., J.O. Kiggans, D.M. Yang, Y. Ouch, R.W. Tennant, A. Brown and R.H. Bassin (1980). Synthesis and Circularization of N and B tropic retroviral DNA in Fv-1 permissive and restrictive mouse cells. Proc. Natl. Acad. Sci. U.S.A. 77: 2994-2998.

Yoshimura, F.K. and R.A. Weinberg (1979). Restriction endonuclease cleavage of linear and closed circular murine leukemia viral DNAs: discovery of a smaller circular form. Cell 16: 323-332.

Ziff, E. and N. Fraser (1978). Adenovirus type 2 late mRNAs: structural evidence for 3'-coterminal species. J. Virol. 25: 897-906.