ROLE OF AD5 EARLY REGION I FUNCTIONS DURING TRANSFORMATION OF RAT CELLS

THE ROLE OF EARLY REGION I FUNCTIONS DURING BIOCHEMICAL TRANSFORMATION OF RAT CELLS BY HUMAN ADENOVIRUS TYPE 5

By

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ABSTRACT

The purpose of this study was to assess the effect of early region 1 functions of Ad5 on the ability of viruses containing a selectable marker in early region 3 (E3) to transform mammalian cells. To do this I have constructed and characterized five recombinant viruses containing the thymidine kinase (tk) gene from Herpes Simplex Virus type 1 (HSV1) inserted in E3. The biochemical transformation assay performed using these recombinant viruses allowed the separation of the selection process (incorporation and expression of tk) from the requirement for expression of E1 functions.

The method of isolating the desired recombinant viruses was *in vivo* recombination following mixed infection of 293 cells. The parental viruses used were: hr 1 which expresses a truncated version of the E1a 243R product lacking amino acids 166 to 243; pm975 which fails to express the E1a 289R product; dl312 from which the majority of the E1a coding region has been deleted; hr6 which fails to express a wild type E1b 496R protein; and wild type Ad 5. Each coinfection was done with dlE1,3tk, a previously constructed recombinant virus (Haj-Ahmad and Graham, 1986). Using the resulting recombinant viruses, semipermissive tk^- Rat 2 cells were infected and selected for conversion to the tk^+ phenotype, as well as being assayed for viability post infection. Comparisons were made of tk transformation frequencies with and without

correction for differential cell viability measured after infection with different viruses. Correction for differential cell viability greatly reduced the differences in transformation frequencies observed directly. However hr1tk remained able to induce tk^+ transformation at a significantly greater frequency than hr6tk, pm975tk, or Ad5tk. The mutants d1312tk and dlE1,3tk gave statistically indistinguishable results corresponding to an intermediate level of transformation, while hr6tk, pm975tk, and Ad5tk were grouped together as being least efficient at transformation. The infected Rat2 cell viability assays provide evidence of a correlation involving expression of the early region 1a (E1a) 289 residue product, efficient viral DNA replication, and cell death.

Recombination frequencies obtained during the isolation of the recombinant viruses varied greatly depending on the combination of infecting parental viruses. The following factors appeared to affect recombination frequency: 1. the input ratio of the coinfecting viruses; 2. interference in the replication of dlE1,3tk relative to the other virus present; and 3. the presence of small numbers of mismatched base pairs (seven) near the left terminus of some of the viruses used in coinfection with dlE1,3tk.

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LIST OF ABBREVIATIONS

Ad2, 5, 12, etc.	adenovirus type 2, 5 12, etc.
bp	base pairs
$CaCl_2$	calcium chloride
$cDNA^2$	copy DNA
cm	centimetre
cpe	cytopathic effect
cpm	counts per minute
dATP, dCTP, dGTP, dTTP	deoxynucleotide triphosphate
dl	deletion
DNA	deoxyribonucleic acid
DNAse	deoxyribonuclease
E1, E2, E3, E4	early regions 1, 2, 3, and 4
EDTA	ethylenediamine tetracetic acid
et al	and co-workers
FBS	fetal bovine serum
Fig.	figure
g	gram(s)
HCl	hydrochloric acid
HMT	hypoxanthine, methotrexate, thymidine
hr	host range
HS	horse serum
HSV	herpes simplex virus
ITR	inverted terminal repeat
kDa	kilodaltons
M	molar
min	minute(s)
$MgCl_2$	magnesium chloride
MEM^2	minimal essential medium
mg	milligram(s)
ml	millilitre
mM	millimolar
moi	multiplicity of infection
mRNA	messenger RNA
mu	map unit(s)
MW	molecular weight
N	normal
NaCl	sodium chloride
NCS	newborn calf serum
nm	nanometre(s)
PBS	phosphate-buffered saline
pfu	plaque forming units
pi	post infection
pm	point mutant
RNA	ribonucleic acid

rpm	rotations per minute
SDS	sodium dodecyl sulphate
SSC	saline sodium citrate
SSPE	saline sodium phosphate EDTA
TE	tris-EDTA buffer
tk	thymidine kinase
Tris	tris (hydroxymethyl) aminomethane
UV	ultraviolet radiation
wt	wild type
^{32}P	phosphorus-32

Introduction

1.1 Adenoviruses

1.1.1 General Background

Human adenovirus type 5 is a member of the family Adenoviridae, of which there are two genera: aviadenovirus and mastadenovirus based on whether the isolation was from an avian or mammalian species respectively. The original discovery of adenoviruses was by Rowe et al. (1953) and by Hilleman and Werner (1954). The various antigenically distinct human adenoviruses isolated to date can cause "acute respiratory, ocular, gastrointestinal and urinary diseases" (Smith, 1984).

Despite a wide diversity of hosts and etiologies these viruses are classified as a single family on the basis of their many similarities including structural morphology, immunological cross-reactivity, and genomic DNA homology (Matthews, 1982). Many of the human serotypes also have varying amounts of oncogenic potential and have been further classified according to this characteristic as well as DNA GC content, epidemiology, and other criteria (Table 1). The adenoviruses used in this study, serotype 5, and to some extent serotype 2, have been and continue to be studied extensively and the following descriptions apply primarily to these group C viruses.

Table 1. Properties of human adenovirus subgenera A to G

Subgenus	Species	DNA			Apparent molecular weight of the major internal polypeptides			Hemagglutination pattern[c]	Oncogenicity in newborn hamsters
		Homology (%)[a]	G+C (%)	Number of *Sma*I fragments	V	VI	VII		
A	12, 18, 31	48–69 (8–20)	48	4–5	51 to 51.5K 46.5 to 48.5K[d]	25.5 to 26K	18K	IV	High (tumors in most animals in 4 months)
B[e]	3, 7, 11, 14 16, 21, 34, 35	89–94 (9–20)	51	8–10	53.5 to 54.5K	24K	18K	I	Weak (tumors in few animals in 4–18 months)
C[e]	1, 2, 5, 6	99–100 (10–16)	58	10–12	48.5K	24K	18.5K	III	nil
D[e]	8, 9, 10, 13, 15, 17, 19, 20, 22, 23, 24, 25, 26, 27, 28, 29, 30, 32, 33, 36, 37, 38, 39	94–99 (4–17)	58	14–18	50 to 50.5K[f]	23.2K	18.2K	II	nil
E	4	(4–23)	58	16–19	48K	24.5K	18K	III	nil
F	40	n.d.	n.d.	9	46K	25.5K	17.2K	IV	nil
G	41	n.d.	n.d.	11–12	48.5K	25.5K	17.7K	IV	nil

n.d., not done

[a] Per cent homology within the subgenus. Figures in brackets: homology with members of other subgenera

[b] The restricted DNA fragments were analyzed on 0.8–1.2% agarose slab gels. DNA fragments smaller than 400 bp were not resolved

[c] I, Complete agglutination of monkey erythrocytes; II, complete agglutination of rat erythrocytes; III, partial agglutination of rat erythrocytes (fewer receptors); IV, agglutination of rat erythrocytes discernible only after addition of heterotypic antisera

[d] Polypeptide V of Ad31 was a single band of 48K

[e] Only DNA restriction and polypeptide analysis have been performed with Ad32 to Ad39

[f] Polypeptides V and VI of Ad8 showed apparent molecular weights of 45K and 22K respectively. Polypeptide V of Ad30 showed an apparent molecular weight of 48.5K

Reprinted from Wadell (1984)

1.1.2 Virion Structure

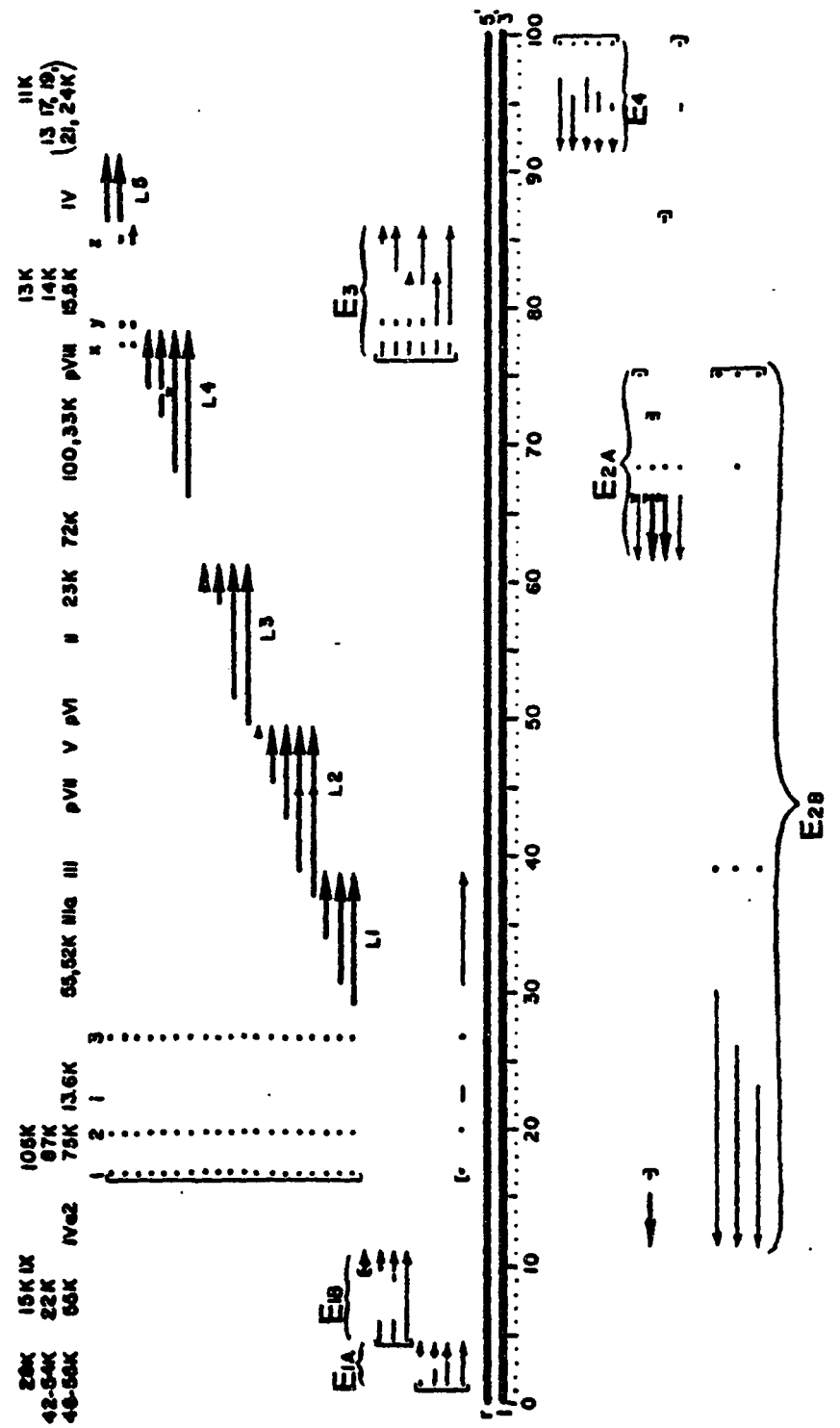
Adenoviridae have a characteristic icosahedral shape made up of twenty triangular surfaces with twelve vertices. This outer capsid structure is composed of at least nine different polypeptides. These structural products associate in the form of capsomers, of which there are 252: 240 hexons forming the twenty triangular "facets" and 12 pentons at the vertices. The pentons are in turn made up of a base inserted within the capsid, and a fiber, which has the appearance of a projecting antenna. The overall size of a hydrated virus particle has been calculated to be approximately 880 Angstroms (Nermut, 1984).

1.1.3 The Adenovirus Genome and Viral DNA Replication

The adenovirus genome is a linear double stranded DNA molecule (van der Eb and van Kesteren, 1966; Green et al., 1967; Younghusband and Bellett, 1971), approximately thirty six kilobase pairs in length. The molecular weight of the DNA varies from serotype to serotype, but the approximate weight for Group C viruses is 2.3 to 2.4 x 10^7 (Green et al., 1967). The genome is divided into early and late transcription units containing genes which start being expressed either before or after viral DNA replication respectively. There are five major regions of early transcription called E1, E2a, E2b, E3, and E4 and one major late transcription unit (Fig. 1).

All human adenovirus serotypes characterized to date have inverted terminal repeat (ITR) sequences from 100 to 200 base pairs in

Figure 1 Transcription Map of Group C Adenoviruses. A schematic illustration of the transcription units of the Ad2 genome. The messenger RNAs (mRNAs) of blocks of genes transcribed early in infection are designated E1a, E1b, E2a, E2b, E3, and E4, whereas late mRNAs are called L1, L2, L3, L4, and L5. The r strand and l strand of the genome are transcribed rightward and leftward respectively. The arrow heads indicate polyadenylation sites and promoters are shown as square brackets. The molecular weights of some of the proteins generated are shown in kilodaltons (K); in other instances the proteins are identified by Roman numerals. The three segments spliced to form the tripartite leader of the late mRNAs are also indicated (1,2,3). Adapted from Tooze (1981).


E1A
E1B
E3
E2A
E4
E2B
L1
L2
L3
L4
L5
0
10
20
30
40
50
60
70
80
90
100
5'
3'
28K
42-54K
48-58K
15K IX
22K
55K
IVa2
105K
97K
75K
13.6K
55,52K
IIIa
III
pVII
V
pVI
II
23K
72K
100,33K
pVIII
IIIa
13K
14K
15.5K
IV
11K
(13, 19, 21, 24K)

length. The Ad2 and Ad5 ITRs have been sequenced and found to be identical in nucleotide composition and length - 103 base pairs (Steenbergh et al., 1977; Shinagawa and Padmanabhan, 1979). Lechner and Kelly (1977) demonstrated that these structures play a role in viral DNA replication.

Another striking feature of the adenovirus genome is the presence of a 55 kDa terminal protein (TP) bound to the 5' terminus of each DNA strand (Robinson et al., 1973; Robinson and Bellett, 1974) via a phosphodiester linkage between the beta-OH of a serine residue in the protein and the 5'-OH of the terminal deoxycytidine nucleotide. The association of the 80 kDa precursor terminal protein (pTP) bound to dCTP via the reaction described above is the primary initiation event in viral DNA synthesis. This complex which is associated with the terminus of a viral genome, initiates strand displacement by allowing the dCMP residue to serve as a primer for chain elongation (Rekosh et al., 1977; Pincus et al., 1981).

A model for the replication of adenovirus genomes has been proposed by Lechner and Kelly (1977). They suggest that replication can be initiated at either end of a linear double stranded parental DNA molecule because of the identical sequences making up the ITRs at the two termini. Following initiation a daughter strand is synthesized in the 5' to 3' direction (type I replication) causing displacement of the identical parental strand. This displaced parental strand can also serve as a template for viral DNA synthesis after forming a stem and loop structure, in which the stem corresponds to the hybridization of the

terminal ITR sequences (type II replication). An illustration of this model is shown in Figure 2.

1.1.4 Transcription Unit E1

The first gene products to be expressed after infection are encoded by E1a (Nevins, 1979). This region together with E1b makes up E1 (Fig. 3). Five differentially spliced messenger (m)RNAs are expressed from the E1a region. They are named according to their sedimentation coefficients 13S, 12S, 11S, 10S, and 9S. The 11S and 10S mRNAs encode proteins of 35 kDa and 30 kDa respectively (Stevens and Harlow, 1987; Ulfendahl et al., 1987), and are expressed at late times during infection. The 9S mRNA product is also not expressed to a significant extent until late in infection (Virtanen and Peterson, 1983). All five products share common 5' and 3' sequences, differing only in the size of the internal segments removed by splicing. The 13S and 12S mRNAs encode polypeptides containing 289 and 243 amino acid residues respectively (Perricaudet et al., 1979). These two products are phosphorylated at a number of different sites (Yee et al., 1983). Furthermore when subjected to one dimensional (Yee et al., 1983; Rowe et al., 1983) or two dimensional gel electrophoresis (Harlow et al., 1985) each mRNA produces a number of different species in terms of electrophoretic mobility. These variants are thought to be due to posttranslational modifications.

The E1b transcription unit expresses two major mRNAs called 22S and 13S, and at least three minor products with sedimentation coefficients of 14.5S, 14S, and 9S (Anderson et al., 1984; Virtanen and Pettersson, 1985). The 22S message is translated into a 496R (Ad5)

Figure 2 Model of Adenovirus DNA Replication. Chain elongation, indicated by bold lines displaces a parental strand of like polarity (indicated by light lines) during type I replication. The bracketed circular stem and loop structures are theoretical intermediates leading to type II replication. Adapted from Lechner and Kelly (1977).

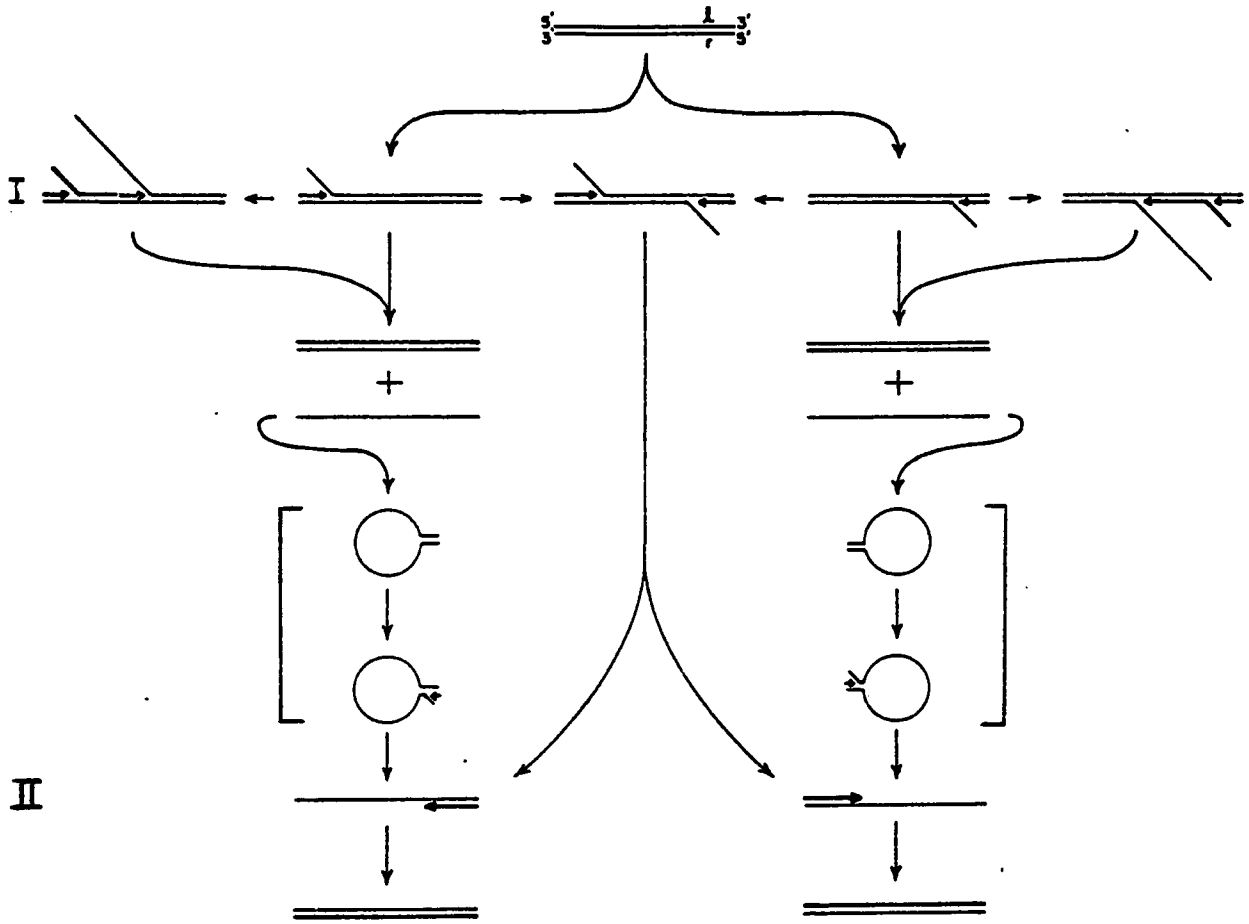
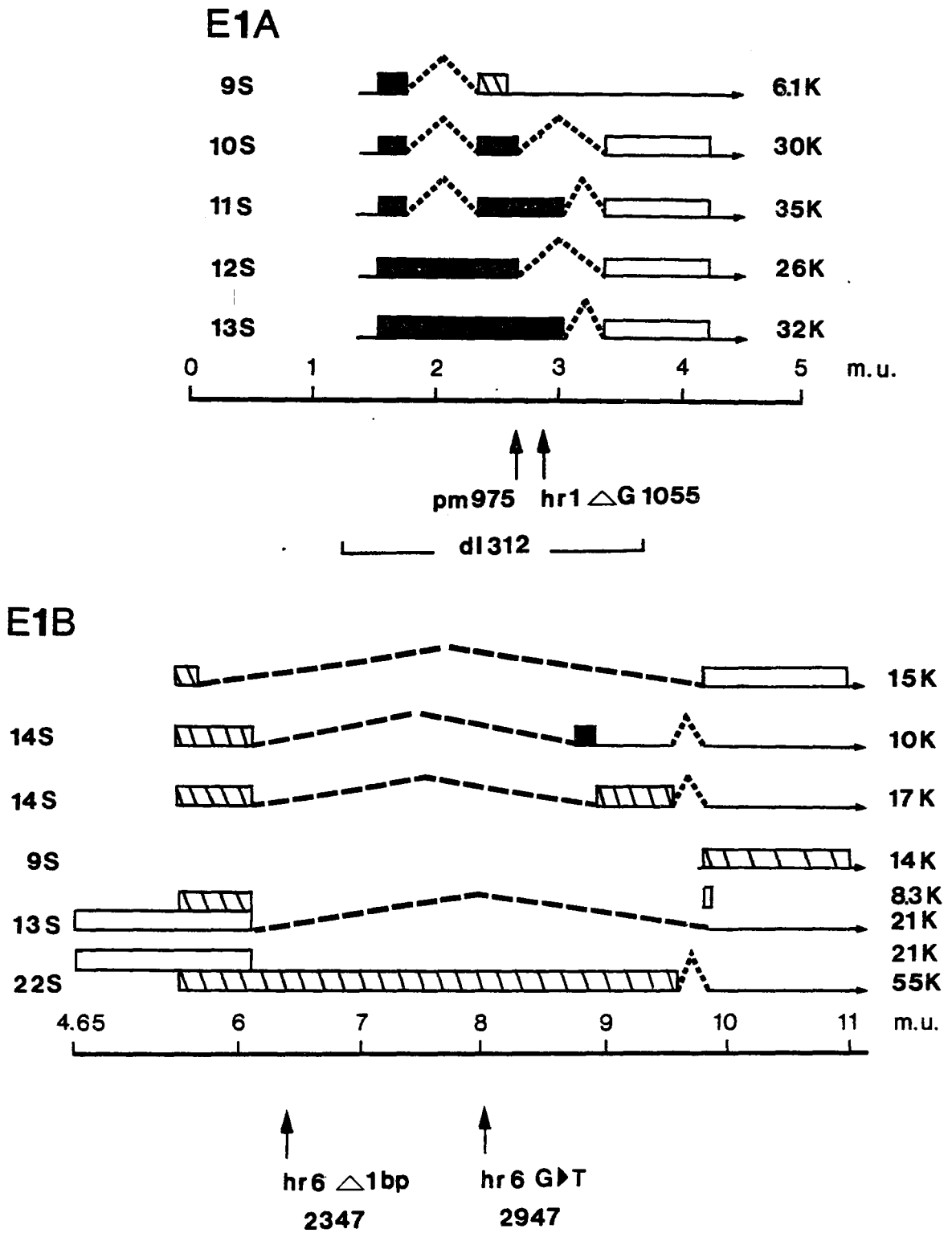

I
II

Figure 3 The Adenovirus Ela and Elb Transcription Units. The two independently promoted El transcription blocks - Ela and Elb are shown. The coding sequences of the genome are indicated in map units (mu). The mRNAs expressed, are shown as open, closed, or hatched bars, indicating transcription from reading frames 1,2, or 3 respectively. The molecular weights of the translation products are shown in parentheses beside the corresponding mRNAs. The locations of the lesions of four El mutants used in this study are also illustrated. Adapted from Stephens and Harlow (1987), and Lewis and Anderson (1987).


E1A
9S
6.1K
10S
30K
11S
35K
12S
26K
13S
32K
0
1
2
3
4
5
m.u.
pm975
hr1 △G 1055
dl312
E1B
15K
14S
10K
14S
17K
9S
14K
8.3K
13S
21K
21K
22S
55K
4.65
6
7
8
9
10
11
m.u.
hr6 △1bp
2347
hr6 G▶T
2947

protein having a predicted molecular weight of 55 kDa, and from a different but overlapping reading frame, a 176R or 21 kDa (Ad5) protein (Esche et al., 1980; Bos et al., 1981). The observed molecular weights of these proteins are about 58 kDa (Levinson and Levine, 1977ab; Lassam et al, 1979) and 19 kDa (Schrier et al., 1979; Jochemsen et al., 1981) respectively as determined by SDS-polyacrylamide gel electrophoresis. The 13S mRNA also encodes the 19 kDa product. The Elb products will subsequently be referred to by their observed molecular weights of 58 kDA and 19 kDA.

The E1 region is the object of intensive investigation in part because it is known to contain the information which is necessary and sufficient for oncogenic transformation of primary rodent cells (Gallimore et al., 1974; Graham et al., 1974).

1.2 E1 Mutants

1.2.1 Characterization of E1 Mutants

The E1 regions of a number of human adenovirus serotypes, in particular Ad2, Ad5, and Ad12 are being extensively characterized by mutational analysis. The development of a permissive human cell line called 293 simplifies the isolation of E1 mutants. This line obtained by transforming human embryonic kidney cells with sheared Ad5 DNA (Graham et al., 1977), expresses integrated E1 sequences (Aiello et al., 1979; Ruben et al., 1982; Spector, 1983). A series of host range mutants were isolated using 293 cells and were identified as being defective for lytic replication in HeLa cells (Harrison et al., 1977). These mutants

defined two complementation groups, hrI and hrII, which have subsequently been identified as transcription units Ela and Elb.

The exact location of the El lesions of hrl and hr6 are now known. The hrl lesion at nucleotide 1055, a one base pair deletion, results in a frameshift and introduction of an extraneous stop codon near the 3' end of exon 1 of the 1.1 kb mRNA (Ricciardi et al., 1981). This in turn prevents expression of a full sized 289 residue ElA protein, although a truncated product encoded by the mRNA has been detected by in vitro translation. This truncated product contains the amino terminal 165 amino acids of the wild type product joined to 10 novel amino acids generated by the shift in reading frame at nucleotide 1055. Sequencing of the El region of hr6 reveals a one base pair deletion at nucleotide 2347, as well as a one base substituton of a guanidine residue by a thymidine residue at nucleotide 2947 (Williams et al., 1986). These mutations are within and unique to the coding region of the Elb 58 kDa protein.

The d1312 virus is one of a series of Ad5 El mutants isolated by Jones and Shenk (1979). It lacks DNA sequences extending from nucleotide 448 to 1349, which removes the bulk of the Ela coding region. The virus has been shown by Jones and Shenk to be defective for lytic replication on HeLa and human embryonic kidney (HEK) cells.

The mutant pm975 has a mutation within the 5' splice site of the Ela 12S mRNA which prevents proper splicing and expression of this message as well as its product, the 243 residue protein (Montell et al., 1982). The mutant pm975 is a chimeric virus consisting of the left 3.8 map units of adenovirus type 2 (Ad2), with the remainder composed of Ad5

sequences from the mutant d1309 (Jones and Shenk, 1979). The final construction of pm975 was by the method of Stow (1981).

1.2.2 Oncogenic Transformation by E1 Mutants

The hr1 virus transforms primary BRK cells at an apparent efficiency approximately 10 to 50 times greater than wild type virus (Graham et al., 1978). These transformants are called abnormal or semiabortive since they fail to grow in semisolid medium and are morphologically different than cells transformed with wild type virus (Ruben et al., 1982). The mutant transformed cells are fibroblastic in appearance whereas wild type transformants are epithelioid in morphology. Furthermore hr1 transformed cells are unable to induce tumours when injected into nude mice. Finally this mutant is unable to transform either rat embryo or rat brain cells unlike the wild type virus (Graham et al., 1978).

Due to the recent findings of a number of groups, the role of the 58 kDa protein in oncogenic transformation is uncertain and controversial. Much of the controversy concerns the activity of truncated proteins postulated to be generated by mutant viruses with lesions in the 58 kDa coding region. There are a number of examples of this. The hr6 virus can potentially express a short truncated product corresponding to the amino terminal 109 amino acids of the wt 496R product. This product has not been detected directly but cells transformed by the Ad5 HindIII G fragment which includes coding sequences for the amino terminal half of the 58 kDa product, and used to inject rodents sometimes result in antibodies specific for the 58 kDa

protein (Rowe et al., 1984; Zantema et al.,1985). A mutant virus called pm2022 constructed by Barker and Berk (1986) expresses a mutant 22S mRNA which undergoes premature termination of translation after the first two amino acids. However there is evidence of reinitiation at a nearby AUG triplet resulting in expression of reduced levels of a truncated product with an apparent molecular weight of 46 to 50 kDa. Bernards et al. (1986) also constructed a 58 kDa mutant, Ad5d158, which potentially expresses a 13 kDa truncated product. Both Ad5d158 and pm2022 are able to transform rodent cells although at reduced levels relative to wild type virus (25% and 20% respectively). In contrast hr6 which potentially expresses a shorter truncated product, displays a barely detectable ability to transform rodent cells in virus mediated assays (Graham et al., 1978). A second virus constructed by Barker and Berk, dl1522, is almost fully defective in virus mediated assays. This virus has the pm2022 mutation, but in addition has a deletion of 327 nucleotides and an insertion of a second premature termination codon, in a further attempt to prevent expression of any 58 kDa related product. Together these data suggest that the size of these putative truncated products does affect the outcome of transformation assays.

Attempts to compare the results of virus mediated transformation assays using 58 kDa mutants to results of transfection assays using viral DNA containing the same lesions have also produced conflicting results. In contrast to the lack of transformation observed with hr6 virus, the DNA extracted from this mutant is capable of inducing focus formation by primary BRK cells at a rate equivalent to wild type DNA (Rowe and Graham, 1983). In comparison pm2022 and dl1520 DNA give

results consistent with those of the corresponding viruses, i.e. dl 1520 DNA is almost completely defective, while pm2022 DNA produces a significant amount of transformation but at reduced levels relative to wild type DNA. Transformation by these mutants in DNA mediated assays does not correlate directly with the size of potential truncated products.

The results obtained in hr6 virus and DNA mediated transformation assays led to the suggestion (Rowe and Graham, 1983) that initiation of transformation during viral infection involves a different pathway or mechanism than does initiation of transformation by viral DNA. This difference in initiation is postulated to involve the 58 kDa product, which appears to be required for the latter process but not the former. An alternate explanation has been proposed by Barker and Berk (1986). They suggest that the requirement for expression of some function provided by the 58 kDa protein is the same in either type of assay, but that only after transfection with hr6 DNA is there enough of the truncated product present to be biologically active in transformation. I will present results which show that the hr6tk virus is not defective in its ability to biochemically transform semipermissive Rat2 cells to the tk^+ phenotype when compared with Ad5tk which is wild type with respect to E1 sequences.

The mutant pm975 is partially defective for oncogenic transformation. Virion mediated transformation assays using cloned rat embryo fibroblast (CREF) cells produce approximately five fold fewer foci than wt Ad2 virus, and reduced values are also obtained relative to wt virus after infection of primary BRK cells. Assays for growth in soft

agar also reveal a defect in the ability of pm975 transformed CREF cells to undergo anchorage independent growth. Colonies obtained with pm975 transformants are much smaller than those obtained with either wt Ad2 or hr1 transformed cells (Montell et al., 1984).

The mutant d1312 was shown by Jones and Shenk (1979) to be completely defective for transformation as measured by focus formation in infected primary rat embryo or rat embryo brain cells. Subsequently numerous assays have been done using a variety of rodent cell types with the same results, showing that the Ela gene products are essential for oncogenic transformation (Montell et al., 1984; Lillie et al., 1986; Barker and Berk, 1987; etc.).

1.2.3 Rationale for Testing El Mutants Using a Biochemical Transformation Assay

Oncogenic transformation assays of numerous viral mutants have provided the information required to establish conclusively that products encoded within the El region of adenovirus are necessary and sufficient to induce transformation of suitable cellular hosts (Gallimore et al., 1974; Graham et al., 1974). However these assays also reveal mutants which only partially transform various cell types. For example the Ela mutants hr1 and pm975 described here induce some of the properties of the fully transformed phenotype. This type of evidence suggests that oncogenesis is a multi-step process involving more than one gene product from Ela. One of the early steps in transformation presumably involves integration of at least the part of the viral genome containing essential El sequences into the host chromosomal DNA. These

viral sequences have been found by sensitive DNA hybridization procedures based on the technique developed by Southern (1975), by a number of groups (Flint et al., 1976; Johansson et al., 1978; Ruben et al., 1982, etc.). An assay which measures the efficiency of the biochemical transformation process would be helpful in determining whether or not viral products play a role, or if incorporation of viral DNA is mediated by cellular products only. Furthermore, by selecting for the stable uptake and expression of a biological marker inserted within the viral genome but distant from E1, one can avoid the experimental bias produced by selection for a particular oncogenic phenotype. These are the main attributes of the transformation assay used here.

A major problem in comparing transforming abilities of various E1 mutants is the possibility of differential cytopathic effects post infection i.e. each virus used may cause different amounts of cell mortality. To more accurately measure relative transformation frequencies these differences in cytopathic effect must be measured and accounted for. This necessitates the use of viability assays incorporated within each transformation experiment.

1.3 Isolation of Recombinant Adenoviruses

1.3.1 In Vivo Recombination as a Method for Creating Recombinant Adenoviruses

The parental viruses chosen for mixed infection with dlE1,3tk in subsequent experiments were: host range 1 (hr1), host range 6 (hr6), wild type adenovirus 5 (wt Ad5), deletion mutant 312 (d1312), and point

mutant 975 (pm975). Intraspecific recombination between identifiably different adenoviruses during mixed infection has been shown to occur relatively frequently by several groups (Williams and Ustacelebi, 1971; Takemori, 1972). The mechanism by which this recombination occurs is unclear but experimental evidence indicates a correlation between replication and recombination (Wolgemuth and Hsu, 1981; Young et al., 1984). The electron microscopy studies of Wolgemuth and Hsu show replication intermediates simultaneously undergoing recombination, but also provide evidence of a strand exchange process by pairs of viral genomes which would not require DNA replication in order to occur. This study therefore provides evidence for the occurrence of recombination by mechanisms described by the Holliday and the Meselson-Radding models (Holliday, 1964; Meselson and Radding, 1975).

The role of viral products in recombination is also unclear but Young et al. have shown that blocking early protein synthesis abolishes detectable adenovirus recombination if the blocking agent (anisomycin) is added no later than 5 hours post infection. They conclude that their data provide evidence for a virus specified product or products synthesized early in infection (i.e. before five hours post infection) with a role in recombination (Young et al., 1984). The authors also found that in two different types of experiments designed to measure both adenovirus DNA replication and recombination, the bulk of the recombination occurred after the initiation of replication.

1.3.2 The Recombinant Virus dlE1,3tk

The recombinant virus called dlE1,3tk was constructed by Yousef Haj-Ahmad (Haj-Ahmad and Graham, 1986). This virus lacks the portions of the viral genome extending from 1.0 to 10.6 map units and from 78.5 to 84.7 map units. These two deletions remove sequences from E1 and E3 respectively. The lack of E1 sequences means this virus will replicate efficiently only when E1 products are provided in *trans*, as is the case in 293 cells. The products of the E3 region are nonessential for replication of Ad5 in cell culture (Berkner and Sharp, 1983; Saito *et al.*, 1985).

The orientation of the tk gene is opposite to the direction of transcription of the deleted E3 genes in this specific virus (formerly called $Adtk_4$). The tk gene and its upstream regulatory sequences were obtained from pTK173, a plasmid previously constructed in our laboratory (McKinnon, 1984). The HSV 1 tk promoter is unresponsive to transactivation by E1a products in transient expression assays (Weeks and Jones, 1983). The dlE1,3tk virus is capable of biochemically transforming several different established cell lines including mouse fibroblasts (LTA), and human cells from a line called 143 which are also tk^-. Thymidine kinase expression by dlE1,3tk was measured indirectly by measurement of tritiated thymidine incorporation into the DNA of replicating 143 or LTA cells infected with the virus; and directly by enzymatic assay of tk activity in cell free extracts from infected 143 cells. Furthermore tk activity can be increased significantly by superinfection with a tk^- strain of HSV 1, indicating that the gene

remains responsive to positive regulation by HSV products (Haj-Ahmad and Graham, 1986).

1.4 Purpose of the Study

The objective of the first part of this study was to recombine the right end of dlE1,3tk containing the HSV 1 tk gene into a number of different viruses with mutations in E1. This was done by coinfection of 293 cells with dlE1,3tk and several previously isolated and well characterized E1 mutants and wild type Ad5, allowing *in vivo* recombination to occur. The resulting recombinants and the parental virus dlE1,3tk were then used in biochemical transformation assays to determine the effect of mutations in E1 on the efficiency of transformation of semipermissive Rat2 cells to the tk^+ phenotype. Each assay included determinations of the viability of infected and mock infected cells in order to compensate for variability in the lethality of the viruses. Comparisons were then made of transformation efficiencies without and with compensation for differential cell viability. The nature of the semipermissive virus host interraction was further characterized by measuring the extent of viral DNA synthesis and viral replication in Rat2 cells with four of the recombinant viruses. The last section of the study describes an investigation of some observations made during the isolation of the recombinant viruses. The unexpected results were: variations in the frequency of recombination obtained with various parental viruses; and assymetry in the number of recombinants of two basic types i.e. those containing the tk gene and

those with viral sequences in the right portion of the genome including E3 but with the E1 deletion. Possible explanations of these observations are discussed with reference to current models of recombination.

Materials and Methods

2.1. Cell Lines

The Rat2 cell line used for biochemical transformation experiments was obtained from Dr. S. Bacchetti. These cells are an established rat fibroblast line deficient in expression of thymidine kinase (Topp, 1981). HeLa cells used for plaque titration assays of host range (hr) viruses and one recombination experiment were originally obtained from Dr. J. Williams. The 293 cells used for plaque titration assays and mixed infections were created by transfection of human embryonic kidney cells with sheared DNA (Graham et al., 1977), using the calcium phosphate technique (Graham and Van der Eb, 1973).

Rat2 cells were grown in alpha minimal essential medium (MEM) supplemented with 10% fetal bovine serum (FBS, Gibco Laboratories). HeLa cells were cultured in alpha minimal essential medium plus 10% newborn calf serum (NCS, Gibco). The 293 cells were maintained in Joklik's modified MEM plus 10% heat inactivated horse serum (HS, Gibco). All media also contained 100 units per millilitre (U/ml) penicillin, 100 micrograms/ml streptomycin (Gibco), and 1.2 mg/ml L-glutamine. Culture conditions were 37^{o}C in a 5% CO2 and 95% air mixture.

2.2 Virus Strains and Infection Procedures

The wild type Ad5 strain (Williams, 1970) used here was from a previously prepared laboratory stock, as were hr1 and hr6 (Harrison et al., 1977), d1312 (Jones and Shenk, 1979), and pm975 (Montell et al., 1982). The mutant dlE1,3tk was constructed by Haj-Ahmad and Graham (1986). The viruses VpKGO-007, VpKGO-13S, and VpKGO-12S were created from plasmids pKGO-007, pKGO-13S, and pKGO-12S, provided by Dr. G. Akusjarvi (Svensson et al., 1983). The plasmid pKGO-007 contains the 7.9 map unit HindIII G fragment of Ad2. The other two plasmids were created by replacing a short segment containing the 13S and 12S mRNA coding sequences and introns with cDNA copies of the 13S or 12S mRNAs.

2.3 tk^+ Transformation Assay

Rat2 cells were seeded on 60mm or 100mm dishes at densities of approximately 2 x 10^5 (60 mm) or 5 x 10^5 (100 mm) cells per dish. The following day two dishes of cells were trypsinized, suspended in 5 or 10 mls of alpha MEM, and counted using an improved Neubauer counting chamber (Hausser Scientific). Based on the average cell count obtained, the cells in the remaining dishes were mock infected or infected with the recombinant viruses at various multiplicities of infection (moi), usually 10, 15, or 20 plaque forming units per cell (pfu/cell). The virions were suspended in 0.25 ml (for 60 mm dishes) or 0.75 ml (for 100 mm dishes) of phosphate buffered saline containing $MgCl_2$ and $CaCl_2$ (PBS^{++}). The cells were incubated at 37°C for 45 minutes and then re-fed

with 5 ml (60 mm), or 15 ml (100 mm) alpha MEM plus 10% FBS. The following day one dish of mock infected cells, and one dish of cells infected with each virus at each moi was trypsinized, and counted. All remaining dishes were then trypsinized and the cells were re-suspended in appropriate volumes of alpha MEM plus 10% FBS (with or without selection), and re-seeded on 60 mm or 100 mm dishes. The densities of cells to be selected with medium containing hypoxanthine, methotrexate, and thymidine (HMT) were calculated to be approximately equivalent per unit area for 60 mm or 100 mm dishes i.e. 2×10^5 cells/dish or 5×10^5 cells/dish respectively. The concentrations of the compounds in the media were: 10 micrograms/ml hypoxanthine; 7 micrograms/ml thymidine; and 330 nanograms/ml methotrexate. Cell densities for the viability assays varied depending upon the virus used. In general mock infected cells, and cells infected with nonlethal viruses were seeded at densities of 150 cells per 100 mm dish at each moi, whereas cells infected with lethal viruses such as Ad5tk were plated at increasing densities with increasing moi. The medium on cells exposed to selection was changed twice per week, whereas viability assay dishes required only one medium change. The viability assay dishes were stained with crystal violet ten days post infection. Beyond this time colonies became so large that they often merged with one another, making accurate counting difficult. The dishes containing selection medium were stained 15 or 16 days post infection, also with crystal violet and counted. Those tk^+ colonies with less than 50 cells were not included.

2.4 In Vivo Recombination

2.4.1 Mixed Infection Procedure

The cells to be used for the mixed infections (usually 293) were seeded on 60 mm dishes at densities of about 5 x 10^5 cells per dish (in Joklik's modified MEM in the case of 293 cells). The following day two dishes of cells were trypsinized and counted to obtain an average cell count per dish. Based on this value the remaining dishes were infected with a suspension of the two chosen viruses in PBS^{++}. Aliquots of the virus stocks were initially diluted in PBS^{++} separately, then mixed together immediately prior to infection. Each 60 mm dish received a total volume of 0.30 ml of mixed viral suspension (containing equal amounts of each virus). The adsorption period was 45 minutes at 37°C. The infected cells were then re-fed with F-11 plus 5% HS. The infected cells were harvested when the cytopathic effect (cpe) was complete - usually 48 hours post infection. The medium was removed from each dish, and a portion was saved for freezing at -80°C. To each infected dish (usually a total of five dishes for each mixed infection) 0.4 ml of PBS^{++} plus 10% glycerol was added. The cells were then removed from the dishes by scraping with a sterile rubber policeman. The cell suspensions from all of the dishes were combined, mixed, and aliquoted into 4 ml sterile glass vials for storage at -80°C.

2.4.2 Isolation and Characterization of Recombinant Progeny

2.4.2.1 Plaque Isolation

The infected cell suspensions were freeze-thawed three times prior to use, to ensure complete lysis of the cells. Aliquots of these lysates were added to PBS^{++} to obtain serial dilutions. Subconfluent 60 mm dishes of 293 cells were then infected with 0.25 ml volumes from the successive dilutions. After the 45 minute incubation period the cells were overlaid with 10 mls of F-11 plus 5% HS plus 0.5% agarose. Seven to nine days post infection well isolated plaques were picked from dishes with appropriate dilutions. Care was taken to choose randomly from the entire range of plaque sizes present. The plaques were suspended in 1 ml aliquots of PBS^{++} plus 10% glycerol.

2.4.2.2 Expansion of Plaque Isolates and DNA Extraction

Aliquots (0.25 ml) of the individual plaque isolate suspensions were used to infect subconfluent dishes of 293 cells in order to expand the amount of virus available for analysis. At full cpe (generally 3 to 7 days post infection), the medium was removed with care to minimize the loss of infected cells. To each dish 0.4 ml of a solution containing 0.5 mg/ml Pronase B (Boehringer Manheim Ltd.) in 0.01 M Tris-HCl pH 7.4, 0.01 M EDTA, and 0.4% SDS was added. The dishes were incubated for a period of time ranging from 4 to 18 hours at 37°C. The digests were then treated with Tris-buffer saturated phenol, shaken vigourously for 2 to 5 minutes, and then centrifuged for about 5 minutes at 13,000 rpm. The

upper, aqueous phase was then carefully removed. To this solution 96% ethanol was added to precipitate the DNA present. The DNA was then pelleted, washed with 96% ethanol and dried for about 15 minutes at 37°C. The pellets were finally suspended in 100 microlitres of 10 mM Tris pH 7.4 and 1 mM EDTA.

2.4.2.3 Analysis of Extracted DNA

Samples of DNA from each viral isolate were digested with the restriction endonuclease Hind III(Bethesda Research Laboratory). The digested DNA samples were electrophoresed in horizontal 1% agarose slab gels (15 cm x 30 cm x 0.7 cm) for approximately 18 hours at about 1 V/cm. The electrophoresis buffer was an aqueous solution containing 40 mM Tris-HCl pH 7.9, 5 mM sodium acetate, 0.8 mM EDTA, and 0.2 micrograms/ml ethidium bromide. The UV illuminated gels (Fotodyne Inc.) were photographed using Type 57 high speed Polaroid land film and a red filter. The restriction endonuclease profiles of each sample were examined and categorized as parental or recombinant. Recombinants with the tk gene were also analyzed with EcoRI (BRL) to confirm the identification.

2.4.2.4 Plaque Purification, Expansion, and Titration of Recombinant Viral Stocks

Two plaque isolates of each desired recombinant type showing no evidence of parental contamination in their restriction enzyme profiles were chosen for plaque purification. Aliquots of the original plaque isolate suspensions were diluted in PBS^{++} and used to infect 60 mm

dishes of 293 cells. Seven to nine days later 6 to 12 well isolated plaques were picked, expanded and analysed as already described. If all plaques picked resulting from the same original isolate showed the correct restriction enzyme profile with no parental contamination, one of these isolates was then arbitrarily chosen for expansion to provide the final stock of each recombinant virus.

The expansion process involved the infection of four or five large (150 mm) dishes of 293 cells with a desired recombinant virus. When full cpe was achieved, all medium was removed and 1.5 mls of PBS^{++} plus 10% glycerol was added to each dish. The infected cells were then scraped with sterile rubber policemen to detach them from the surface of the dish. The suspensions from all of the dishes were pooled and mixed. Ampoules of the cell suspension were stored at $-80^{o}C$ and subjected to three cycles of freezing and thawing prior to use.

All recombinant viral stocks were titred at least twice on 293 cells, and the average value of these titres was used. The two host range mutants hr1tk, and hr6tk, were also titred on HeLa cells to determine if they were reduced in replication efficiency as expected.

2.5 Viral Replication Assay

In order to determine whether or not any viral replication occurred upon infection of Rat2 cells with the recombinant adenoviruses, infected Rat2 cell lysates were also subjected to plaque assay on 293 cells. The moi chosen for the infections was 20 pfu/cell. The cells were seeded on 60 mm dishes in alpha MEM plus 10% FBS at densities of about

2.5 10^5 cells per dish. Four viruses - hr1tk, d1312tk, pm975tk, and Ad5tk were chosen for the assays. The medium on the cells was changed 24 hours post infection (pi) to remove unadsorbed viruses. The fresh medium was alpha MEM supplemented with 5% FBS. The infected cells were harvested 72 hours pi by removal of medium, scraping with sterile rubber policemen and suspension in PBS^{++} plus 10% glycerol. Two mock infected dishes were treated in the same manner, with the exception of the presence of virus. In order to control for the presence of residual input virions, two dishes of Ad5tk infected Rat2 cells in the second assay (Table 3) were rinsed with fresh medium and then harvested eight hours pi, before viral replication could occur. The plaque assay procedure was identical to that already described. The plaques were counted for the final time nine days pi.

2.6 Assay for Viral DNA Replication in Rat2 Cells

2.6.1 DNA Extraction and Agarose Gel Electrophoresis

Some of the dishes of mock infected and infected cells from the two viral replication assays described above were used to determine the extent of viral DNA replication during the infection period of 72 hours. The procedure for extraction of DNA from infected cells has already been described. The pronase/SDS digestions in this case were incubated for a minimum of 14 and a maximum of 18 hours. The total concentration of nucleic acids present in each sample was determined by measurement of UV absorption at 260 nm using a Beckman Du-7 Spectrophotometer. Two different amounts (0.5 microgram and 2.5 micrograms) of total cellular

plus viral nucleic acid were loaded and electrophoresed for each virus analyzed. The 1% agarose gels were electrophoresed for about 20 hours at 1 volt per centimetre (V/cm). In order to increase the sensitivity of detection of viral DNA, the electrophoresed samples were transferred to nitrocellulose and probed with ^{32}P labelled banded wt Ad5 DNA.

2.6.2 Southern Transfer

Samples of total nucleic acid from infected or mock infected Rat2 cells were loaded on 1% agarose gels. Various amounts of banded HindIII cut Ad5 DNA were also loaded as size markers and controls for viral DNA concentration in the infected Rat2 DNA. A control for viral DNA from residual input virions was also included. The gels were electrophoresed overnight using the conditions already described.

The transfer of the electrophoresed samples to nitrocellulose (Schleicher and Schuell, BA85 0.45 micron) was by the method of Southern (1975). The gels were soaked in 0.25 N HCl for 10 minutes at room temperature with shaking to cause random depurination of the DNA. After briefly rinsing the gel in double distilled water, the DNA was denatured and cleaved at sites of depurination by soaking the gels in 0.5 M NaOH/1.5 M NaCl for 1 hour at room temperature with shaking. A 1 hour wash in 1 M Tris pH 7.5/1.5 M NaCl at room temperature with agitation was used to neutralize the gels. The nitrocellulose filters were soaked in 2x SSC (from 20x stock containing 3.0 M NaCl, 0.30 M sodium citrate, pH 7.0) for 5 minutes at room temperature. The gel was rinsed briefly in double distilled water prior to stacking the nitrocellulose, 2 pieces of

3MM filter paper soaked in 2x SSC and 4 pieces of 3MM paper soaked in 10x SSC on top of it. The transfer buffer into which 2 pieces of 3MM paper placed under the gel were dipped consisted of 10x SSC. Finally a stack several inches high of paper towels, a glass plate and a heavy weight were added to the stack to maximize capillary transfer. The transfer apparatus was left overnight. The gel was removed the following day, and examined under UV light to ensure that transfer of the DNA had occurred. The nitrocellulose filter was washed for 5 minutes in 6x SSC at room temperature with shaking. The filter was then air dried for 0.5 hour at room temperature, then baked at 80°C between 2 pieces of 3MM paper for 2 hours.

2.6.3 In Vitro Labelling of Ad5 DNA

Banded Ad5 DNA (500 ng) was nick translated using a kit purchased from Bethesda Research Laboratories containing nick translation buffer, a DNase I/Pol I enzyme mixture, and unlabelled dATP, dTTP, and dGTP. The radiolabel was in the form of alpha - ^{32}P dCTP. The reaction mixture was incubated at 18°C for 1 hour. Unincorporated radiolabelled nucleotides were separated from the labelled DNA by passage of the reaction mixture through a Sephadex G-50 spin column. Five microlitre aliquots of the nick translation reaction mixture were removed before and after passage through the spin column to determine the amount of ^{32}P incorporation. These samples were counted in a Beckman LS 1801 liquid scintillation counter. The specific activity obtained was about 6 x 10^7 cpm/microgram of DNA. The labelled probe was denatured by addition of 0.1 volume of 1 N NaOH and incubation at 37°C for 5 minutes.

2.6.4 Hybridization and Autoradiography

The hybridization procedure used was that of Wahl et al., (1979). The filters were incubated for a minimum of 4 hours in a prehybridization solution containing: 5x SSPE from 20x stock containing 3.6 M NaCl, 0.2 M $NaPO_4$, pH 7.7, and 20 mM EDTA; 5x Denhardt's solution I (Denhardt, 1966); 50% formamide; 0.5% SDS; and 200 micrograms/ml denatured herring sperm DNA, at 42°C. To this solution the denatured and labelled probe was added to give a final concentration of 5×10^5 cpm/ml. The hybridization was done at 42°C for approximately 16 hours. After hybridization the filters were washed twice in 1x SSPE, 0.1% SDS for 30 minutes at room temperature, and twice in 0.1x SSPE, 0.1% SDS for 30 minutes at 65°C. The filters were then air dried, wrapped in polyvinyl film and autoradiographed at -80°C with Kodak X-OMAT AR (XAR-5) or RP (XRP-1) film.

2.6.5 Densitometry Scanning of Autoradiographs

Scanning of autoradiographs was done using a Hoefer Scanning Densitometer, Model GS300 using Program GS350. No attempt was made to preflash the X ray film prior to exposure to ensure sensitivity within the linear range. The values derived from scanning are therefore only approximate.

RESULTS

3.1 Characterization of Rat2 Infections

This study involved the characterization of three major aspects of adenovirus infection of semipermissive Rat2 cells. These were: the effect of recombinant viruses containing wild type or mutant E1 regions on cell viability; the ability of these viruses to transform tk^- cells to tk^+; and the ability of the viruses to replicate, in terms of viral DNA synthesis and production of infectious progeny.

3.1.1 Construction of Recombinant Viruses

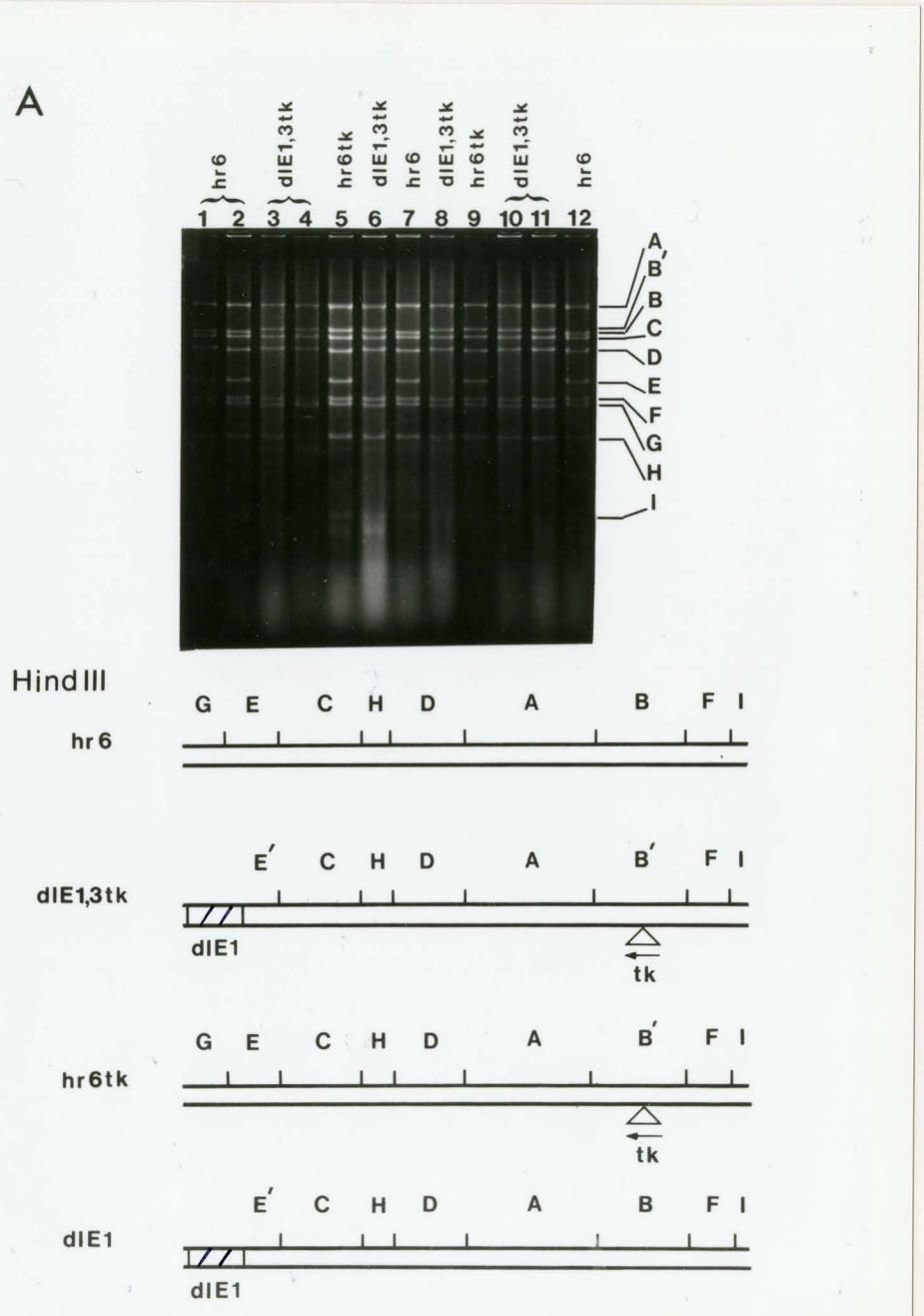
Five recombinant viruses containing the HSV 1 tk gene inserted in E3 were isolated by *in vivo* recombination. The parental viruses used in separate coinfections with dlE1,3tk were: hr1, hr6, d1312, pm975, and wt Ad5. The screening of plaque isolated progeny involved digestion of DNA extracted from infected 293 cells with the restriction enzymes HindIII and EcoRI followed by agarose gel electrophoresis. In each case the initial screening was done with HindIII which allowed the identification of four types of progeny: those with restriction enzyme profiles of either parental type, and two different products of recombination (Fig. 4, panel A, and Fig. 8). The two types of recombinant molecules correspond to the expected products of a homologous recombination event between the two parental viruses.

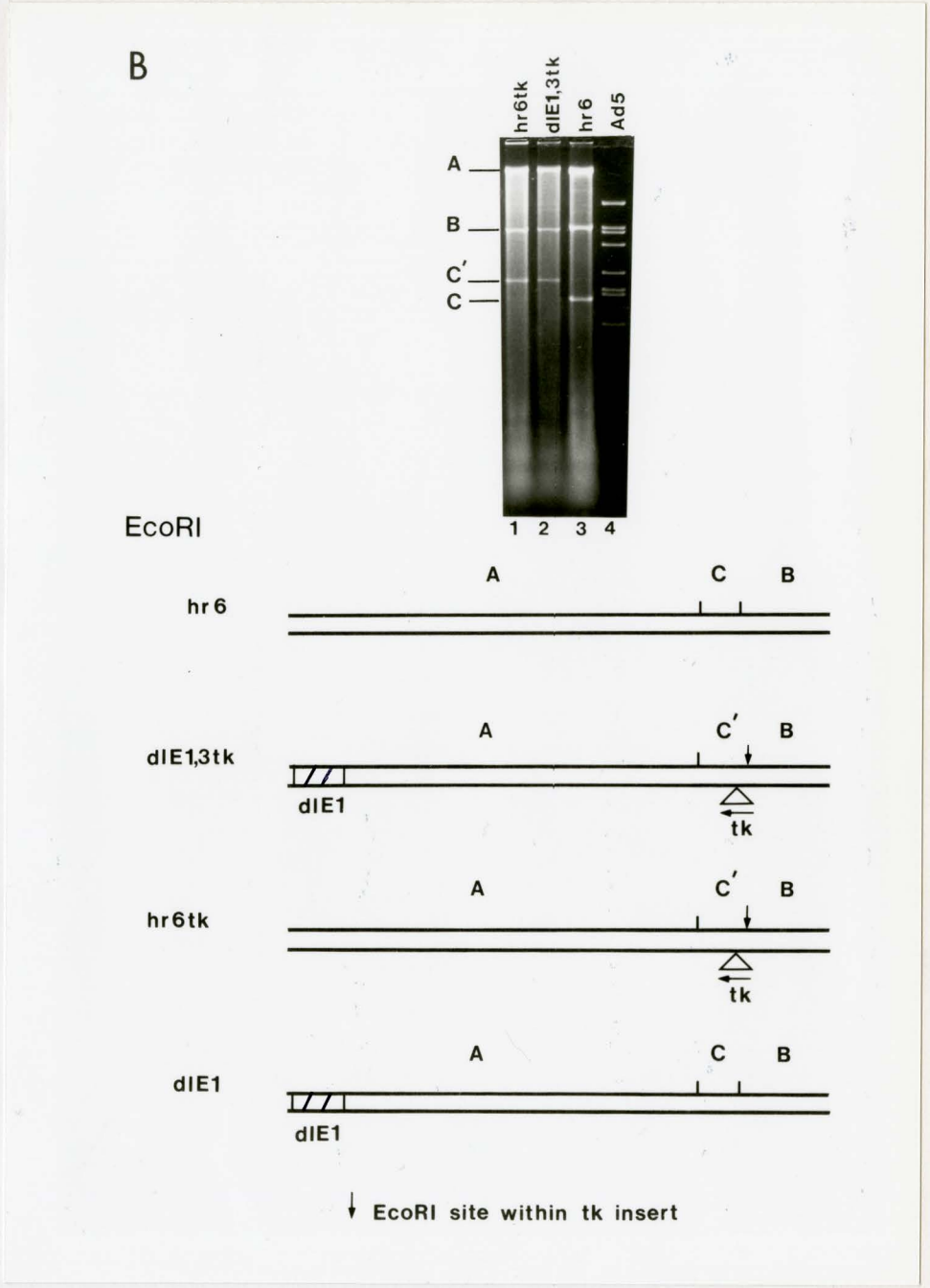
The insertion of the tk gene in E3 in each recombinant virus caused a shift in the migration of the HindIII B fragment observed following agarose gel electrophoresis due to an increase in size. This fragment labelled B' in Fig. 4 panel A was sufficiently different in size (6 kb vs 5.8 kb for the wild type B fragment) to be used to tentatively identify recombinant progeny of this type (lanes 5 and 9). The other feature of the HindIII restriction enzyme profile which made it diagnostic for recombinant viruses was the presence or absence of the wild type E fragment. This characteristic made it possible to discriminate between the parental virus dlE1,3tk (Fig. 4 lanes 3,4,8,10, and 11) which lacks the E fragment, and recombinant tk progeny which all contained the normal sized fragment. The E1 deletion in dlE1,3tk also produced a G-E fusion fragment (indicated as E' in Fig. 4) which comigrated with the wt G fragment. A second type of recombinant virus recognizable by this type of analysis, dlE1, was identified by the presence of a wild type Hind III B fragment and the absence of the Hind III E fragment (Fig.8 lanes 1 to 6 of inset).

One characteristic which could not be determined in these experiments was the location of the crossover events which produced the recombinant viruses. The parental viruses are entirely homologous from the 3' end of the E1 deletion of dlE1,3tk to the 5' end of the tk insertion. It was within this region that the crossover events occurred to generate the recombinant viruses described here.

To ensure correct identification of recombinant viruses containing the tk gene, a second anaylsis was done with EcoRI. The size difference between the wt EcoRI C fragment (2.6 kb) and the novel

Figure 4 Restriction enzyme analysis of viral progeny from mixed infection of 293 cells. Panel A: identification of hr6tk recombinant progeny by analysis of HindIII restriction enzyme profiles. Lane 1: isolate 41 (hr6 parental profile); lane 2: isolate 40 (hr6); lane 3: isolate 39 (dlE1,3tk parental profile); lane 4: dlE1,3tk control; lane 5: isolate 38 (hr6tk recombinant profile); lane 6: isolate 37 (dlE1,3tk); lane 7: isolate 36 (hr6); lane 8: isolate 35 (dlE1,3tk); lane 9: isolate 34 (hr6tk); lane 10 isolate 33 (dlE1,3tk); lane 11: isolate 32 (dlE1,32tk); and lane 12: hr6 control. Panel B: verification of hr6tk recombinant progeny by analysis of EcoRI restriction enzyme profiles. Lane 1: isolate 34 (hr6tk profile); lane 2: dlE1,3tk control; lane 3: hr6 control; lane 4: HindIII digested Ad5 marker. HindIII and EcoRI restriction enzyme maps of all parental and recombinant viruses are shown below. The E1 deletion is indicated in each case by a hatched bar, and the position and orientation of the tk insert is also shown.


A
hr6
dlE1,3tk
hr6tk
dlE1,3tk
hr6
dlE1,3tk
hr6tk
dlE1,3tk
hr6
1 2 3 4 5 6 7 8 9 10 11 12
A
B'
B
C
D
E
F
G
H
I
HindIII
G E C H D A B F I
hr6
E' C H D A B' F I
dlE1,3tk
dlE1
tk
G E C H D A B' F I
hr6tk
tk
E' C H D A B F I
dlE1
dlE1


B
hr6tk
dlE1,3tk
hr6
Ad5
A
B
C'
C
1 2 3 4
EcoRI
hr6
A
C
B
dlE1,3tk
A
C'
B
dlE1
tk
hr6tk
A
C'
B
tk
dlE1
A
C
B
dlE1
EcoRI site within tk insert

fragment called C' (3.2 kb) generated by the presence of tk gene in E3 was readily apparent (Fig. 4 panel B, lanes 1 and 2).

Isolates of each recombinant type i.e. hr1tk, hr6tk, d1312tk, pm975tk, and Ad5tk were subsequently plaque purified and expanded to produce high titre stocks (Materials and Methods). Each stock was titred at least twice on 293 cells, and the average value was used in subsequent experiments. The two host range mutants, hr1tk and hr6tk, were also titred on HeLa cells to determine if they were reduced in replication efficiency as expected. The results of all of the plaque titrations performed are shown in Table 2.

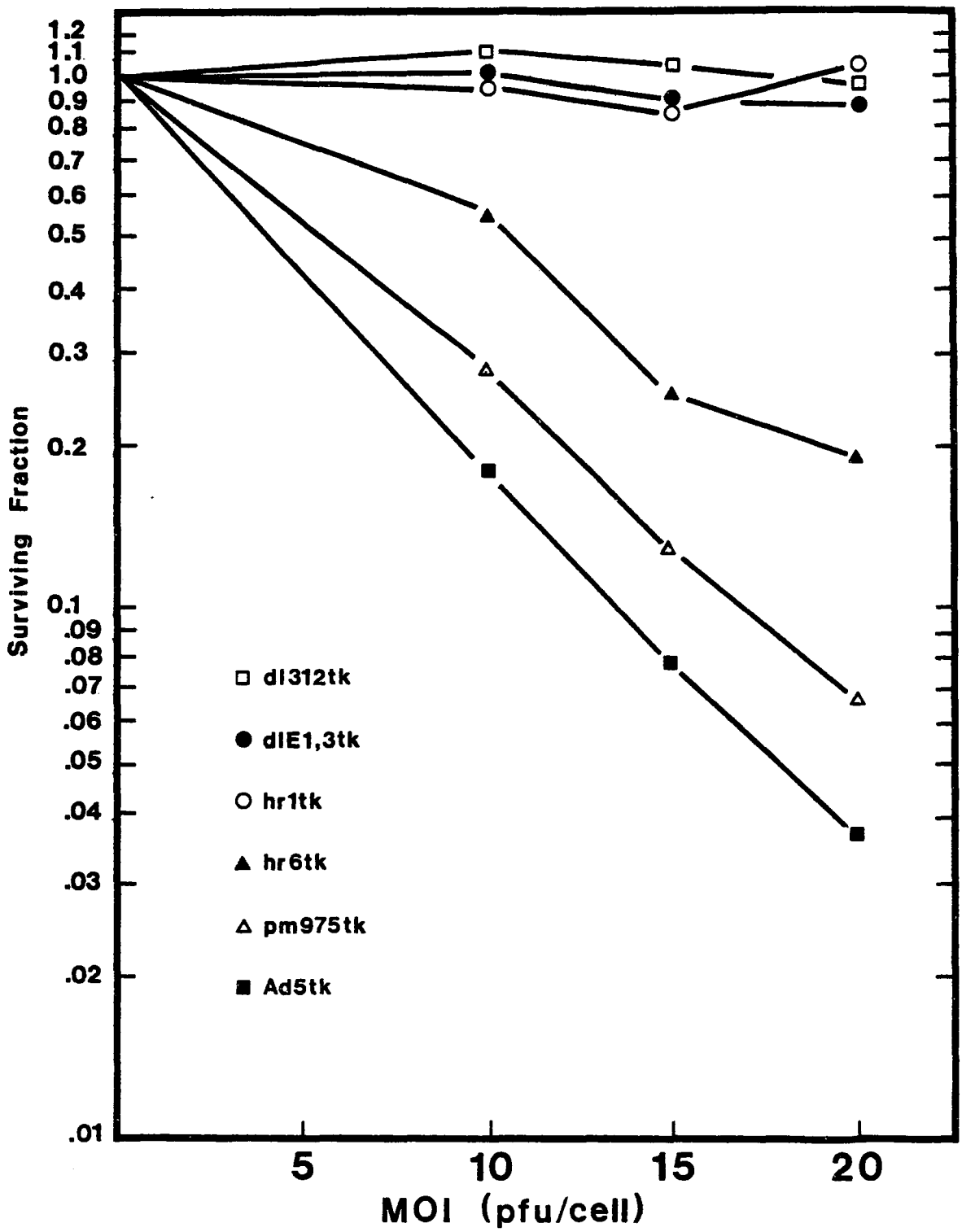
3.1.2 Viability Assays

The viability assays described in Materials and Methods were included as a part of each Rat 2 transformation experiment. However the results of these assays also provided additional information concerning the potential role of Ad5 proteins in reducing cell viability post infection. Examples of the results obtained are shown in Fig. 5, and Table 4. These assays clearly indicate that hr1tk, d1312tk, and dlE1,3tk had no significant effect on cell viability over the range of moi tested. The remaining viruses, hr6tk, pm975tk, and Ad5tk all caused significant and increasing cell mortality over this same range of moi. The virus hr6tk was apparently somewhat less lethal to Rat2 cells than either Ad5tk or pm975tk at an moi of 20 pfu/cell. Infection by hr6tk allowed for a 3 to 6 fold larger surviving fraction than infection by Ad5tk or pm975tk in two experiments (Table 4). In general pm975tk

Table 2 Plaque Titration of Recombinant Adenovirus Stocks

Virus	Titre		
	293 (pfu/ml)		HeLa (pfu/ml)
		mean	
hr1tk	2.9×10^9		1.7×10^5
	1.6×10^9		
	1.5×10^9		
		2.0×10^9	
hr6tk	6.4×10^{10}		2.5×10^7
	2.7×10^{10}		
		4.5×10^{10}	
Ad5tk	2.8×10^{10}		
	2.0×10^{10}		
		2.4×10^{10}	
dl312tk	3.8×10^9		
	4.4×10^9		
		4.1×10^9	
pm975tk	9.6×10^9		
	1.3×10^{10}		
		1.1×10^{10}	

Figure 5 Rat2 cell viability assays. Each point represents the average value obtained from at least two experiments. Colonies were counted nine or ten days post infection after staining with crystal violet.


1.2
1.1
1.0
0.9
0.8
0.7
0.6
0.5
0.4
0.3
0.2
0.1
.09
.08
.07
.06
.05
.04
.03
.02
.01
Surviving Fraction
dl312tk
dlE1,3tk
hr1tk
hr6tk
pm975tk
Ad5tk
5
10
15
20
MOI (pfu/cell)

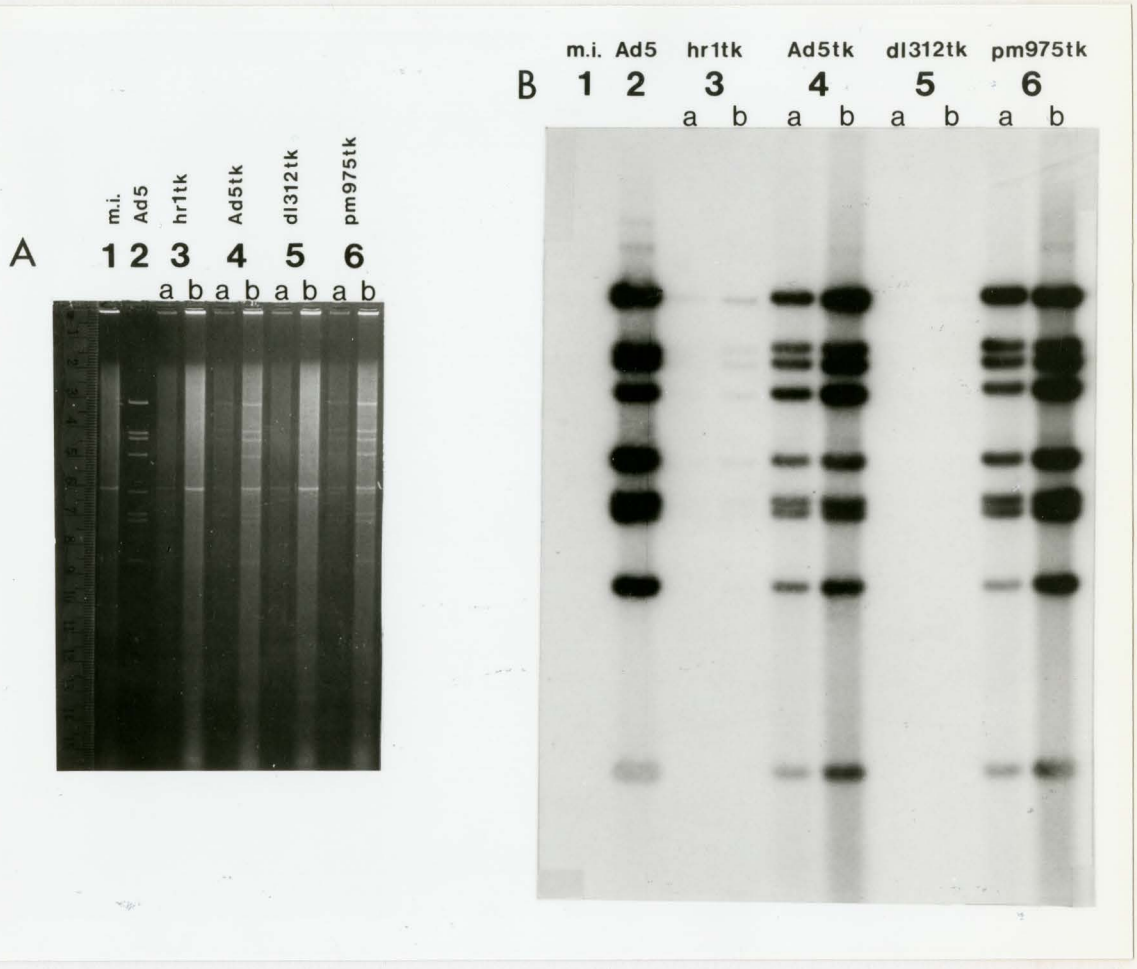
infection leaves a slightly larger surviving fraction of Rat2 cells than Ad5tk infection does but this difference is not consistently statistically highly significant.

The three nonlethal viruses all share a common defect i.e. they do not express the Ela 289R product. Conversely all three lethal viruses do express this polypeptide. Based upon the evidence presented here there is a definite correlation between expression of the wild type Ela 289R product and Rat2 cell death post infection, however a cause and effect relationship has not been established. Two other assays were also carried out to further characterize two viruses from each group.

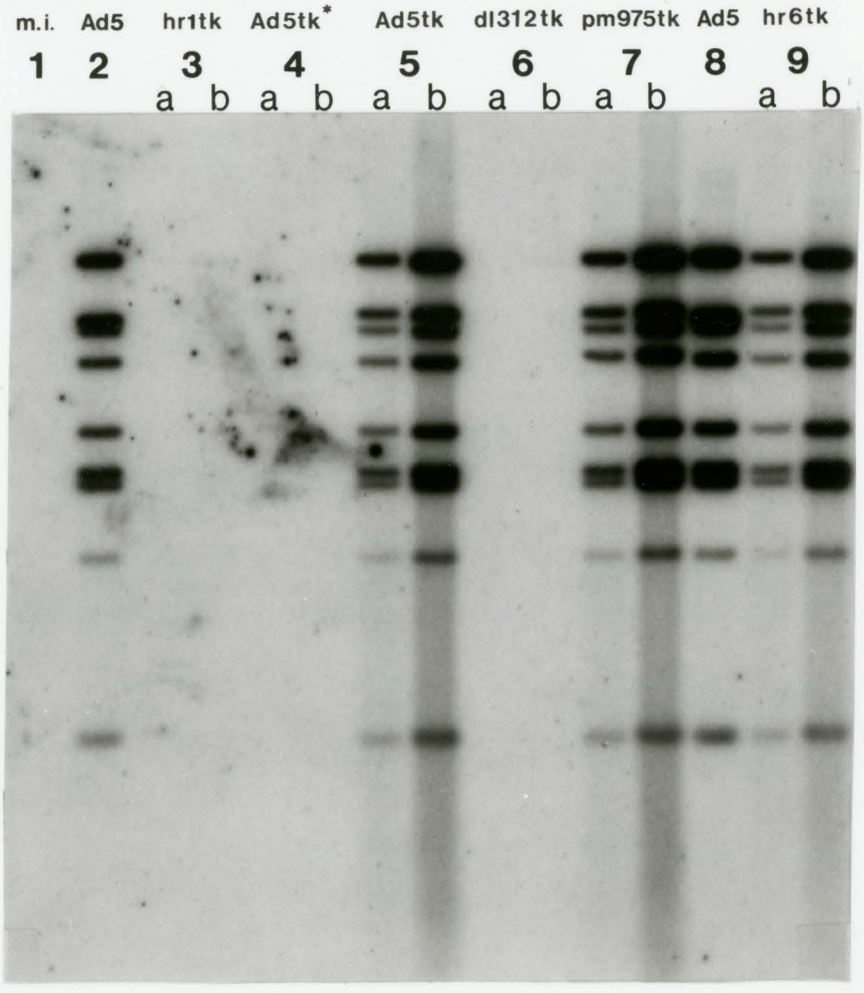
3.1.3 Determination of the Amount of Viral DNA Synthesis in Infected Rat2 Cells

Four viruses - hr1tk, d1312tk, pm975tk, and Ad5tk were tested for their ability to undergo viral DNA synthesis pi. This involved infection of Rat 2 cells with each of the viruses, extraction of DNA and RNA, and analysis for the presence of viral DNA by hybridization of labelled Ad5 probe to Southern blots of electrophoresed samples of total nucleic acid digested with the restriction enzyme Hind III (Materials and Methods). The results of two experiments are shown in Fig. 6. Panel A of Fig. 6 shows a photograph of the electrophoresed samples from the first experiment prior to Southern transfer. Two different amounts of total intracellular nucleic acid - 0.5 micrograms and 2.5 micrograms, were loaded in adjacent lanes for each virus tested. Bands migrating at the positions predicted for HindIII restricted viral DNA are clearly

Figure 6 Assay for viral DNA replication in Rat2 cells. The cells were infected at an moi of 20 pfu/cell with recombinant adenoviruses and harvested 72 hours post infection. Panel A: photograph of the ethidium bromide stained agarose gel from which the Southern blot shown in panel B was obtained. Panel B: Southern blot of 0.5 (a) or 2.5 (b) micrograms of total extracted nucleic acid digested with HindIII. The hybridizing probe was wt Ad5 DNA labelled with ^{32}P. The cells were infected with: lane 3: hr1tk; lane 4: Ad5tk; lane 5: d1312tk; or lane 6: pm975tk. Lane 1: nucleic acid from mock infected Rat2 cells (2.5 micrograms); and lane 2: 100 ng of wt Ad5 DNA digested with HindIII. Panel C: HindIII digest of 0.5 (a) or 2.5 (b) microgram samples of total nucleic acid extracted from Rat2 cells infected with: hr1tk (lane 3); Ad5tk and extracted 8 hours post infection (lane 4); Ad5tk (lane 5); d1312tk (lane 6); pm975tk (lane 7); or hr6tk (lane 9). Mock infected Rat2 cell nucleic acid (2.5 micrograms): lane 1. Banded wt Ad5 DNA: lane 2 (50 ng); lane 8 (100 ng).


A
m.i.
Ad5
hr1tk
Ad5tk
dl312tk
pm975tk
1 2 3 4 5 6
a b a b a b a b
B
m.i. Ad5 hr1tk Ad5tk dl312tk pm975tk
1 2 3 4 5 6
a b a b a b a b

C



* 8 hr control

evident in the 2.5 microgram samples of nucleic acids extracted from Ad5tk and pm975tk infected cells (lanes 4b and 6b respectively), whereas no viral DNA is apparent in the hr1tk or d1312tk lanes (3a and b, and 5a and b respectively). A 2.5 microgram sample extracted from mock infected Rat2 cells (lane 1) and a 100 nanogram sample of banded wt Ad5 DNA (lane 2) were also included. Panel B shows the results of autoradiography of the subsequent Southern transfer. Faint bands corresponding in size to viral DNA fragments are apparent in lanes 3b (hr1tk) and 5b (d1312tk) indicating a barely detectable level of viral DNA replication relative to that observed with Ad5tk and pm975tk. Panel C shows an autoradiograph from a second experiment, identical to the first, except that hr6tk was also assayed (lanes 9a and 9b). Densitometry scans of the autoradiographs indicated that Ad5tk, pm975tk, and hr6tk induced approximately equal amounts of viral DNA synthesis, and that this level of viral DNA accumulation was about 50 fold greater than was induced by hr1tk (data not shown).

The results of these assays demonstrate that there is a direct correlation between lethal effect and viral DNA replication in infected Rat 2 cells. Lack of expression of the E1a 289R product prevents the lethal effect, possibly as a consequence of the inability to replicate the viral genome. In addition, the E1b 58 kDa protein may also play a direct or indirect role in cell killing, since a mutation in the gene encoding this product as specified by the hr6tk virus caused a significant reduction in cell mortality post infection.

3.1.4 Determination of the Amount of Viral Replication in Infected Rat2 Cells

The assay for viral replication involved titres of stocks of infected Rat 2 cell lysates on 293 cells as described in Materials and Methods. The results of two experiments are shown in Table 3. The initial experiment, in which all cell lysates were harvested 72 hours pi, indicated that although measureable numbers of infectious virus particles were present in each of the lysates (with the exception of the mock infected control), the amount of de novo viral replication must have been very limited, or nonexistent. This is clear when comparing the virus yield to the input, which is reduced on average by a factor of approximately five thousand in experiment 1. To further clarify the question of de novo replication, experiment 2 included a control for the presence of input virus particles remaining in, or adsorbed to, the cells after infection and rinsing with fresh medium. The control cell lysate, indicated as Ad5tk^{b}, was harvested 8 hours pi, whereas the other lysates were harvested 72 hours pi, as in experiment 1. This control clearly demonstrated that virus particles from the initial input did remain associated with the cells pi. These infectious particles present in the Ad5tk control titre could not have been due to de novo synthesis because the cells were harvested 8 hours post infection, which is insufficient time for viral replication to occur. A comparison of the magnitude of the control titre to the other viral titres in experiment 2 shows that virus yield decreases by an additional factor of 20 to 50 fold on incubation to 72 hours. This provides further evidence that no

Table 3. Assays of Recombinant Adenovirus Replication in Rat2 Cells[a]

Expt. No.	Infecting Virus	Virus Input (pfu/dish)	Cell Lysate Dilution	Avg Plaque Titre (plaques/ dish)	Viral Yield (pfu/ dish)	Input/Yield
1	hr1tk	$9x10^6$	10^{-1}	30	$1.2x10^3$	$1.3x10^{-4}$
	dl312tk		10^{-1}	48	$1.9x10^3$	$2.1x10^{-4}$
	Ad5tk		10^{-1}	42	$1.7x10^3$	$1.9x10^{-4}$
	pm975tk		10^{-1}	57	$2.1x10^3$	$2.3x10^{-4}$
2	hr1tk	$7.2x10^6$	10^{-1}	11	$4.4x10^2$	$6.1x10^{-5}$
	dl312tk		10^{-1}	17	$6.8x10^2$	$4.4x10^{-5}$
	Ad5tk		10^{-1}	19	$7.6x10^2$	$1.1x10^{-4}$
	Ad5tk[b]		10^{-2}	41	$1.6x10^4$	$2.2x10^{-3}$
	pm975tk		10^{-1}	8	$3.2x10^2$	$4.4x10^{-5}$

[a] viral input was equivalent to 20 pfu/cell in all cases; incubation period was 72 hrs pi.

[b] 8 hr incubation period pi.

significant viral replication occurred with any of the four viruses tested, i.e. hr1tk, d1312tk, pm975tk, and Ad5tk.

3.2. Biochemical Transformation

3.2.1 Comparison of Transformation Results without Correction for Differential Cell Viability

The results of the biochemical transformation experiments are summarized and subjected to statistical analysis in Tables 4, 5, and 6. In Table 4, two experiments in which all six recombinant viruses were assayed, are presented. The results obtained without correction for differential cell viability in Table 4, under the heading average number of tk^+ colonies per dish, show that each virus tested can be assigned to one of two groups: lethal viruses which transform with relatively low efficiency (hr6tk, pm975tk, and Ad5tk,); and nonlethal viruses which transform with relatively high efficiency (dlE1,3tk, d1312tk, and hr1tk). The ratio of high to low transformation frequencies has a maximum value of 260 (Table 4 E1, hr1tk versus Ad5tk) and a minimum value of 24 (E1, dlE1,3tk versus pm975tk). Other conclusions which were made based on direct comparisons of the raw data were: 1. hr1tk, d1312tk and dlE1,3tk were not consistently significantly different in ability to induce transformation, and 2. hr6tk, pm975tk, and Ad5tk did not significantly differ from one another in transformation efficiency. Graphs illustrating the transformation results obtained from pairwise comparisons of the recombinant viruses without and with correction for

Table 4. Biochemical Transformation Assays which Include All Recombinant Adenoviruses Isolated

Virus	Avg no. of tk^+ colonies per dish[a]		Surviving Fraction		Avg. no. of tk^+ colonies per 5×10^5 viable cells	
	E95	E98	E95	E98	E95	E98
hr1tk	79+/-3	30+/-3	0.89	0.68	84+/-3	41+/-3
dl312tk	76+/-1	23+/-2	1.1	0.95	66+/-1	23+/-2
dlE1,3tk	19+/-1	20+/-2	0.97	0.69	19+/-1	28+/-2
Ad5tk	.3+/-.3	.5+/-.3	0.05	0.07	6+/-.3	7+/-.3
pm975tk	.8+/-.4	.7+/-.3	0.06	0.08	13+/-.4	8+/-.3
hr6tk	3+/-1	.8+/-.5	0.34	0.24	8+/-1	3+/-.5

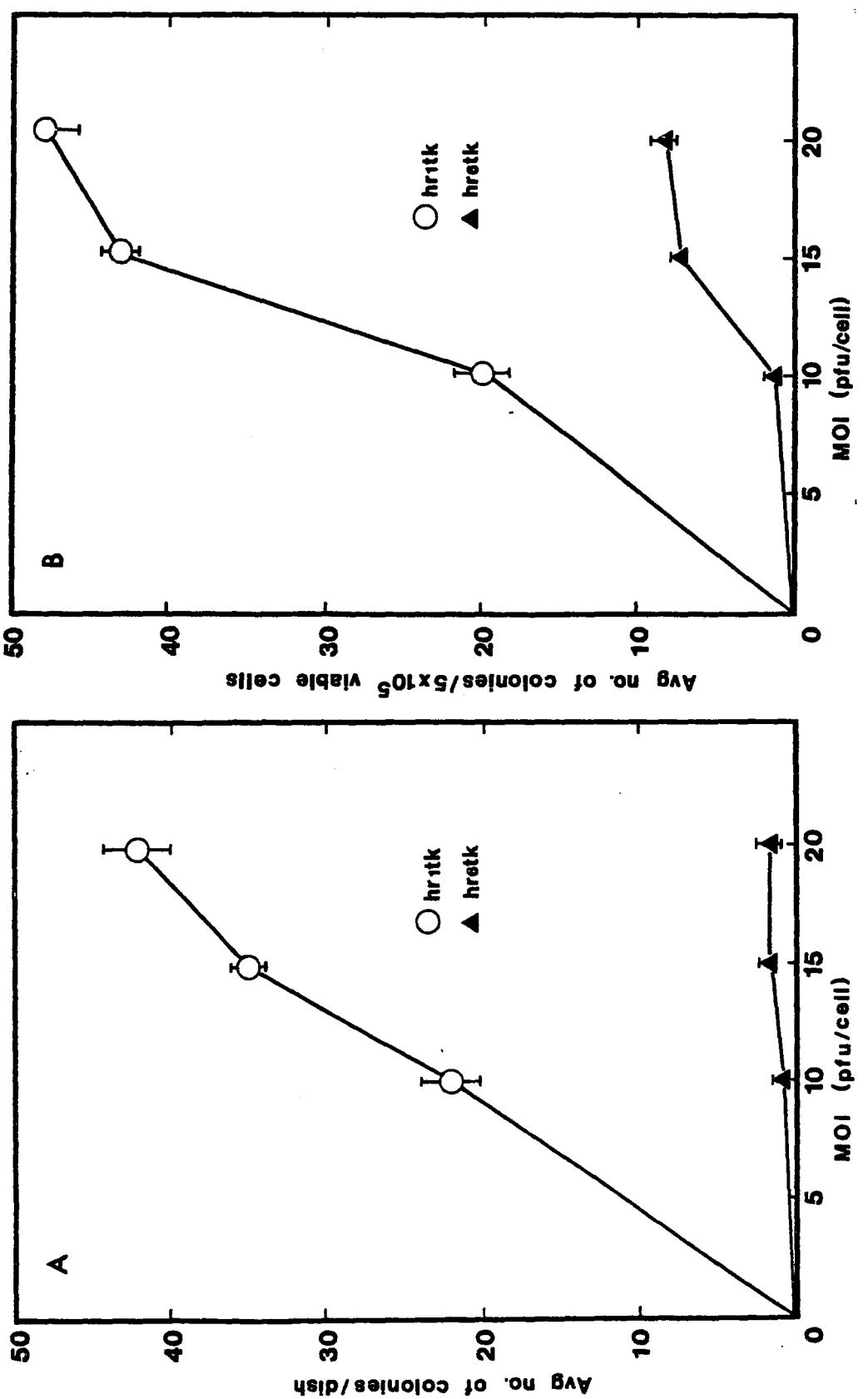
[a] all infections done at an moi of 20 pfu/cell; standard error of each transformation value is indicated.

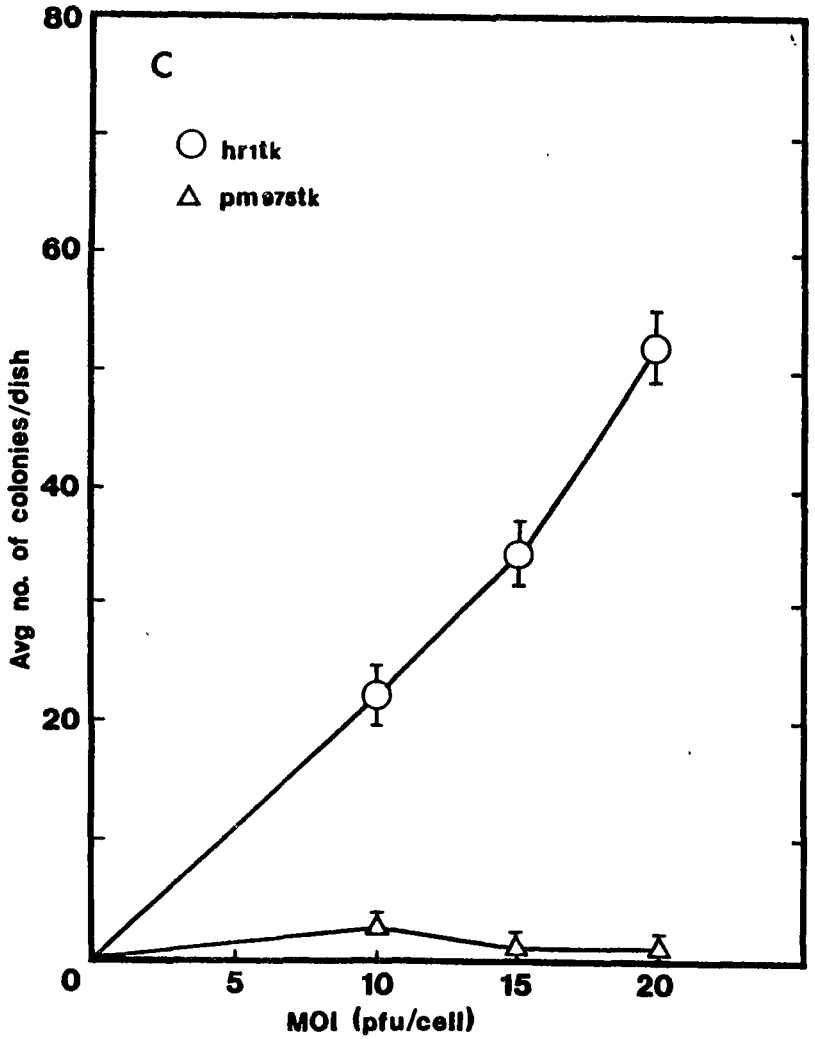
differential cell viability are shown in Fig. 7, panels A through H. Three pairs of graphs show comparisons of hr1tk with the three lethal viruses: hr1tk versus hr6tk (panels A and B); hr1tk versus pm975tk (C and D); and hr1tk versus Ad5tk (E and F). The remaining pair of graphs (panels G and H) illustrate results obtained with d1312tk and d1E1,3tk, the remaining nonlethal viruses.

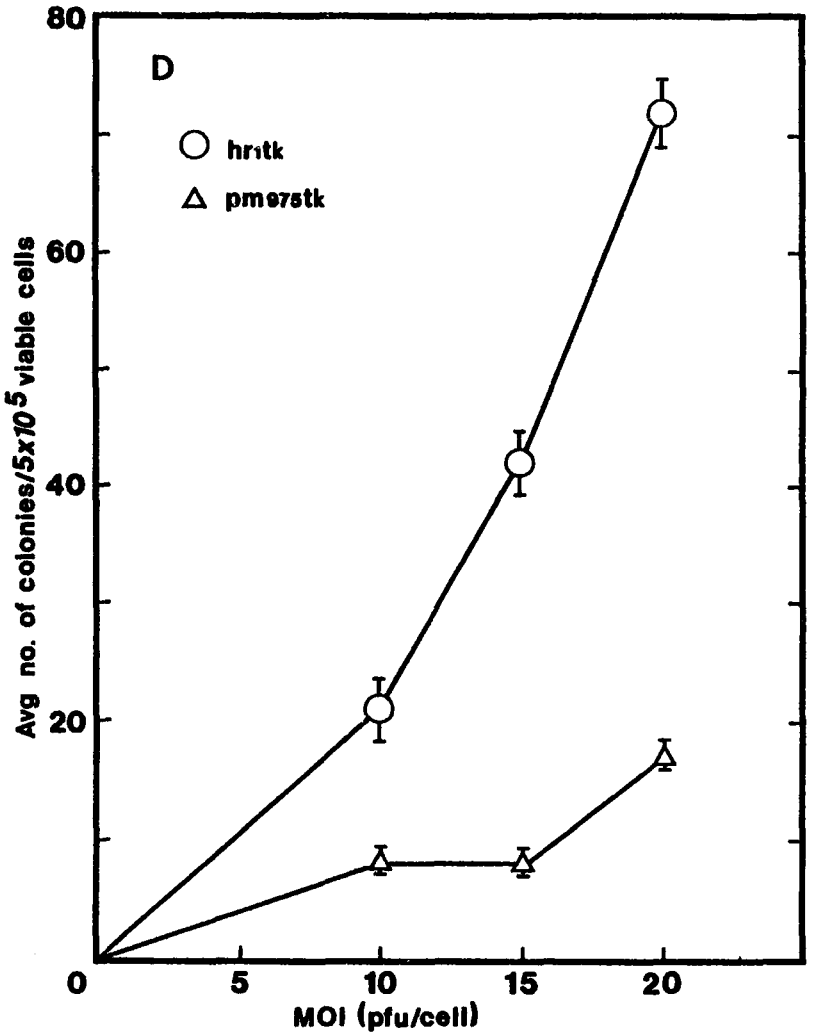
3.2.2 Comparison of Transformation Results After Correction for Differential Cell Viability

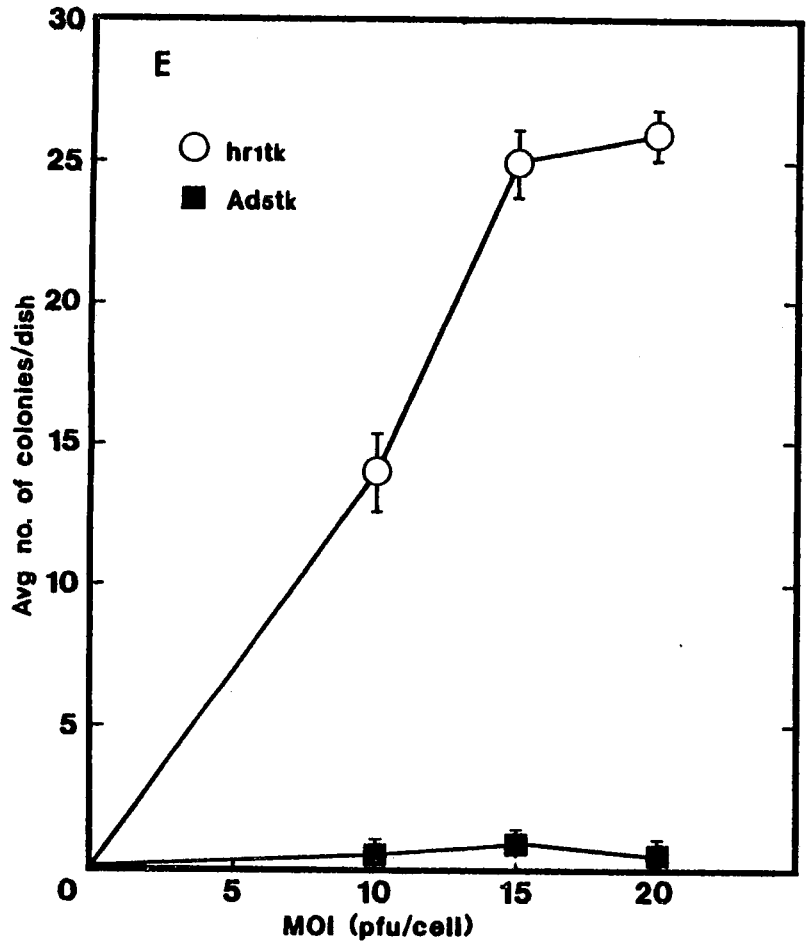
The prime effect of compensating for differential cell viability was to increase the apparent transforming efficiency of the lethal viruses such that there were no longer two clearly distinct groups of three viruses (Table 6). For example d1E1,3tk and pm975tk which differed significantly prior to removal of viability bias (Table 5) were not statistically different after this correction was introduced (Tables 5 and 6). This weakened the direct correlation of increased cell death post infection with reduced transformation efficiency. The transformation ratios obtained when hr1tk was compared to the three lethal viruses were reduced dramatically. The maximum value after these controls were introduced was a factor of fifteen (Table 4, expt. 1, hr1tk versus Ad5tk, and expt. 2, hr1tk versus hr6tk), reduced from a value of 260. Despite the reduction hr1tk continued to have greater potential for inducing tk^+ transformation than Ad5tk or pm975tk. This is also apparent in Table 6 where mean transformation values obtained from a number of experiments are presented and compared after controlling for

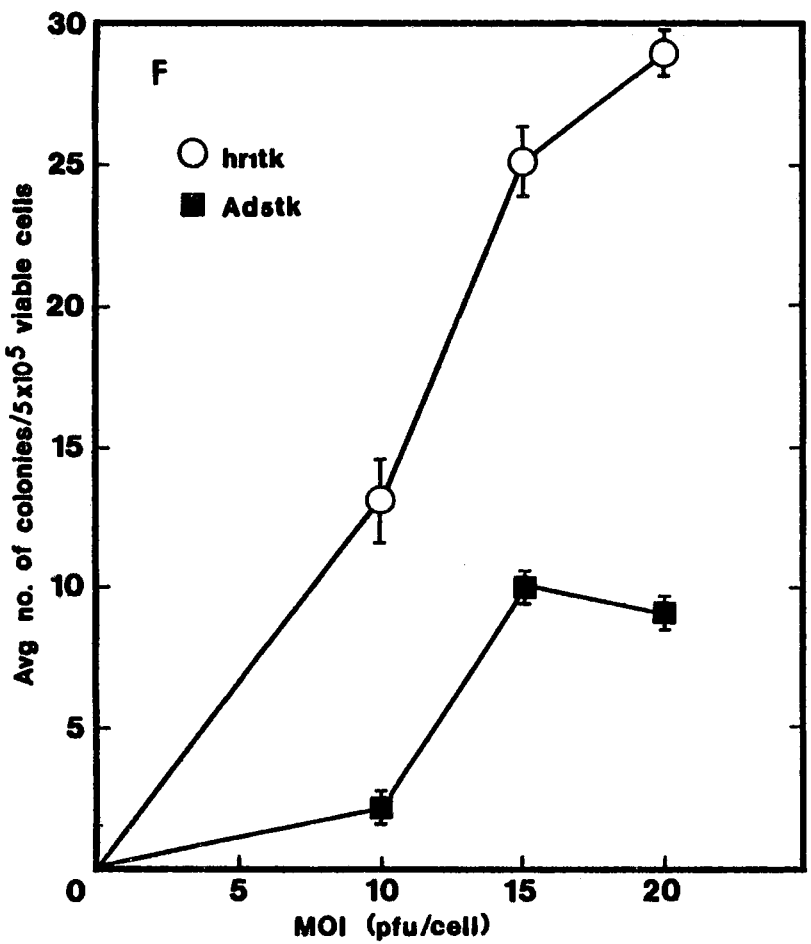
Figure 7 Examples of biochemical transformation assay results shown prior to (Panels A, C, E, and G) and after (Panels B, D, F, and H) correction for differential Rat2 cell viability. Each point represents the average value obtained from six dishes.

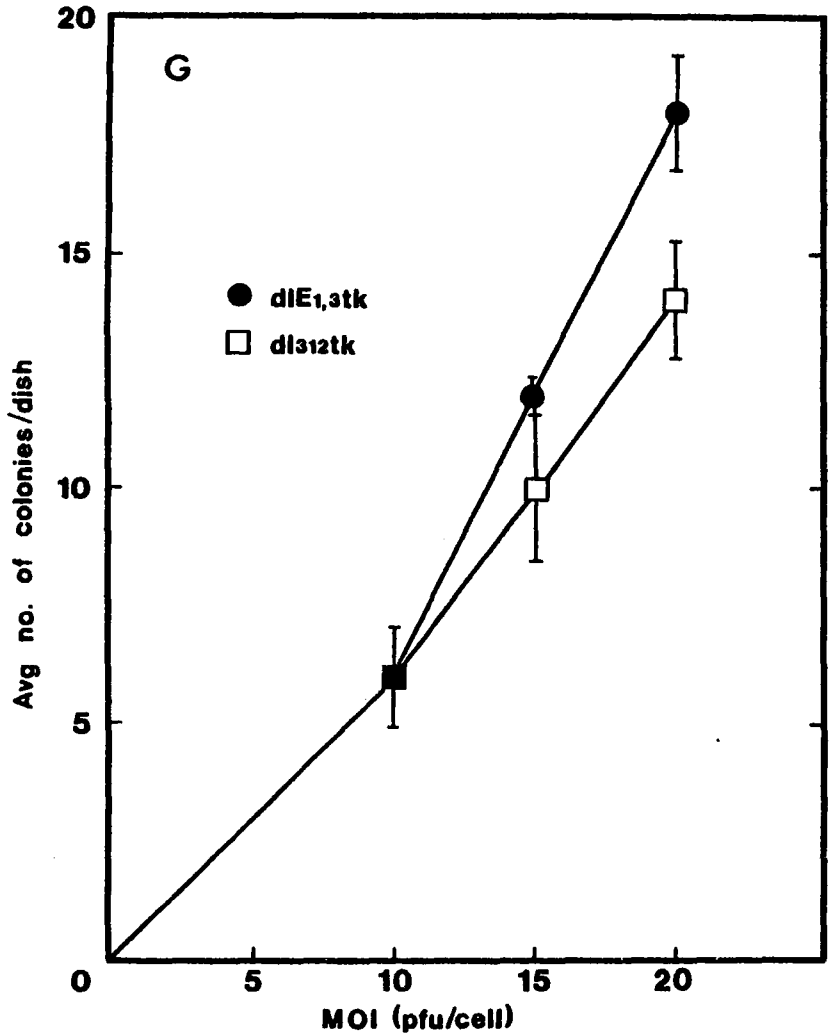

A
B
hr1tk
hr0tk
Avg no. of colonies/dish
Avg no. of colonies/5x10^5 viable cells
MOI (pfu/cell)
0
10
20
30
40
50
5
15

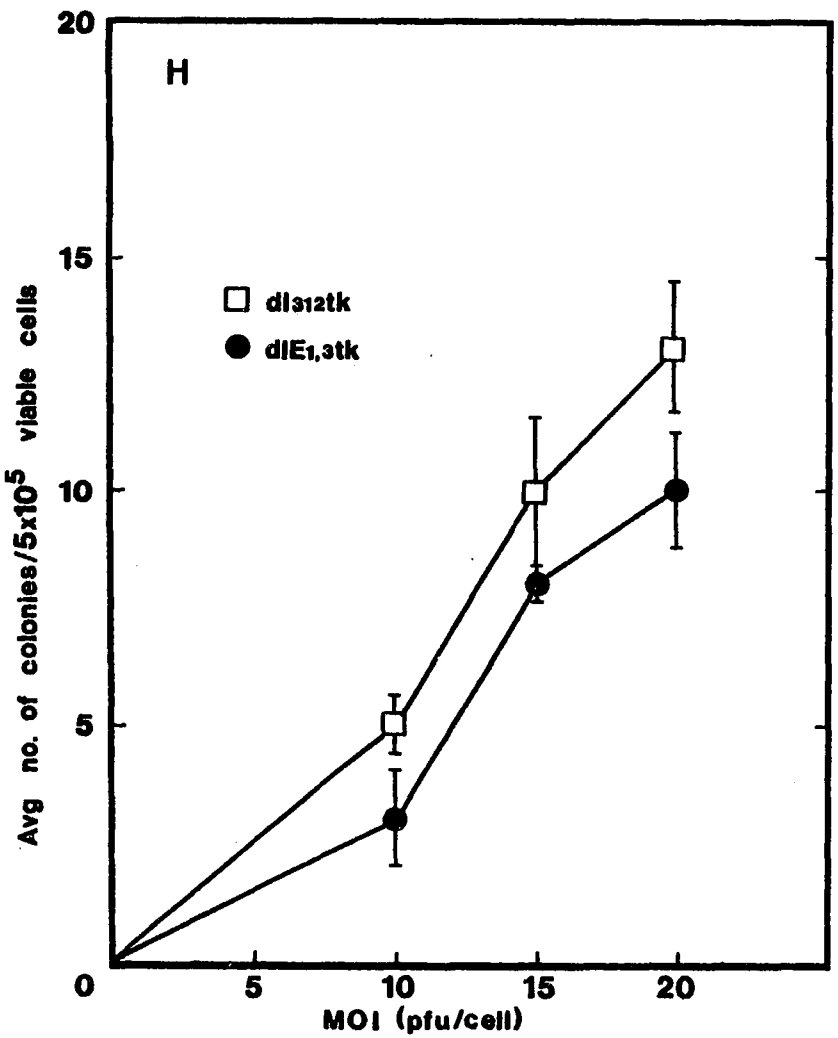

C
hr1tk
pm97stk
Avg no. of colonies/dish
MOI (pfu/cell)
0
20
40
60
80
5
10
15
20


D
hr1tk
pm97stk
Avg no. of colonies/5x10^5 viable cells
MOI (pfu/cell)
0
20
40
60
80
5
10
15
20


E
hr1tk
Ad5tk
Avg no. of colonies/dish
MOI (pfu/cell)
0
5
10
15
20
25
30


F
hr1tk
Ad5tk
Avg no. of colonies/5x10^5 viable cells
MOI (pfu/cell)
0
5
10
15
20
25
30


G
dlE1,3tk
dl312tk
Avg no. of colonies/dish
MOI (pfu/cell)
0
5
10
15
20


H
dl312tk
dlE1,3tk
Avg no. of colonies/5x10^5 viable cells
MOI (pfu/cell)
0
5
10
15
20

differential cell viability. The mutant hr1tk produced about five times as many transformants as pm975tk, and about eight times as many as Ad5tk at an moi of 20 pfu/cell. This suggests that the E1a 289R protein may interfere with biochemical transformation but it is not clear whether the effect is direct or indirect.

There is insufficient evidence to indicate a role for the E1a 243R product in enhancing biochemical transformation since results obtained with hr1tk and d1312tk did not consistently differ by more than a factor of two (Table 4). Because of the uncertainty associated with viral titres, a difference of this magnitude cannot be considered to be indicative of a significant biological effect. Similarly the approximately equal transformation frequencies obtained with d1312tk and dlE1,3tk (Fig. 7, panels G and H) suggest that the E1b region does not encode products which affect biochemical transformation, a conclusion also supported by the hr6tk results which suggest that lack of expression of the E1b 58 kDa protein does not alter the efficiency of biochemical transformation (Tables 4 and 6, Ad5tk versus hr6tk). However the conclusions made concerning E1b may not be valid due to possible differences in the level of expression of E1b products by d1312tk compared to Ad5tk, and because of uncertainty as to whether or not hr6tk expresses a truncated protein related to the wt 58 kDa product.

3.2.3 Experimental Variability and Statistical Analyses

In order to determine whether or not the differences in transformation frequencies were statistically significant, the results

Table 5. Statistical Analysis of Transformation Results by Student's t Test

Expt. No.	Viruses Compared[a]	t_s (with bias)	Stat. Sig.[b]	t_s (without bias)	Stat. Sig.	Critical Value ($t_{0.05}$)
95	hr1tk/hr6tk	22.4	++	16.4	++	2.306
	hr1tk/pm975tk	10.7	++	9.9	++	2.228
	hr1tk/Ad5tk	29.2	++	11.3	++	2.228
	hr1tk/dlE1,3tk	20.6	++	17.5	++	2.228
	hr1tk/dl312tk	0.9	-	4.5	++	2.262
	dl312tk/dlE1,3tk	31.4	++	26.2	++	2.262
	hr6tk/Ad5tk	4.6	++	0.3	-	2.306
	dlE1,3tk/pm975tk	15.1	++	0.9	-	2.228
98	hr1tk/hr6tk	10.2	++	8.6	++	2.228
	hr1tk/pm975tk	10.3	++	5.8	++	2.228
	hr1tk/Ad5tk	10.4	++	5.5	++	2.228
	hr1tk/dlE1,3tk	3.0	++	2.8	+	2.228
	hr1tk/dl312tk	1.9	-	3.9	++	2.228
	dl312tk/dlE1,3tk	1.1	-	1.5	-	2.228
	hr6tk/Ad5tk	0.5	-	0.6	-	2.228
	dlE1,3tk/pm975tk	10.8	++	4.3	++	2.228

[a] all infections done at an moi of 20 pfu/cell.

[b] ++ highly significant, + significant, - not significant.

Table 6. Summary of Biochemical Transformation Results and X^2 Analysis

Transformation[a]			X^2 Analysis		
Virus	No. of tk^+ Colonies/ $5x10^5$ Viable Cells	mean	Viruses Compared	X^2	Stat. Sig.[b]
hr1tk	49;77;52;72; 29;84;41	58			
			hr1tk/d1312tk	27.1	++
d1312tk	30;17;66;23	34			
			d1312tk/d1E1,3tk	6.5	+
d1E1,3tk	16;10;8;19;28	16			
			d1E1,3tk/pm975tk	3.7	-
pm975tk	6;17;13;8	11			
			pm975tk/Ad5tk	1.8	-
Ad5tk	9;6;7;3	7			
			Ad5tk/hr6tk	0.3	-
hr6tk	8;1;8;3	5			

[a] each transformation value represents an average of six dishes; all experiments done with an moi of 20 pfu/cell.

[b] ++ highly significant, + significant, - not significant.

obtained with pairs of viruses were compared using the Student's t test (Table 5). Each t_s value (called the sample statistic of the t distribution) in Table 5 indicates the statistical significance of the difference between two mean biochemical transformation values. For example in the first comparison of experiment 1, Table 5, the mean transformation values obtained with hr1tk and hr6tk as shown in Table 4 were compared, both with (t_s with bias) and after the removal of (t_s without bias) the bias associated with differential cell viability post infection. The transformation frequencies obtained with hr1tk and hr6tk without removal of bias were 79 and 3 colonies per dish respectively (Table 4, experiment 1). This produced a t_s of 22.4, indicated in the third column of Table 5. This value showed that the difference in means was statistically highly significant (indicated as ++). After removal of the viability bias the transformation frequencies were: hr1tk - 84 colonies per $5x10^5$ viable cells; and hr6tk - 8 colonies per $5x10^5$ viable cells. The corresponding Student's t test gave a t_s of 16.4 (column six of Table 5), also indicative of a statistically highly significant difference in mean values. Finally in the last column of Table 5 the critical value ($t_{0.05}$) defines the limits of the 95% confidence interval, i.e. only t_s values larger than or equal to the critical value are considered indicative of mean values which are significantly different (indicated by a single + in the appropriate columns). The critical values for 99% confidence intervals which determine differences in means which are statistically highly significant are not shown. It is important to note that comparisons of results obtained from different experiments by the t test were not statistically significant. The amount

of variance in transformation at any moi as measured between experiments for each virus was large, often amounting to a factor of two. This large variation is primarily due to variation in the condition of the Rat2 cells from the successive passages used for the transformation experiments. Biological variability of this sort cannot be eliminated or entirely controlled for.

The only statistical comparisons done using results of more than one experiment are shown in Table 6. Here X^2 analyses of average transformation values for each recombinant virus obtained from several experiments at an moi of 20 pfu/cell are shown. These comparisons of mean values are valid and useful.

3.3 Recombination

3.3.1 Analysis of Mixed Infection Progeny

During the construction of the various recombinant viruses we obtained certain anomalous results in terms of recombination frequencies. These observations were extended in more exhaustive analyses, the results of which are presented here. With one exception these recombination assays were performed on 293 cells, and involved screening of individual plaque isolates from infected cell lysates, as with construction of the viruses. There is a considerable amount of information to be gained from assays of this type. Since individual isolates were analyzed, a quantitative estimate of the frequency of occurrence of four different types of progeny were obtained from each mixed infection. This allowed for an estimate of the recombination

frequency in each mixed infection, based upon the recombination frequency of the progeny sampled, as well as providing an indication of the relative replication efficiencies of the parental viruses.

3.3.2 Interference in the Replication of dlE1,3tk Correlates with Reduced Recombination Frequency

A comparison of the frequency of occurrence of progeny of each parental type (Table 7) revealed an interesting correlation. In general in mixed infections with equal input of the two parental viruses an expected outcome was equal numbers of progeny viruses of the two parental types. Deviation from this 1:1 ratio of parental viruses would suggest that replication of one of the viruses was being interfered with, presumably by some mechanism involving replication of the other virus. All of the viruses used here could be grown to high titre in 293 cells in single infections, demonstrating that as expected they have no growth defects in this host. In order to determine if interference was occurring, the results from each coinfection were subjected to a X^2 analysis (Sokal and Rohlf, 1969). In each case the data were analyzed for significant deviations from a 1:1 ratio of progeny of each parental type. Four of the viruses tested -Ad5, pm975, dl312, and in a single experiment VpKGO-13S, showed a statistically significant level of interference in the replication of the coinfected virus dlE1,3tk when infections were done at the standard input ratio (Table 7). Mixed infections involving these four viruses with dlE1,3tk also resulted in the lowest levels of recombination - varying from an average of 8.5%

Table 7. Recombination Frequencies Obtained by Mixed Infection of 293 Cells at Standard Input of Adenoviruses

Parental Virus Used With dlE1,3tk	Progeny: Parental Type dlE1,3tk	Progeny: Parental Type Other	Progeny: Variation from 1:1[b]	Progeny: Recombinant Type tk	Progeny: Recombinant Type dlE1	Recomb. Freq. (%)
hr1	5	1	*	22	1	79
hr1	14	15	-	6	1	19
hr1	12	20	-	5	0	14
hr6	7	10	-	5	0	23
hr6	18	13	-	6	0	16
dl312	14	20	-	0	0	0
dl312	5	42	++	0	0	0
Ad5	12	22	-	3	0	8
Ad5	9	30	++	4	0	9
pm975[a]	12	25	+	2	1	8
pm975	3	39	++	1	0	2
VpKGO-007[a]	17	9	-	0	0	0
VpKGO-12S[a]	16	22	-	0	0	0
VpKGO-13S[a]	6	24	++	0	0	0

[a] chimeric Ad2/Ad5 virus.

[b] ++ highly significant, + significant, - not significant
* sample size too small for analysis.

with Ad5, to 0% with dl312 and VpKGO-13S. Four viruses - hr1, hr6, VpKGO-12S, and VpKGO-007 showed no interference.

The infections done at varying input ratios of the parental viruses (Table 8) provided the initial dl312tk and pm975tk isolates essential for the transformation assays. The dl312, dlE1,3tk crosses provided additional evidence of the interference effect in all three assays. However in contrast to the infections done at standard conditions, recombinant viruses were successfully isolated, indicating that the block in recombination previously observed with dl312 was not absolute. The mixed infection involving Ad5 (E33) was done inadvertently at the indicated input moi due to an error in the Ad5 viral titre. The experiments involving mixed infections of HeLa cells with the VpKGO viruses were done at an input ratio of 50:50 pfu per cell in an attempt to ensure that the infections would be productive in terms of yield of viral progeny. It is known that mutants which do not express the E1a 289R product (such as VpKGO-12S) do not replicate efficiently in HeLa cells, particularly at low moi (Montell et al., 1982). Subsequent plaque assays to isolate progeny from these crosses revealed that the VpKGO-12S, dlE1,3tk coinfection was not productive whereas the other two mixed infections of HeLa cells were (data not shown). The analysis of progeny from the HeLa mixed infections revealed about 5% recombination with VpKGO-007, 3% recombination with VpKGO-12S, and 0% recombination with VpKGO-13S.

Table 8. Recombination Frequencies Obtained by Mixed Infection of 293 or HeLa Cells with Varying Input of Parental Viruses

Expt. No.	Virus Used with dlE1,3tk	Input Ratio dlE1,3tk: Other (pfu/cell)	Progeny: Parental Type dlE1,3tk	Progeny: Parental Type Other	Progeny: Recombinant Type tk	Progeny: Recombinant Type dlE1	Recomb. Freq. (%)
A. 293 Cells							
33	Ad5	10:15	0	28	2	0	7
59	dl312	10:35	1	22	0	1	3
64	dl312	35:35	4	17	3	0	13
64	dl312	40:30	3	16	3	0	14
65	pm975	40:35	1	15	0	0	0
72	pm975	40:35	5	30	1	2	8
72	pm975	50:35	11	15	1	0	4
B. HeLa Cells							
92	VpKGO-007	50:50	31	5	1	1	5
92	VpKGO-12S	50:50	23	10	1	0	3
92	VpKGO-13S	50:50	16	22	0	0	0

3.3.3 Ad2/Ad5 Chimerism at the Left Viral Terminus Correlates with Reduced Recombination Frequency

Before the correlation between replication interference and recombination frequency was established, the results obtained from mixed infections of dlE1,3tk with pm975 or dl312 (5% and 0% average recombination respectively as indicated in Table 7) suggested an alternate hypothesis. This was that lack of expression of the Ela 243R protein by pm975 and dl312 was reducing recombination. In an attempt to address this possibility we undertook the isolation of three more viruses, VpKGO-007, VpKGO-13S, and VpKGO-12S. These viruses were isolated using a procedure developed in the laboratory (McGrory et al., 1988) from three plasmids pKGO-007, pKGO-13S, and pKGO-12S provided by Dr. G Akusjarvi (Svensson et al., 1987). These plasmids included the Ad2 complementary DNA sequences for the 13S or 12S mRNA inserted within the Ad2 HindIII G fragment to replace the Ela coding sequences (pKGO-13S, and pKGO-12S respectively), or the wt Ad2 HindIII fragment (pKGO-007). The plasmids were rescued into an Ad5 viral background by a technician in the lab, J. Rudy. The intention was to observe the effect of expressing either the 289R or 243R protein independently of the other, on the recombination frequencies resulting from mixed infections with dlE1,3tk, with VpKGO-007 serving as the control for expression of both products. The mixed infections were done in 293 cells and HeLa cells with surprising results (Tables 7 and 8). No measureable recombination occurred with any of the viruses in 293 cells. The lack of recombination with VpKGO-007 was incompatible with the results obtained with Ad5. It

had already been shown (Table 7) that Ad5 underwent significant recombination (an average value of 8.5%) in mixed infections of 293 cells with dlE1,3tk. The only difference between Ad5 and VpKGO-007 is the presence of Ad2 sequences at the left terminus of the latter virus. It should be noted that the viruses used here can be categorized in the following way: viruses containing serotype 5 sequences only - hr1, hr6, d1312, and Ad5; and chimeric Ad2/Ad5 viruses - pm975, VpKGO-007, VpKGO-13S, and VpKGO-12S. As with the mutant pm975 only the Ad2 sequences extending from nucleotide 1 to 360 were relevant with respect to homologous recombination with dlE1,3tk. Downstream from this region the E1 deletion of dlE1,3tk begins and no further homology exists until the 3'end of the deletion.

The X^2 test for interference was also applied to the data gathered from the VpKGO, dlE1,3tk mixed infections (Table 7). The VpKGO-13S, dlE1,3tk cross produced significant interference as had the analogous virus pm975. The VpKGO-12S, dlE1,3tk coinfection produced no significant interference, nor did the mixed infection involving VpKGO-007.

The results showed that interference, and heterology due to chimerism, were distinct and separable phenomena in terms of their effects on recombination. The viruses VpKGO-12S and VpKGO-007 did not induce significant interference in the replication of dlE1,3tk, and they also did not recombine to a measureable extent. Conversely d1312tk has no Ad2, sequences but induced highly significant interference effects, and also had no measureable recombination frequency using the same parameters.

To summarize, there was a strong correlation between interference in the replication of dlE1,3tk and recombination frequency. Significant reductions in the frequency of occurrence of dlE1,3tk relative to the coinfecting virus coincided with reduced recombination. There was a second correlation between the presence of Ad2 sequences (seven mismatched base pairs) near the left terminus of the virus used in coinfection with dlE1,3tk, and reduced recombination.

3.3.4 Frequency of Occurrence of dlE1 Recombinant Viruses

The data concerning recombinant viruses has to this point been discussed in terms of the overall recombination frequency arising from each mixed infection. However as mentioned previously two different types of recombinant viruses arose and could be identified by their restriction enzyme profiles. These were the "tk" recombinants, characterized by wild type or mutant E1 regions as well as the tk gene inserted in E3, and the dlE1 recombinant viruses, which lacked the E1 region and the tk insert. Recombinant DNA molecules of either type could have arisen by either of two mechanisms described by Holliday, and Meselson and Radding i.e. homologous strand exchange or strand invasion respectively (refer to Introduction and Discussion for further details). Considering recombination due to homologous strand exchange first, a prediction that can be made is that equal numbers of "tk" and dlE1 recombinants should arise as a consequence of this mechanism. Any crossover event between the two markers i.e. the E1 deletion and the tk gene, will generate both a "tk" recombinant and a dlE1 recombinant molecule. The same prediction can be made for "tk" and dlE1 recombinants

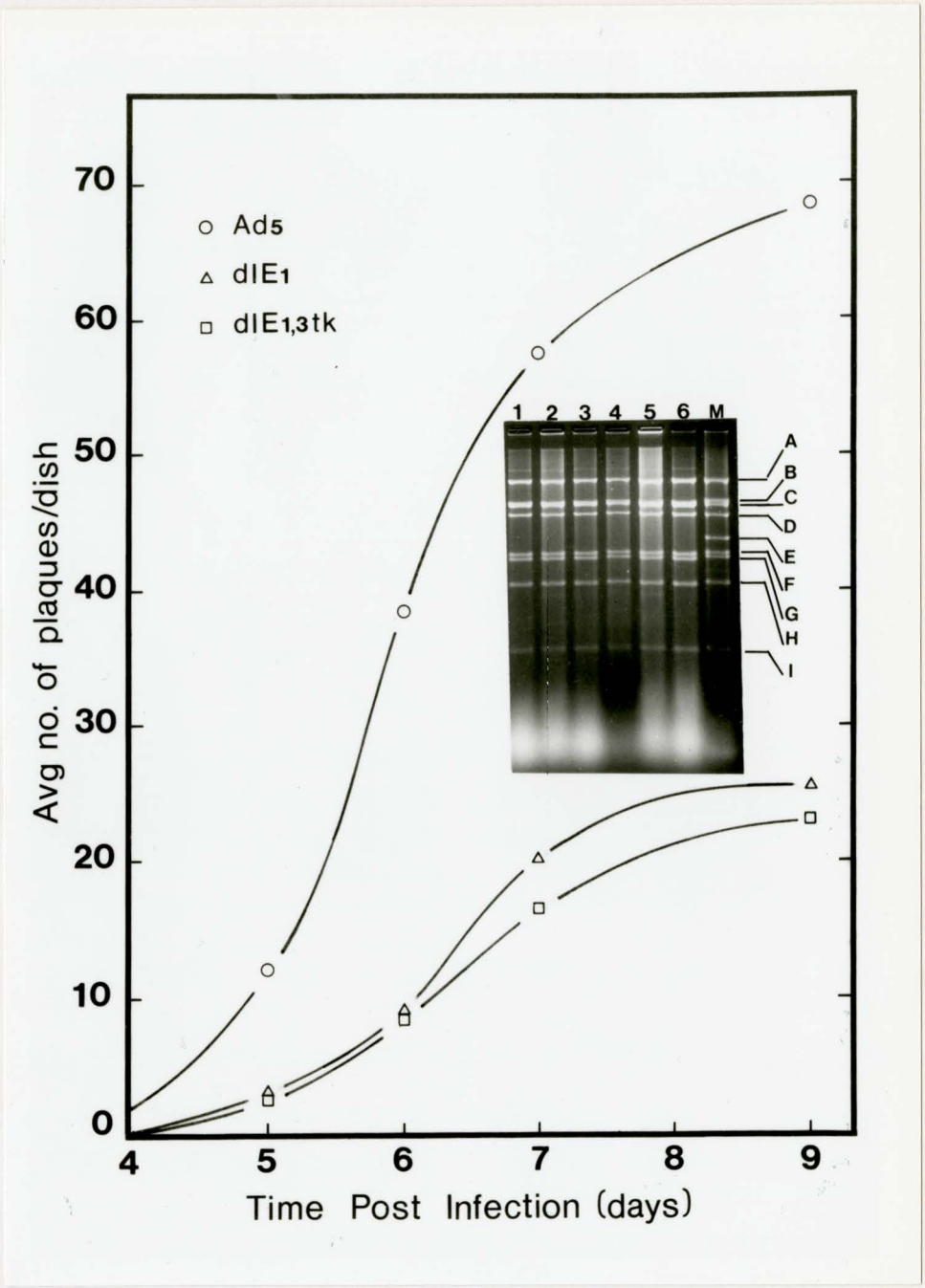
arising from recombination via the strand invasion mechanism. Either dlE1,3tk or the coinfecting virus can supply the invading strand as a consequence of strand displacement during DNA replication. In addition, in the replicating molecule, the region of single stranded DNA can extend away from the right or left terminus depending upon which terminus underwent the replication initiation event. These structures will be described in more detail in the Discussion section. Current understanding of adenovirus replication suggests that initiation of replication is equally likely to occur from the left or right terminus (Challberg and Kelly, 1982; Wang and Pearson, 1985). Therefore the expected outcome of the mixed infections described here is the isolation of approximately equal numbers of "tk" and dlE1 recombinant viruses regardless of what proportion of the recombinants are provided by either mechanism.

The results clearly demonstrated that "tk" recombinants were much more frequent than dlE1 recombinants. The ratio generated by mixed infection of 293 cells with equal numbers of parental viruses as shown in Table 7 was 32:2 in favour of "tk" recombinants, ignoring the anomalous E27 results. This deviation from a 1:1 ratio is statistically highly significant. The ratios representing the sums of recombinants from all mixed infections of 293 cells except E27 (42:5), and the sums of recombinants from all mixed infections including HeLa cells (44:6) are also statistically highly significant deviations from the predicted ratio. The extremely small number of dlE1 recombinants, and the small number of "tk" recombinants recovered relative to the total sample size, made it impossible to apply X^2 or G tests for goodness of fit to the

recombinant data from individual experiments. This is based on the general rule that if a particular class of samples has an expected frequency of occurrence of less than five, statistical analysis is not valid (Sokal and Rohlf, 1969).

The trivial explanation that recombination was skewed from the predicted ratio due to a delay in the kinetics of dlE1 plaque formation relative to the other viruses was investigated. In a plaque assay experiment summarized in Figure 8, the kinetics of appearance of dlE1,3tk, Ad5, and dlE1 plaques on 293 cells were measured. Small dishes (60 mm) of 293 cells were infected with serial dilutions of dlE1,3tk, Ad5, or dlE1 virions, and subjected to the plaque assay procedure described previously (Materials and Methods). The dishes were monitored daily from day four to day nine post infection. Plaques became visible by day four and increased in numbers until approximately day eight in all three cases. Ten dlE1 plaques were picked and analyzed after the assay was completed to ensure that they displayed the dlE1 Hind III restriction profile. This precaution was taken because the dlE1 virus used in the infection was from an original isolate rather than a plaque purified stock. All ten isolates were identified as being dlE1. HindIII digests of six dlE1 isolates are shown as an inset in Figure 8. This experiment ruled out the possibility of delayed appearance of dlE1 plaques in mixed infections.

Figure 8 Kinetics of Ad5, dlE1,3tk, and dlE1 plaque formation on 293 cells. Each point represents the average value from two dishes. The Ad5 and dlE1,3tk infections were from dilutions of plaque purified stocks of virus. The dlE1 infection was done with viral progeny harvested from an original recombinant isolate. The inset shows the Hind III analysis of DNA from six plaque isolates from the dlE1 infection.


70
60
50
40
30
20
10
0
Avg no. of plaques/dish
○ Ad5
△ dlE1
□ dlE1,3tk
1 2 3 4 5 6 M
A
B
C
D
E
F
G
H
I
4
5
6
7
8
9
Time Post Infection (days)

DISCUSSION

4.1 Characterization of Semipermissive Infection of Rat2 Cells by Recombinant Adenoviruses

4.1.1 A Mutation in the Ela 13S mRNA Product Affects Viral DNA Synthesis and Rat2 Cell Viability Post Infection

The results presented here showed that 3 viruses (hrltk, dl312tk, and dlEl,3tk) had no significant effect on cell viability over the range of moi used, whereas hr6tk, pm975tk, and Ad5tk were significantly and increasingly lethal to the cells over this same range. Furthermore, in subsequent DNA replication assays, the two nonlethal viruses tested, hrltk and dl312tk, did not replicate their DNA efficiently in Rat2 cells, whereas pm975tk, hr6tk, and Ad5tk did undergo relatively efficient viral DNA replication (Fig. 6). The 3 nonlethal mutants presumably have a defect in common based on the characterization by others (Introduction) of their parental counterparts hrl, dl312, and dlEl,3tk, i.e. they are unable to express the Ela 289R protein, whereas the three lethal viruses express this product, suggesting that it plays an indirect or direct role in cell killing. In permissive infections the 289R protein has been suggested to be primarily responsible for

transactivation of other viral early genes (Montell et al., 1982 and 1984; Lillie et al., 1986) as well as some cellular genes (Kao and Nevins, 1983; Stein and Ziff, 1984). The region of the protein most strongly implicated by accumulating evidence from a number of groups is the unique region of 46 amino acids (Svensson and Akusjarvi, 1984; Glenn and Ricciardi, 1985; Lillie et al., 1987). The coding sequences for the carboxy-terminal 20 amino acids of this region are lacking in hr1tk (see Introduction). The relationship suggested by the observations above is that Rat2 cell death is correlated with expression of a wild type 289R protein which is able in turn to transactivate other viral genes, including those required for viral DNA replication. The results obtained with pm975tk suggest that the 243R product plays no role in the induction of the lethal effect since this virus lacks expression of this product and yet induces cell killing at levels comparable to Ad5tk.

It has been noted in a previous study (Ruben de Campione, 1983) that replication of hr1 DNA in rat cells varies with the type of cell used. When primary BRK cells were infected, viral DNA replication was measurably greater than after infection of rat 3Y1 cells, an established cell line (Kimura et al., 1975). There was slight but detectable hr1tk DNA replication in Rat2 cells as indicated by autoradiographs of nitrocellulose filters probed with labelled viral DNA (Fig. 6, panel B, lane 3b). Similar results were obtained by Ruben de Campione with 3Y1 cells. The suggestion then is that established cell lines (Rat2 and 3Y1) prevent efficient viral DNA replication when a wt 289R product is absent, whereas primary BRK cells do not.

4.1.2 Elb 58 kDa Expression may also Reduce Rat2 Cell Viability

There are published reports demonstrating that the wild type Elb 58 kDa protein is involved in the accumulation and stabilization of late viral messenger RNA transcripts in the infected cell cytoplasm (Pilder et al., 1986; Williams et al., 1986) during permissive infections, as well as being involved in the shut-off of host protein synthesis (Babiss and Ginsberg, 1984). If the protein is not prevented from performing the same functions in semipermissive infections it could directly contribute to the lethal effect of Ad5tk or pm975tk infection on Rat2 cells observed here. The absence of the lethal effect observed with hr1tk and d1312tk which can potentially express the 58 kDa product, would then be related to the transactivation effects of the Ela 289R protein. Since the wild type 13S mRNA product is not expressed by either virus, the level of expression of the 58 kDa protein may then have been greatly diminished, thereby preventing cell killing.

There was an apparent reduction in lethality observed with hr6tk when compared to the results of infection of Rat2 cells with Ad5tk or pm975tk. However this reduction was not of a large enough magnitude to conclude that 58 kDa expression correlates strongly with cell killing. The effect may have been due to uncertainty in the titres of the recombinant viruses. For example in experiment 1 of Table 4, about three times as many cells survived infection with hr6tk than with either Ad5tk or pm975tk, a difference of uncertain significance.

It was not possible, based on the results reported here to determine an exact cause for Rat2 cell death post infection. Although

cell killing correlates with expression of the Ela 289R product, and with viral DNA synthesis, expression of other proteins including late products may be involved.

4.1.3 Viral Replication in Rat2 Cells

Replication of infectious Ad5tk, pm975tk, dl312tk, or hr1tk particles by 72 hours post infection in Rat2 cells was either reduced or completely absent, as measured by plaque formation by infected cell lysates on 293 cells (Table 3). Although some infectious virus was detectable in each case, it was of approximately the same magnitude as was produced by the lysate which measured the presence of input Ad5tk virions after rinsing the infected cells with fresh medium and harvesting 8 hours post infection (see Materials and Methods). These infectious particles cannot be the result of viral replication because of the lack of sufficient time for viral replication to occur. Eggerding and Pierce (1986) also showed this inhibition of infectious wt Ad2 viral replication in their study of various rat cell types. They also used the same control for the presence of residual input virions. These results demonstrated that the differential cell viability observed with various recombinant adenoviruses cannot be accounted for by viral replication since there is no correlation of lethal effect with production of infectious viral progeny.

4.2 Biochemical Transformation

4.2.1 Transformation Frequencies

All of the transformation experiments described here clearly showed that the mutant viruses hr1tk and dl312tk induced tk^+ transformation at a greater frequency than Ad5tk, hr6tk, or pm975tk. These results have been analyzed by the Student's t and X^2 tests and have been found to be statistically significant (Tables 5 and 6). This observation holds true whether or not the differential cell viability bias is compensated for. Ad5tk, hr6tk, and pm975tk are indistinguishable from one another using the same criteria.

The remaining conclusions concerning the categorization of the six viruses according to their ability to induce the transformed phenotype must be drawn from the results obtained after controlling for differential cell viability post infection. The validity of this approach has already been discussed (Introduction). The actual transformation frequency obtained with hr1tk was in a range between approximately 40 and 120 transformants per one million viable cells infected, in a range of mutiplicity of infection between 10 and 20 pfu/cell respectively. All of the viruses tested showed a dose response over the range of moi used i.e. with increasing moi one generally obtained increasing numbers of transformants. With the lethal viruses this dose response effect was clearly evident only when cell viability was controlled for (Fig. 7, panels B,D, and F). The narrow range of moi for which it was possible to obtain transformation data makes it

impossible to more fully characterize the dose response of the six viruses. Given the increasing lethality of hr6tk, pm975tk, and Ad5tk viruses in infections of Rat2 cells, transformation data at moi greater than 20 pfu per cell would be difficult if not impossible to obtain. Conversely at moi less than 10 pfu per cell, the extreme inefficiency of the transformation process, particularly as induced by hr6tk, pm975tk, or Ad5tk, would make detection of significant numbers of tk^+ transformants unlikely. Furthermore the Poisson distribution allows the prediction that less than 1 percent of the Rat2 cells remain uninfected at an moi of 10, whereas at moi less than 10 significant numbers of cells would remain uninfected, thereby further reducing the frequency of occurrence of transformation.

4.2.2 The Role of E1a in Biochemical Transformation

Based on the results obtained with hr1tk versus those obtained with pm975tk, hr6tk, and Ad5tk, there is evidence for a direct or indirect role for the E1a 289R product in interfering with biochemical transformation of Rat 2 cells. The latter three viruses express wt 289R products and display reduced ability to biochemically transform Rat2 cells relative to hr1tk. Evidence is accumulating which supports the hypothesis that both the E1a 289R and 243R products are multifunctional proteins with different functions assigned to distinct domains (Montell et al., 1984; Moran et al., 1986; Lillie et al., 1986; Whyte et al., 1988).

The distinct functional domains thought to be present in the Ela 243R protein include regions responsible for repression of enhancer mediated transcription (Lillie et al., 1986) and induction of efficient viral replication in growth arrested permissive cells (Montell et al., 1984). Since all of the amino acids of the 243R polypeptide are also present in the the 289R product it would seem likely that the larger product would also express these functions. However it is possible that the presence of the 46 amino acids of the unique region changes the conformation of the resulting polypeptide enough to prevent this. In fact Lillie, Green, and Green (1986) have proposed that the two products are antagonistic in their functions. They have correlated the enhancer mediated repression function of the 243R product with oncogenic transformation, suggesting that expression of the 289R polypeptide may interfere with the transformation process.

It is interesting to note the parallel which occurs with respect to the present study and previous experiments done with hr1. Enhanced tk^+ transformation only occurs when a complete 289R protein is not expressed (as with hr1tk or d1312tk). This result compares favourably with the oncogenic transformation results obtained with hr1 infections of primary BRK cells versus wt Ad5 infections (refer to Introduction). However lack of expression of both major Ela products as with d1312tk also leads to enhanced transformation relative to Ad5tk, whereas the parental virus d1312 is completely defective for oncogenic transformation of primary BRK cells. This is due to the type of assay used in each case - oncogenic transformation of primary BRK cells

requires Ela products, biochemical transformation with a selectable marker does not (Van Doren et al., 1984).

The relatively small difference in transformation frequency observed with hr1tk versus d1312tk is difficult to interpret in a biological sense. Many of the results (Tables 4 and 6) suggest an approximate difference of a factor of two but this is not entirely consistent. Differences of this magnitude have been demonstrated here to be statistically significant (Tables 5 and 6), but these analyses do not allow for the fact that the virus titres employed here have a considerable uncertainty. Therefore there is insufficient evidence to conclude that the Ela 243R product is enhancing the biochemical transformation frequency.

4.2.3 The Role of Elb in Biochemical Transformation

In order to determine if Elb products are involved in tk^+ transformation we compared the biochemical transformation efficiencies of d1312tk and dlE1,3tk. However the parental virus d1312 expresses only a low level of Elb mRNA transcripts in permissive infections relative to wt Ad5 due to the lack of Ela transactivion (Nevins, 1981), and it is likely that this holds true for d1312tk infection of Rat2 cells. Therefore although dlE1,3tk and d1312tk did not differ significantly in the experiments described here (Tables 5 and 6) it is not necessarily true that Elb products when expressed at wild type levels have no effect on the efficiency of biochemical transformation.

The results obtained with hr6tk are difficult to interpret due to the uncertainty as to whether or not a truncated 58 kDa product is

expressed. No such product has been detected by immunoprecipitation of hr6 infected or transformed cell lines with appropriate anti-tumour sera (Lassam et al., 1979; Rowe and Graham, 1983), however changes in conformation and or stability of the protein compared to the wild type product may account for this. The results described here do not mirror those obtained with hr6 in induction of foci formation by infection of primary BRK cells however. The mutant hr6 was found to be almost completely defective in this assay (Graham et al., 1979). In subsequent experiments it was found that DNA extracted from hr6 virions could induce formation of foci at levels approximating wt Ad5 DNA when used to transfect primary BRK cells (Rowe and Graham,1983). This led to the proposal that the 58 kDa product plays a role in the initiation of transformation by adenovirus during infection of semipermissive cells, but that the product was not needed for transformation by viral DNA (Rowe et al., 1984). The results presented here do not support this suggestion, since hr6tk was capable of inducing tk^+ transformation with an efficiency approximating that of the wild type control. Several factors must be considered when comparing hr6 and hr6tk results however, such as differences in cell type used, or the possibility of defects in putative transformation steps which might be reflected by the results of assays for oncogenicity but not by the tk transformation assay.

4.2.4 Viral DNA Replication and Biochemical Transformation

The results of the viral DNA replication experiments were significant with respect to biochemical transformation. As mentioned previously all six viruses exhibited a dose response i.e. increasing the

moi led to increased transformation frequency. This dose response was possibly due to an increased likelihood of integration of viral DNA into the host genome when larger numbers of viral genomes were present. Yet inhibition of viral DNA replication during infection did not reduce biochemical transformation frequency; in fact the opposite effect was observed. Two viruses - Ad5tk and pm975tk, which have been shown to undergo significant viral DNA replication in Rat2 cells, induced reduced levels of transformation relative to hr1tk and d1312tk which underwent little measureable DNA replication. This observation again provides evidence that the 289R product when expressed is antagonistic to the biochemical transformation process, despite also playing a role in increasing the number of viral genomes present in each cell post infection.

4.2.5 The Importance of Controlling for Differential Cell Viability

The significance of measuring the variation in cytopathic effect induced by the six recombinant adenoviruses is best demonstrated with an example. A comparison of the transformation frequencies observed with hr1tk and Ad5tk without correction for differential cell viability reveals differences of at least a factor of sixty (Table 4, E98). This difference is reduced to a factor of six after compensation for differential cell viability. Therefore ninety per cent of the apparent difference in transformation frequency was actually the result of another effect. This type of differential cell viability effect is equally likely to lead to incorrect conclusions concerning the oncogenic transformation potential of E1 mutants, particularly if the results are

based on virus mediated assays without inclusion of suitable viability controls. Since it is difficult to undertake viability assays when primary cells are used, this suggests a reason for the use of established cell lines such as cloned rat embryo fibroblasts (CREF) in virus mediated oncogenic transformation assays.

4.3 Recombination

4.3.1 The Input Ratio of Coinfecting Viruses Affects Recombination Frequency

The mixed infections of 293 cells were originally intended only to provide the desired recombinant adenoviruses. In general, the recombinant progeny made up a considerable portion of the total number of progeny analyzed, and therefore this method was usually found to be an efficient means of introducing the tk gene into both mutant and wild type genomes. However, the results also suggested that the following factors played important roles in determining recombination frequency: 1. the input ratio of the parental viruses, 2. the type of E1 mutant used in the coinfection with dlE1,3tk, and 3. the presence of even small numbers of mismatched base pairs in the coinfecting viruses. The first factor is readily explained. If the coinfection is done in a manner such that a great majority of one type of virus is used, the probability of recombination with a second much less numerous virus would be much lower than if both viruses were present in equal numbers. This hypothesis is based on the assumption of equal viability and rate of replication of

the two viruses in the host 293 cells. An example of the results of unequal input of two parental viruses is shown in Table 8, E59. Here the ratio of dlE1,3tk to dl312 was 10:35 pfu/cell respectively. The resulting progeny analyzed were highly skewed in favour of dl312 (22 dl312 isolates, 1 dlE1,3tk virus and 1 dlE1 recombinant). Another example involves a dlE1,3tk, Ad5 mixed infection with an input ratio of 10:15 pfu/cell respectively. Here again the frequency of occurrence of specific viral progeny reflected the relative abundance of the two parental viruses i.e. 28 Ad5 isolates, no dlE1,3tk progeny, and 2 Ad5tk recombinants.

4.3.2 Reduced Replication of dlE1,3tk Correlates with Reduced Recombination Frequency

To determine if either virus used in a mixed infection of 293 cells was interfering in the replication of the other virus, the progeny were analyzed to determine the ratio of each parental type. If cells are infected with equal numbers of two viruses, analysis of the progeny should provide a measure of the relative replication frequencies of these viruses. Furthermore, if either of the coinfecting viruses does not interfere with the replication of the other, equal numbers of progeny of the two parental types should occur. Conversely interference will result in a statistically significant variation from this 1:1 ratio. The results of all coinfections of 293 cells done at equal moi (i.e. 10 pfu/cell of each parental virus) are shown in Table 7. The number of progeny of each parental type have been shown for each experiment. The results were analyzed by X^2 test for departure from the

1:1 ratio (column 4). Significant interference occurred in mixed infections of dlE1,3tk with pm975, d1312, or Ad5. In each case dlE1,3tk replicated less efficiently than the coinfecting virus.

These interference results can in turn be compared to the recombination frequencies obtained in the same experiments. A strong correlation results. The three viruses which produced the lowest recombination frequencies in mixed infections with dlE1,3tk were the three viruses which produced the interference effect discussed above. Interference in viral replication after mixed infection of 293 cells has been observed previously. Berkner and Sharp (1983) noted that the replication efficiency of wt Ad5 was impaired by a coinfecting virus called Ad5delta, which had a deletion extending from 78.9 to 84.3 map units. This result is the opposite of that presented here. In the present study replication of the deletion mutant dlE1,3tk was interfered with by wt Ad5 and other viruses.

4.3.3 A Small Amount of DNA Heterology also Correlates with Reduced Recombination Frequency

The results of mixed infections of VpKGO-007, VpKGO-12S or VpKGO-13S with dlE1,3tk in 293 cells, along with the results of pm975, dlE1,3tk experiments, provided evidence that a small amount of DNA heterology could have a large effect on recombination frequency (Table 7). For example it was shown that wt Ad5 could undergo significant recombination (an average value of 8.5%) in mixed infections of 293 cells with dlE1,3tk, whereas VpKGO-007 could not (Table 7). The only difference between Ad5 and VpKGO-007 is the presence of Ad2 sequences at

the left terminus of the latter virus. As already described (Results), only the segment extending from nucleotide 1 to 360 is relevant with respect to recombination with dlE1,3tk. Within this short segment of 360 base pairs, there are seven mismatches as determined by DNA sequence comparison. These mismatches are scattered randomly throughout the segment, and consist of one mismatched adjacent pair and five single mismatches. The fact that recombination resulting from mixed infection of dlE1,3tk with VpKGO-007 was undetectable, provides evidence that the seven mismatched base pairs interfere significantly with recombination. Failure to detect recombination with VpKGO-12S also supports this hypothesis. In comparison the average value of recombination observed with hr1 using the same parameters of infection was 17% (Table 7). This difference in recombination frequency must be due to the mismatched base pairs in VpKGO-12S, or to the expression of the truncated 243R product by hr1 or to some combination of these two factors. Finally, VpKGO-13S was also unable to recombine to any detectable extent with dlE1,3tk, but in this case there was an alternate explanation for the lack of recombination, i.e. interference.

The lack of data from mixed infections of HeLa cells using hr1, hr6, dl312, pm975, and Ad5 made it impossible to determine whether or not the mismatched base pairs would also inhibit recombination in HeLa cells. Mixed infection experiments using mutants analogous to VpKGO-13S and VpKGO-12S but consisting entirely of Ad5 sequences would help to resolve this question.

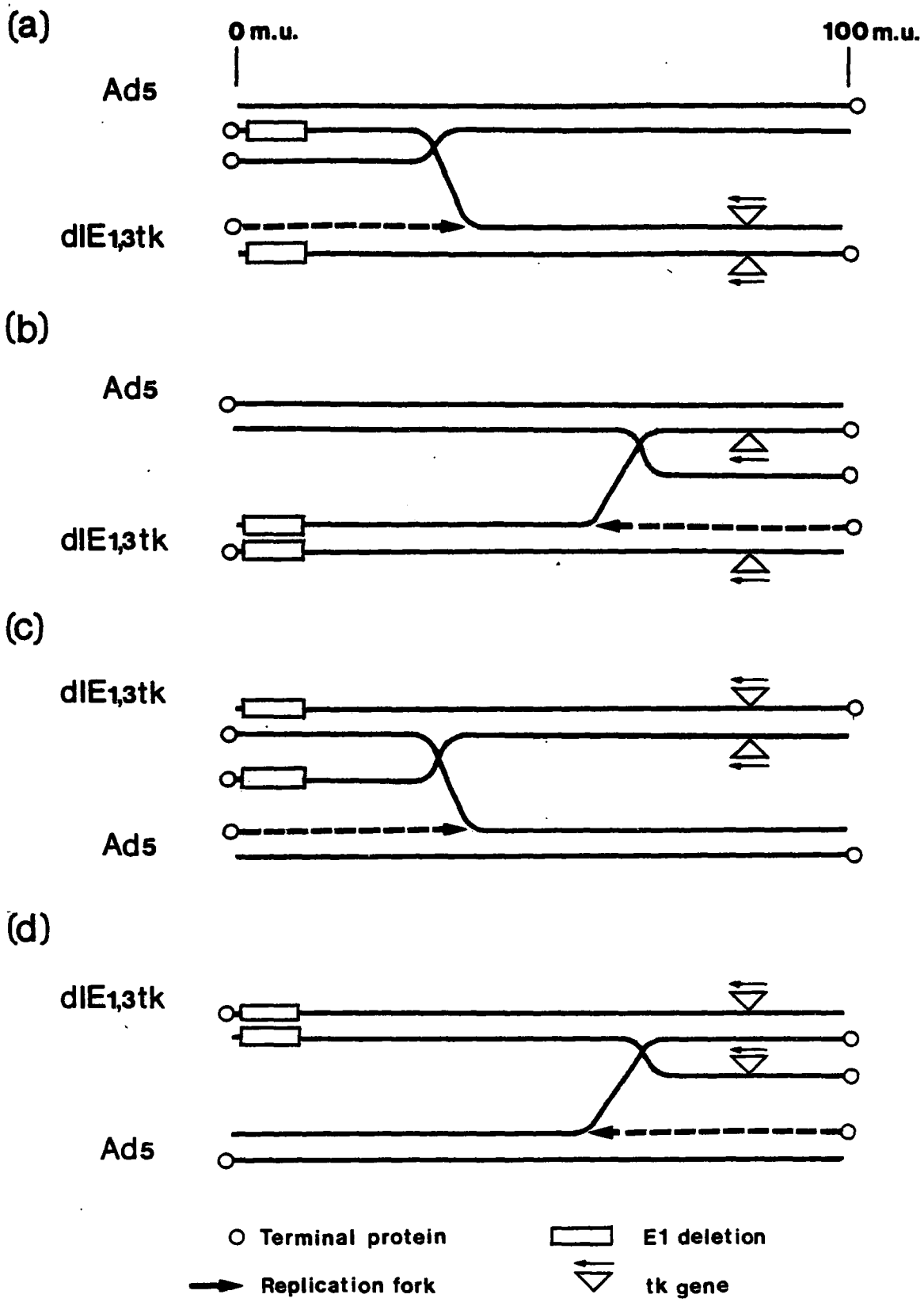
4.3.4 The Low Frequency of Occurrence of dlE1 Viruses Correlates with Missing E1 Sequences in Recombinant Heteroduplexes

There are at least two mechanisms by which recombination has been proposed to occur during mixed infection of permissive cells by adenoviruses. One of these - homologous recombination by strand exchange, does not require concurrent replication of the viral DNA in order to occur. This general model has been proposed by Holliday (1964). The other mechanism, described as the "strand invasion model" by Meselson and Radding (1975), requires replication by one of the participating genomes. Structures resembling the intermediates predicted to occur by both models during recombination have been observed in electron micrographs of nucleoprotein complexes isolated from Ad2 infected HeLa cells by Wolgemuth and Hsu (1980, 1981). The proportion of recombinants generated by either mechanism is not known.

With respect to the current study, both mechanisms of recombination described above would predict that dlE1 recombinant viruses and "tk" recombinant viruses should occur with equal frequency during the mixed infections described here. However the Meselson Radding mechanism does require concommitant replication by one of the molecules participating in the recombination event. This complicates analysis of recombination by increasing the number of ways recombinant molecules can arise. Adenovirus DNA is known to be capable of undergoing initiation of replication from either terminus (reviewed by T. Kelly, 1984). Therefore in each mixed infection there are four different replication intermediates which can participate in recombination. These are: two

different dlE1,3tk intermediates produced by initiation of replication from either the right or left viral terminus; and two different intermediates resulting from initiation from either terminus of the coinfecting viral DNA molecule. This is a somewhat simplified view because it considers only type I replication. The replicating molecule provides the single strand of DNA which displaces the identical strand on the other molecule up to a breakage point. Consequently heterologous duplex DNA is generated. The application of this type of recombination mechanism as it might function during adenovirus mixed infections is illustrated in Figures 9 and 10. The illustrations in Figure 9 were adapted from similar drawings used by Wolgemuth and Hsu (1981) to demonstrate Ad2 recombination intermediates. The heterologous duplex DNA structures shown in Figure 10 should be of varying stability. Heteroduplexes initiated at the left end of the recipient virus will have a larger region of nonhomology than those initiated at the right end of the recipient molecule. This is because the E1 deletion of dlE1,3tk is much larger (about 3,800 base pairs) than the tk insert in E3 (about 2,200 base pairs). Furthermore and perhaps more significantly, the region of heterology present in heteroduplexes (b) and (d) is much further from the closer terminus of the viral genome than is the corresponding region of heterology shown in (a) and (c). The nonhomologous regions may reduce the frequency of heteroduplex formation and/or the likelihood that the heteroduplex remains intact long enough to generate recombinant viral genomes. Despite the difference in the size of the nonhomologous regions, this should not of itself affect the ratio of dlE1:"tk" recombinants, because the generation of both

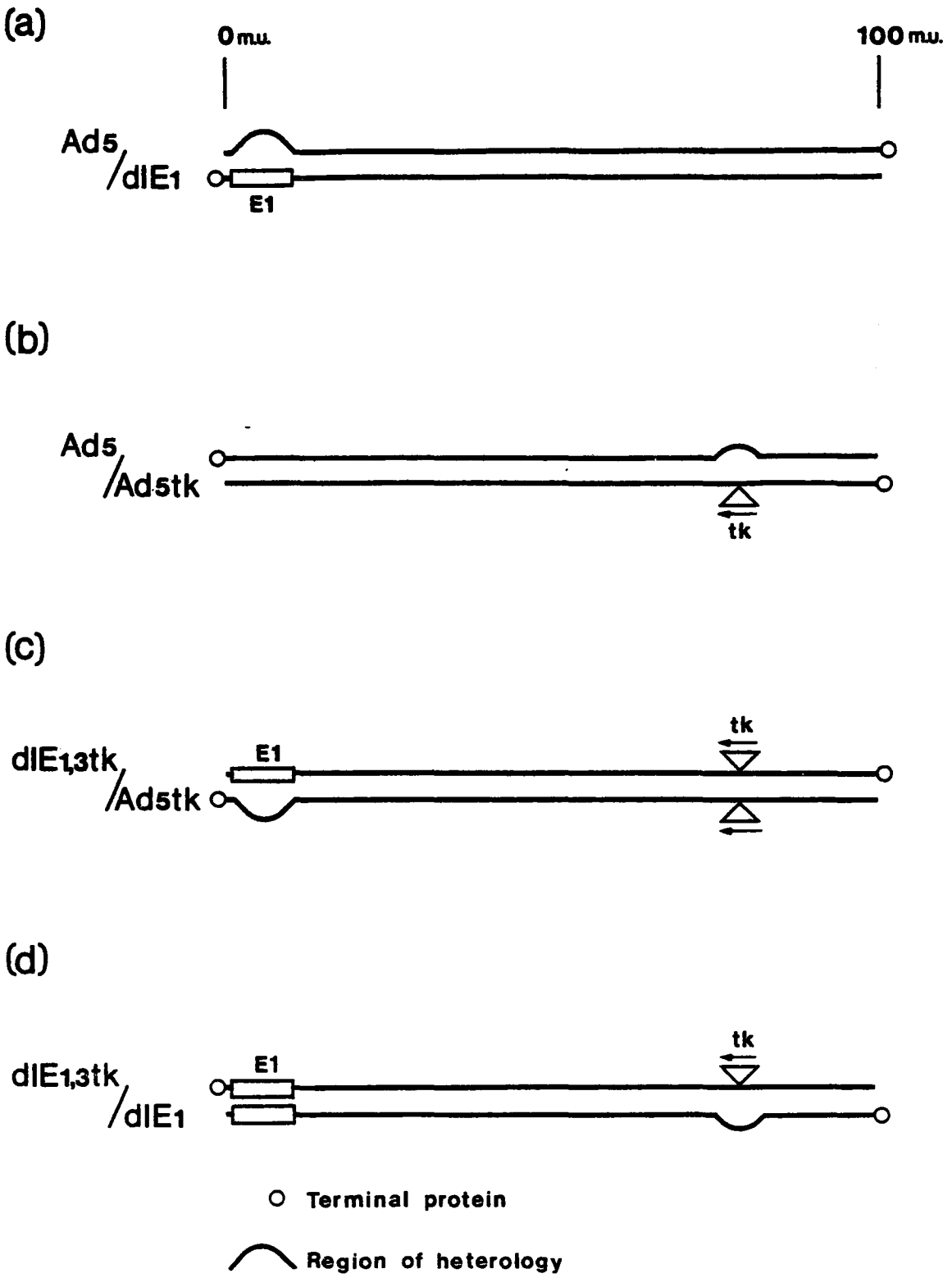
Figure 9 Meselson Radding model of recombination by strand invasion resulting in the formation of adenovirus recombinant heteroduplexes. In each of the four examples illustrated one of the two parental viruses, Ad5 or dlE1,3tk, undergoes replication initiated either at the left ((a) and (c)) or right ((b) and (d)) terminus of the virus. The displaced parental strand interracts with the genome of a nearby virus of the other parental type resulting in the formation of a recombinant heteroduplex. Adapted from Wolgemuth and Hsu (1981).


(a)
0 m.u.
100 m.u.
Ad5
dlE1,3tk
(b)
Ad5
dlE1,3tk
(c)
dlE1,3tk
Ad5
(d)
dlE1,3tk
Ad5
Terminal protein
E1 deletion
Replication fork
tk gene

recombinants should be equally affected by instability due to regions of heterology.

Despite the prediction that "tk" and dlE1 progeny viruses should occur with equal frequency after the mixed infections described above, the experimental evidence does not provide support for this hypothesis. The only hypothesis which is consistent with the experimental results already described is one which states that any putative heteroduplex which has an E1 deletion on one or both strands is more unlikely to give rise to a recombinant progeny than a recombinant molecule in which E1 is intact on both strands. Again referring to Figure 10, in this example involving an Ad5/dlE1,3tk mixed infection, the "tk":dlE1 ratio will depend upon the frequency of occurrence of Ad5/Ad5tk and Ad5tk/dlE1,3tk heteroduplexes versus the number of dlE1/Ad5 and dlE1/dlE1,3tk heteroduplexes. The only apparent factor which correlates with an assymetry in the output of "tk" versus dlE1 recombinants is the absence of E1 DNA on one or both strands in three of the four heteroduplexes formed. The remaining molecule - Ad5/Ad5tk is intact in the E1 region and would generate an Ad5tk recombinant homoduplex in the next round of DNA replication. It is not clear why a heteroduplex which has identical E1 deletions on both strands (such as dlE1,3tk/dlE1 in Fig. 10) would be less viable than a heteroduplex with an intact E1 region (Ad5/Ad5tk in Fig. 10). In the case of the former molecule, E1 DNA is missing from both strands, there is no region of heterology requiring a "looping out" of single stranded DNA, and therefore the stability of the heteroduplex should not be reduced. If however specific DNA sequences within the E1

Figure 10 Putative heteroduplex structures formed by strand invasion recombination during Ad5/dlE1,3tk coinfection of 293 cells. Regions of nonhomology are indicated by curved lines representing looped out regions of DNA.


(a)
0 m.u.
100 m.u.
Ad5
/dlE1
E1
(b)
Ad5
/Ad5tk
tk
(c)
dlE1,3tk
/Ad5tk
E1
tk
(d)
dlE1,3tk
/dlE1
E1
tk
Terminal protein
Region of heterology

region were required in cis during heteroduplex formation or subsequent replication of the heteroduplex, any molecule lacking this region, or having the sequences present as a looped out single strand would perhaps be less viable.

4.3.5 A Summary of Recombination Results

In summary, mixed infections of 293 cells using viruses with Ad5 sequences only, produced variable recombination frequencies. This variation was correlated with interference in the replication of dlE1,3tk by the coinfecting virus. Maximum recombination frequencies (hr1 - 17%; hr6 - 20%) occurred when there was no significant interference. Mixed infections of 293 cells by dlE1,3tk and chimeric Ad2/Ad5 viruses frequently resulted in reduced or undetectable recombination frequencies. This reduction or lack of recombination could be correlated with two characteristics: 1. a small number (seven) of mismatched base pairs close to the left terminus of the viruses; and 2. interference in the replication of dlE1,3tk by VpKGO-13S, and by pm975. Finally, the reduced frequency of occurrence of dlE1 recombinant viruses may reflect a requirement for E1 sequences in cis, and/or differences in the relative size and position of major regions of heterology in the genomes of recombinant adenoviruses.

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