ROLE OF ITRs IN THE PRESERVATION OF THE ENDS OF ADENOVIRUS

THE ROLE OF TERMINAL REPEAT SEQUENCES IN THE

PRESERVATION OF THE ENDS OF THE ADENOVIRUS GENOME

By

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A Thesis

Submitted to the School of Graduate Studies

in Partial Fulfillment of the Requirements

for the Degree

Master of Science

McMaster University

March, 1989

MASTER OF SCIENCE (1989)
(Biology)

McMASTER UNIVERSITY
Hamilton, Ontario

TITLE:            The Role of Terminal Repeat Sequences in the
                  Preservation of the Ends of the Adenovirus Genome

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NUMBER OF PAGES:  xiii, 159

## ABSTRACT

The requirement for identical inverted terminal repeats (ITRs)
for viral viability and the role of internal viral sequences in the
specification of the sequences of the termini were investigated. The
viral strains used in this study were a variant Ad2 strain Ad2 (mac) and
the wild type Ad5 strain which was very similar to the former one in
sequence except at the extreme end of the terminal repeat. A hybrid
virus (sub54), obtained by recombination between Ad2 (mac) and Ad5,
derived the left 41-51% of its genome from Ad2 (mac) and the right 59-49%
from Ad5. The identity of the termini was determined by Southern
blotting analysis using $^{32}$P end labeled oligodeoxynucleotides. Analysis
of the sub54 isolate indicated that both Ad2 (mac) and Ad5 ITRs were
present. Plaque purification of sub54 demonstrated that viruses with non
identical terminal sequences were viable and allowed their
characterization. This analysis also indicated that Ad5 ITRs are
converted to Ad2 (mac) ITRs possibly as a result of repair of the ends to
yield viruses with identical termini. A model involving replication and
emphasizing the importance of panhandle formation as a replicative
intermediate is proposed. These results also indicated a possible role
of the internal sequences of adenovirus in the selection and maintenance
of serotype specific ITRs. The preference for Ad2 (mac) termini observed
during repair of the ends of sub54 may be related to the origin of the

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genes coding for the adenoviral polymerase and/or the terminal protein both of which were derived from Ad2 (mac). Further investigation would be required to determine whether these replicative proteins are actually involved in ITR conversion.

Transformation of Escherichia coli with a DNA preparation from sub54 infected rat embryo cells resulted in the isolation of the plasmid pFG154. This plasmid contained the entire adenovirus genome with an Ad2 (mac) ITR at the "left" terminus covalently linked to an Ad5 ITR at the "right". Analysis of the viral progeny generated upon transfection of mammalian cells with pFG154 indicated that the Ad2 (mac) ITRs were very efficiently converted to Ad5 termini. These results, although apparently contradictory to those initially obtained from the plaque purification of sub54, may be explained by an ITR repair model which is specific for infectious circles.

## ACKNOWLEDGEMENTS

I would like to thank my supervisor, Dr. F.L. Graham, for his support and advice throughout the course of this study as well as the other members of my graduate committee, Dr. S. Bacchetti and Dr. L. Prevec, for their help, constructive comments, and availability.

I am grateful to the Medical Research Council of Canada for providing financial support throughout most of my graduate studies. Special thanks to Pamela Brinkley, Gary Wilson, Joel McGrory, Dody Bautista, Frank Lee, Xiankun Bao, John Rudy, and Dr. Mary Hitt for technical advice, friendship and cheerful discussions. I am also indebted to Dr. Mary Hitt for proofreading this thesis.

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## INTRODUCTION

## RESULTS

## ABBREVIATIONS

| | |
|---|---|
| $\gamma^{32}P$-ATP | gamma $^{32}P$ adenosine triphosphate |
| 2—5, 5—2 | virus with Ad2 (mac) ITR at left end and Ad5 ITR at right end (vice versa for 5—2) |
| 5—5, 2—2 | virus with homologous Ad5 or Ad2 (mac) ITRs |
| Ad | adenovirus |
| Ad2 (mac) | adenovirus type 2 (strain "mac") |
| Adpol | adenoviral polymerase |
| A,T,G,C | the nucleotides of adenosine, thymidine, guanosine, and cytidine, respectively (in DNA) |
| ATP | adenosine triphosphate |
| bp | base pair(s) |
| Ci | curies |
| CPE | cytopathic effect |
| DBP | DNA binding protein |
| dCMP | deoxycytidine monophosphate |
| ddH$_2$O | deionised distilled water |
| dl | deletion |
| DNA | deoxyribonucleic acid |
| ds | double stranded |
| E1, E2, ... | early region 1, 2, ... |
| EDTA | ethylenediamine tetracetic acid |
| EtBr | ethidium bromide |
| HeBS | Hepes Buffered Saline |
| Hepes | N-2-hydroxyethylpiperazine-N'-2-ethanesulphonic acid |
| ITR(s) | inverted terminal repeat(s) |
| KAc | potassium acetate |
| kb | kilobase pairs |
| kDa | kilodaltons |
| L1, L2, ... | late region 1, 2, ... |
| M | molar |
| MEM | minimal essential medium |
| MgAc | magnesium acetate |
| mM | millimolar(s) |
| mmol | millimoles |
| N | any nucleotide (A, G, T, or C) |
| NaAc | sodium acetate |
| NFI, NFII, NFIII | nuclear factor I, II, III |
| PBS | phosphate buffered saline |
| PBS$^{--}$ | PBS without magnesium and calcium |
| pmoles | picomoles |
| pTP | terminal protein precursor |
| RNA | ribonucleic acid |
| SDS | sodium dodecyl sulphate |

| | |
|---|---|
| ss | single stranded |
| SSC | saline sodium citrate |
| SSPE | saline sodium phosphate EDTA |
| TAE | tris acetate electrophoresis buffer |
| $T_H$ | hybridization temperature |
| TP | terminal protein |
| Tris | tris (hydroxymethyl) aminomethane |
| Tris Ac | Tris acetate |
| UV | ultraviolet radiation |

# INTRODUCTION

## 1.1 Adenoviruses

### 1.1.1 Classification

Since the discovery of adenoviruses in 1953 by Rowe et al. and in 1954 by Hilleman and Werner, forty one distinct antigenic types of human adenoviruses have been identified (Kasel, 1979). They are associated with a variety of diseases, primarily respiratory, ocular, and gastrointestinal. A number of adenoviruses which infect other animal species are also known (Ishibashi and Yasue, 1984) and belong, with the human adenoviruses, to the Adenoviridae family which is composed of two genera: 1) Mastadenovirus (adenoviruses infecting mammals) and 2) Aviadenovirus (adenoviruses with avian hosts). These are further subdivided on the basis of the adenoviral hosts (ex.: ovine, bovine, porcine, murine, fowl, turkey, pheasant adenoviruses) (Ishibashi and Yasue, 1984). There may also be adenoviruses growing in poikilotherms which would represent a group distinct from the above two (Ishibashi and Yasue, 1984).

Numerous classification schemes exist for human adenoviruses including classifications based on hemagglutination properties (Rosen, 1960), oncogenicity (Green, 1970), restriction cleavage patterns (Wadell et al., 1980), molecular weight of the polypeptides V, VI and VII (Wadell, 1979), GC content (Piña and Green, 1965) and DNA homology (Green

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## TABLE 1.1:  CLASSIFICATION OF ADENOVIRUSES

| Sub-genus | Species | DNA Homo-logy (%)[a] | G+C (%) | Number of *SmaI* frag-ments | Apparent molecular weight of the major internal polypeptides V | VI | VII | Hem-agglu-tination pattern[c] | Oncogenicity in newborn hamsters |
|---|---|---|---|---|---|---|---|---|---|
| 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 (1980)

et al., 1979). Table 1.1 shows an overall classification of human adenoviruses.

### 1.1.2  Structure of the Genome

Adenoviruses are non-enveloped viruses containing double-stranded (ds) linear DNA (van der Eb and van Kesteren, 1966; Green et al., 1967; van der Eb, van Kesteren, and van Bruggen, 1969; Younghusband and Bellett, 1971) with an icosahedral capsid (Nermut, 1984). The DNA is closely associated with virus-coded proteins (Laver et al., 1967, 1968; Maizel, White, and Scarff, 1968; Prage et al., 1968, 1970) and assumes a nucleosomelike configuration (Mirza and Weber, 1982). All human and non-human serotypes studied so far share the same general genetic organization (Sussenbach, 1984). They have a molecular weight of 16–30 X $10^6$ (Ishibashi and Yasue, 1984; Sussenbach, 1984), contain five early transcription regions (E1, E2a, E2b, E3, and E4), which are expressed prior to DNA synthesis, and five late transcription blocks (L1-L5) expressed following initiation of replication (Sussenbach, 1984). Utilization of both strands, translation of the three coding frames, and differential splicing allow an economical and efficient use of the coding capacity of the virus (Fig. 1.1).

The 5' end of each DNA strand of adenoviruses has a covalently attached terminal protein (TP) (Robinson, Younghusband, and Bellett, 1973; Robinson and Bellett, 1974) which is encoded by the L strand (bottom strand in Fig. 1.1) of the E2b region of the viral genome (Stillman et al., 1981). The adenovirus genome has another very interesting feature:  the presence of inverted terminal repeats (ITRs)

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Figure 1.1: Transcription Map of Group C Adenoviruses. The genome is
divided into 100 map units. The DNA strands designated R
and L are transcribed rightward and leftward respectively.
Three classes of messenger RNAs are depicted based on the
kinetics of their appearance during lytic infection. RNAs
shown in bold lines are detected early in infection
(before the onset of DNA replication), RNAs in double-
lined arrows are late RNA species (detected only after
the onset of DNA replication), and RNAs in single light
lines are synthesized at early as well as at late times.
The capped 5' ends of the cytoplasmic RNAs indicate the
positions of transcriptional promoters while the
arrowheads represent the 3' polyadenylation sites. Gaps
in arrows indicate intervening sequences, which are
removed from the RNAs by splicing. The proteins
translated from these RNAs are designated by their
molecular weights in kilodaltons or by Roman numerals
(virion components). Reprinted from Sussenbach (1984).
Not shown is the 120 kDa open reading frame extending from
24.1 map units to 14.2 map units in E2b and coding for the
adenoviral polymerase. The 87 kDa open reading frame (E2b)
corresponds to the 80 kDa terminal protein precursor
(section 1.2.2).
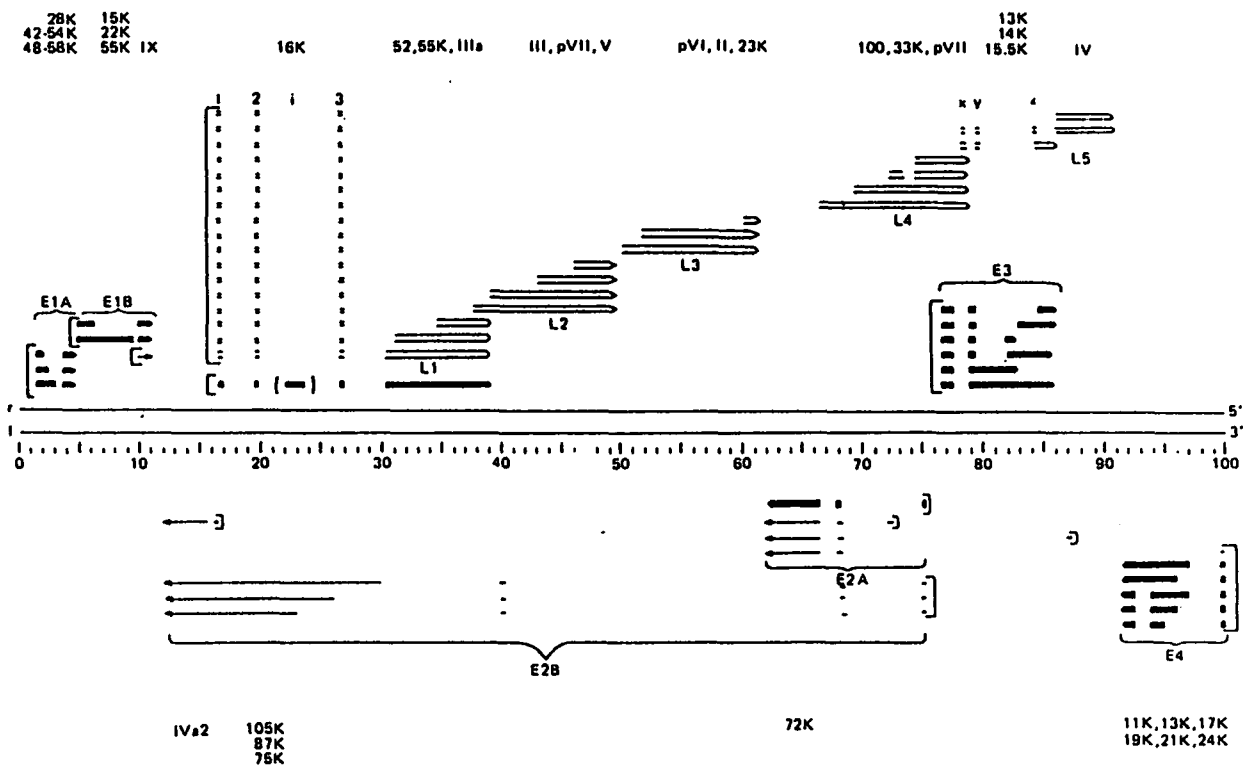
ranging from 102-165 base pairs (bp) depending on the serotype of the virus (Kelly, 1984; Sussenbach, 1984). Although most serotypes only have one type of repeats, different isolates of some of the serotypes may have different, yet similar, repeats (Kelly, 1984; Graham, personal communication).

## 1.2 Replication

### 1.2.1 Models of Replication

Lechner and Kelly (1977) proposed the following widely accepted model of replication (Fig. 1.2). Replication of the double-stranded genome is initiated at either ITR by protein priming (see section 1.2.2) and is semi-conservative. As replication proceeds from one end of the genome to the other the duplicated parental strand is displaced and is subsequently used as a template for the second round of replication. The mechanism of initiation for the second round of replication at the 3' end of the displaced strands remains unclear. Daniell (1976) proposed that the two ITRs of the displaced single-stranded molecule might hybridize together and form a panhandle structure. Such a structure would have an end identical to the ones found in the double-stranded genome thus allowing initiation of replication to proceed by the same mechanism. The existence and use of the panhandle is mechanistically attractive but only theoretical at present since no experimental proof has been established. Furthermore the presence of a mechanism of initiation of replication involving single-stranded 3' ends as templates has not been ruled out.

Other models of replication were also proposed, none of which are fully satisfactory. A model which relies on the formation of hairpin

Figure 1.2:  Model of Replication of Adenovirus (Lechner and Kelly).

Bold lines indicate DNA synthesis, whose direction is

indicated by arrows, and light lines, parental strands.

Types I and II represent replicative intermediates whereas

the bracketed molecules are theoretical and may not

exist. The presence of the terminal protein at the 5'

end of each strand is shown by a closed circle. See text

for details. Adapted from Lechner and Kelly (1977).

TYPE I

TYPE II
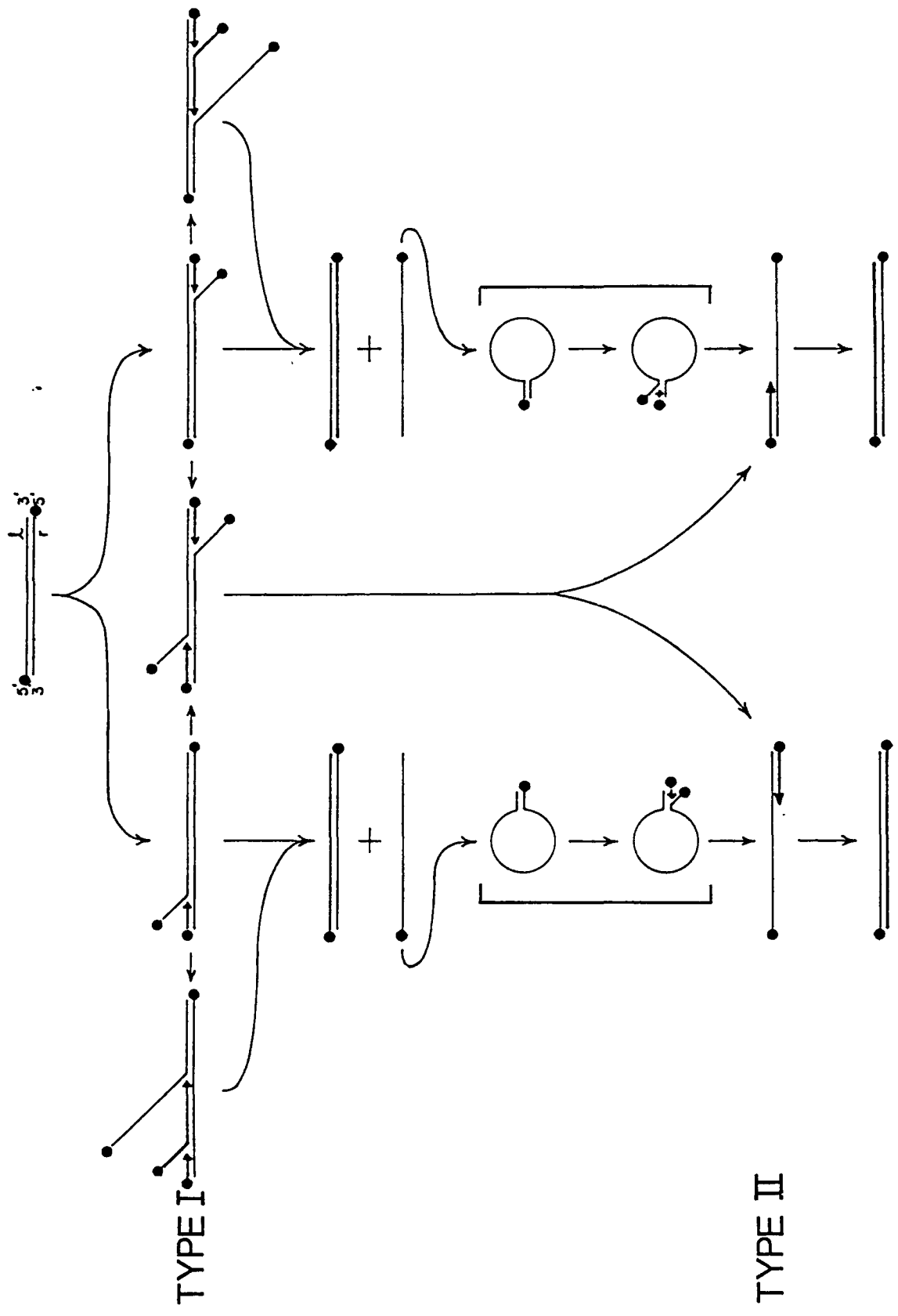
structures by base pairing of putative palindromic sequences at the ends of adenovirus has been considered (see Rekosh et al., 1977) but later ruled out since sequencing data of the ITRs of numerous adenovirus serotypes has shown no palindromic sequences present at the ends (Fütterer and Winnacker, 1984; Kelly, 1984).

Another model has been proposed which relies on initiation of replication by a RNA primer (Bellett and Younghusband, 1972). The authors have suggested that a protein present in infected cells binds to two replicating viral molecules simultaneously and brings them together such that the 3' end of one molecule could be used as a primer to fill in the gap, caused by the removal of the RNA primer, in the other molecule. This would result in the concatemerization of the genomes. The absence of replicating molecules longer than unit length (Bellett and Younghusband, 1972) rules out this model and any other models of replication which rely on a concatemerization mechanism.

Other models invoke the formation of a circular template and are supported by the discovery of the TP-DNA complex at both ends of the genome. Robinson et al. (1973) and Robinson and Bellett (1974) first suggested a rolling circle model in which TPs produce covalently closed circles by joining the two ITRs through their ability to bind to both ends of the genome and to each other. This model is unlikely to be true since there is no evidence for the existence of replicating rolling circles either by electron microscopy studies (Ellens, Sussenbach, and Jansz, 1974; Lechner and Kelly, 1977; Revet and Benichou, 1981) or by analyses of the replicative intermediates of adenovirus by velocity and density sedimentation (Bellett and Younghusband, 1972).

A second model involving the formation of covalently closed molecules replicating according to the rolling circle model was also proposed by Pearson et al. (1981). This model is improbable since it relies on replicating circular templates for which no evidence has been found.

More recently Friefeld et al. (1984) has suggested a model where the TPs from the two ends of adenovirus cause the formation of non covalently closed circles. Following initiation, the polymerase would proceed only to the end of the strand. The displaced parental strand could form a panhandle structure from which replication could be initiated. Such model is consistent with the current data and with the Lechner and Kelly (1977) model of replication of adenovirus.

## 1.2.2 Initiation

DNA polymerases exclusively synthesize DNA in the $5' \rightarrow 3'$ direction from the 3' hydroxyl group of a primer which is base paired to the template being copied (Weissbach, 1975). This primer is RNA in many replication systems. In eukaryotic genomic replication, after priming, the RNA is removed by cellular nucleases and replaced by DNA to complete the replication of the genome. In the replication of circular genomes removal of the RNA primer poses no problems since the DNA polymerases can continue synthesis up to and beyond the point at which priming occurred (Fig. 1.3). However in the replication of linear genomes, removal of the RNA primer from the 5' ends of newly synthesized strands results in the loss of several nucleotides which cannot be replaced by DNA polymerases (Fig. 1.3). Absence of an appropriate mechanism of duplication of those

Figure 1.3:   DNA Synthesis Using a RNA Primer.  Replication of circular

(panel A) or linear DNA genomes (panel B) using a RNA

primer are shown.  The parental strands are indicated by

light lines, newly synthesized strands by bold lines, and

the RNA primer by an asterisk.  See text for details.

A
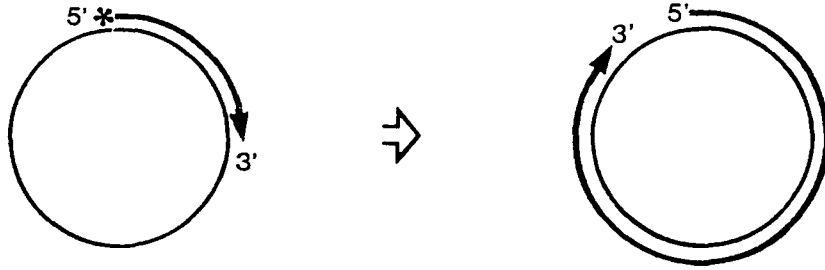


B

sequences would result in the shortening of the genome at each replication cycle and eventually eliminate the entire coding sequences (Watson, 1972).

Viruses have exploited a number of ways to solve this problem such as the formation of concatemers (e.g., bacteriophage T7 (Watson, 1972)), hairpin structures (e.g., Parvovirus (Hauswirth, 1984)) or circularization of the genome (e.g., phage lambda (Tomizawa and Ogawa, 1968)). Extension of telomeric sequences by a terminal transferase-like activity has also been reported for Tetrahymena (Greider and Blackburn, 1985).

A different mechanism has been proposed for priming adenovirus DNA synthesis which Rekosh et al. (1977) have named "protein priming" (Fig. 1.4). During initiation of replication the 80 kDa terminal protein precursor (pTP) is covalently coupled by a phosphodiester bond from the $\beta$ hydroxyl group of its serine at position 577 to the 5' hydroxyl of deoxycytidine monophosphate (dCMP) (Desiderio and Kelly, 1981). The "initiation complex" pTP-dCMP, non covalently coupled to the DNA template, primes replication of the linear genome with the dCMP constituting the first nucleotide of the new strand (Fig. 1.4) (Rekosh et al., 1977; Stillman and Bellett, 1979; Challberg, Desiderio, and Kelly, 1980; Challberg, Ostrove, and Kelly, 1982). Elongation follows with chain synthesis to the end of the genome. Processing of the pTP into its 55 kDa mature form (TP) is not required for DNA replication and is done late in the infection cycle by a virus coded protease (Challberg and Kelly, 1981). This mechanism for initiation of replication is particularly compelling since all human serotypes have a dCMP at the 5'

Figure 1.4:  Priming of Adenovirus Replication.  The details of the
             initiation of replication can be found in the text.
             Sequences at one end of the double-stranded adenoviral
             genome are shown (e.g., Ad5, Ad2).  The 55 kDa terminal
             protein and its 80 kDa precursor (pTP) are represented by
             short and long rectangles respectively.  'Ser' designates
             the serine residue of TP/pTP to which the deoxycytidine
             triphosphate 'pppC-OH' is coupled prior to or following
             association of pTP with the DNA template (pTP + dCTP ->
             pTP-dCMP).  Elongation, subsequent to initiation, and
             strand displacement are also shown.  Reprinted from
             Challberg et al. (1980).
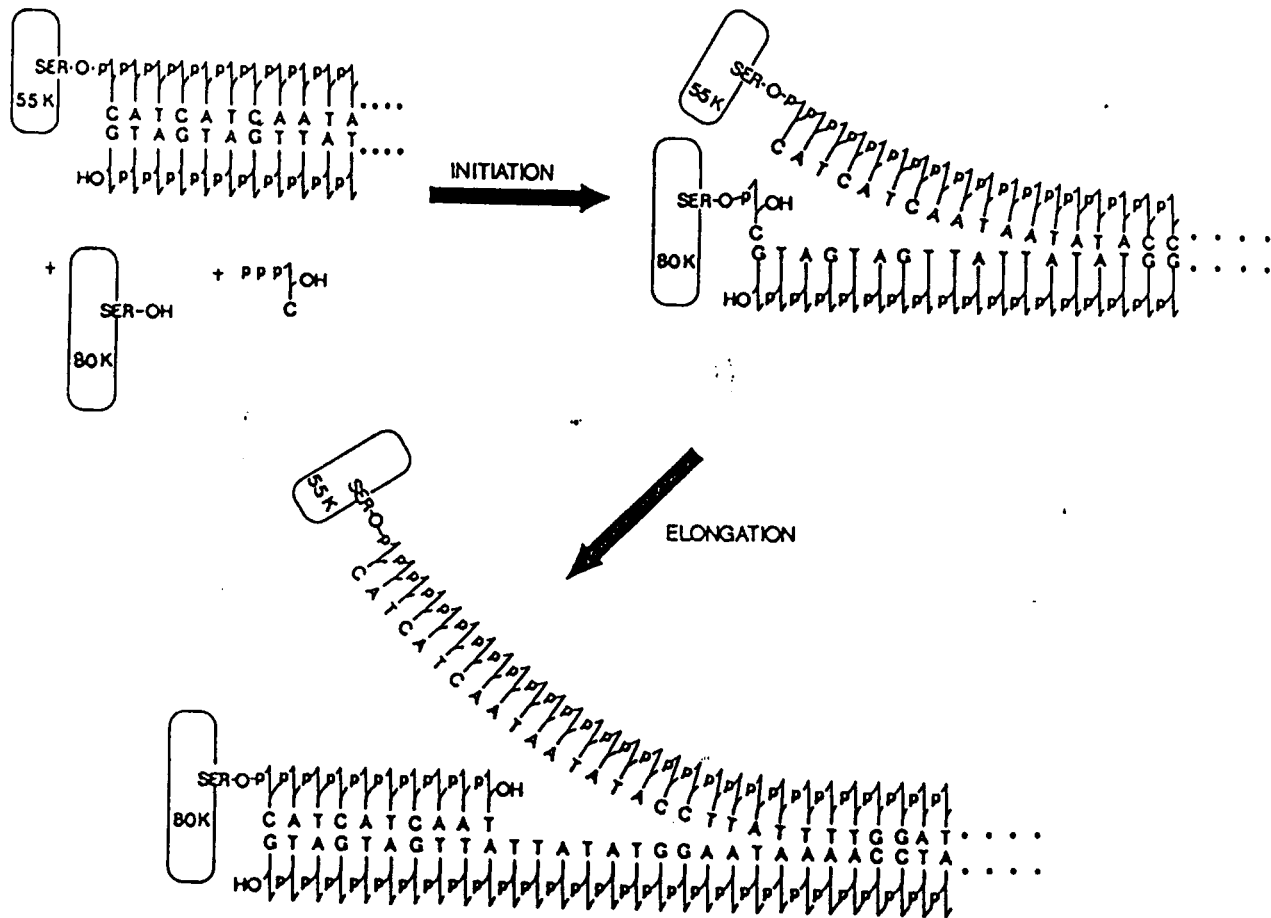
end of each strand (Fütterer and Winnacker, 1984; Kelly, 1984).

An in vitro replication assay developed by Challberg and Kelly (1979) that allows both initiation and elongation has been a very useful tool for the understanding of the details of the replication of adenovirus. It has permitted the identification of factors, both cellular and viral, that play an important role in initiation and/or elongation during DNA synthesis. Using this in vitro replication system it was determined that the formation of the pTP-dCMP complex is dependent on the presence of adenosine triphosphate (ATP; De Jong et al., 1983). It was also shown that there is no absolute need for the presence of a TP on the parental strand but that its presence enhances both replication efficiency and infectivity (Sharp, Moore, and Haverty, 1976; Stillman et al., 1981; Challberg et al., 1982; Tamanoi and Stillman, 1982; Rijnders et al., 1983a; van Bergen et al., 1983a; Guggenheimer et al., 1984b). The partial removal of TP by proteinase K (Lichy, Horwitz, and Hurwitz, 1981; Tamanoi and Stillman, 1982) or pronase (Tamanoi and Stillman, 1982) inhibits the pTP-dCMP formation. This inhibition was attributed by Tamanoi and Stillman (1982) to the presence of residual amino acids attached to the ITRs since complete removal of TP with piperidine restores the ability of the DNA template to support replication. Consistent with these observations is the fact that linearized plasmids containing adenoviral terminal sequences (no TP) are substrates for replication but are not used as efficiently as TP-linear DNA. The role that TP from the parental DNA plays in replication is unknown. Rekosh et al. (1977) have suggested that TP is involved in the formation, positioning and/or stabilization of the initiation complex (adenoviral

polymerase-pTP-dCMP) on the DNA template. In any case more work is required to elucidate the function of TP in the replication of adenovirus.

A second viral gene product, N protein, is required for the formation of the pTP-dCMP complex. The 140 kDa N protein, also referred to as the adenoviral polymerase (Adpol), catalyzes the initiation step of the replication cycle (Lichy et al., 1982; Stillman, Tamanoi, and Mathews, 1982a; Friefeld et al., 1983b; van Bergen and van der Vliet, 1983b). Adpol is encoded by the E2b region of the adenovirus genome (Stillman et al., 1982a) and was shown to complex with pTP (Enomoto et al., 1981). It is distinct from all known cellular polymerases on the basis of its sensitivity to aphidicolin, dideoxy nucleotides, N-ethylmaleimide, NaCl and 1-$\beta$-D arabinofuranosyl cytosine triphosphate as well as its template preference (Ikeda et al., 1980; Enomoto et al., 1981; Kowalski and Denhardt, 1982; Lichy et al., 1982; Ariga, 1983; Pincus and Rekosh, 1984).

In addition to the viral gene products mentioned above, the efficient initiation of adenovirus replication requires cellular factors. To date three such factors, isolated from uninfected HeLa cells, have been identified: Nuclear factors I and III (NFI and NFIII) and origin recognition protein A (ORP-A) (Enomoto et al., 1981; Nagata et al.,1982; Rosenfeld and Kelly, 1986). Nuclear factor I is a 47 kDa protein that enhances the initiation of replication of Ad2 by a factor of 10 (Nagata et al., 1982). This activation is dependent upon the presence of a specific DNA sequence in the template (Leegwater, van Driel and, van der Vliet, 1985; Guggenheimer et al., 1984b; Rawlins et al., 1984) to which

NFI specifically binds (Nagata et al., 1983b; de Vries et al., 1985; Leegwater et al., 1985; Schneider et al., 1986). The NFI binding site is not present in the genomes of all adenovirus serotypes nor is it exclusive to adenovirus. For instance, the replication of Ad4, which carries no NFI binding site, is not enhanced by NFI (Hay, 1985b). Gronostajski et al. (1984, 1985) have identified NFI binding sites in human genomic DNA and nuclear factor I activity has also been detected in porcine liver and Saccharomyces cerevisiae by Schneider et al. (1986). Nuclear factor I has no detectable nuclease, RNA or DNA polymerase, ATPase or topoisomerase activity (Nagata et al., 1982). Despite the absence of any known activity for NFI, it was proposed that NFI facilitates the interaction between the DNA template and the proteins involved in initiation of replication. This facilitation may occur by increased binding or stabilization of the proteins, by unwinding the DNA, or by altering DNA conformation in some other way (Leegwater et al., 1985; Rosenfeld and Kelly, 1986). Further investigation is required to elucidate its exact role and mode of action.

Nuclear factor III is another cellular factor that increases the efficiency of the initiation process (Pruijn, van Driel, and van der Vliet 1986; Pruijn et al., 1987; O'Neill and Kelly, 1988). It also binds to a specific DNA sequence present in adenovirus (Pruijn et al., 1987), is identical to ORP-C, a factor independently identified by Rosenfeld et al. (1987), and may be the same as the nuclear factor A1 (NF-A1) reported by Singh et al. (1986). It has an apparent molecular weight of 92 kDa by SDS-polyacrylamide gel electrophoresis and 78 kDa in sucrose gradients (O'Neill and Kelly, 1988). Apart from its role in the

replication of adenovirus, NFIII may be involved in the transcriptional control of a number of genes, since binding to several eukaryotic promoter and enhancer elements, such as the histone 2B, immunoglobulin's light and heavy chains and U1 and U2 small RNAs, has been demonstrated (Pruijn et al., 1987).

The third cellular factor involved in the initiation of replication of adenovirus is ORP-A, which Rosenfeld et al. (1987) have shown binds specifically to sequences present within the first twelve base pairs of adenovirus. ORP-A is required, along with NFI and NFIII, for optimal initiation of replication.

In addition to the cellular and viral gene products described, the replication of adenovirus is dependent on particular sequences in the DNA template i.e., the origin of replication, most of which, if not all, are contained within the ITRs (Fig. 1.5). A number of investigators (Enns et al., 1983; Tamanoi and Stillman, 1983a; Challberg and Rawlins, 1984; Rawlins et al., 1984; Hay, 1985a; Hay and McDougall, 1986; Wides et al., 1987) have shown by mutational analyses that the origin is contained within the first 40-67 bp at both ends of the genome. The origin has been further subdivided into a minimal essential origin of replication (nucleotides 1-18) and a complete origin of replication (nucleotides 1-40 or 1-67 depending on the investigator).

Nucleotides 9-18 are conserved among all human serotypes and are partially conserved in non-human serotypes (Rijnders, 1983b). This sequence contains part or all of the binding sites for pTP-Adpol (Rijnders et al., 1983b), for ORP-A (Rosenfeld et al., 1987), and possibly for other proteins.

Figure 1.5:   Origin of Replication of Adenovirus.  A complete
inverted terminal repeat (103 bp for Ad2 or Ad5) is shown
along with the minimal essential and complete origin of
replication.  The numbers below the ITR represent the
distance, in base pairs, from the end of the genome.
Black boxes depict sequences conserved among human
adenoviruses which may also be found elsewhere in the
genome or whose positions in the ITRs vary depending on
the serotype (with the exception of the conserved
sequence 'ATAATATACC' (9-18)).  Brackets delimit the
known binding sites of proteins which bind to the origin
of replication.  Dashed lines reflect the variation of
the boundaries of those binding sites (and complete
origin of replication) reported by different authors.
Covalent attachment of the terminal protein at the 5' end
of the genome is also shown.  See text for details.
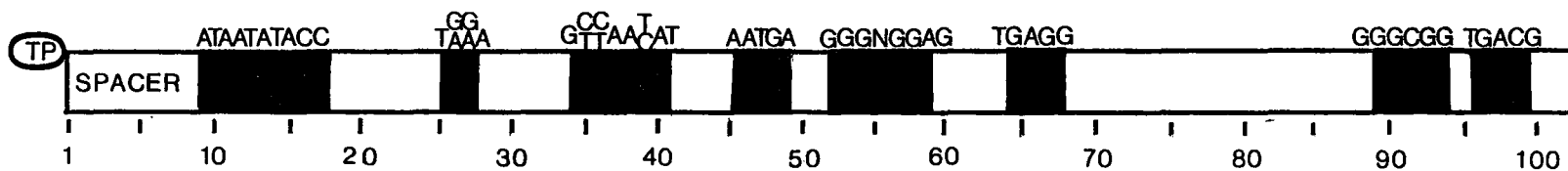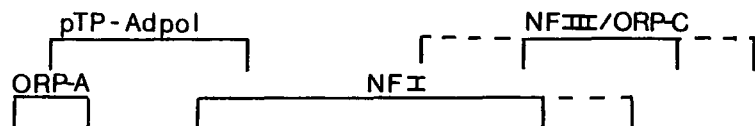
Point mutations of nucleotides 1 to 8 do not affect replication indicating that their exact sequence is not important (Enns et al., 1983; Rawlins et al., 1984). Their role may be to provide the appropriate spacing between the conserved decamer 9-18 and the end of the genome as suggested by Stillman, Topp, and Engler, (1982b) based on in vitro replication studies of a number of serotypes. In that investigation, they compared replication of adenovirus types 2, 4, 7, 9 and 31 which differ in their terminal DNA sequences except for the conserved sequences 9-18. All five serotypes could replicate in vitro in the presence of an Ad2 infected nuclear extract thus indicating that the sequences 9-18 are indeed a sufficient minimal origin of replication. This result is consistent with the idea of a "spacer element" spanning nucleotides 1 to 8 since this region is different among the serotypes used. In contrast, the mouse Adf1 and human Ad2 genomes share the first 17 nucleotides but the Adf1 infected nuclear extract cannot substitute for the Ad2 extract, and vice versa, in in vitro replication assays suggesting that the eighteenth nucleotide is part of the minimal adenovirus origin (Lally et al., 1984).

The sequences spanning nucleotides 19 to 42 (Rawlins et al.,1984; Leegwater et al., 1985) or 48 (Nagata et al., 1983b; Guggenheimer et al., 1984c) and 34-41 to 51-56 (Pruijn et al., 1986; Rosenfeld et al., 1987; O'Neill and Kelly, 1988) are known to be the binding sites for NFI and NFIII respectively. The importance of those sequences in the optimal origin of replication can be accounted for by the stimulation of adenovirus DNA replication by both NFI and NFIII.

Stow (1982), Tamanoi and Stillman (1982, 1984), Guggenheimer et

al. (1984a), and Leegwater et al. (1985) have shown the importance of the proximity of the origin to the end of the genome by demonstrating that replication of plasmids containing an adenovirus ITR required cleavage of the plasmids such that the ITR was present at or near the end of the molecule. Hay, Stow, and McDougall (1984) reported the in vivo replication of minichromosomes with internal ITRs when cotransfected with helper wild type Ad2 thus suggesting that internal origins of replication are active. However, the replication efficiency of these minichromosomes with internal origins of replication is considerably reduced compared to that of the same minichromosomes linearized such that the ITRs are near the ends. Furthermore the authors could not rule out recombination between the helper Ad2 and the minichromosomes, generating minichromosomes with terminal ITRs. Their results therefore indicate that internal origins are at most a poor substrate for initiation of replication, which is consistent with the idea that the origin must to be near the end of the genome to be fully functional.

Although the origin of replication has been delimited to 1 to 40-67, the ITRs contain other sequences that are completely or partially conserved (Stillman et al., 1982b; Tamanoi and Stillman, 1983b; Fütterer and Winnacker, 1984; Hay and McDougall, 1986). Such sequences include 'GGGCGG' found in multiple copies in all human serotypes as well as simian and equine adenoviruses, 'GGGNGGAG' also found in multiple copies, and the sequence 'TGACG' present in all human serotypes at or near the internal border of the ITRs. Also conserved, the sequences 'AATGA' ('AATAA' in Ad4), 'G(C/T)(C/T)AA(T/C)AT', and 'T(G/A)(G/A)A' (absent in Ad4) are found once in the ITRs of all human serotypes (Fig. 1.5). Their

roles, if any, in replication are unknown at the moment.

These are the known requirements for the efficient initiation of replication of adenovirus. It has been suggested that RNA (van der Vliet, van Dam, and Kwant, 1984) and the cellular transcriptional factor Sp1 (Schneider et al., 1986) may also be factors needed for optimal replication of adenovirus. Further fractionation of in vitro replication systems should reveal other requirements, if any.


### 1.2.3 Elongation

Once the initiation complex is formed and positioned on the DNA template, elongation proceeds at an approximate rate of 1700 nucleotides/min (Bodnar and Pearson, 1980) producing full length viral genomes. The synthesis of DNA is semi-conservative (Bellett and Younghusband, 1972) and results in the displacement of the strand that is not being used for template (Lechner and Kelly, 1977).

Pulse and pulse-chase experiments revealed the presence of molecules, interpreted as replicative intermediates, with an increased sedimentation rate in sucrose gradients and an increased buoyant density in cesium chloride gradients (Pearson and Hanawalt, 1971; Sussenbach et al., 1972; van der Eb, 1973; Schilling, Weingärtner, and Winnacker, 1975). The presence of replicative intermediates was confirmed by their sensitivity to ss DNA nucleases (Pettersson, 1973; Robin, Bourgaux-Ramoisy, and Bourgaux, 1973) and by analysis on benzoyl-naphthoyl-diethylaminoethyl (BND-DEAE) cellulose columns (Sussenbach et al., 1972; Robin et al., 1973). Two types of replicative intermediates have been observed by electron microscopy (Ellens et al., 1974; Lechner and Kelly,

1977) (Fig. 1.2). Type I intermediates are full length double-stranded molecules with single-stranded branches of variable sizes and at different positions along the DNA template. These intermediates presumably result from the progressive displacement of strands from the replicating ds DNA templates. Type II molecules, in which part of the genome is single-stranded and part is double-stranded, are presumably generated by initiation and elongation at the 3' end of the displaced strands. Together types I and II molecules constitute the majority of replicative intermediates. A small proportion of molecules with the characteristics of both types I and II replicative intermediates were also observed.

In electron microscopy studies it is possible to distinguish the two ends of adenovirus after partial denaturation because the right end contains AT rich stretches (Doerfler and Kleinschmidt, 1970). In this way, Lechner and Kelly (1977) determined the number of molecules replicating in either direction (left to right or vice versa). They found an equivalent quantity of molecules replicating in each direction indicating that replication can initiate at both ITRs at approximately the same frequency. These findings are identical with those obtained by the electron microscopy studies conducted by Revet and Benichou (1981). Pulse labeling adenovirus DNA and isolation of mature adenovirus genomes (Schilling et al., 1975; Tolun and Pettersson, 1975; Sussenbach and Kuijk, 1977) or replicative intermediates (Kowalski and Denhardt, 1982) indicated that the specific radioactivity of the two terminal fragments were equivalent. These results contradict those of Sussenbach, Ellens, and Jansz (1973) and Ellens et al., (1974) who reported that initiation

only occurs at the right terminus. Lechner and Kelly (1977) attributed this discrepancy to inappropriate conditions (in the latter case) when separating the replicative intermediates from the mature genomes (no precautions were taken to prevent branch migration or reannealing, possibly resulting in the preferential annealing of the single strands at the left terminus over the right).

The minimal DNA template required for efficient initiation of replication supports a maximal rate of elongation (Rawlins et al., 1984) indicating that no additional specific sequences are required for the latter process. Nevertheless, as for initiation, a number of viral gene products are needed, the first and most important being Adpol which synthesizes the nascent strand (van Bergen and van der Vliet, 1983b). The dependence on ATP is not as strict as for initiation since reduced concentrations of ATP have only a slight effect on elongation (De Jong et al., 1983).

The second protein essential for elongation is the viral DNA binding protein (DBP) which, contrary to Adpol, is not needed for efficient initiation of replication (Challberg et al., 1982; Friefeld, Krevolin, and Horwitz, 1983a; Guggenheimer et al., 1984a,b; Prelich and Stillman, 1986). It is encoded by the E2a region of adenovirus (Kruijer, van Schaik, and Sussenbach, 1982) and has a molecular weight of 72 kDa (van der Vliet and Levine, 1973). The 27 kDa N-terminal fragment generated by chymotrypsin digestion was shown to be phosphorylated, unlike the 44 kDa C-terminal fragment which was shown to contain the replicative function of DBP (Klein, Maltzman, and Levine, 1979; Linné and Philipson, 1980). The availability of several temperature sensitive

mutants (for a map of all the available mutants see Prelich and Stillman, 1986) allowed the clarification of the role of this ss DNA binding protein (van der Vliet and Levine, 1973; van der Vliet et al., 1975; van der Vliet, Zandberg, and Jansz, 1977; Fowlkes et al., 1979; Nass and Frenkel, 1980; Schechter, Davies, and Anderson, 1980; Prelich and Stillman, 1986). Mutations that resulted in the loss of binding also resulted in the absence of replication thereby indicating that the two functions are interrelated. Substitution of the adenoviral DBP by host, Escherichia coli or bacteriophage fd single-stranded DNA binding proteins does not support the complete replication of adenovirus (van Bergen and van der Vliet, 1983b; Friefeld et al., 1984). In addition to the ability of DBP to protect ss DNA from nucleases, it was postulated that DBP may alter the configuration of DNA such that the elongation rate is optimal (Süssenbach and van der Vliet, 1983). The analysis of the crystallized carboxy terminal 44 kDa fragment (Tsernoglou, Tucker, and van der Vliet, 1984) may reveal the precise location of the replicative domain.

Elongation requires a cellular factor (NFII) for replication to proceed beyond approximately one third of the genome (Nagata, Guggenheimer, and Hurwitz, 1983a). Replication of the entire genome depends of the presence of both NFII and NFI (Nagata et al., 1982; Nagata et al., 1983a; Friefeld et al., 1984). Nuclear factor II, isolated from uninfected HeLa cells, has a molecular weight between 25 and 45 kDa and is composed of several subunits (Friefeld et al., 1984). NFII does not appear to be a sequence specific binding protein nor does it possess a polymerase, an ATPase or a nuclease activity. However, a topoisomerase I activity has been detected in purified NFII preparations

(Friefeld et al., 1984). Topoisomerase I from calf tissue or HeLa cells
(molecular weight of 100 kDa (Liu and Miller, 1981), which is much larger
than the molecular weight of NFII), can substitute in replication assays
for NFII but topoisomerase I from Escherichia coli cannot (Nagata et al.,
1983a; Friefeld et al., 1984).

The role of NFII in the replication cycle of adenovirus has not
yet been elucidated but the discovery of its topoisomerase I activity
has led to the suggestion that NFII may interact with the replication
complex Adpol-pTP-dCMP-DNA template in the following manner. The link
between Adpol and pTP-dCMP on the nascent strand and/or to TP on the
displaced parental strand may create topological problems as new DNA is
synthesized (Fig. 1.6). The role of NFII may be to alter the
conformation of the template at or near the replication fork and relieve
such topological constraints which may prevent the polymerase from
proceeding (Friefeld et al., 1984; Guggenheimer et al., 1984b). This
could explain why DNA synthesis is blocked after replicating only one
third or so of the entire genome's length in the absence of NFII.

As for initiation, further dissection of the in vitro replication
assays should provide more information about eventual additional
requirements for elongation and also allow a better and more detailed
understanding of the replication process.

## 1.3 Circular Forms of Adenovirus

### 1.3.1 Isolation and Characterization

Although adenovirus has a linear genome and is presumed to

Figure 1.6:   Role of NFII in Replication.  Following initiation,

elongation might proceed by one of three ways.  The

terminal protein from the displaced parental strand might

be bound to Adpol, proceeding along the template,

resulting in the first structure shown.  Alternatively

Adpol may be attached to pTP of the nascent strand (second

structure), or both TP and pTP (third structure).  As

elongation proceeds, the structures may become

increasingly constrained until the polymerase is

completely prevented from moving along the template.

NFII may be able to remove such block and allow Adpol to

resume DNA synthesis.  Reprinted from Sussenbach and van

der Vliet (1983).

Initiation

1

Elongation 2

3
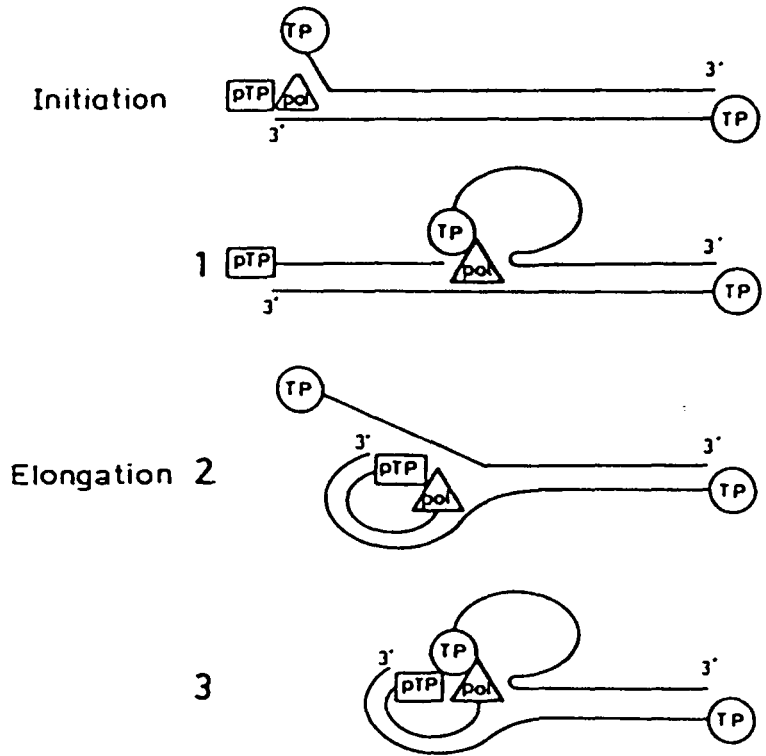
replicate without circularization, circular forms of adenovirus have been identified in which the two ends of the genome are covalently joined head-to-tail (Ruben, Bacchetti, and Graham, 1983). Insertion of a bacterial origin of replication and a resistance gene, usually $\beta$ lactamase, into the adenoviral genome allows such circles to be propagated in Escherichia coli (Graham, 1984a). Those plasmids, when transfected into mammalian cells, generate infectious virions with no detectable circular forms of adenovirus (Graham, 1984a). Hanahan and Gluzman (1984) described the construction of another plasmid containing the entire adenovirus genome and showed that this particular construct is not infectious upon transfection of mammalian cells. This lack of infectivity has been attributed to the presence of bacterial sequences at the junction between the ITRs (Rudy and Graham, personal communication) indicating that covalently linked ITRs joined head-to-tail are required for infectivity. The exact significance of the junction for infectivity of those circles remains to be established.

It is not clear whether the circular forms of adenovirus replicate in a manner similar to linear molecules and their function, if any, in the replication cycle of the virus is also unknown. While the significance of circles in replication is uncertain, they have been proposed to play an important role in the integration process. It is well established that adenovirus can transform non permissive or semi-permissive cells (for reviews see Graham, 1984b; Branton, Bayley, and Graham, 1985). It may do so through a circular intermediate. Although most adenovirus transformed cell lines contain only the left end of adenovirus in their genome, cell lines with all or practically all of

adenovirus are also obtained (Green et al., 1976; Dorsch-Hasler et al., 1980; Ibelgaufts et al., 1980; Kuhlman et al., 1982; Fisher et al., 1982; Ruben, Bacchetti, and Graham, 1982). In some cases, analysis of the integrated viral sequences has revealed that the two ends are joined together (Sambrook et al., 1979; Stabel, Doerfler, and Friis, 1980; Vardimon and Doerfler, 1981; Visser et al., 1981, 1982; van Doren, Hanahan, and Gluzman, 1984) suggesting that circularization may occur prior to integration. Also consistent with the hypothesis that circularization is a prerequisite for integration is the observation that optimal conditions for the production of circles correlate with a high frequency of transformation (Graham, 1984a). Despite these observations, the role of circles in both replication and integration remains to be determined.

Although the role of circles in the life cycle of adenovirus is still unclear, these molecules have proven to be very useful as cloning vectors allowing relatively easy manipulations of the adenovirus genome (Haj-Ahmad and Graham, 1986a; Ghosh-Choudhury et al., 1986) and construction of potential recombinant vaccines (Christie, Graham, and Prevec, personal communication).

## 1.3.2 Regeneration of the Termini

A very interesting feature of the infectious circles resides at the junction formed by the two ends where sequencing data has revealed that a few base pairs are missing at either or both ITRs at the junction (Graham, 1984a; Graham et al., submitted). However, the linear progeny produced after transfection of mammalian cells have complete ITRs

(Graham, 1984a) indicating that restoration of the ITRs has occurred. The timing and mechanism of regeneration of the termini are not known but regeneration of missing base pairs from one ITR can occur through the second ITR by using it as a template, a process known to happen (Hay et al., 1984). Nevertheless this process cannot account for the regeneration of nucleotides missing from both ends of the genome. Therefore a mechanism involving slippage of the replication complex on the DNA template has been proposed (Graham et al., submitted).

## 1.4 Importance of the Inverted Terminal Repeats

The ITRs have been associated with a variety of functions which underline the importance of the exact DNA sequence of the ITRs and the positions of the various binding sites within them. As described above, the ITRs contain the origin of replication of the virus and their strategic position at both ends of the genome allows replication of the terminal sequences by protein priming. The presence of the ITRs also potentially enables the displaced strands produced during replication to assume a panhandle configuration which may be required for initiation of replication of the single-stranded adenoviral intermediates. The circular forms of adenovirus require both the presence of the ITRs and their particular arrangement (covalently linked adjacent ITRs) for infectivity once again indicating that the terminal repeats are indispensable. The regeneration of missing nucleotides in both linear and circular forms strongly suggests that the integrity of the ITRs is important for adenovirus. Finally, their presence and stability among the different serotypes also argue in favour of a significant and vital

role for the termini. The presence of ITRs in numerous and yet very different viruses (Salas, 1983; Gerendasy and Ito, 1987) may indicate that the importance of ITRs is a general feature. This is particularly relevant considering that other viruses, such as Φ29 (Salas, 1983) and PRD1 (Gerendasy and Ito, 1987), have adopted a replication strategy similar to adenovirus which relies on the presence of inverted and terminal repeats.

## 1.5 Problem and Approach

The problem under consideration relates to the ITRs and is twofold. First, granted the importance of and the different roles attributed to the ITRs, it is very reasonable to assume that the presence of exactly identical inverted terminal sequences is essential for adenovirus. It was my interest to determine the validity of this statement and the approach pursued is described in detail in the results section.

Second, the stability of the ITRs in viruses and their precise regeneration from infectious circles suggested that the different adenoviral serotypes may have the ability to specify, to some extent, the DNA sequence of their respective ITRs. I therefore tested the hypothesis that internal viral DNA sequences specify the type of ITRs present at the ends of the genome. If this were true, the serotype of those internal sequences would determine the exact sequence of the termini, ensure that both ITRs are identical, and presumably ensure survival of the virus.

## MATERIAL AND METHODS

### 2.1  Tissue Culture

Human embryonic kidney 293 cells (Graham et al., 1977) were grown at 37°C in Joklik's modified medium supplemented with 10% horse serum. The human cervical carcinoma cell line HeLa was maintained at the same temperature in alpha minimal essential medium ($\alpha$MEM) plus 10% newborn calf serum. All media were supplemented with 0.03% (w/v) L-glutamine, $10^2$ units/ml of penicillin, and $10^2$ $\mu$g/ml of streptomycin (Gibco Laboratories). All sera, purchased from Gibco Laboratories, were heat inactivated for 30 minutes at 56°C.

### 2.2  Infection of Mammalian Cells

### 2.2.1  Plaque Purification

Plaque assays were performed according to Harrison, Graham, and Williams (1977) using 60 mm dishes of 293 cells. Briefly, after removal of the medium, one quarter of a milliliter of appropriate dilutions of virus (diluted in phosphate buffered saline with magnesium and calcium (PBS) (137 mM NaCl - 2.6 mM KCl - 8.3 mM $Na_2HPO_4$ - 1.5 mM $KH_2PO_4$ - 0.5 mM $MgCl_2$ - 0.7 mM $CaCl_2$)) was added to 60 mm dishes of subconfluent 293 cells and incubated 40 minutes at 37°C. After adsorption each dish was overlaid with 10 ml of 0.5% agarose in F11 medium supplemented with penicillin and streptomycin (see above) as well as 5 $\mu$g/ml fungizone

(Squibb), 2% (w/v) yeast extract (Difco), and 5% horse serum, and then incubated at 37°C. Approximately 7 days post-infection, well isolated plaques were picked with the small end of pasteur pipettes and resuspended in 1.25 ml of 10% glycerol in PBS. The viruses were frozen (-70°C) and thawed (room temperature) three times to break up the cells and finally stored at -70°C.

## 2.2.2 Transfection

After preincubation in F11 - 10% horse serum, 60 mm dishes of subconfluent 293 cells were transfected using the calcium technique (Graham and van der Eb (1973)). Briefly, 28.6 $\mu$g hamster carrier DNA in 10 ml HeBS (137 mM NaCl - 5 mM KCl - 0.9 mM $Na_2HPO_4$ - 21 mM Hepes - 5.6 mM dextrose, pH 7.1) was vortexed in a 15 ml Corning tube for 1 minute to shear the DNA. Either 20 or 40 $\mu$g plasmid DNA and 100 $\mu$l of 2.5 M $CaCl_2$ (final concentration of 125 mM) were added to 2 ml (5.7 $\mu$g) sheared carrier DNA in HeBs. After standing 15 minutes at room temperature to allow precipitation of the DNA, 0.5 ml of this mixture was added to each 60 mm dish from which the medium had been removed. Following adsorption at 37°C for 4.5 hours the cells were overlaid with 10 ml of 0.5% agarose in F11 (containing fungizone, penicillin, streptomycin, yeast extract, and horse serum; see plaque purification), left at room temperature to allow solidification of the agarose, then incubated at 37°C. Plaques were picked 10-14 days post-infection as described before (see plaque purification).

## 2.2.3 Liquid Infection

Subconfluent monolayers of HeLa cells (60 mm dishes) were infected with undiluted virus as described (see plaque purification) except in this case incubated in 5 ml αMEM - 2% horse serum. When more than 90-95% of the cells showed cytopathic effect (full CPE), the DNA was harvested (see below).

## 2.3 DNA Extraction

Dishes with full CPE (see liquid infection) were left standing in a biohazard hood for approximately 20 minutes before harvesting to allow floating cells to settle in order to increase the DNA recovery. The medium was then carefully removed from the dishes and 0.5 ml of pronase-SDS (0.5 mg/ml pronase in 0.01 M Tris - 0.01 M EDTA - 0.8% SDS, pH 7.4) was added. If required, approximately 3-4 ml of medium from the infection was saved in a small vial containing sterile glycerol (to give a final concentration of 10%) and stored at -70°C. Dishes were incubated at 37°C for 6 to 15 hours. Samples were transferred to Eppendorf tubes, phenol extracted with 0.5 ml of 0.5 M Tris pH 8.0 saturated phenol containing hydroxyquinoline, then ethanol precipitated and centrifuged using an Eppendorf microfuge for 5 minutes. The nucleic acid pellets were washed first with 80% ethanol, then with 96% ethanol, and finally were air-dried at 37°C. The resulting nucleic acid preparations were redissolved in 100 $\mu$l of 0.1 X SSC (15 mM NaCl - 1.5 mM sodium citrate, pH 7.0) and stored at 4°C.

## 2.4  Restriction Enzyme Analysis

### 2.4.1  Restriction of DNA

All restriction enzymes were purchased from Bethesda Research Laboratories (BRL) with the exception of AccI which was obtained from Boehringer Mannheim.  A single restriction buffer was used for all digests (10 X buffer:  0.5 M KCl - 0.1 M MgCl$_2$ - 0.1 M Tris, pH 7.5) except for AccI, for which the 10 X restriction buffer 'A' (33 mM Tris Ac - 10 mM MgAc - 66 mM KAc - 0.5 mM dithiothreitol) provided by the manufacturer was used.

Typically 15 $\mu$l of the DNA extract obtained from a 60 mm dish of infected HeLa cells was digested with 10 units of HindIII, unless otherwise specified in a total volume of 70 $\mu$l for 6 to 12 hours at 37°C.  The reaction was then stopped by the addition of 20 $\mu$l of loading buffer (50% sucrose - 10 mM EDTA - 1% SDS - 0.1% bromophenol blue).  A second aliquot of enzyme (same amount as before) was usually added several hours after the beginning of the reaction to ensure total digestion of the DNA.  Complete digestion of DNA was important since the subsequent Southern blotting analysis was sensitive enough to detect even a few partially digested DNA fragments, which would have complicated the densitometric analysis of the data (see results).

### 2.4.2  Agarose Gel Electrophoresis

Restricted DNA samples were subjected to electrophoresis for 10 to 12 hours at 30-40 volts through 1% agarose (BRL) gels (unless otherwise specified) in Tris acetate electrophoresis buffer (1 X TAE:  40 mM Tris - 5 mM NaAc - 1 mM EDTA, pH 7.9) containing 0.17 $\mu$g/ml of EtBr

(Sigma). Following electrophoresis the gels were photographed using a UV light box, a Polaroid land camera (with a monochromatic red filter), and Polaroid type 57 film. The amount of viral DNA in each lane was estimated by comparison to a known quantity of HindIII digested Ad5 DNA marker. Approximately 0.5 $\mu$g of each viral DNA sample was then loaded onto identical agarose gels, subjected to electrophoresis, and photographed as before. HindIII digested Ad5 and Ad2 (mac) DNA were included on each gel as controls for oligodeoxynucleotide probing.

## 2.5 Southern Blotting Analysis

### 2.5.1 Transfer to Nitrocellulose

The method used to transfer the electrophoretically separated DNA fragments to nitrocellulose was adapted from that described by Southern (1975). Gels were gently agitated at room temperature in 0.25 M HCl for 10 minutes followed by denaturation in 0.5 M NaOH - 1.5 M NaCl for 60 minutes and then neutralization in 1 M Tris - 1.5 M NaCl, pH 7.5 for an hour. After each step, the gels were rinsed with deionized distilled water (ddH$_2$O) to completely remove the different solutions. The transfer apparatus consisted of two layers of 3 MM Whatman chromatography paper, soaked in 10 X SSC prior to transfer, disposed onto a glass plate and bent at the ends such that the ends were in glass dishes containing the transfer solution (10 X SSC). The gels were then laid upside down on the 3 MM paper and the exposed surface of the paper covered with saran wrap to eliminate short circuiting. A piece of nitrocellulose (BA85; 0.45 $\mu$m, Schleicher & Schuell) previously soaked in 2 X SSC and approximately 5 mm larger and longer than the gels was put on each of the gels followed by

two layers of 3 MM paper wetted in 2 X SSC (same size as the gels) and two stacks of absorbing paper (one on each of the duplicate gels). Two weights (total ≈ 4 kilograms) were then placed on a glass plate positioned on top of the transfer setup and the DNA unidirectionally transferred for 12 to 15 hours. The following day the nitrocellulose filters were washed in 6 X SSC to remove agarose particles (5 minutes with gentle agitation), air-dried, baked for 2 hours at 80°C (no vacuum), and stored dry at 4°C in sealed hybridization bags (Micro-Seal bags from Dazey). After transfer, the gels were stained in TAE - EtBr for a minimum of 30 minutes and examined under UV light to verify complete transfer of the DNA. Duplicate gels were treated together in a single Tupperware container during the several washes and transferred in parallel to ensure identical conditions.

## 2.5.2  Preparation of Probes

### 2.5.2.1  Radioisotope

Fresh gamma $^{32}$P-ATP (adenosine triphosphate) was obtained from New England Nuclear (specific activity:  3000 Ci/mmol).

### 2.5.2.2  Oligodeoxynucleotides

AB 12 and AB 335 (formerly SAM 28) were synthesized by the Institute for Molecular Biology and Biotechnology (McMaster University) using an Applied Biosystems (or Synthesis Automation Machine) DNA synthesizer (Biosearch) and purified by FPLC (Fast Proteins, Polypeptides, Polynucleotides Liquid Chromatography). Both oligodeoxynucleotides were single stranded 15-mers with 5' hydroxyl

groups, suitable for end labeling. AB 12 was homologous to the extreme 15 bases of the Ad2 (mac) termini whereas AB 335 (SAM 28) was homologous to the extreme 15 bases of the Ad5 ITRs (Table 2.1). The two oligodeoxynucleotides were only 60% homologous to each other so that, under appropriate conditions, cross hybridization could be minimized.

### 2.5.2.3 End Labeling of Oligodeoxynucleotides

AB 12 and AB 335 (or SAM 28) were end labeled with a two to five fold molar excess of $r^{32}P$-ATP using 1.4 unit of $T_4$ polynucleotide kinase (BRL)/pmole of DNA in 50 mM Tris - 10 mM $MgCl_2$ - 5 mM dithiothreitol-0.1 mM spermidine - 0.1 mM EDTA, pH 7.6 in a volume of 50 $\mu$l for 60 minutes at 37°C. The mixtures were then immediately purified by chromatography through Sephadex or stored at -20°C.

### 2.5.2.4 Preparation of Sephadex G-50 Columns

Two 5 ml (0.6 cm diameter x 29 cm long) Pyrex pipettes (one for each probe) were siliconized by treatment with a solution of 5% dichlorodimethylsilane in trichloroethane, then air-dried 1-2 minutes, rinsed with 96% ethanol, and dried at 65°C for an hour. A 20 gauge needle was then fused by heat to the tip of each pipette and a bit of cotton placed inside to retain the Sephadex. Fine Sephadex G-50 (Pharmacia) was boiled for an hour in 10 mM Tris pH 8.0 - 1 mM EDTA to allow swelling of the beads, cooled, and carefully poured into the inclined pipettes (approximately 6 ml) to avoid trapping of air bubbles. The columns, vertically held on a stand, were packed and equilibrated with two to three volumes of $ddH_2O$ and slices of a rubber cork were used

TABLE 2.1:   HYBRIDIZATION PROBES

| PROBE | LENGTH | SEQUENCE (nucleotide #) |
|-------|--------|-------------------------|
| AB 12 | 15 mer | (1)                    (15)<br>3'- GAT AAG ATT ATT ATA -5' |
| AB 335 (SAM 28) | 15 mer | 3'- GTA GTA GTT ATT ATA -5'<br>(1)                    (15) |

N.B.:   Ad2 (mac) ITR -> 5'- CTA TTC TAA TAA TAT ...
                        3'- GAT AAG ATT ATT ATA ...

        Ad5 ITR        -> 5'- CAT CAT CAA TAA TAT ...
                        3'- GTA GTA GTT ATT ATA ...

to stop the flow. After each use, the columns were flushed with ddH$_2$O and stored at room temperature.

### 2.5.2.5 Purification of Probes

The level of the eluant (ddH$_2$O) was brought down to the surface of the Sephadex, the flow stopped, the phosphorylated oligodeoxynucleotides (diluted to 100 μl in ddH$_2$O) loaded onto their respective column using siliconized pasteur pipettes, and the level of the mobile phase once again brought down to the surface of the Sephadex. The process was repeated once with an equal volume of eluant allowing the oligodeoxynucleotides to penetrate the columns and give sharp, tight bands of radioactive material. The columns were then filled with ddH$_2$O, the flow restored and regulated by a beaker of eluant on a height adjustable support (0.1 - 0.3 ml/min). For each column, twenty five fractions of twenty drops were collected in 1.5 ml Eppendorf tubes. A 10 μl aliquot of each fraction was counted, without added scintillation fluid, in a Beckman LS1801 scintillation counter (Cerenkov radiation). Two peaks of radioactivity (oligodeoxynucleotide then ATP) were reproducibly obtained under these conditions. The four or five most radioactive fractions of the first peak were pooled and used as probe in Southern blotting analyses.

### 2.5.3 DNA:DNA Hybridization

Duplicate blots (section 2.5.1) were soaked in hybridization bags at room temperature with ≈ 15 ml of 6 X SSPE (20 X: 3 M NaCl - 200 mM NaH$_2$PO$_4$ - 20 mM EDTA disodium, pH 7.4). After complete rehydration,

the 6 X SSPE was removed and the blots were then prehybridized for approximately 8 hours in 6 X SSPE - 5 X Denhardt's (0.02% ficoll - 0.02% polyvinylpyrrolidone - 0.02% bovine serum albumin) - 0.5% SDS (0.05 ml/cm$^2$ of nitrocellulose) at $T_H$ (5°C below the calculated melting temperature of the probe). The melting temperature was estimated by the following formula, according to Suggs et al. (1981): 0°C + (2°C for each adenosine or thymidine) + (4°C for each cytidine or guanosine); i.e. $T_H$ for AB 12 is 29°C, and for AB 335 (SAM 28) is 31°C. The prehybridization solution was then completely removed and replaced with an equal volume of hybridization solution, consisting of fresh prehybridization solution and 10$^5$ - 10$^6$ counts per minute of Sephadex purified end labeled oligodeoxynucleotide per ml of solution. One of the duplicate blots was hybridized to the AB 12 probe, and the other to the AB 335 probe by incubating them submerged in a water bath at $T_H$ for 12 to 14 hours. Following hybridization the blots were transferred to Tupperware containers and washed in 2 X SSPE - 0.1% SDS three times at room temperature using a rotary shaker and once at $T_H$ (20 minutes/wash).

2.5.4 Autoradiography

The probed blots were air-dried, laid (face up) on pieces of 3 MM paper, wrapped in Saran Wrap, and put in X-ray cassettes with intensifying screens in presence of Kodak's X-Omat AR (XAR-5) or X-Omat RP (XRP-1) film. The films were exposed at -70°C and processed in an automatic Kodak RP X-Omat "rapid processing" processor.

## 2.6 Densitometry

The autoradiograms were scanned twice using a Hoefer GS-300 scanning densitometer (transmittance, speed = 6.5 cm/min, gain = minimal) hooked to an Altex (model CR1A) integrator (width = 3, slope = 65000, drift = 0, minimum = 500, T-DBL = 999, lock = 0, stop time = 999, attenuator = 9, speed = 50, method = 41) and the average of the two scans computed. The intensity of the signal for each band (darkness of the X-ray film) was measured in arbitrary "area" units (area below the curve).

## 2.7 Mathematical and Computer Analysis of the Densitometry Data

Since a large number of samples were screened for the presence of either Ad5 or Ad2 (mac) ITRs, all densitometry data (over 3000 scans) were processed by an IBM XT compatible computer via a homemade program written in "C language" (see appendix).

Each experiment (agarose gels, transfers, Southern blotting analyses, scans, and computer analyses) was repeated twice to ensure reproducibility. The computer output is the average of the independent duplicate experiments.

# RESULTS

## 3.1 Isolation of Sub54

In order to ascertain the role of internal viral sequences in the determination of the sequence of the termini as well as the need for identical ITRs at the ends of the genome, the isolation and characterization of viral hybrids using two adenoviral serotypes with distinct ITRs were undertaken. This endeavor was aided by the discovery of an Ad2 strain (Ad2 (mac)) which had an altered sequence within the first seven base pairs of the viral genome (Brinkley and Graham, personal communication). This sequence was different from the published sequences of other Ad2 strains (Arrand and Roberts, 1979; Shinagawa and Padmanabhan, 1979) and in fact was completely different from that of any other human adenovirus reported (Fütterer and Winnacker, 1984; Kelly, 1984).

Sub53 was an Ad5 virus derived from the virus dlE1,3 (Haj-Ahmad and Graham, 1986a) into which a bacterial origin of replication and ampicillin resistance gene (pMX$_2$) was inserted at the XbaI site. Due to its E1 deletion, sub53 could not replicate in HeLa cells. This constituted an easy and effective way to screen out sub53 while trying to isolate recombinants. Unfortunately no such defective Ad2 (mac) viruses were available, resulting in the isolation of parental Ad2 (mac) virus along with the putative recombinants.

46

Sub53 and Ad2 (mac) were used to coinfect 293 cells, which allow both viruses to grow and recombine. Virions from the infected cell lysate were grown on HeLa cells (plaque assay) eliminating one of the parental genomes (sub53), and the recombinants isolated (Fig. 3.1). Unless they resulted from multiple crossovers, the initial recombinants were expected to contain both types of ITRs, one provided by each parental genome. If identical terminal repeats were essential for viral replication, it should have been impossible to isolate such recombinants and only viruses which underwent repair or replacement of their ends should have been obtained.

Mapping of serotype specific internal sequences using viral hybrids was possible due to the similar but non identical genomic structure among all characterized adenoviral serotypes (Sussenbach, 1984). Presumably, any "ITR specifying sequence" would be in the same position in both the Ad2 (mac) and sub53 genomes and therefore the recombinants would be unlikely to contain the hypothetical "ITR specifying sequence" of both parents. As a result, the nature of the "ITR specifying sequence" from either of the two serotypes would determine the DNA sequence of the ITRs in the recombinant viruses. A strong correlation between the location of the crossover in the recombinant viruses (determined by restriction enzyme analysis) and the type of ITRs used by the viruses (assayed by Southern blotting analysis using oligodeoxynucleotidic probes) would suggest the presence of such internal ITR coding sequences.

Figure 3.1:   Isolation of Ad2 (mac)/Ad5 Viral Hybrids.   Examples of

possible locations of crossovers between sub53 and Ad2

(mac) yielding recombinants viable on HeLa cells are

shown.   Ad2 (mac) sequences are indicated by closed boxes

whereas sub53 sequences, containing an E1 deletion (shaded

area) and bacterial sequences (origin of replication and

ampicillin resistance gene) (triangle), are depicted by

open boxes.   '5', '2' and '?' refer to Ad5, Ad2 (mac) and

undetermined ITR, respectively.   'A', 'B', 'C' are

three putative recombinants and 'sub54' an actual

recombinant isolated from such a co-infection.   In each

case the location of the crossover and the type of

termini must be determined by restriction enzyme and

Southern blotting analysis respectively.

pMX₂

dlE1

sub 53          5

Ad 2 (MAC)     2

A     ?

B     ?

C     ?
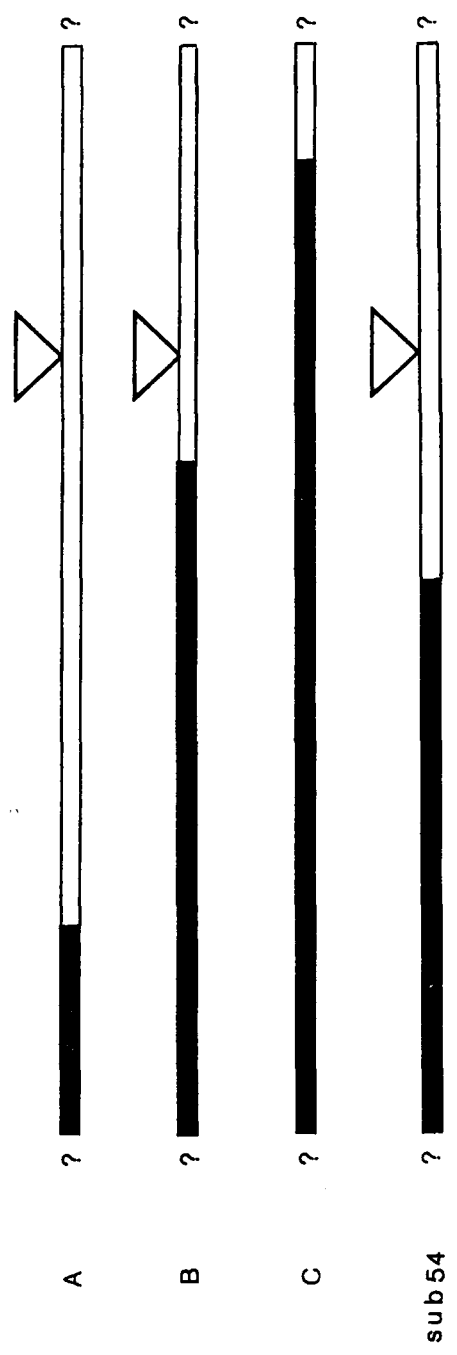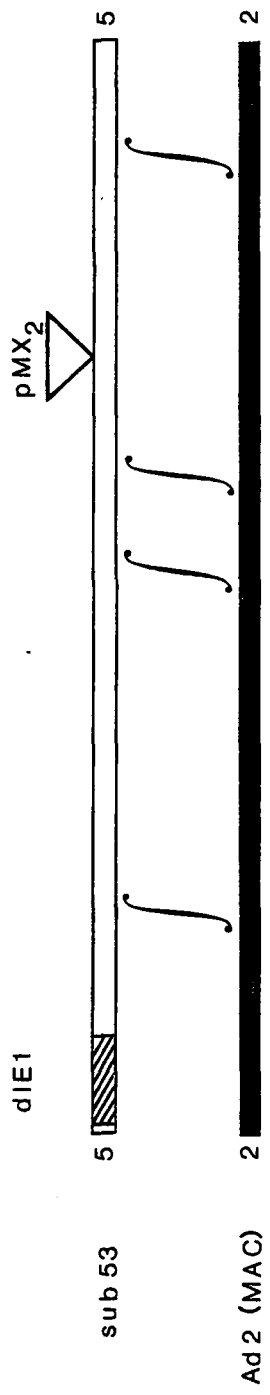
sub 54   ?

### 3.2  Requirement for Identical Termini

### 3.2.1  Characterization of Sub54

The recombinant virus sub54, previously isolated and available in the lab, resulted from a recombination event between Ad2 (mac) and sub53 (Graham, personal communication) (Fig. 3.1). The left end was provided by Ad2 (mac) and the right end by sub53 with a crossover between 41.0% and 51.9% as determined by restriction enzyme analysis. HeLa cells were infected with sub54 as described in materials and methods (liquid infection), the DNA extracted, cut with HindIII, and electrophoresed. Hybridization of the resulting DNA fragments to AB 12 (Ad2 (mac) probe) and AB 335 (Ad5 probe) revealed the presence of Ad2 (mac) and Ad5 termini at the ends of the virus (Fig. 3.2). The absence of signal with the negative controls on the nitrocellulose filters indicated that the stringency of hybridization was adequate.

### 3.2.2  Absence of Contaminating Parental Genomes in the Sub 54 Plaque Isolate

The detection of both Ad2 (mac) and Ad5 ITRs in the sub54 plaque isolate could have been due to the presence of contaminating Ad2 (mac) and/or sub53 viruses from which sub54 was derived (mixed plaque). Since Ad2 (mac), which is believed to be identical to Ad2 except for its terminal repeats (Graham, personal communication), and Ad5 have high DNA homology (Green et al. (1979)) they share numerous restriction sites. As a result the HindIII terminal fragments of sub53, Ad2 (mac), and sub54 were identical in size, thus probing a HindIII digest of sub54 could not reveal the presence of any contaminating Ad2 (mac) or sub53 (Fig. 3.3).

Figure 3.2: Presence of Both Ad2 (mac) and Ad5 Inverted Terminal

Repeats in the Sub54 Sample. Sixty millimeter dishes of

HeLa cells were infected with sub54, the DNA harvested

when full CPE was obtained as described in materials and

methods, cut with HindIII and electrophoresed on

duplicate 1% agarose gels. The DNA was transferred to

nitrocellulose filters and probed with either AB 335

(panel A) or AB 12 (panel B). M: Wild type Ad5 HindIII

marker; Ad2: Ad2 (mac) HindIII digest; Mock: Uninfected

HeLa HindIII digested DNA.

## A) Ad5 PROBE

MOCK  M  sub54  Ad 2



## B) Ad2 (MAC) PROBE

MOCK  M  sub 54  Ad 2
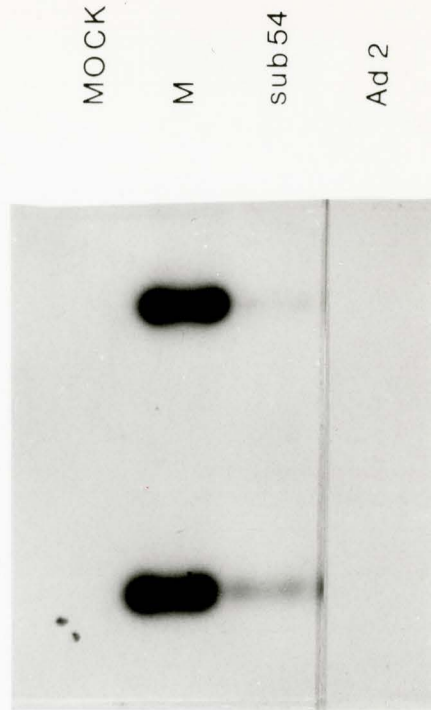
In contrast, the restriction enzyme XhoI allowed discrimination of Ad2 (mac), sub53, and sub54 and was therefore a diagnostic enzyme for contaminating parental genomes (Fig. 3.3). The presence of a 2.2 kilobase pair XhoI fragment in the sub54 sample (hybridizing to AB 335) would have indicated that sub53 was present, whereas a 6.2 kilobase pair XhoI fragment (hybridizing to AB 12) would have been diagnostic of Ad2 (mac) contamination (Fig. 3.3). Therefore, to detect any possible parental virus in the sub54 population, sub54 DNA was cut with XhoI, electrophoresed, and probed with AB 12 and AB 335. As seen in Fig. 3.3 the results indicated that no contamination with Ad2 (mac) or sub53 viruses could be detected by either ethidium bromide staining or Southern blotting analysis. The level of sensitivity of this Southern blotting analysis was approximately 1 ng (500 fold less than the amount of DNA loaded on the gels).

### 3.2.3 Plaque Purification of Sub54

The absence of detectable contaminating Ad2 (mac) or sub53 viruses in the sub54 isolate suggested that the sub54 sample contained a mixture of viruses with either type of ITR. To isolate these different types of molecules, sub54 was plaque purified once, several plaques picked, and their DNA obtained through liquid infections. The DNA was then cut with HindIII, electrophoresed on 1% agarose gels, and probed with AB 12 and AB 335. Six plaques were initially picked and all were found to exclusively have Ad2 (mac) termini. A more extensive plaque isolation was therefore carried out to obtained molecules with Ad5 ends. A series of 60 mm dishes of 293 cells were infected as before, all of the

Figure 3.3:    Analysis of Sub54 DNA for the Presence of Ad2 (mac) or

Sub53 Contaminating Parental Viruses.  Panel A shows the

restriction maps of Ad2 (mac), sub53, and sub54 (vertical

bars: HindIII and small arrows: XhoI).  The shaded area

denotes an E1 deletion (3.5 kb) and the open triangle

indicates bacterial sequences (see Fig. 3.1).  The HindIII

terminal fragments were identical in size for all three

viruses (1.0 kb for the left end and 2.8 kb for the right

end).  Panel B shows the ethidium bromide staining of a

XhoI digest of sub53, sub54, and Ad2 (mac) DNAs submitted

to electrophoresis on a 1% agarose gel whereas panels C

and D shows the corresponding Southern blots using AB 335

or AB 12 (two different exposures) for probe respectively.

Single asterisks indicate fragments containing the left

ITR whereas double asterisks indicate the fragments

containing the right ITRs.  The numbers on the restriction

maps (panel A), along the side of the photograph (panel B)

and the autoradiograms (panels C and D) represent the

sizes of the XhoI terminal fragments in kilobase pairs.

M: Wild type Ad5 HindIII marker; Ad2: Ad2 (mac) HindIII

digest; Mock: Uninfected 293 HindIII digested DNA.  Sub54

RE, used to generate pFG154 (see isolation of plaques from

pFG154), was obtained from infecting rat embryo cells with

sub54.
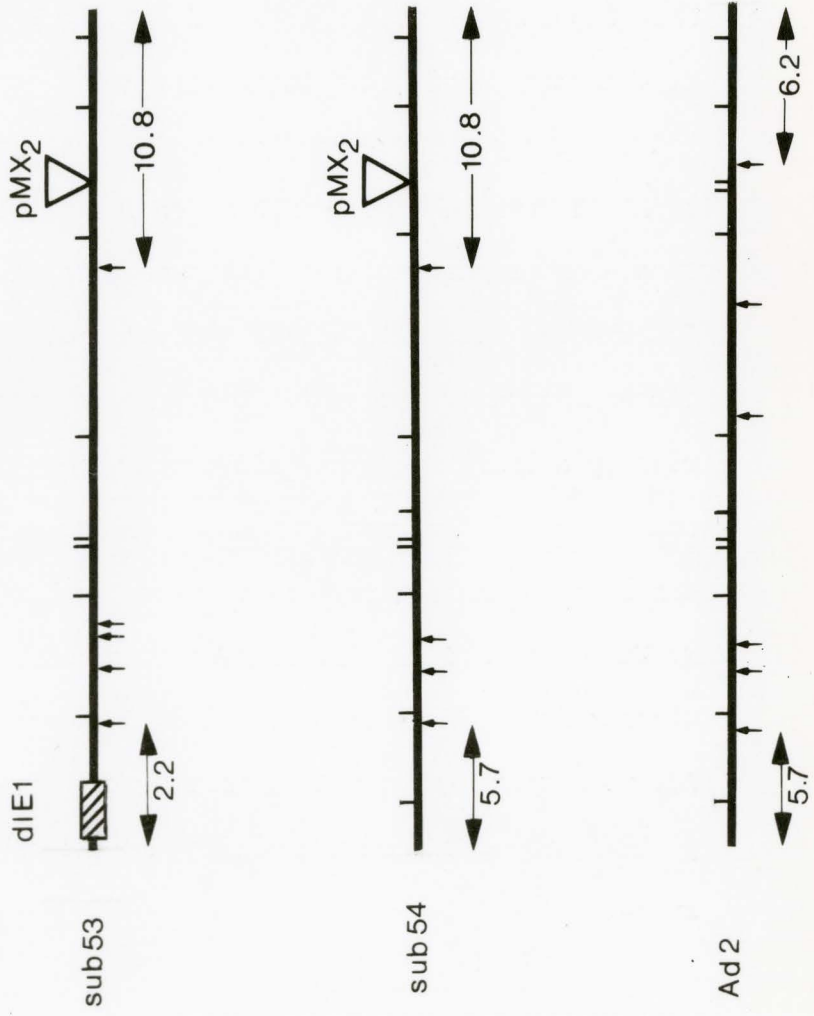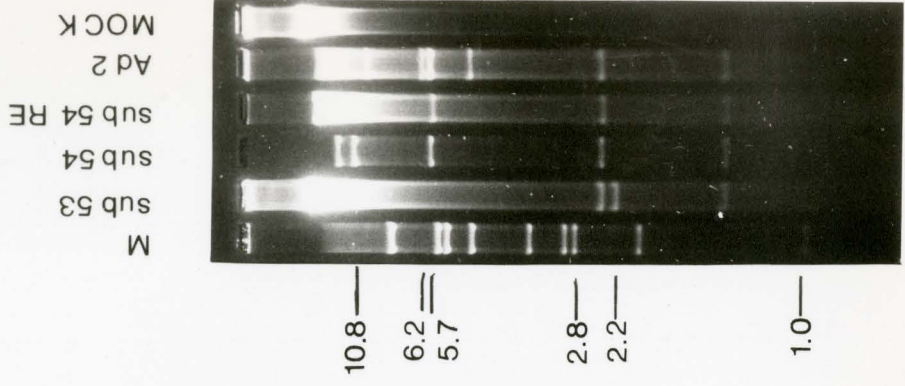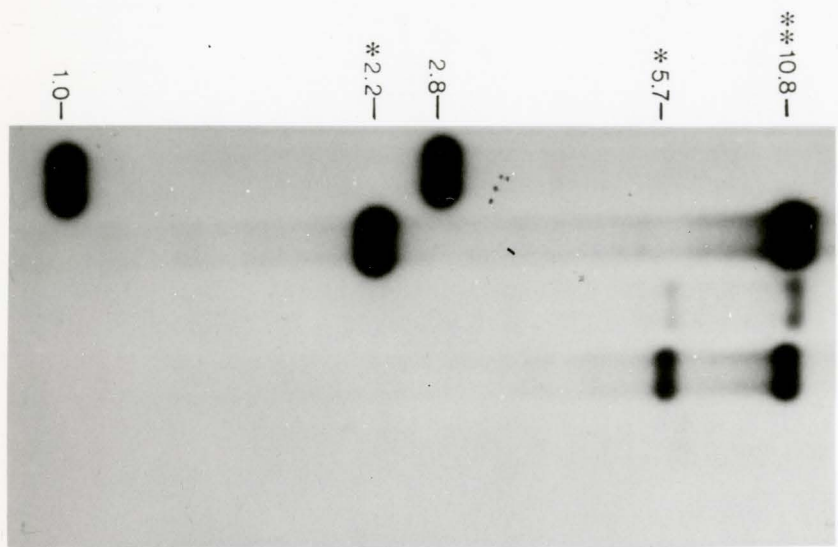
C) Ad5 PROBE

D) Ad2 (MAC) PROBE
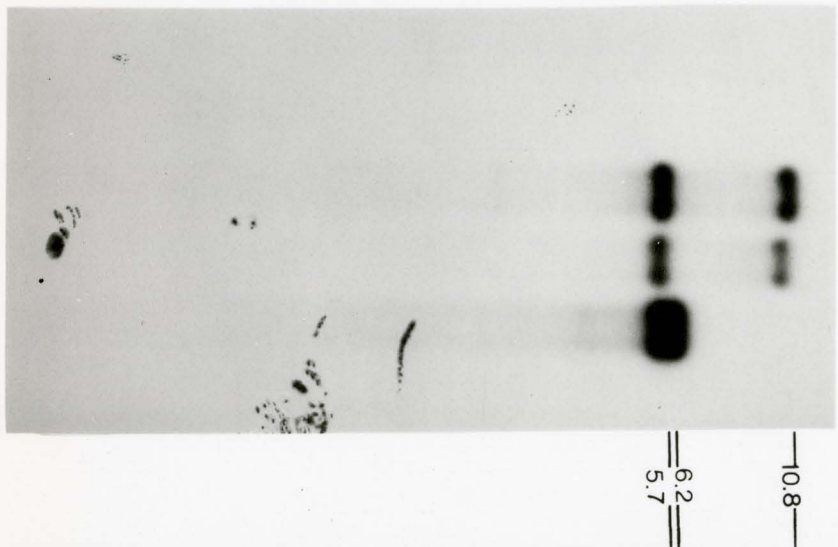
well isolated plaques picked (59 isolates), and their type of ITRs assayed as usual (liquid infections, DNA extractions, agarose gels, and Southern blots). Of the 65 isolates analyzed (59+6), 62 proved to have only Ad2 (mac) ITRs whereas the remaining 3 (sub54-21, sub54-31 and sub54-51) gave a signal with both probes (Fig. 3.4) hereafter referred to as a "composite signal".

In an effort to isolate molecules with Ad5 ITRs, sub54-21, sub54-31, and sub54-51 were each plaque purified once and their progeny (60 samples for each virus) analyzed as for sub54. Figure 3.5 shows a representative analysis of the plaque isolates obtained (progeny of sub54-21). Viruses with identical Ad2 (mac) or Ad5 ITRs (e.g. isolates sub54-21.32 or sub54-21.47 respectively) were obtained as well as numerous samples that gave a composite signal (36, 40 and 11 samples for sub54-21, -31 and -51 respectively). Figure 3.6 shows the relationship among the different progenies isolated.

### 3.2.4   Isolation of Viruses with Non Identical Inverted Terminal Repeats

As shown in Fig. 3.4, the Ad5-specific AB 335 signals for sub54-21, sub54-31, and sub54-51 were much stronger for the HindIII I fragment (right end of the genome) than for the HindIII G fragment (left end). This unequal distribution of Ad5 ITRs indicated that some of the molecules in each isolate had an Ad5 ITR at the right end of the genome and a "non Ad5 ITR", presumably an Ad2 (mac) ITR, at the left end. The detection of Ad2 (mac) ITRs at the left end (by AB 12 hybridization) suggested the presence of "2—5" viruses (viruses with an Ad2 (mac) ITR at the left and an Ad5 ITR at the right). The Ad2 (mac) specific probe

Figure 3.4:  Plaque Purification of Sub54.  Sub54 was plaque purified

once on 293 cells, each plaque isolate used to infect

HeLa cells (liquid infection), and the DNA extracted as

described in materials and methods.  The DNA was then cut

with HindIII, subjected to electrophoresis on duplicate

1% agarose gels, transferred to nitrocellulose,  and

probed with AB 335 (panel A) or AB 12 (panel B).  M: Wild

type Ad5 HindIII marker; 1, 2, 3, 4, 6, 7, 9, 21, 31 and

51: HindIII digested sub54 progeny; Ad2: Ad2 (mac) HindIII

digest; Mock: Uninfected HeLa HindIII digested DNA.

## A) Ad5 PROBE
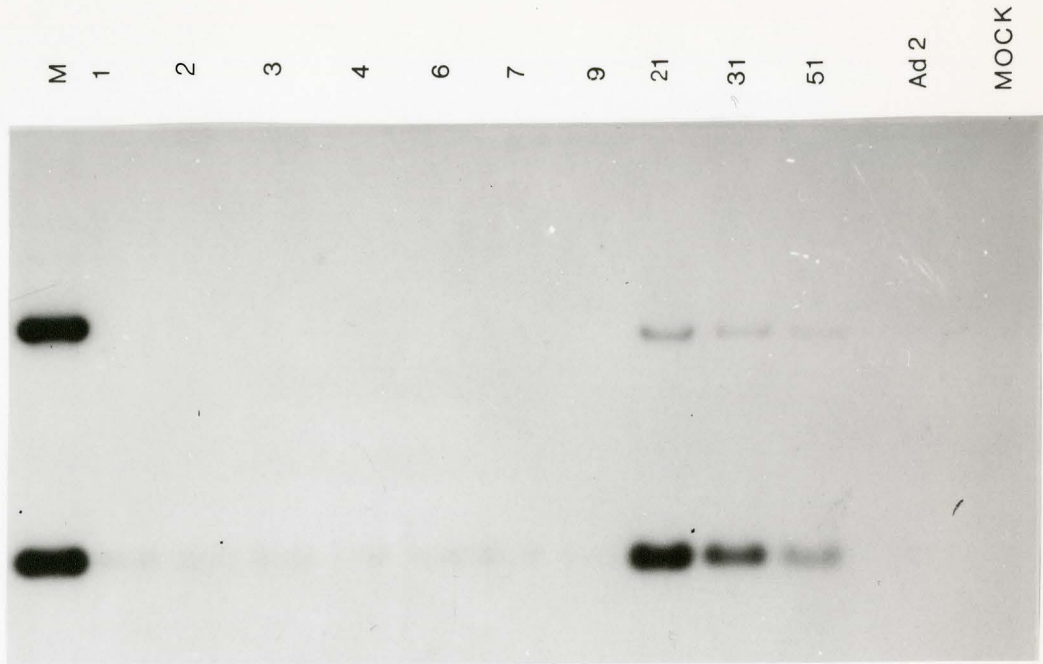


## B) Ad2 (MAC) PROBE

Figure 3.5:  Plaque Purification of Sub54-21.  Sub54-21 progeny was

obtained and analyzed as described in Fig. 3.4.  Panel A:

Probing with AB 335; Panel B: Probing with AB 12; M:

Wild type Ad5 HindIII marker; 31-60: HindIII digest of

sub54-21 progeny; Ad2: Ad2 (mac) HindIII digest.

A) Ad5 PROBE

B) Ad2 (MAC) PROBE

M
31
32
33
34
35
36
37
38
39
40
41
42
43
44
45
46
47
48
49
50
51
52
53
54
55
56
57
58
59
60
Ad2

M
31
32
33
34
35
36
37
38
39
40
41
42
43
44
45
46
47
48
49
50
51
52
53
54
55
56
57
58
59
60
Ad2
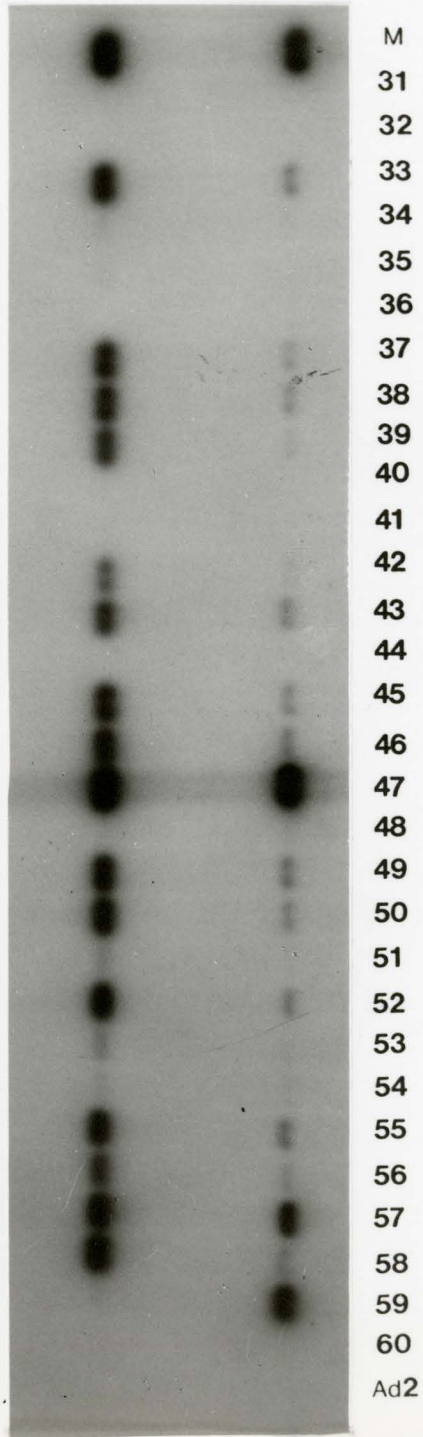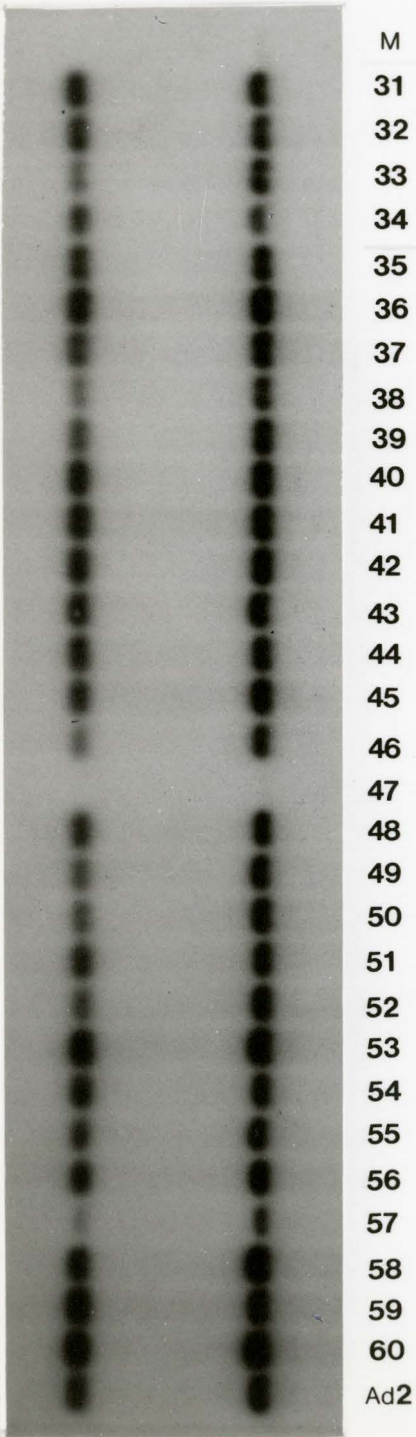
Figure 3.6:  Flow Chart of the Different Viral Progenies Isolated.  The

flow chart includes the plaque isolates discussed so far

as well as all the subsequent ones found in this

manuscript.  The type of isolate (sample exclusively

hybridizing to one of the probes (2---2 or 5---5 viruses) or

giving a composite signal) is indicated in parentheses and

the table or figure where the data can be found in

brackets.  Each vertical continuous arrow represents a

plaque purification in which the indicated number of

plaques were picked and used to infect 60 mm dishes of

HeLa cells (liquid infections), from which the DNA was

extracted, cut with HindIII, electrophoresed, and probed

as usual.  Among those samples, the ones that were

further plaque purified or used for the mixed infection

are indicated directly below the specified amount of

isolates analyzed (⌐─┬─¬).  Sub54-21.47 and sub54-

21.60 were plaque purified once and titrated prior to

utilization in the mixed infection assay (discontinuous

vertical arrows).  Consult the respective section for

details.

```
                        SUB 54              TRANSFORMATION OF E. COLI          pFG154
                   (ORIGINAL SAMPLE) ─────────────────────────────────────►    (2—5)
                      [TABLE 3.1]                                            [FIG. 3.10]

                            │                                                    │
                            ▼                                                    ▼
        ┌─────────────── 65 SAMPLES ───────────────────────┐        ┌─ 65 SAMPLES ─┐
        │                 [TABLE 3.2]                       │        │  [TABLE 3.7] │
        │                     │                             │        │              │

     SUB 54-21            SUB 54-31                     SUB 54-51           1.5 D
    (COMPOSITE)          (COMPOSITE)                  (COMPOSITE)        (COMPOSITE)

         │                    │                             │                  │
         ▼                    ▼                             ▼                  ▼
    ┌─ 60 SAMPLES ─┐      60 SAMPLES                    60 SAMPLES         60 SAMPLES
    │  [TABLE 3.3]  │     [TABLE 3.4]                   [TABLE 3.5]        [TABLE 3.8]
    │      │       │

 SUB 54-21.47  SUB 54-21.47  SUB 54-21.60
    (5—5)     (COMPOSITE)       (2—2)

    ┊            │             ┊
    ▼            ▼             ▼
  MIXED      60 SAMPLES      MIXED
 INFECTION   [TABLE 3.6]    INFECTION
 [FIG. 3.7]                 [FIG. 3.7]
```

hybridized nearly the same to both ends of the viral genome indicating that 2—2 molecules were predominant over 2—5 molecules. The presence of weak Ad5 signals at the left end showed that not all the molecules were of type 2—2 or 2—5 but that some 5—5 or possibly 5—2 were also present.


## 3.3  Data Analysis

To better assess the relative amount of each type of molecules present in each sample, the autoradiograms from the Southern blot analysis were scanned, as described in materials and methods, to quantitate the signals. The details of the data analysis are described below. The reasons for analyzing such a large number of samples by Southern blotting and subsequently by densitometry were to ensure that the results obtained were consistent and reproducible as well as to obtain statistically valid results.


### 3.3.1  Normalization

One problem encountered was the difficulty in obtaining the same signals from the two different oligodeoxynucleotides when probing the positive controls. Even under the same conditions (amount of control Ad5 or Ad2 (mac) DNA loaded, $T_H$, specific radioactivity of the probes, etc.), the AB 335 signal was approximately nine fold greater (on average) than the AB 12 signal. Direct comparison of signals, obtained by hybridizing the samples to the two probes, was therefore impossible unless the values were normalized against the corresponding controls (condition # 1). Thus all the areas obtained with AB 12 probing were multiplied by the ratio of

Ad5 to Ad2 (mac) signal to compensate for the difference in signal observed for the two probes.

Four additional conditions, dictated by simple rules, were required to determine the ratio of molecules containing Ad5 and/or Ad2 (mac) ITRs in each sample. Condition # 2 was that the negative controls (Ad5 when probed with AB 12; Ad2 (mac) when probed with AB 335) should not have given a signal (area = 0), since the presence of a signal would have indicated a lack of stringency and would have represented background hybridization (cross hybridization). Condition # 3 was that the HindIII G signal should have been equal to the HindIII I signal for each of the two positive controls (this is a characteristic of Southern blot analyses when using end labelled probes) since each lane should have contained equimolar amounts of each fragment. Similarly, condition # 4 was that the combination of HindIII G fragment signals (AB 12 + AB 335) had to be equal to that of the HindIII I fragment signals for each sample tested. Finally, condition # 5 was that no value could be negative (i.e. less than zero) since it was a measure of the intensity of the signal (darkness of the autoradiogram). The fulfillment of the third and fourth conditions was required because the analysis of the results was dependent on the ratio of the HindIII G / I signals (see next section).

In summary the conditions are:

1)  Ad5 = Ad2 (mac)          (for the positive controls)
2)  Ad5 = Ad2 (mac) = 0      (for the negative controls)
3)  HindIII G = HindIII I    (for the positive controls)
4)  $\Sigma$ HindIII G (AB 12 + AB 335) =
      $\Sigma$ HindIII I (AB 12 + AB 335) (for all samples tested)
5)  area $\geq$ 0            (for controls and samples)

Although these conditions should theoretically have been met if the transfer to nitrocellulose and hybridization were ideal, in this case they were not. This could have been due to a number of factors such as stringency too low (allowing cross hybridization), non uniform transfer of the DNA fragments to nitrocellulose filters, uneven hybridization (filters not exactly flat), and non uniform distance between films and blots (e.g., presence of air bubbles between the Saran Wrap and the filters). It was difficult to determine which of these factors had a significant impact on the results but the fact was that the above conditions were not met. Consequently adequate corrections were required to determine the ratio of Ad5 vs Ad2 (mac) ITRs present in the samples. Therefore the background signals obtained from each probe were first subtracted for all the samples, the values (areas) for each isolate normalized against the positive controls and the HindIII G and I signals corrected to fulfill the third and fourth conditions. Exact details of these corrections can be found in the appendix (computer program).

### 3.3.2 Estimation of the Ratio of Ad5 and Ad2 (mac) Inverted Terminal Repeats

After normalization of the data, the second part of the computer program determined the ratio of Ad5 to Ad2 (mac) ITRs present in each sample analyzed. As mentioned above, the detection of Ad5 ITRs at the left terminus of the adenoviral genome could mean that not only 5—5 molecules were present but also 5—2 molecules. Similarly, all other signals could have been the result of two possibilities, and from these considerations, four equations were obtained:

1) Left Ad5 signal        = 5—5 + 5—2
2) Right Ad5 signal      = 5—5 + 2—5
3) Left Ad2 (mac) signal = 2—2 + 2—5
4) Right Ad2 (mac) signal = 2—2 + 5—2

i.e. four equations and four unknowns (2—2, 2—5, 5—2 and 5—5) where Left Ad5, Right Ad5, Left Ad2 (mac) and Right Ad2 (mac) were the areas measured by densitometry.

When attempts were made to mathematically solve these equations it was found that the set of equations was degenerate, i.e. had no unique solution. However, it was possible to calculate a range of values for each unknown by applying the "boundary condition" mentioned before (condition # 5 i.e. no negative values) by simple manipulation of the equations 1 to 4:

5) 5—5 =    Left Ad5 signal     - <u>5—2</u>   (from eq. 1)
6) 5—5 =   Right Ad5 signal    - <u>2—5</u>   (from eq. 2)
7) 2—2 = Left Ad2 (mac) signal - <u>2—5</u>   (from eq. 3)
8) 2—2 = Right Ad2 (mac) signal - <u>5—2</u>   (from eq. 4)

From equation 5, one could deduce that 5—2 molecules present in any particular sample could range from a minimum of 0 (none detectable) up to the value of "Left Ad5" (determined by densitometry) otherwise 5—5 would have been negative. This analysis was applied to all four equations and resulted in the following equations:

9)  0 ≤ <u>5—2</u> ≤ Left Ad5     (from eq. 5)
10) 0 ≤ <u>2—5</u> ≤ Right Ad5    (from eq. 6)
11) 0 ≤ <u>2—5</u> ≤ Left Ad2     (from eq. 7)
12) 0 ≤ <u>5—2</u> ≤ Right Ad2    (from eq. 8)

The next step was to substitute both extreme values of 5—2 and 2—5 (from equations 9 to 12) back in equations 5 and 6 to obtain minima

and maxima for 5—5 (or in equations 7 and 8 for 2—2).

Once the range of values of 5—5 (or 2—2) was determined, its substitution in equations 1 to 4 allowed the determination of the other three variables. In this fashion, the proportion of 5—5, 2—5, 5—2 and 2—2 molecules was obtained for each sample scanned. The computer program in the appendix actually determined each of the four variables first and subsequently resolved the equations, therefore solving the equations four times (to make sure that, no matter what variable was first assigned a value, the results remained the same). The terminology used in the computer program is somewhat different than the one used in this section (for example the name of the variables) to fulfill the requirements of the "C language" and to be suitable for the large number of data analyzed (use of "arrays").

Analysis of sub54, its progeny, and the progenies of sub54-21, sub54-31, and sub54-51 (Tables 3.1 to 3.5) was therefore performed as described above. In each case (as well as for samples subsequently analyzed (Tables 3.6 to 3.8)) two independent experiments (consisting of separate agarose gels, Southern blots, and densitometric analyses) were done, except in the case of sub54 for which three independent experiments were performed (Table 3.1). The output of the densitometer/integrator, subsequently analyzed by the computer program, is shown in the tables, which only include the samples that hybridized to both probes and were thus scanned. The data shown below each table represent the samples that only gave a signal with one of the oligodeoxynucleotides (not scanned).

Tables 3.2 to 3.5 indicate that the majority of the plaque isolates exclusively contained 2—2 viruses (153/245 or 63%). Almost all

the samples that gave a composite signal were predominantly composed of 2—2 molecules followed by 2—5 and very few 5—2 or 5—5 molecules. The low levels of 2—5 molecules found in some samples (e.g., sub54-1) may be real or due to cross hybridization. The proportion of samples containing exclusively 2—2 genomes and the abundance of 2—5 viruses relative to 5—2 correlated, to some extent, with the proportion of such molecules in their respective parental plaque. For instance, 61 of the 65 plaques (94%) obtained with sub54 contained genomes with identical Ad2 (mac) ITRs (Table 3.2) whereas the proportion of such molecules in the sub54 plaque isolate itself was 76-83% of the total amount of viruses (Table 3.1). Similarly both the parental sub54 isolate and the isolates of its progeny contained more 2—5 viruses than 5—2. Assuming that each plaque isolate was the progeny of a unique virus, the preponderance of Ad2 (mac) ITRs observed within the plaque isolates giving a composite signal (derived from a 2—5 virus in most cases) suggested a preference for the Ad2 (mac) ITR over Ad5 (2—5 -> 2—2>>5—5).

## 3.4  Preferential Utilization of Type 2 (mac) ITRs

### 3.4.1  Effect of the Orientation of the Non Identical Termini in the Genome

To determine whether the strong bias towards the production of 2—2 molecules by 2—5 viruses was due to the orientation of the two termini in the genome (Ad2 (mac) at the left end and Ad5 at the right end) or to the presence of an Ad2 (mac) ITR irrespective of its location, the progeny of a virus with termini in the opposite orientation was analyzed, i.e. 5—2. As for the other viruses plaque purified to this

TABLE 3.1
COMPUTER ANALYSIS OF SCANNING DATA:  PLAQUE COMPOSITION (AVERAGES)


Sub54 (original sample)

| | SAMPLE | % 2—2 | % 2—5 | % 5—2 | % 5—5 |
|---|---|---|---|---|---|
| 1) | Sub54 | 76 – 83* | 8 – 15 | 0 – 6 | 0 – 6 |

* The values in the table indicate the possible range
of each of the four types of molecules in the plaque
isolate.  For instance sub54 contains between 76% and
83% of molecules with identical Ad2 (mac) ITRs (2—2).




TABLE 3.2
COMPUTER ANALYSIS OF SCANNING DATA:  PLAQUE COMPOSITION (AVERAGES)


Sub54 progeny

| | SAMPLE | % 2—2 | % 2—5 | % 5—2 | % 5—5 |
|---|---|---|---|---|---|
| 1) | Sub54-1 | 95 – 95 | 3 – 3 | 0 – 0 | 0 – 0 |
| 2) | Sub54-21 | 47 – 56 | 34 – 43 | 0 – 8 | 0 – 8 |
| 3) | Sub54-31 | 52 – 57 | 36 – 41 | 0 – 4 | 0 – 4 |
| 4) | Sub54-51 | 83 – 84 | 14 – 14 | 0 – 0 | 0 – 0 |

also:  61 samples —> 100% 2—2
         0 sample  —> 100% 5—5

TABLE 3.3
COMPUTER ANALYSIS OF SCANNING DATA:   PLAQUE COMPOSITION (AVERAGES)


Sub54-21 progeny

|  | SAMPLE | % 2—2 | % 2—5 | % 5—2 | % 5—5 |
|---|---|---|---|---|---|
| 1) | Sub54-21.2 | 45 − 47 | 50 − 52 | 0 − 1 | 0 − 1 |
| 2) | Sub54-21.3 | 49 − 54 | 40 − 45 | 0 − 4 | 0 − 4 |
| 3) | Sub54-21.5 | 94 − 94 | 4 − 4 | 0 − 0 | 0 − 0 |
| 4) | Sub54-21.8 | 51 − 54 | 42 − 45 | 0 − 3 | 0 − 3 |
| 5) | Sub54-21.9 | 51 − 54 | 43 − 45 | 0 − 2 | 0 − 2 |
| 6) | Sub54-21.10 | 42 − 45 | 51 − 54 | 0 − 2 | 0 − 2 |
| 7) | Sub54-21.11 | 49 − 50 | 47 − 48 | 0 − 1 | 0 − 1 |
| 8) | Sub54-21.12 | 58 − 61 | 34 − 38 | 0 − 3 | 0 − 3 |
| 9) | Sub54-21.15 | 33 − 42 | 48 − 57 | 0 − 8 | 0 − 8 |
| 10) | Sub54-21.16 | 76 − 77 | 21 − 22 | 0 − 0 | 0 − 0 |
| 11) | Sub54-21.17 | 61 − 65 | 30 − 33 | 0 − 3 | 0 − 3 |
| 12) | Sub54-21.19 | 60 − 62 | 35 − 37 | 0 − 1 | 0 − 1 |
| 13) | Sub54-21.20 | 86 − 88 | 9 − 11 | 0 − 1 | 0 − 1 |
| 14) | Sub54-21.21 | 85 − 86 | 10 − 12 | 0 − 1 | 0 − 1 |
| 15) | Sub54-21.23 | 85 − 85 | 14 − 14 | 0 − 0 | 0 − 0 |
| 16) | Sub54-21.24 | 61 − 66 | 27 − 32 | 0 − 5 | 0 − 5 |
| 17) | Sub54-21.26 | 64 − 65 | 33 − 34 | 0 − 1 | 0 − 1 |
| 18) | Sub54-21.27 | 71 − 73 | 24 − 26 | 0 − 1 | 0 − 1 |
| 19) | Sub54-21.33 | 29 − 39 | 51 − 60 | 0 − 9 | 0 − 9 |
| 20) | Sub54-21.37 | 62 − 62 | 36 − 37 | 0 − 0 | 0 − 0 |
| 21) | Sub54-21.38 | 57 − 58 | 39 − 40 | 0 − 0 | 0 − 0 |
| 22) | Sub54-21.39 | 71 − 72 | 25 − 27 | 0 − 1 | 0 − 1 |
| 23) | Sub54-21.42 | 95 − 95 | 3 − 4 | 0 − 0 | 0 − 0 |
| 24) | Sub54-21.43 | 83 − 84 | 14 − 15 | 0 − 1 | 0 − 1 |
| 25) | Sub54-21.45 | 73 − 74 | 23 − 24 | 0 − 0 | 0 − 0 |
| 26) | Sub54-21.46 | 48 − 51 | 44 − 47 | 0 − 3 | 0 − 3 |
| 27) | Sub54-21.49 | 55 − 64 | 26 − 35 | 0 − 8 | 0 − 8 |
| 28) | Sub54-21.50 | 52 − 53 | 45 − 46 | 0 − 0 | 0 − 0 |
| 29) | Sub54-21.51 | 97 − 97 | 2 − 2 | 0 − 0 | 0 − 0 |
| 30) | Sub54-21.52 | 59 − 60 | 38 − 39 | 0 − 0 | 0 − 0 |
| 31) | Sub54-21.53 | 98 − 98 | 1 − 1 | 0 − 0 | 0 − 0 |
| 32) | Sub54-21.55 | 61 − 63 | 34 − 36 | 0 − 2 | 0 − 2 |
| 33) | Sub54-21.56 | 79 − 80 | 17 − 18 | 0 − 1 | 0 − 1 |
| 34) | Sub54-21.57 | 0 − 15 | 31 − 47 | 0 − 15 | 36 − 52 |
| 35) | Sub54-21.58 | 65 − 66 | 31 − 32 | 0 − 1 | 0 − 1 |
| 36) | Sub54-21.59 | 59 − 59 | 0 − 0 | 39 − 39 | 0 − 3 |

also:   23 samples —> 100% 2—2
         1 sample  —> 100% 5—5  (sub54-21.47)

TABLE 3.4
COMPUTER ANALYSIS OF SCANNING DATA:   PLAQUE COMPOSITION (AVERAGES)

Sub54-31 progeny

| | SAMPLE | % 2—2 | % 2—5 | % 5—2 | % 5—5 |
|---|---|---|---|---|---|
| 1) | Sub54-31.2 | 43 - 44 | 54 - 55 | 0 - 0 | 0 - 0 |
| 2) | Sub54-31.5. | 25 - 27 | 68 - 71 | 0 - 2 | 0 - 2 |
| 3) | Sub54-31.6 | 31 - 34 | 62 - 65 | 0 - 2 | 0 - 2 |
| 4) | Sub54-31.7 | 51 - 52 | 45 - 46 | 0 - 0 | 0 - 0 |
| 5) | Sub54-31.9 | 97 - 97 | 2 - 2 | 0 - 0 | 0 - 0 |
| 6) | Sub54-31.10 | 27 - 30 | 65 - 68 | 0 - 3 | 0 - 3 |
| 7) | Sub54-31.12 | 55 - 56 | 42 - 43 | 0 - 0 | 0 - 0 |
| 8) | Sub54-31.14 | 50 - 51 | 47 - 48 | 0 - 0 | 0 - 0 |
| 9) | Sub54-31.15 | 55 - 56 | 42 - 43 | 0 - 0 | 0 - 0 |
| 10) | Sub54-31.16 | 36 - 38 | 59 - 61 | 0 - 1 | 0 - 1 |
| 11) | Sub54-31.18 | 88 - 88 | 10 - 10 | 0 - 0 | 0 - 0 |
| 12) | Sub54-31.19 | 70 - 71 | 26 - 28 | 0 - 0 | 0 - 0 |
| 13) | Sub54-31.20 | 54 - 56 | 41 - 43 | 0 - 1 | 0 - 1 |
| 14) | Sub54-31.21 | 61 - 64 | 33 - 35 | 0 - 1 | 0 - 1 |
| 15) | Sub54-31.22 | 61 - 63 | 33 - 36 | 0 - 1 | 0 - 1 |
| 16) | Sub54-31.23 | 32 - 34 | 63 - 65 | 0 - 1 | 0 - 1 |
| 17) | Sub54-31.24 | 64 - 64 | 34 - 34 | 0 - 0 | 0 - 0 |
| 18) | Sub54-31.26 | 66 - 67 | 31 - 32 | 0 - 0 | 0 - 0 |
| 19) | Sub54-31.27 | 22 - 23 | 75 - 76 | 0 - 1 | 0 - 1 |
| 20) | Sub54-31.28 | 29 - 30 | 67 - 69 | 0 - 1 | 0 - 1 |
| 21) | Sub54-31.31 | 79 - 81 | 16 - 18 | 0 - 1 | 0 - 1 |
| 22) | Sub54-31.33 | 55 - 57 | 39 - 41 | 0 - 2 | 0 - 2 |
| 23) | Sub54-31.34 | 59 - 61 | 35 - 37 | 0 - 1 | 0 - 1 |
| 24) | Sub54-31.35 | 60 - 63 | 34 - 36 | 0 - 2 | 0 - 2 |
| 25) | Sub54-31.36 | 50 - 52 | 45 - 47 | 0 - 1 | 0 - 1 |
| 26) | Sub54-31.38 | 27 - 29 | 67 - 69 | 0 - 2 | 0 - 2 |
| 27) | Sub54-31.39 | 87 - 87 | 11 - 11 | 0 - 0 | 0 - 0 |
| 28) | Sub54-31.40 | 99 - 99 | 0 - 0 | 0 - 0 | 0 - 0 |
| 29) | Sub54-31.42 | 80 - 81 | 16 - 18 | 0 - 1 | 0 - 1 |
| 30) | Sub54-31.43 | 87 - 88 | 10 - 11 | 0 - 1 | 0 - 1 |
| 31) | Sub54-31.44 | 67 - 70 | 27 - 29 | 0 - 1 | 0 - 1 |
| 32) | Sub54-31.46 | 40 - 43 | 53 - 55 | 0 - 2 | 0 - 2 |
| 33) | Sub54-31.49 | 63 - 64 | 34 - 35 | 0 - 0 | 0 - 0 |
| 34) | Sub54-31.50 | 66 - 72 | 21 - 27 | 0 - 6 | 0 - 6 |
| 35) | Sub54-31.51 | 78 - 79 | 18 - 19 | 0 - 1 | 0 - 1 |
| 36) | Sub54-31.54 | 23 - 25 | 72 - 74 | 0 - 1 | 0 - 1 |
| 37) | Sub54-31.57 | 50 - 51 | 46 - 48 | 0 - 0 | 0 - 0 |
| 38) | Sub54-31.58 | 52 - 54 | 43 - 44 | 0 - 1 | 0 - 1 |
| 39) | Sub54-31.59 | 32 - 33 | 65 - 66 | 0 - 1 | 0 - 1 |
| 40) | Sub54-31.60 | 96 - 96 | 2 - 2 | 0 - 0 | 0 - 0 |

also:   20 samples --> 100% 2—2
        0 sample  --> 100% 5—5

TABLE 3.5
COMPUTER ANALYSIS OF SCANNING DATA:   PLAQUE COMPOSITION (AVERAGES)

Sub54-51 progeny

|  | SAMPLE | % 2—2 | % 2—5 | % 5—2 | % 5—5 |
|---|---|---|---|---|---|
| 1) | Sub54-51.3 | 17 - 19 | 76 - 78 | 0 - 2 | 1 - 3 |
| 2) | Sub54-51.5 | 64 - 66 | 30 - 32 | 0 - 1 | 0 - 1 |
| 3) | Sub54-51.8 | 71 - 71 | 27 - 27 | 0 - 0 | 0 - 0 |
| 4) | Sub54-51.13 | 93 - 93 | 5 - 5 | 0 - 0 | 0 - 0 |
| 5) | Sub54-51.14 | 57 - 59 | 37 - 39 | 0 - 2 | 0 - 2 |
| 6) | Sub54-51.28 | 12 - 16 | 79 - 83 | 0 - 4 | 0 - 4 |
| 7) | Sub54-51.31 | 50 - 55 | 40 - 44 | 0 - 4 | 0 - 4 |
| 8) | Sub54-51.40 | 54 - 55 | 43 - 44 | 0 - 1 | 0 - 1 |
| 9) | Sub54-51.46 | 60 - 68 | 22 - 31 | 0 - 8 | 0 - 8 |
| 10) | Sub54-51.48 | 99 - 99 | 0 - 0 | 0 - 0 | 0 - 0 |
| 11) | Sub54-51.57 | 23 - 55 | 10 - 42 | 0 - 31 | 0 - 31 |

also:   49 samples —> 100%  2—2
         0 sample  —> 100%  5—5

point, the sub54-21.59 plaque isolate contained a majority of 2—2 molecules and a minimal amount of 5—5 molecules, but in this case there was a preponderance of 5—2 molecules over 2—5 (Table 3.3). Sub54-21.59 was plaque purified and the relative amount of each type of molecule in sixty different progeny plaques was analyzed as usual. Table 3.6 shows that, for the samples hybridizing to both oligodeoxynucleotides, a majority of molecules were of the type 2—2, although a significant proportion of DNA was of the type 5—2, as in sub54-21.59. The table also shows that most samples contained pure 2—2 genomes in a similar proportion to that of 2—2 molecules in the sub54-21.59 plaque isolate itself (respectively 48% (29/60) (Table 3.6) and 59% (Table 3.3)) and that once again, no pure 2—5 or 5—2 populations could be isolated. The results thus indicated that the preference for Ad2 (mac) ITRs was not based on its position at the left end of the genome, since the preference was not affected by reversing the ends, with type 2 (mac) ITR at the right end and type 5 at the left.

### 3.4.2  Coinfection of 2—2 and 5—5 Viruses

The results of the preceding sections suggested that ITRs of type 2 (mac) were preferred over type 5, at least when present at either end of the same molecule. It was of interest to examine that preference with the two ITRs on separate molecules. Sub54-21.47 (an isolate exclusively containing 5—5 viruses) and sub54-21.60 (an isolate exclusively containing 2—2 viruses) (Table 3.3) were therefore plaque purified, titrated, and used at equal multiplicities of infection (10 and 10) to coinfect 293 cells as described in materials and methods (liquid

TABLE 3.6
COMPUTER ANALYSIS OF SCANNING DATA:  PLAQUE COMPOSITION (AVERAGES)

Sub54-21.59 progeny

| | SAMPLE | % 2—2 | % 2—5 | % 5—2 | % 5—5 |
|---|---|---|---|---|---|
| 1) | Sub54-21.59.1 | 45 – 60 | 0 – 15 | 23 – 38 | 0 – 15 |
| 2) | Sub54-21.59.4 | 87 – 89 | 0 – 1 | 8 – 10 | 0 – 1 |
| 3) | Sub54-21.59.6 | 94 – 95 | 0 – 1 | 2 – 3 | 0 – 1 |
| 4) | Sub54-21.59.7 | 62 – 65 | 0 – 2 | 31 – 34 | 0 – 2 |
| 5) | Sub54-21.59.8 | 86 – 88 | 0 – 1 | 9 – 11 | 0 – 1 |
| 6) | Sub54-21.59.10 | 85 – 87 | 0 – 1 | 11 – 12 | 0 – 1 |
| 7) | Sub54-21.59.11 | 65 – 68 | 0 – 2 | 27 – 31 | 0 – 2 |
| 8) | Sub54-21.59.13 | 96 – 96 | 0 – 0 | 3 – 3 | 0 – 0 |
| 9) | Sub54-21.59.14 | 60 – 61 | 0 – 1 | 36 – 37 | 0 – 1 |
| 10) | Sub54-21.59.15 | 83 – 85 | 0 – 1 | 12 – 14 | 0 – 1 |
| 11) | Sub54-21.59.16 | 87 – 89 | 0 – 2 | 7 – 9 | 0 – 2 |
| 12) | Sub54-21.59.17 | 90 – 93 | 0 – 3 | 2 – 5 | 0 – 3 |
| 13) | Sub54-21.59.18 | 84 – 88 | 0 – 2 | 7 – 11 | 0 – 2 |
| 14) | Sub54-21.59.23 | 74 – 76 | 0 – 0 | 21 – 23 | 0 – 0 |
| 15) | Sub54-21.59.24 | 96 – 97 | 0 – 0 | 1 – 1 | 0 – 0 |
| 16) | Sub54-21.59.25 | 70 – 72 | 0 – 2 | 23 – 26 | 0 – 2 |
| 17) | Sub54-21.59.27 | 95 – 95 | 0 – 0 | 3 – 3 | 0 – 0 |
| 18) | Sub54-21.59.31 | 97 – 97 | 1 – 1 | 0 – 0 | 0 – 0 |
| 19) | Sub54-21.59.32 | 94 – 95 | 0 – 1 | 2 – 3 | 0 – 1 |
| 20) | Sub54-21.59.37 | 84 – 85 | 0 – 1 | 12 – 13 | 0 – 1 |
| 21) | Sub54-21.59.38 | 72 – 74 | 0 – 2 | 21 – 24 | 0 – 2 |
| 22) | Sub54-21.59.41 | 98 – 98 | 0 – 0 | 0 – 0 | 0 – 0 |
| 23) | Sub54-21.59.44 | 44 – 49 | 0 – 5 | 44 – 49 | 0 – 5 |
| 24) | Sub54-21.59.45 | 22 – 43 | 0 – 21 | 33 – 55 | 0 – 21 |
| 25) | Sub54-21.59.47 | 76 – 79 | 0 – 2 | 18 – 20 | 0 – 2 |
| 26) | Sub54-21.59.52 | 70 – 73 | 0 – 2 | 22 – 25 | 0 – 2 |
| 27) | Sub54-21.59.54 | 72 – 75 | 0 – 2 | 21 – 23 | 0 – 2 |
| 28) | Sub54-21.59.56 | 85 – 87 | 0 – 1 | 10 – 11 | 0 – 1 |
| 29) | Sub54-21.59.57 | 93 – 94 | 0 – 1 | 3 – 4 | 0 – 1 |
| 30) | Sub54-21.59.58 | 95 – 96 | 0 – 0 | 1 – 2 | 0 – 0 |
| 31) | Sub54-21.59.60 | 75 – 78 | 0 – 1 | 19 – 21 | 0 – 1 |

also:  29 samples —> 100% 2—2
        0 sample  —> 100% 5—5

infection), except that the two viral samples were diluted in PBS to obtain the required quantity of viruses per 60 mm dish of subconfluent 293 cells (0.125 ml of each viral sample was simultaneously added to the cells instead of 0.25 ml). At full CPE the DNA was harvested, the medium saved and used to reinfect 60 mm dishes of 293 cells (0.25 ml of undiluted medium/dish), and the process repeated for a total of ten successive infection cycles. The DNA samples were then cut with HindIII, electrophoresed, and probed as usual. The computer analysis of the resulting data differed from the one described in the appendix as follows: 1) The total signal (AB 12 + AB 335) for each sample was normalized to account for slightly different amounts of DNA in each lane, thus allowing a direct comparison of the samples across the blots; 2) The determination of 2—2, 2—5, 5—2, and 5—5 molecules, as described in pages 135-145 of the appendix, was not done. Instead, for each sample, the proportion (%) of AB 12 signal ((AB12 HindIII G + I) X 100/total signal obtained with both probes) or AB 335 signal in each lane was directly plotted, thus indicating the total amount of Ad5 or Ad2 (mac) ITRs present in each sample. Figure 3.7 indicates that viruses with Ad2 (mac) ITRs replicated slightly faster than viruses with Ad5 termini during the first infectious cycles, but more or less at the same rate in the last cycles. The preponderance of type 5 termini over type 2 in the first cycle for both coinfections may have been due to slightly incorrect titers (the accuracy of titers is within a factor of two).

Figure 3.7: ITR Preference by Viruses with Identical Termini. Two

independent coinfections of 293 cells were done using

sub54-21.47 and sub54-21.60 at a multiplicity of

infection of 10 for each virus. Ten successive liquid

infections were performed using the medium to reinfect

cells. At each step DNA was extracted from the infected

cell monolayer. HindIII restricted DNA was loaded on

duplicate gels, probed with AB 12 or AB 335, scanned, and

the data analyzed according to the changes specified in

the text. Open circles: Ad2 (mac) ITRs; Open squares:

Ad5 ITRs. For each of the two independent coinfections

(panels A and B) the liquid infections 3-10 were split

into two duplicate series (left and right).

## A) COINFECTION # 1
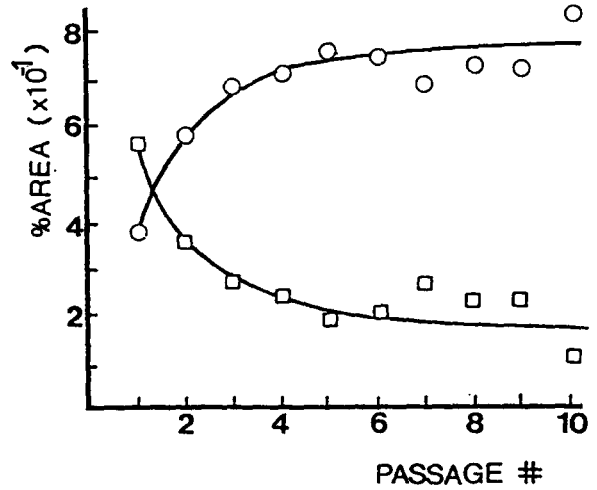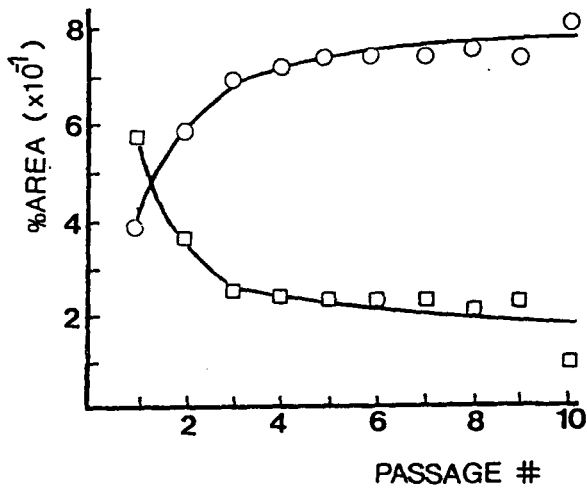


## B) COINFECTION # 2
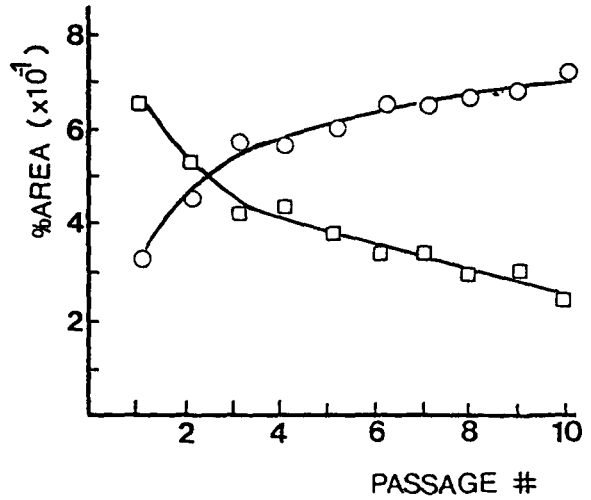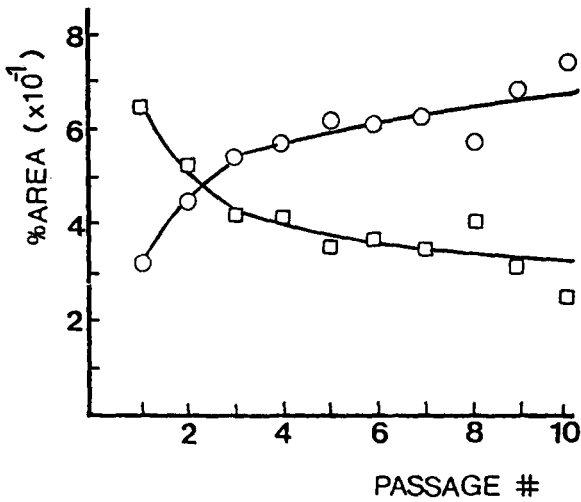


□ Ad 5

○ Ad2 (MAC)

### 3.4.3  Isolation and Characterization of Viruses with Complete Ad5 Replication Machinery

All viruses described so far have the same genetic background, i.e. sub54, in which the position of the crossover was such that the Ad2 (mac) sequences were encoding Adpol and pTP/TP but not DBP (encoded by the Ad5 sequences). Since replication of adenovirus is dependent on these gene products (see introduction) it was of interest to determine whether the preference for the type 2 termini observed was due to the presence of an Ad2 (mac) Adpol and/or pTP/TP. To address this problem, the technique developed by McGrory, Bautista, and Graham (1988) was used to rescue recombinant viruses carrying only the first few kilobases of the left end of Ad2 (mac) in a background of Ad5 sequences. Briefly, 293 cells were coinfected with pJM17 (a plasmid carrying the entire Ad5 genome plus a large insert at 3.7 map units) and pFG154-G8 or pFG154-G8Δ6 (plasmids derived from the left 7.7 map units of Ad2 (mac)). Recombination by a crossover event between the plasmids resulted in viral hybrids with Ad5 sequences encoding the replicative proteins Adpol, pTP/TP and DBP. These recombinants could be detected by analysis with the restriction enzyme AccI (appearance of a ≈ 0.2 kb terminal fragment) which cleaves at nucleotides 196 and 1108 of Ad2 (mac) but only at nucleotide 1108 of dl309 (from which pJM17 is derived) (Fig. 3.8). The presence of a BamHI linker in pFG154-G8Δ6 at a SmaI site at nucleotide 1008 (Brinkley and Graham, personal communication) was also diagnostic for recombinants when using pFG154-G8Δ6 in the coinfection (shift of the 21.4 kb terminal fragment to a 1.0 kb band containing the ITR sequences) (Fig. 3.9).

Five or ten micrograms of both pJM17 and pFG154-G8 (or pFG154-G8Δ6) were used to transfect 293 cells as described in materials and methods (kindly performed by J. Rudy and G. Wilson). Twenty seven putative recombinant plaques (14 for pFG154-G8 and 13 for pFG154-G8Δ6) were picked, from which the DNA was isolated (liquid infections and DNA extractions), cut with either AccI (when using pFG154-G8) or BamHI (when using pFG154-G8Δ6), electrophoresed, and the termini probed as usual. Figures 3.8 and 3.9 show that 26 of the 27 isolates were recombinants and AccI restriction analysis of the recombinants obtained from coinfection with pFG154-G8Δ6 indicated that the Ad2 (mac) sequences extended to nucleotide 196 (Fig. 3.9). Figures 3.8 and 3.9 also show that all the recombinants had exclusively Ad5 ITRs (no detectable Ad2 (mac) termini). In several lanes, AccI partially digested fragments were detected as indicated by the presence of both a 0.2 kb and a 1.1 kb fragment (no cut at nucleotide 196) and/or by an additional band ($\approx$ 3-4 kb) above the right terminal fragment ($\approx$ 2 kb fragment). The low intensity of the 0.2 kb fragment relative to the other bands is probably due to inefficient hybridization as it is smaller than 500 bp (Southern, 1975).

The plasmid pJM17 has a small deletion of Ad5 sequences at the junction of the covalently linked ITRs identical to the deletion in pFG140 (McGrory et al., 1988; Graham, 1984a). As a consequence, pJM17 has no complete template for the Ad5 termini since both ITRs lack sequences. Yet the viruses rescued using pJM17 had restored the missing Ad5 sequences, as confirmed by Southern blotting analysis, presumably despite the presence of an Ad2 (mac) ITR at the left end of the genome (Fig. 3.8 and 3.9).

Figure 3.8:  Ad2 (mac)/Ad5 Viral Hybrids with an Ad5 Replication

Machinery (using pJM17 and pFG154-G8). Panel A shows a

possible recombination event between pJM17 and pFG154-G8

in which the open box depicts Ad5 sequences, the closed

box Ad2 (mac) sequences, while the undulated lines

indicate bacterial sequences. Both genomes are

covalently closed circles but are shown as linear

molecules for simplicity. The vertical bars represent the

AccI sites present in the left 3.7% of the genome whose

positions are indicated in base pairs. The numbers along

the side of the autoradiograms (panels B and C) indicate

the sizes of the fragments containing ITR sequences (in

kb). Fourteen plaques were obtained, their DNA cut with

AccI, subjected to electrophoresis on duplicate 2%

agarose gels, and probed with AB 335 (panel B) or AB 12

(panel C). M: Wild type Ad5 HindIII marker; Ad2: Ad2

(mac) HindIII digest; 1-14: HindIII digests of plaque

isolates obtained from the cotransfection; Mock:

Uninfected 293 HindIII digested DNA. Some partially

digested fragments can be seen.

A)

pBRX
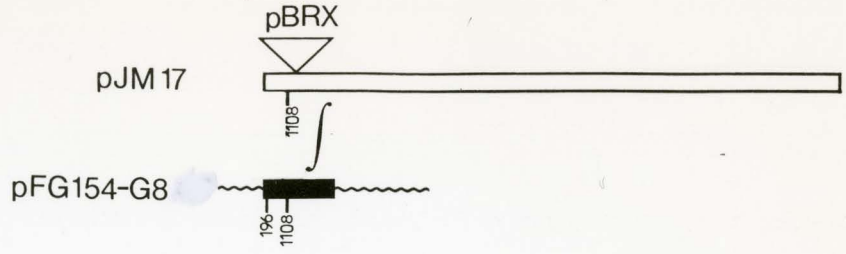
pJM17

pFG154-G8

B) Ad5 PROBE
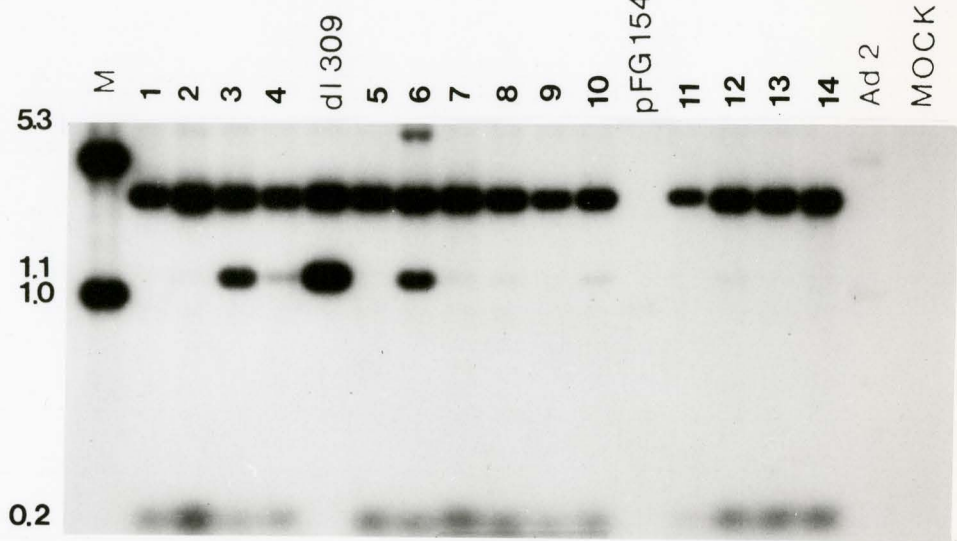
C) Ad2 (MAC) PROBE
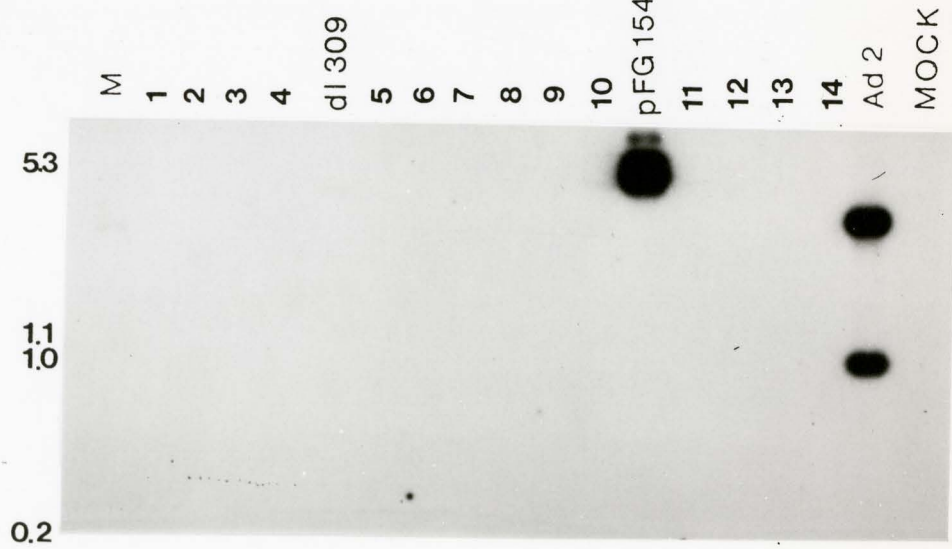
Figure 3.9:  Ad2 (mac)/Ad5 Viral Hybrids with an Ad5 Replication

Machinery (using pJM17 and pFG154-G8A6). The symbols used

are as described in Fig. 3.8. In addition to the AccI

sites present at the left 3.7% of the genome of adenovirus

(vertical bars), the BamHI sites are shown for the entire

genome (arrows). Thirteen plaques were obtained, and

their DNA cut with BamHI, submitted to electrophoresis on

duplicate 0.8% agarose gels, and probed with AB 335 (panel

B) or AB 12 (panel C). Alternatively, the DNA samples

were cut with AccI, subjected to electrophoresis on

duplicate 2% agarose gels and probed with AB 335 (panel

D) or AB 12 (panel E). M: Wild type Ad5 HindIII marker;

Ad2: Ad2 (mac) HindIII digest; 15-27: HindIII digests of

plaque isolates obtained from the cotransfection; Mock:

Uninfected 293 DNA digested with HindIII.

A)

pFG154-G8Δ6

pJM17

pBRX

B) Ad5 PROBE

M dl309 15 16 17 18 19 Ad2 MOCK

M pFG154-G8Δ6 20 21 22 23 24 25 26 27 dl309 Ad2 MOCK

C) Ad2 (MAC) PROBE

M dl309 15 16 17 18 19 Ad2 MOCK

M pFG154-G8Δ6 20 21 22 23 24 25 26 27 dl309 Ad2 MOCK
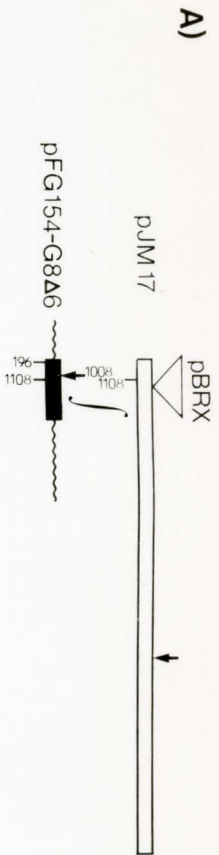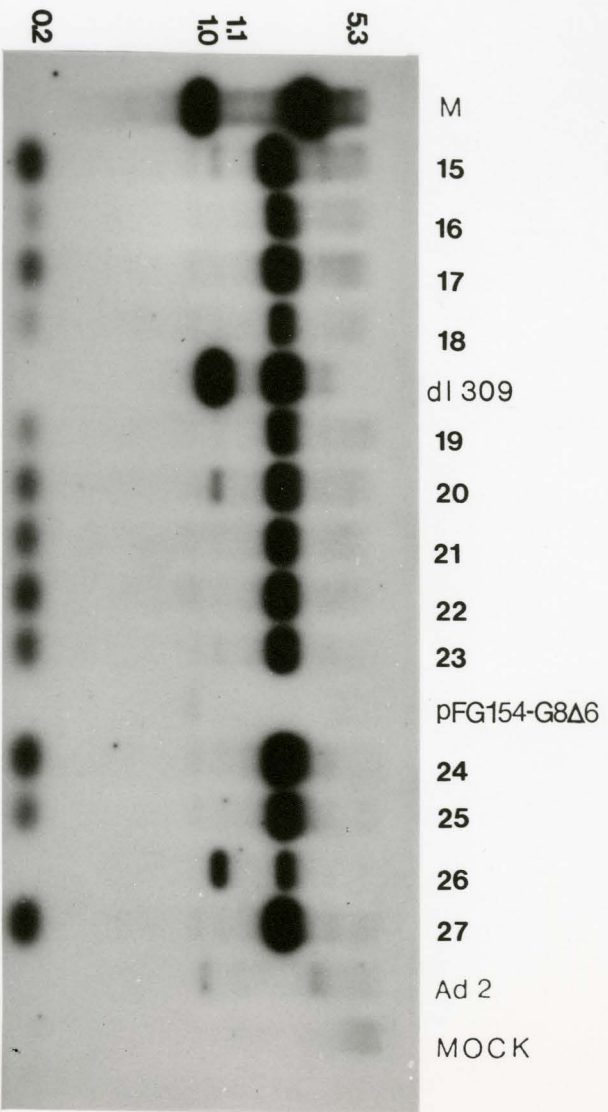
E) Ad2 (MAC) PROBE

D) Ad5 PROBE
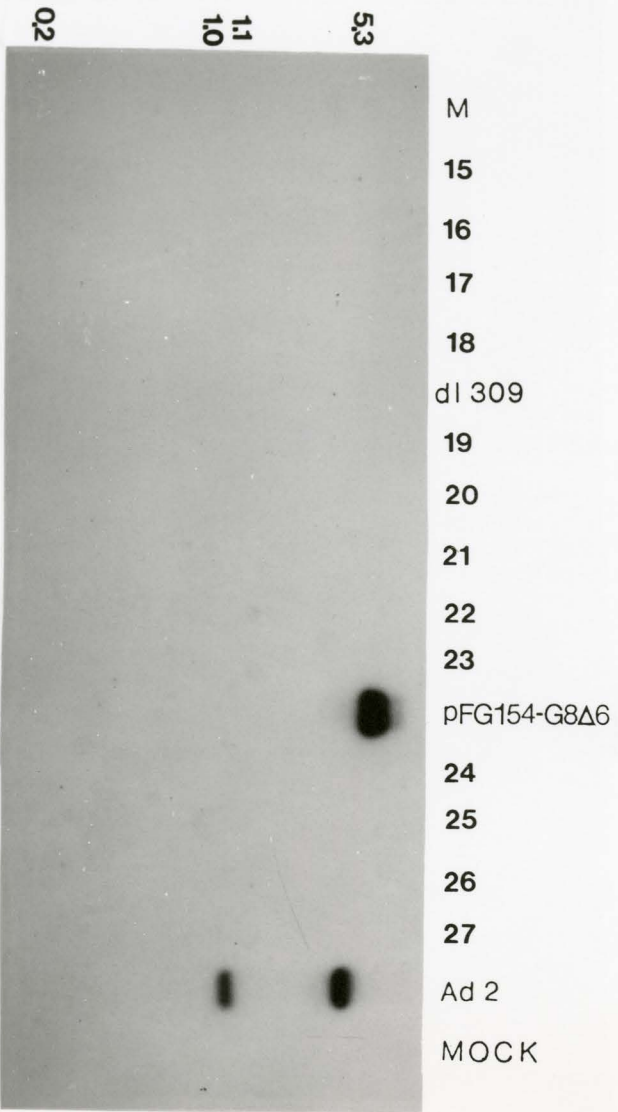
## 3.5  Isolation of Plaques from a Pure 2—5 sample

Although the results indicated that 2—5 or 5—2 viruses existed and were capable of replication, it was impossible to isolate a pure population of either species.  By the time a virus preparation could be expanded from a pure plaque and its DNA analyzed the population was always a mixture of viruses (2—2 and 2—5 in most cases).  It was therefore impossible to precisely ascertain the progeny of such viruses and definitively determine whether 2—5 and 5—2 viruses could yield 2—2 and 5—5 viruses upon replication.  As previously shown (Ruben, Bacchetti, and Graham, 1983; Graham, 1984a; Ghosh Choudhury et al., 1986), it is possible to isolate circular forms of adenovirus by transformation of bacteria with DNA extracted from infected baby rat kidney cells (or rat embryo cells).  This approach was used to obtain a plasmid, pFG154, which was derived from a sub54 infected rat embryo cell DNA extract (sub54RE) (Graham, personal communication) which also gave a composite signal when probed with AB 12 and AB 335 (Fig. 3.2).  Both pFG154 and sub54 shared the same genomic structure (Ad2 (mac) sequences at the left half of the genome and Ad5 sequences at the right) except for the very ends of the genome where the circular form carried an extra three base pairs joining the two covalently linked ITRs.  The "left end" of pFG154 had an Ad2 (mac) ITR and the "right end" an Ad5 ITR (Fig. 3.10).  Several cycles of subcloning of pFG154 ensured that the plasmid was derived from a unique colony.

To determine the progeny of 2—5 viruses upon replication, 293 cells were transfected with pFG154, thirteen plaques picked, and an additional fifty two plaques subsequently isolated to allow the analysis

Figure 3.10: Sequence at the Junction of pFG154. The partial sequence
of the L strand across the junction formed by the two
covalently linked termini in pFG154 is shown. The
sequence 'CTT' found between the left (type 2 ITR) and
right ends (type 5 ITR) was not present in sub54 and
probably arose due to the presence of the palindrome
created by the attachment of the two ITRs in pFG154, as
observed with a number of other infectious circles
(Graham, 1984a). The conserved decamer found in all human
adenoviral serotypes characterized so far is underlined.
The sequence was obtained from Brinkley and Graham
(personal communication).

...GGTATATTAT  TGATGATG  CTT  CTATTCTA  ATAATATACC...

◄──── RIGHT TERMINUS ────►    ◄──── LEFT TERMINUS ────►

of all the possible types of genome. The DNA of each plaque isolate was then amplified (liquid infection), extracted, cut with HindIII, electrophoresed, and probed (Table 3.7). Although most of the isolates contained pure 5—5 viruses (51/65), all four combinations of ITRs were detected. Of all the samples that gave a composite signal none contained pure 2—5 or 5—2 molecules, indicating that 2—5 and 5—2 viruses could generate 2—2 and 5—5 virions.

In contrast to the results obtained with linear genomes, there was no apparent preference for either type of terminus in the samples that gave a composite signal. Nevertheless, the preponderance of pure 5--5 viral isolates, despite the fact that pFG154 was a pure 2—5 sample, indicated a very efficient conversion of type 2 (mac) ITRs to type 5 suggesting a strong preference for the latter type of terminus.

## 3.6  Influence of Mixed Plaques on the Results

With all the viruses that were plaque purified to this point, it was found that 2—2 molecules were predominant whenever 2—5 or 5—2 molecules were detected, but in each case the parental plaque isolate had contained a large proportion of 2—2 molecules. To rule out the possibility that the abundance of 2—2 molecules in the samples containing 2—5 or 5—2 genomes was actually due to aggregation of parental virions resulting in mixed plaques, the progeny of the virus 1.5D was analyzed. This virus was identical to sub54 (linear Ad2 (mac)/Ad5 hybrid genome) and was one of the progeny viruses obtained from pFG154. The 1.5D isolate was primarily composed of 5—5 viruses, but also contained some 5—2 viruses, and possibly minute amounts of 2—5 and

TABLE 3.7
COMPUTER ANALYSIS OF SCANNING DATA: PLAQUE COMPOSITION (AVERAGES)

pFG154 progeny

| | SAMPLE | % 2—2 | % 2—5 | % 5—2 | % 5—5 |
|---|---|---|---|---|---|
| 1) | 1.5 D | 0 – 1 | 0 – 1 | 7 – 8 | 88 – 90 |
| 2) | JR1295 B | 51 – 54 | 0 – 2 | 42 – 45 | 0 – 2 |
| 3) | JR1295 C | 95 – 95 | 3 – 3 | 0 – 0 | 0 – 0 |
| 4) | JR1295 E | 4 – 30 | 0 – 25 | 22 – 48 | 19 – 45 |
| 5) | pFG154-3 | 5 – 73 | 0 – 8 | 17 – 26 | 0 – 8 |
| 6) | pFG154-7 | 59 – 69 | 0 – 8 | 21 – 30 | 0 – 8 |
| 7) | pFG154-21 | 92 – 94 | 1 – 3 | 0 – 2 | 0 – 1 |
| 8) | pFG154-37 | 95 – 95 | 2 – 3 | 0 – 1 | 0 – 0 |
| 9) | pFG154-38 | 98 – 98 | 0 – 0 | 0 – 0 | 0 – 0 |
| 10) | pFG154-41 | 96 – 97 | 0 – 2 | 0 – 1 | 0 – 1 |
| 11) | pFG154-44 | 0 – 5 | 1 – 6 | 0 – 6 | 86 – 92 |
| 12) | pFG154-47 | 46 – 59 | 27 – 40 | 0 – 12 | 0 – 12 |
| 13) | pFG154-51 | 88 – 93 | 1 – 6 | 0 – 4 | 0 – 4 |

also      1 sample  —-> 100%  2—2  (1.10 B)
         51 samples —-> 100%  5—5

2—2 viruses (Table 3.7). The sample 1.5D was thus analogous to sub54-21.59 except that 5—5 molecules were predominant rather than 2—2. If the results reported so far were mainly due to mixed plaques then the 1.5D plaque isolates (progeny) containing 2—5 or 5—2 viruses should also contain large quantities of 5—5 viruses. 1.5D was therefore plaque purified and sixty plaques were picked, from which the DNA was isolated, restricted with HindIII, and analyzed as before. Table 3.8 indicates that most of the progeny were pure 5—5 plaques, as in the initial 1.5D plaque, but that the samples containing 2—5 or 5—2 viruses were once again predominantly composed of 2—2 molecules thus indicating that mixed plaques did not significantly contribute to the preference for the Ad2 (mac) ITR by the sub54 isolates. The results also provided further evidence that 2—5 and 5—2 viruses could yield 2—2 and 5—5 viruses upon replication and suggested that the contradictory results obtained with the progeny of pFG154 might have been related to its circular structure.

## 3.7 Rate of ITR Conversion

As shown in the previous tables, the progeny of 2—5 and 5—2 viruses were largely composed of viruses which repaired their ends such that they contained identical terminal sequences. Although the quantity of each type of molecules was approximate (see next section), it was of interest to roughly evaluate the rate of ITR conversion (Table 3.9). Each value reported in Tables 3.2 - 3.8 is the end result of several replication cycles (1 virus -> plaque -> dish (liquid infection)), thus estimation of the rate of conversion of the termini based on these data

TABLE 3.8
COMPUTER ANALYSIS OF SCANNING DATA:    PLAQUE COMPOSITION (AVERAGES)

1.5 D progeny

| | SAMPLE | % 2—2 | % 2—5 | % 5—2 | % 5—5 |
|---|---|---|---|---|---|
| 1) | 1.5D-1 | 90 – 91 | 0 – 0 | 8 – 8 | 0 – 0 |
| 2) | 1.5D-2 | 94 – 96 | 0 – 1 | 1 – 3 | 0 – 1 |
| 3) | 1.5D-5 | 91 – 93 | 0 – 2 | 3 – 5 | 0 – 2 |
| 4) | 1.5D-9 | 96 – 96 | 0 – 0 | 2 – 2 | 0 – 0 |
| 5) | 1.5D-10 | 92 – 92 | 0 – 0 | 6 – 6 | 0 – 0 |
| 6) | 1.5D-11 | 98 – 98 | 0 – 0 | 1 – 1 | 0 – 0 |
| 7) | 1.5D-13 | 73 – 75 | 0 – 2 | 20 – 23 | 0 – 2 |
| 8) | 1.5D-15 | 86 – 87 | 0 – 0 | 10 – 12 | 0 – 0 |
| 9) | 1.5D-16 | 95 – 97 | 0 – 1 | 1 – 2 | 0 – 1 |
| 10) | 1.5D-23 | 85 – 86 | 0 – 1 | 11 – 12 | 0 – 1 |
| 11) | 1.5D-26 | 90 – 93 | 0 – 2 | 3 – 6 | 0 – 2 |
| 12) | 1.5D-39 | 94 – 94 | 0 – 0 | 5 – 5 | 0 – 0 |
| 13) | 1.5D-40 | 41 – 47 | 0 – 6 | 45 – 51 | 0 – 6 |
| 14) | 1.5D-41 | 67 – 73 | 0 – 5 | 21 – 26 | 0 – 5 |
| 15) | 1.5D-48 | 54 – 61 | 32 – 38 | 0 – 6 | 0 – 6 |
| 16) | 1.5D-49 | 74 – 77 | 0 – 2 | 19 – 22 | 0 – 2 |
| 17) | 1.5D-51 | 84 – 90 | 0 – 6 | 3 – 9 | 0 – 6 |
| 18) | 1.5D-53 | 62 – 68 | 0 – 5 | 24 – 30 | 0 – 5 |
| 19) | 1.5D-55 | 96 – 96 | 0 – 0 | 3 – 3 | 0 – 0 |
| 20) | 1.5D-56 | 81 – 83 | 0 – 1 | 14 – 16 | 0 – 0 |
| 21) | 1.5D-59 | 87 – 89 | 0 – 3 | 4 – 8 | 0 – 3 |

also:    1 sample  —> 100%  2—2  (1.5D-38)
         38 samples —> 100%  5—5

TABLE 3.9:   RATE OF ITR CONVERSION

| Source of Data | Number of Samples[*] | Overall Repair | Repair/ Replication Cycle[**] |
|---|---|---|---|
| Table 3.2 | 4 | 73% | 3% |
| Table 3.3 | 36 | 65% | 2% |
| Table 3.4 | 40 | 57% | 2% |
| Table 3.5 | 11 | 56% | 2% |
| Table 3.6 | 31 | 81% | 3% |
| Table 3.8 | 21 | 85% | 3% |
| Total: 143  Averages: | | 69% | 2% |

[*] : Number of plaque isolates with composite signals obtained from the plaque purification of sub54, sub54-21, sub54-31, sub54-51, sub54-21.59, and 1.5D (Table 3.2 to 3.6 and 3.8 respectively).

[**]: Each virus underwent an estimated 28 rounds of replication (virus -> plaque -> dish).
a) plaque purification:     1 pfu -> $3.1 \times 10^7$ pfu/plaque
b) liquid infection: $6.2 \times 10^6$ pfu -> $4.9 \times 10^7$ pfu/dish
(1/5 of total plaque) <⎯⎯
average of (a) 4 titers and (b) 13 titers

Rounds of replication:  Final pfu = Initial pfu X $2^{rounds}$
a) 25 rounds
b)  3 rounds

N.B.: See text for details.

represents cumulative conversion for all the replication cycles. Therefore the overall efficiency of conversion was first calculated for each sample (amount of viruses with identical ends/total amount of viruses in the isolate, i.e. (2—2 + 5—5) / (2—2 + 2—5 + 5—2 + 5—5)) and the average computed for all samples of a particular group (e.g. progeny of sub54). Since ranges of values of 2—2, 2—5, 5—2, and 5—5 were available for each sample, the values used in the estimation of the repair efficiency were the averages of minimum and maximum reported for each sample (e.g. sub54-21: {(47+56)/2 + (0+8)/2} / {(47+56)/2 + (34+43)/2 + (0+8)/2 + (0+8)/2} = 56.6% repair) (see table 3.2). The overall efficiency of repair was then divided by the estimated total number of replication cycles each virus undergoes to yield the efficiency of repair / replication cycle.

## 3.8 Validity of the Results

Considering the pipetting error while loading DNA on the duplicate gels, the normalization of the raw data (areas obtained from the densitometer/integrator), the imperfect linearity of the response of X-ray film to $^{32}P$ using intensifying screens, and the sensitivity of the densitometer used, the output generated by the computer program should be analyzed with care. The numbers obtained should not be considered exact but rather approximate. This caution does not affect the qualitative conclusions derived from the data since those conclusions were based on the general trends of the results rather than their exact values.

# DISCUSSION

## 4.1 Requirement for Identical Termini

The studies presented in the previous sections show that hybridizing the ends of sub54 with serotype specific probes revealed the presence of both types 2 (mac) and 5 termini. It was further shown that this composite signal was not due to the presence of contaminating parental Ad2 (mac) or sub53 viruses, from which sub54 was derived (Fig. 3.3), but rather was due to a mixture of sub54 viruses (Fig. 3.4). Southern blotting of sub54 and its progeny and subsequent analysis of the blots by densitometry indicated that the composite signal obtained with sub54 was not the result of a simple mixture of viruses with type 2 (mac) termini and viruses with type 5 termini, but that viruses with non identical ends (2—5 and/or 5—2) were also present in the plaque isolates (Tables 3.1 - 3.2).

The identification of viruses containing both Ad2 (mac) and Ad5 ITRs unequivocally proves that adenovirus can have non identical termini and that the presence of non identical ends is not lethal for the virus since 2—5 and 5—2 viruses were shown to replicate their DNA and to produce virions (cytopathic effect and formation of plaques). Further evidence of the viability of molecules with non identical termini was provided by the cloning of sub54 as a molecule replicating as a bacterial plasmid (pFG154), which was exclusively composed of 2—5 genomes. The

isolation of 2—2, 2—5, 5—2 and 5—5 viruses from pFG154-transfected cells (Table 3.7) confirmed that genomes with non identical termini are biologically active and once again indicated that the results with sub54 were not due to contaminating Ad2 (mac) or sub53 viruses.

The heterogeneity between the type 2 (mac) and type 5 termini resided at nucleotides 2-7 (Table 2.1), falling within the sequence that appears to function as a spacer element (introduction). The ITR heterogeneity present in sub54 does not overlap with the conserved sequence 9-18, the binding sites for NFI, NFIII (ORP-C) or ORP-A, or any other partially or fully conserved sequence identified to date (Fig. 1.5). The presence of these different binding and conserved sequences may suggest that, although viruses with heterogeneous terminal sequences were shown to be infectious, the location and extent of the heterogeneity may be critical for the viability of such viruses. It is not known whether viruses with non identical termini with heterogeneity within a conserved or binding sequence would be viable or not. It is possible that the minimal requirement for viability is one complete and intact (i.e. wt) terminus.

## 4.2  Interconversion of the Inverted Terminal Repeats

Although viruses with non identical termini were detected, a pure population of 2—5 or 5—2 molecules could never be isolated, i.e. 2—2 and/or 5—5 molecules were always isolated as well. Nevertheless pure 2--2 or 5—5 plaque isolates were obtained indicating that the ends of such viruses were stable. Therefore, interconversion of the termini appears to be characteristic of viruses with non identical ends and

requires Ad2 (mac) and Ad5 terminal templates (i.e. no spontaneous mutations). This was further confirmed by the stability of the ends of Ad5 viruses over the years and Ad2 (mac) viruses over seven successive passages (data not shown).

### 4.2.1 Models (Viruses)

The exact mechanism by which the ITRs were interconverted is unknown and a number of models involving replication or recombination were considered. Homologous recombination among the 2—5 viruses of a plaque isolate can efficiently occur since the genomes are perfectly homologous but recombination within unique sequences would not result in molecules other than 2—5. Therefore production of 2—2, 5—5, and 5—2 viruses requires that left and right ITRs recombine. Two proposed recombination models were considered. Recombination by the Holliday model (Holliday, 1964) does not require replication of the molecules to recombine and is reciprocal, thus predicting equivalent amounts of 2—2 and 5—5 genomes from recombining 2—5 molecules (Fig. 4.1). This model also predicts that subsequent recombination between 2—2 and 5—5 genomes would yield 2—5 and 5—2 viruses in the same ratio, resulting in the presence of numerous 5—2 viruses in plaques originating from 2—5 viruses. Densitometric analyses showed that the progeny of 2—5 viruses did not contain equivalent amounts of 2—2 and 5—5 viruses and that 5—2 molecules were rare (Tables 3.2 - 3.5). Therefore, if a Holliday-type mechanism is responsible for the observed ITR interconversion, one must invoke some sort of growth advantage for viruses with Ad2 (mac) sequences. 5—2 molecules can be the result of a double recombination

Figure 4.1: Repair of the Termini by Recombination Via the Holliday
Model (Linear Molecules). Reciprocal recombination
within two ITRs is shown where light and heavy lines
distinguish the two double stranded parental genomes.
Arrows indicate the orientation of the recombining
molecules (pointing away from the E1 transcription region
and towards the E4 transcription block). Closed circles
represent pTP/TP; 2 and 5 depict Ad2 (mac) and Ad5 ITRs
respectively. The 3' end of the heteroduplexes (2/5
ITRs) are repaired via nucleases and subsequent filling of
the gap by a DNA polymerase (DNA pol), possibly assumed by
Adpol (removal of the terminal 5' nucleotides may occur
but cannot be repaired by subsequent DNA synthesis due to
the absence of a 3' hydroxyl group). Following the
production of 2—2 and 5—5 genomes, 5—2 viruses could
arise by homologous recombination within the termini or
anywhere in the genome. Preferential replication of
molecules with Ad2 (mac) termini would result in more 2—2
than 5—5 viruses. The skewed ratio of 2—2 vs 5—5 would
then result in few 5—2 viruses.
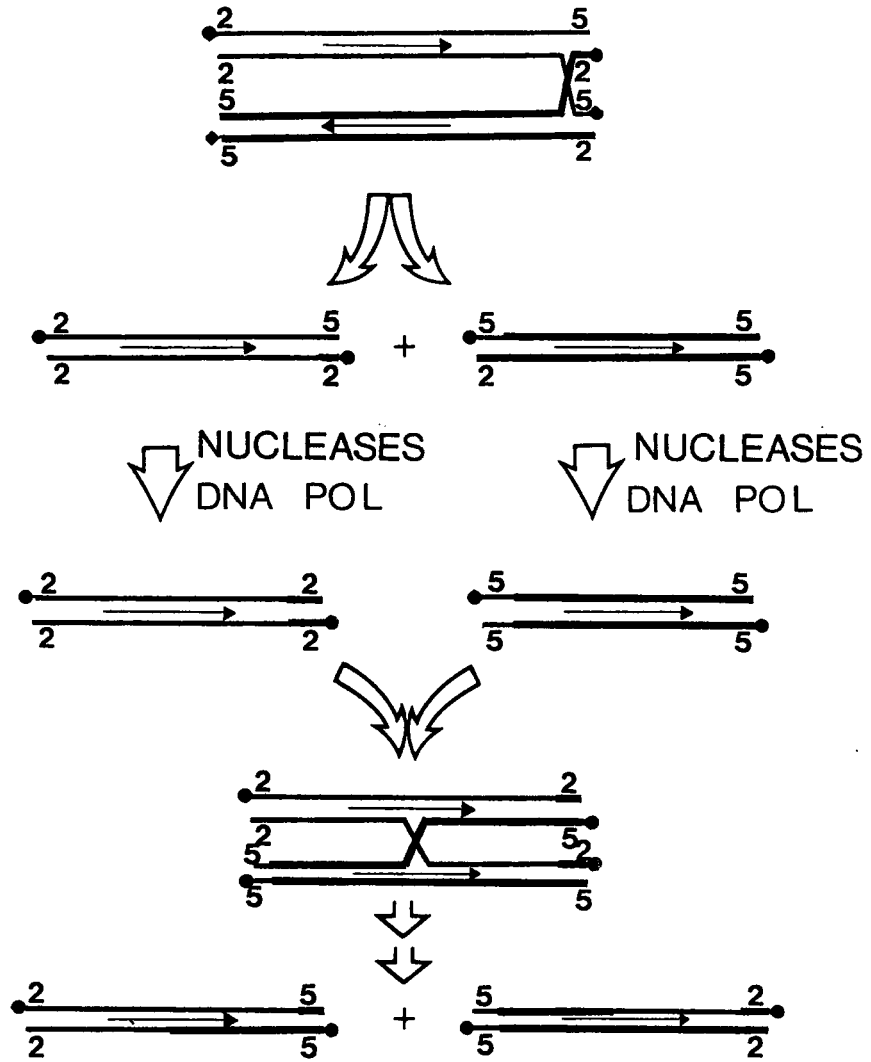
event where both left and right ends of 2—5 viruses recombined or most likely by homologous recombination between 2—2 and 5—5 viruses anywhere in the genome ($\approx$ 36 kb of homology). If viruses with Ad2 (mac) ITRs had a strong growth advantage over those with Ad5 termini, then there would be few 5—2 molecules produced due to the lack of 5—5 viruses (rate limiting factor).

Recombination by the Meselson-Radding model (Meselson and Radding, 1975) is based on the invasion of a dsDNA genome by a single DNA strand. Recombination is asymmetrical since only the "invaded" molecule is modified by the recombination event. The Meselson-Radding model does not predict equivalent amounts of 2—2 and 5—5 viruses by recombination of 2—5 molecules if replication is asymmetrical. Initiation at the Ad2 (mac) termini would produce type 2 (mac) "invading single strands" (Fig. 4.2) which would base pair to the Ad5 ITR. This heterologous region would be repaired to double-stranded Ad2 (mac) ITR whereas the molecules which donated the invading single strand would remain Ad2 (mac). Thus, 2—2 molecules would be produced from recombination between two 2—5 viruses, if replication were initiated at the Ad2 (mac) terminus. In a similar way, during recombination of two 2—5 molecules, an invading single strand carrying an Ad5 ITR would produce 5—5 molecules. Therefore, if the rate of initiation from Ad2 ITRs were greater than that from Ad5 ITRs, one would expect more 2—2 viruses to be produced than 5—5 viruses. This model therefore predicts that, conditional to asymmetry of replication, more 2—2 than 5—5 viruses should be present in the plaques obtained from 2—5 viruses (or 5—2) and is in agreement with the data.

Figure 4.2:  Repair of the Termini by Recombination Via the Meselson-

Radding Model (Linear Molecules).  Recombination occurs

within the ITRs but is not reciprocal.  Preferential

initiation of replication at the Ad2 (mac) ITR (left hand

side of the figure) results in a higher overall conversion

of 2—5 to 2—2 than 5—5.  The skewed ratio of 2—2 vs 5

—5 viruses results in the prediction of few 5—2 viruses.

Refer to Fig. 4.1 for the symbols used.
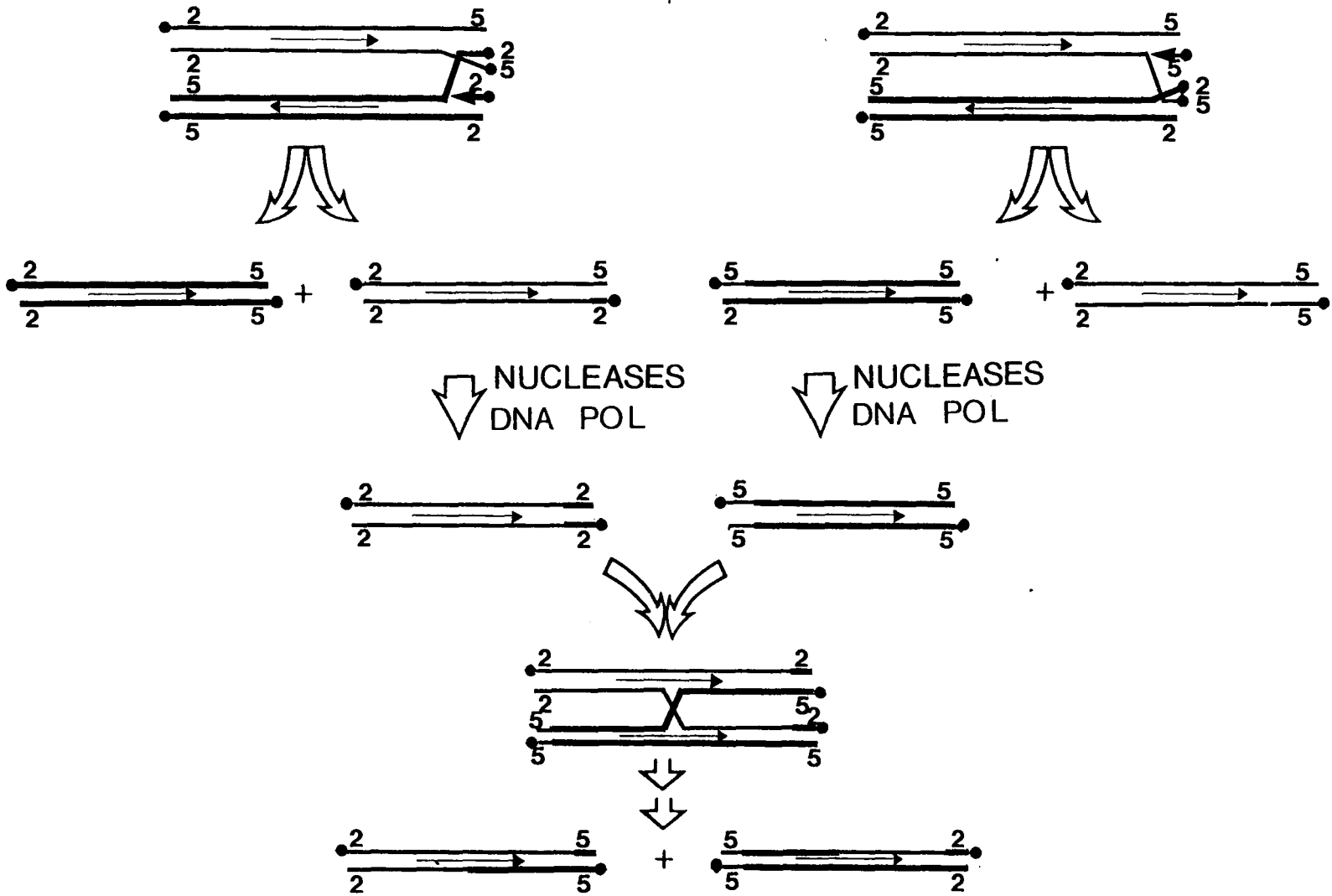
Although the above recombination models could explain the data, they probably do not contribute significantly to the conversion of the termini. First, previous studies have shown that adenovirus can recombine efficiently with recombination frequencies ranging up to ≈40% depending on the systems studied (Young and Silverstein, 1980; Meinschad and Winnacker, 1980; Wolgemuth and Hsu, 1980, 1981; d'Halluin, Cousin, and Boulanger, 1982; Volkert and Young, 1983; Mautner and Mackay, 1984). However, in these studies relatively large regions of homology were involved, ranging from approximately 4 kb to 36 kb, whereas recombination within the ITRs involves the small region of homology present at the ends (96 bp). It is likely that homologous recombination within the ITRs would occur at a relatively low frequency, unless some site-specific recombination process is acting, whereas the conversion of the ITRs observed was quite efficient.

Second, Munz and Young (1984) proposed that recombination is initiated at one of the ITRs followed by branch migration. DasGupta and Radding (1982), and Munz and Young (1984) showed that the presence of heterologous sequences blocks branch migration and results in inhibition of recombination. Although wtAd2 and Ad5 can recombine efficiently (Ginsberg and Young, 1977), during the isolation of sub54 (sub53 X Ad2 (mac) recombinant) it was observed that the frequency of recombination obtained was surprisingly low (Graham, personal communication). The presence of heterologous sequences at the very ends of the two viruses (type 2 (mac) vs type 5 ITRs) might be responsible for that low efficiency of recombination. This explanation would suggest that the high frequency of conversion of the ends of the 2—5 or 5—2 viruses

could not be due to recombination within the terminal 96 bp. Nevertheless recombination models cannot be ruled out.

To reconcile all the results in a single model, a mechanism based on the current model of adenovirus DNA replication was considered (Fig. 4.3). As an example, the replication of a 2—5 virus is depicted but the same model can be applied to 5—2 viruses provided that the two ITRs are switched in Fig. 4.3. In agreement with the Lechner and Kelly model of adenovirus replication, initiation occurs at either end of the 2—5 molecule displacing one of the strands, which is subsequently used as a template for replication from the 3' end. The core of the proposed model relies on the presence and use of the panhandle to allow initiation of replication off the displaced strand. In the case of a 2—5 (or 5—2) virus it is assumed that the panhandle can be formed since 97/103 nucleotides of the termini are complementary, generating an imperfect dsDNA origin of replication which would be repaired at a certain frequency. Repair would consist of the removal of the terminal seven nucleotides (or more) by host nucleases (endo- or exonucleases) or Adpol, thus eliminating the region of heterogeneity at the end of the panhandle, and filling of the gap by a host DNA polymerase or Adpol. The protection by TP from exonuclease digestion of the 5' end (Carusi, 1977) and the inability of polymerases to synthesize DNA in the absence of a 3' hydroxyl group indicates that repair must exclusively take place at the 3' end of the panhandle. Generation of a 2—2 or 5—5 molecule would therefore depend on whether initiation occurred at the left or the right end of the 2—5 dsDNA template, respectively (Fig. 4.3). Production of 5—2 viruses directly from 2—5 viruses (or vice versa) cannot occur by

Figure 4.3: Repair of the Termini Via Panhandle Formation (Linear

Molecules). Replication proceeds as described according

to the Lechner and Kelly model of replication and relies

on the formation of panhandles as replicative

intermediates. The heterogeneity at the duplex end of the

panhandle results in the repair of the 3' ITR (see boxes)

by nucleases and subsequent DNA synthesis to fill in the

gap (DNA pol), both of which are possibly carried out by

Adpol. The repair mechanism is not 100% efficient and

therefore allows some amplification of 2—5. Production

of 2—2 or 5—5 molecules depends on which strand is

displaced and 5—2 viruses presumably result from the

homologous recombination between 2—2 and 5—5 viruses.

Closed circles: TP/pTP present at the 5' end of each

strand; Bold lines: Replicating DNA; Light lines: DNA

templates; Arrows: Direction of DNA synthesis; 2: Ad2

(mac) ITR; 5: Ad5 ITR.

REPLICATION
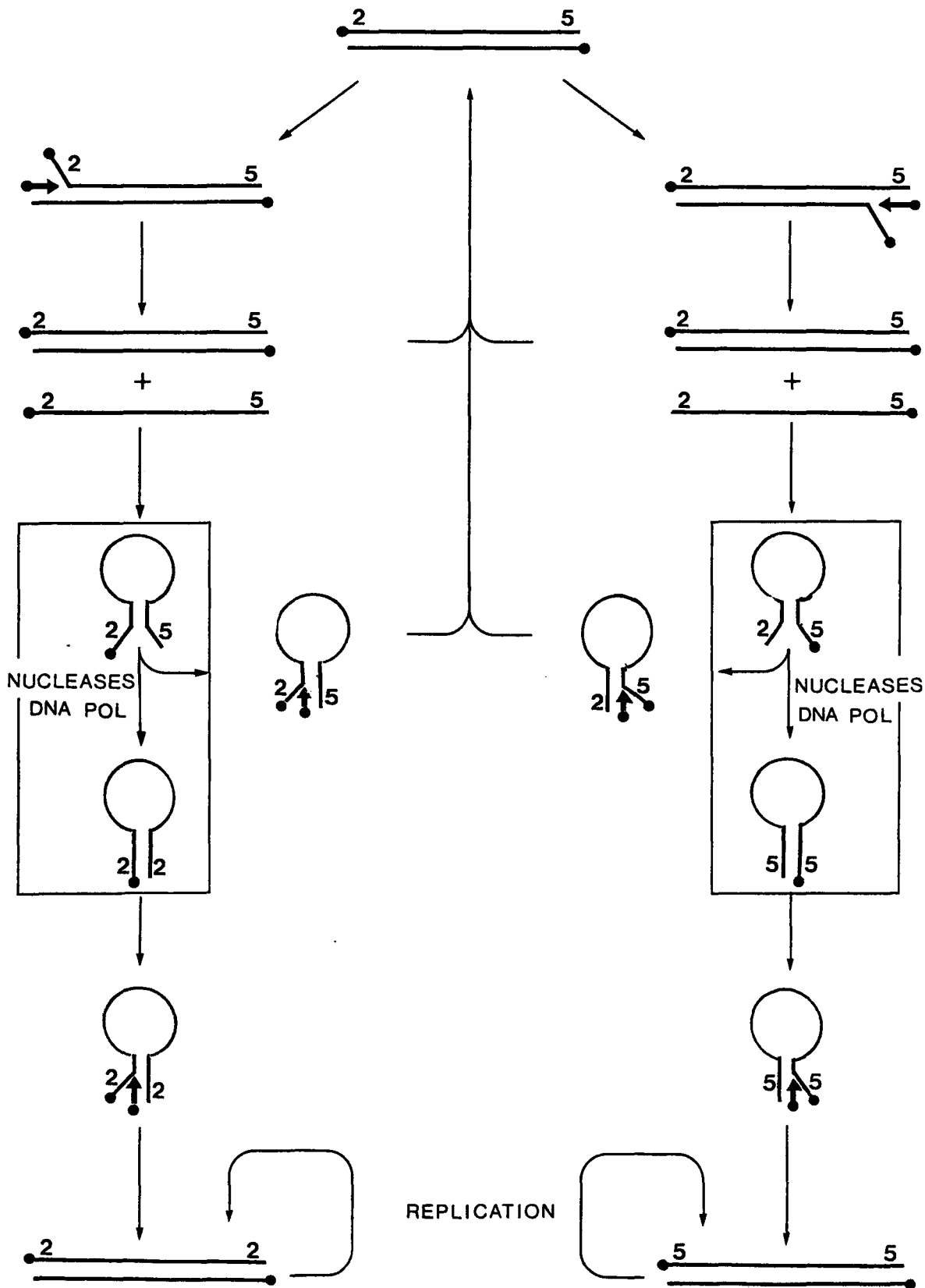
this mechanism and they are likely to arise by homologous recombination between 2—2 and 5—5 viruses (the recombination does not need to be within the ITRs). The low abundance of 5—2 viruses most likely reflects the lack of 5—5 molecules produced by 2—5 viruses, as can be seen in Tables 3.2 - 3.5, rather than a low recombination efficiency.

Tables 3.2 - 3.5 show that the progeny of 2—5 viruses were predominantly 2—2 virions, as opposed to 5—5 virions (this issue is discussed in section 4.3), and that, quite often, the isolates contained more 2—2 than 2—5 viruses. This latter observation can be incorporated into the model in the following way. Although 2—5 viruses are amplified, they produce 2—2 viruses at a certain frequency due to conversion of the ends of 2—5 viruses. Since 2—2 molecules are also amplified but cannot generate 2—5 viruses, 2—2 viruses are amplified at a rate greater than 2—5 viruses. The extent of the 2—5 replication prior to repair of the first ITR (to produce a 2—2 virus) probably accounts for the wide range of ratios of 2—2 vs 2—5 viruses observed, which varied from ≈ 95:5 to ≈ 20:80 (Tables 3.2 - 3.5).

## 4.2.2  Evidence for Panhandle Formation

If the model is correct (Fig. 4.3), the high frequency of conversion of ITRs would suggest that panhandle formation is important for the replication of adenovirus. The results of a number of other investigations also directly or indirectly support the idea that the panhandle is important and active during the replication of adenovirus. First, in their characterization of Ad5 mutants with duplicated ITRs, Haj-Ahmad and Graham (1986b) concluded that the interconversion of

dlE1,3-1, dlE1,3-2 and dlE1,3 can be best explained by hybridization of the ITRs from each end of the molecules through the formation of panhandle structures. Second, in his deletion analysis of the origin of adenovirus replication, Stow (1982) reported the restoration of nucleotides deleted from the left ITR. He proposed that the terminal sequences are regenerated by the use of the right ITR as a template following panhandle formation. Such a proposition is substantiated by the absence of repair when one of the ITRs is completely absent. Third, Hay et al. (1984) showed that the panhandle is a possible configuration in mammalian cells using minichromosomes with Escherichia coli palindromic repeats. They also showed that such a template is used for DNA synthesis. Finally, it was shown that Adpol replicates ΦX174 ssDNA templates using as origin of replication the heptamer 'TATTTG', present at two different locations in the ΦX174 genome (Ikeda, Enomoto, and Hurwitz, 1982; Guggenheimer et al., 1984a). This sequence is present in the ITR of Ad2 and Ad5 but is not in the essential origin of replication (nucleotides 20-26). This indicates that the specificity of replication may be different for single stranded and double stranded templates, which in turn suggests that the generation of a dsDNA origin of replication through panhandle formation may be required to obtain type II adenovirus replicative intermediates. Despite the circumstantial evidence for panhandle formation, direct proof of its existence and role in replication remains to be established.

## 4.3 Preference for Ad2 (mac) Over Ad5 Termini

With respect to the ITR repair model proposed (Fig. 4.3), the

results in Tables 3.2 - 3.5 would suggest that the replication of sub54 is asymmetric, and that initiation at the left end is more frequent than at the right. However, this is in contradiction with the literature (Schilling et al., 1975; Tolun and Pettersson, 1975; Lechner and Kelly, 1977; Kowalski and Denhardt, 1982) and, according to the current model of replication of adenovirus (Lechner and Kelly, 1977), both ends serve as origin of replication at equal frequency. Furthermore if the strong bias for the conversion of 2—5 to 2—2 viruses (rather than to 5—5) were due to asymmetrical replication, then virus isolates having "switched" ITRs, i.e. 5—2 (sub54-21.59), should have generated predominantly 5—5 progenies. The results shown in Table 3.6 indicated that this was not the case and argued strongly against a replication bias favoring the left end of adenovirus.

Although aggregation of viruses (i.e. mixed plaques) has not been reported as a major problem with adenovirus, the preponderance of 2—2 viruses in the plaque purified isolates suggested that aggregation might be responsible for the abundance of type 2 (mac) ITRs in the progeny of those isolates. However, the isolation and characterization of the progeny of sample 1.5D, containing a mixture of 5—2 viruses and a large amount of 5—5 genomes relative to 2—2, revealed that the plaque isolates with composite signals contained an excess of 2—2 molecules over 5—5 (Table 3.8), thus confirming that the interconversion of the termini was a real phenomenon and was not due to production of mixed plaques.

Results from the coinfection of sub54-21.47 (5—5) and sub54-21.60 (2—2) indicated that, over several cycles, viruses with type 2

(mac) ITRs slowly but significantly outgrew viruses with type 5 ends (Fig. 3.7). Plaque purification of sub54-21.47 and subsequent liquid infection (prior to the coinfection) did not result in any detectable Ad2 (mac) ITRs (and no detectable Ad5 ITRs with sub54-21.60) (data not shown) thus once again indicating that the Ad5 and Ad2 (mac) termini were stable in viruses with identical termini. This preference for type 2 (mac) termini was consistent with the results obtained from viruses with non identical ends. However, the rate of conversion (change in the relative quantity of Ad2 (mac) and Ad5 termini during an infection) of Ad2 (mac) to Ad5 was not constant throughout the ten passages but decreased after several passages.

Based upon the results obtained with 2—5 and 5—2 viruses, two mechanisms could account for the conversion of the termini: 1) Repair does not prefer either ITR but initiation of replication is preferential at the type 2 (mac) ends (left pathway at top of Fig. 4.3) or 2) Conversion of type 5 to type 2 (mac) termini in genomes with non identical termini, assuming that 2—2 and 5—5 viruses recombine and generate 2—5 and 5—2 molecules, is favoured by the repair mechanism via the panhandle (left box of Fig. 4.3) and is independent of replication. Preferential conversion of type 5 to type 2 (mac) termini by either preferential replication or preferential repair should result in the disappearance with time of the Ad5 ITRs (one should get an asymptote at 0% for Ad5 ITRs and at 100% for Ad2 (mac) ITRs). The plateaus observed in Fig. 3.7 suggest that, although there was a preference for the Ad2 (mac) ITRs, a second mechanism, in favour of type 5 ends, existed and maintained viruses with those ITRs in the population. Since the

coinfection data suggest that two mechanisms maintain the ITRs, each specific for one of the termini, it is tempting to suggest that the replication machinery preferentially uses the Ad5 ITRs and repair favours the Ad2 (mac) ITR (5 -> 2) (the only possiblity compatible with the ones mentioned above). The selective advantage of the preferential replication of the Ad5 termini may account for the presence of ITRs similar to the Ad5 terminus at the ends of numerous adenoviral serotypes.

Munz et al. (1983) reported that the recombination frequency decreases as the ratio of the parental genomes deviates from 1, an observation consistent with the results obtained in our laboratory (Wilson, personal communication). This decrease is caused by the lower probability of recombination between one of the parental genomes, present in large amounts, and the other parental genome only present in small amounts. The resulting variation in the total amount of recombinants (2--5 and 5--2) vs the ratio of the parental viruses (2--2/5--5) may explain why the net conversion of Ad5 to Ad2 (mac) ITRs decreased with time (i.e. more 2--5 and 5--2 molecules were initially produced than in subsequent liquid infections). Although the ratio of 2--2 and 5--5 viruses were similar in the first and second liquid infections (1st coinfection), conversion of Ad5 ITRs to Ad2 (mac) ITRs in the first passages yielded a ratio of 2--2/5--5 closer to 1 and thus presumably resulted in the production of even more 2--5 and 5--2 viruses (greater conversion by the repair mechanism) whereas in the second passages, repair of the Ad5 termini resulted in a ratio of 2--2/5--5 deviating from 1, thus yielding less 2--5 and 5--2 viruses (lower conversion by the repair mechanism). The balance between the two possibly opposite

conversion mechanisms (replication: 2 -> 5 and repair following recombination between 2—2 and 5—5: 5 -> 2) would determine the net conversion of termini. In the first passages (# 1-3; Fig. 3.7), conversion of the termini by repair might have been more efficient than by replication whereas in the subsequent liquid infections, conversion of the ends by both mechanisms reached an equilibrium (recombination then repair ≈ replication) which was maintained in subsequent liquid infections (net conversion = 0).

The present model remains very speculative. There is to date no direct evidence that the replication machinery of sub54 preferentially uses Ad5 over Ad2 (mac) ITRs. If real, this preference may be relatively slight but could possibly be detected by comparing 2—2 and 5—5 replication rates (time courses). Since the recombination frequency in the assay (coinfections) is unknown, it is not possible to estimate the relative rates of replication of 2—2 and 5—5 viruses using the present data.

## 4.4 Role of Viral Sequences in the Selection of ITRs

### 4.4.1 Possible Selection of Termini by the Gene Products Involved in Replication

The process by which proteins involved in replication and repair of the ITRs might select particular viral termini is unknown. As stated in materials and methods the difference between the two termini is located at nucleotides 2-7 and resides in the spacer element. Since it has been shown that the exact sequence of the spacer element is not critical for replication (introduction), the hypothesis that either type

of ITR (Ad2 (mac) vs Ad5) is preferred over the other is hard to sustain. The spacer element may nevertheless have a subtle and indirect effect on replication or repair by affecting binding of viral or cellular factors to their respective sites. Such an effect may be sufficient to favour the use of particular termini by either of the ITR conversion mechanisms. In vitro studies showed that the Ad2 polymerase can be used to replicate DNA templates of a variety of serotypes but that replication is less efficient than with Ad2 templates (Stillman et al., 1982b), thus suggesting that the Ad2 polymerase cannot efficiently use ITRs from other serotypes. These observations, in appearance contradictory with the possible preference of sub54 for Ad5 termini during replication, may be reconciled with our data if the Ad2 (mac) polymerase is identical to the wild type Ad2 (which has ITRs identical to that of Ad5) polymerase.

Rekosh et al. (1977) proposed that TP plays a role in the formation, positioning and/or stabilization of the initiation complex. Such function(s) may require the recognition of particular adenoviral sequences and may enable pTP/TP to distinguish between Ad2 (mac) and Ad5 ITRs. More precise mapping is required in order to determine whether sequences involved in the selection of the ends map to Adpol, pTP/TP, DBP or any other gene(s). In vitro studies using purified Adpol, pTP, DBP and templates of different serotypes may also be revealing.


4.4.2   Mapping of Internal Viral Sequences Involved in the Selection
        of Termini

Analysis of the sub54 genome reveals that, with the exception of

DBP, the replicative machinery is encoded by the Ad2 (mac) sequences. Therefore, viral hybrids, containing a complete Ad5 replication machinery (Adpol, pTP and DBP), were isolated by recombination of pJM17 and pFG154-G8 or pFG154-G8Δ6 to verify whether the Ad2 polymerase, or pTP/TP, may somehow prefer either ITRs. As shown in Fig. 3.8 and 3.9, all the hybrids possessed Ad5 ITRs.

The absence of any detectable Ad2 (mac) termini among the progeny of the above viral hybrids was probably due to the approach used to generate recombinants with an Ad5 replication machinery. The mechanism by which recombinants arise using the pJM17 technique is unknown but it was suggested by McGrory et al. (1988) that pJM17 recombines as a replicating linear molecule whereas the coinfecting plasmid remains circular. Recombination by the Meselson-Radding model (1975) requires the invasion of the pJM17 plasmid (into which the Ad2 (mac) sequences are to be rescued) by a pFG154-G8 single strand displaced during replication. Replication of pFG154-G8 is unlikely due to the low replication efficiency of templates with an embedded origin of replication, therefore recombination by the Meselson-Radding model is improbable (Fig. 4.4). Alternatively, recombination could proceed by the Holliday model (1964) and generate two types of viral hybrids depending on whether the L or the R single strands of pFG154-G8 and pJM17 were exchanged (Fig. 4.5). In the case of molecules where the L strands were exchanged by recombination (right panel), repair of the ends may produce two different types of molecules. If the 5' end of the L strand were not digested by nucleases, repair of the left ITR would result in the replication of the bacterial sequences. This would inactivate the adenovirus origin of replication at

Figure 4.4: Fate of ITRs in the Viral Hybrids Rescued by the pJM17 Technique (Meselson-Radding Model). Invasion of pJM17 by pFG154-G8 or pFG154-G8Δ6 (left panel) is not possible because the latter plasmids cannot replicate due to their embedded origin of replication. Invasion of pFG154-G8 or pFG154-G8Δ6 by replicating pJM17 (right panel) yields recombinants which only contain the left 7.7% of the adenoviral genome and are therefore not viable. The triangle represents a bacterial insert and the undulated lines the plasmid sequences into which the adenoviral HindIII G fragment was cloned (see Fig. 3.8 and 3.9). The Ad5 and Ad2 (mac) sequences are represented by light and bold lines respectively. '5' and '2' refer to type 5 and type 2 (mac) ITRs respectively. Closed circles depict the 5' terminal protein.
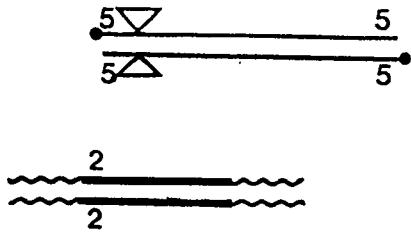
pJM 17

pFG 154 - G8 or pFG154-G8Δ6

NO RECOMBINATION

NOT VIABLE

Figure 4.5:   Fate of ITRs in the Viral Hybrids Rescued by the pJM17

Technique (Holliday Model). Rescue of viral hybrids

resulting from recombination between pJM17 and pFG154-G8

(or pFG154-G8Δ6) is shown. Both possible heteroduplexes

produced by the recombination event, depending on whether

the R (far left pathway) or L strand (right pathway) were

exchanged, are depicted. Repair presumably proceeds as

indicated in Fig. 4.1 - 4.3 i.e. via removal of the

heterologous sequences by nucleases and subsequent DNA

synthesis (DNA pol) to obtain a double-stranded origin of

replication. Replication can initiate at either the left

(1) or the right terminus (r) of the heteroduplex DNA

genomes. The triangle represents a bacterial insert and

the undulated lines the plasmid sequences into which the

adenoviral HindIII G fragment was cloned (see Fig. 3.8

and 3.9). The Ad5 and Ad2 (mac) sequences are represented

by light and bold lines respectively. '5' and '2' refer

to type 5 and type 2 (mac) ITRs respectively whereas '2—

2' and '5—5' refer to viruses with identical Ad2 (mac) or

Ad5 ITRs respectively. Closed circles depict the 5'

terminal protein.
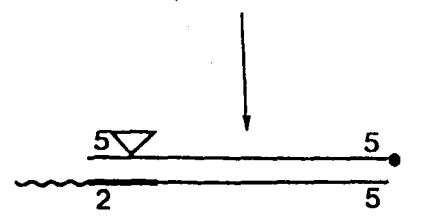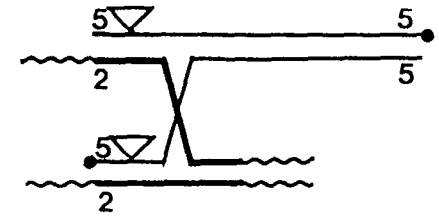
that end and initiation of replication at the right end would result in the exclusive production of 5—5 viruses. Thus all virions produced by the recombinants may have Ad5 termini irrespective of their preference for either ITR. If the bacterial sequences present at the left end were removed by nucleases, then 2—2 or 2—5 viruses would be produced. Such nuclease activity is unlikely though because it would have to be specific for the bacterial sequences, since removal of viral sequences at the 5' end of the left termini could not be replaced by subsequent DNA synthesis (DNA polymerases cannot add nucleotides at 5' ends).

## 4.5  Repair of Termini from Infectious Circles

The results obtained with the circular pFG154 were quite surprising. Although pFG154 was a "pure" 2—5 molecule it generated, from a total of 65 plaques, 51 pure 5—5 plaques as well as several plaques with composite signals containing 2—5 and/or 5—2 genomes. Since large quantities of either 2—2 or 5—5 viruses were found in the composite plaques (Table 3.7), there was no clear indication of ITR preference. These findings significantly differed from the data obtained with linear molecules and did not appear at first to be consistent with the ITR conversion model discussed above. Since pFG154 and sub54 were identical, except for their structure (circular vs linear), the rates of conversion of the termini were expected to be the same for pFG154 as for sub54. As was the case for sub54, repair of the termini of pFG154 was expected to yield 2—2 viruses in large quantities. Eighty percent of the plaque isolates (52/65) did not show any sign of 2—5 genomes (Table 3.7) and an additional five or six samples had low levels of 2—5 and 5—

2 molecules and could indeed have been pure 2—2 or 5—5 plaques. The extremely efficient repair of the ends, much more efficient than that observed with 2—5 and 5—2 linear molecules, the absence of amplification of the circular templates without repair in most cases (no detectable 2—5 viruses), and the preference for Ad5 termini rather than Ad2 (mac) suggested that a different repair mechanism may be involved with pFG154.

The mode of replication of adenoviral circles is unknown. It is assumed that they replicate in a fashion similar to replication of linear molecules, in that initiation occurs with the pTP-dCMP complex followed by elongation. Despite the isolation of linear progenies upon transfection of mammalian cells with infectious circles (Graham, 1984a), actual linearization of the input DNA has never been shown and the mechanism by which linear progeny is produced from circles is unclear. Linearization of pFG154 at the junction of the two ITRs, if it happened, would yield linear 2—5 molecules which should subsequently produce plaques containing abundant quantities of both 2—2 and 2—5 viruses (as described before with viruses with non identical termini). The absence of any 2—5 amplification in most cases therefore suggested that circular templates do not normally linearize and that the production of linear progeny by circles proceeds by a mechanism other than linearization of the input DNA. Pearson et al. (1983) has shown that adenoviral circular templates containing a unique ITR can produce rolling circles upon replication in vitro, suggesting that termination of replication does not take place with a circular substrate. In contrast, termination of replication of linear molecules is inevitable since the Adpol runs off

the template at the end of the genome. Further evidence that replication is terminated by the run off of Adpol at the end of the genome is provided by Hay et al. (1984) who characterized the in vivo replication of minichromosomes containing internal origins of replication. Their results show that Adpol does not terminate DNA synthesis at the internal ITRs since molecules resulting from the run off of the Adpol at the ends of minichromosomes are detectable. They proposed that precise and subsequent reinitiations of replication at the adenoviral origin of replication rather than termination are responsible for the generation of linear molecules off mini-chromosomes with embedded termini.

If running of Adpol off the DNA template is required to terminate a replication cycle, then pFG154 and other infectious circles, unless linearized, should produce adenoviral rolling circles, as observed by Pearson et al. (1983). Since the production of rolling circles is unlikely (see introduction), a different replication model was considered. In the proposed model (Fig. 4.6) replication is preferentially initiated at the Ad5 termini of the circular template, as previously proposed for linear molecules. Following replication of the Ad5 ITR (5' end of the newly synthesized DNA strand) replication proceeds all the way around the circular template. If replication continued up to the Ad2 (mac) ITR (3' end of the newly synthesized DNA strand), then any further replication would yield rolling circles. Alternatively Adpol may switch templates prior to replication of the Ad2 (mac) ITR such that it uses the ITR present at the 5' end of the newly synthesized strand. The Adpol would therefore fall off the template, DNA synthesis stop at that terminus, and a subsequent round of replication

Figure 4.6: Repair of the Termini (Infectious Circles). Replication
is initiated at the Ad5 origin of replication in either
one of the configurations possible for infectious circles
i.e. the two ends may be joined head to tail ("flushed
ends") or assume a cruciform structure (Graham, 1984a).
When replication has proceeded all the way around the
template (except for the 3' ITR) the DNA strands assume a
configuration (possibly base pairing of the ITRs of the
template strand) that allows Adpol to switch templates
and use the 5' ITR from the newly synthesized strand to
complete the first replication cycle. As a result Adpol
falls off the template and DNA synthesis ends. A
subsequent replication cycle produces a linear double
stranded genome. Closed circles: pTP/TP; Vertical bar:
Junction of the two ITRs; E1: Early region 1; E4: Early
region 4; 2: Ad2 (mac) ITR; 5: Ad5 ITR; L: L strand; R: R
strand.
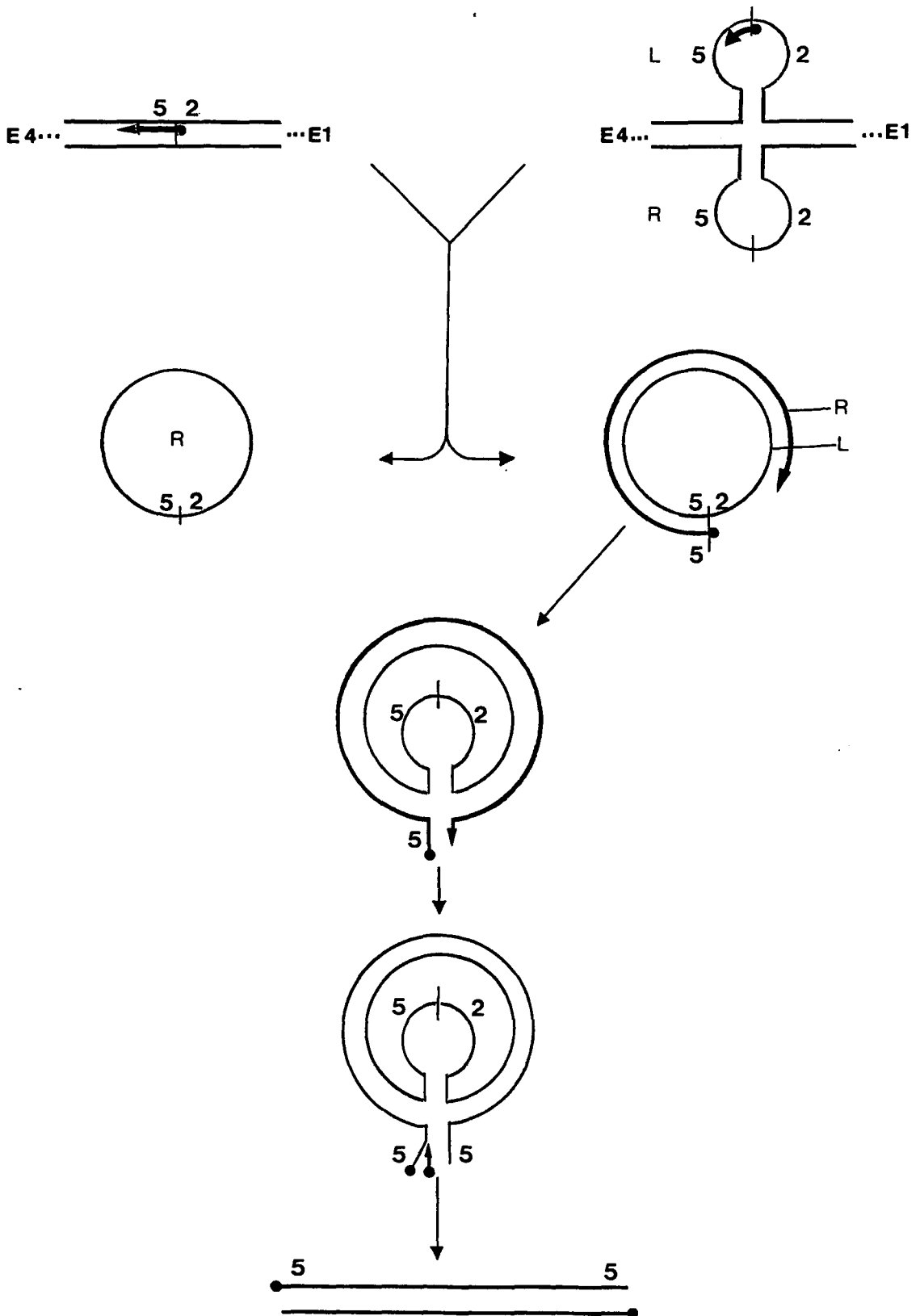
displace the strand and yield a linear molecule. As a result of the template switch, both termini of the genome have the same sequence, thus explaining the repair of the ITRs. If switching occurred at each replication of the circular templates, then the preferential initiation of replication at the Ad5 terminus would produce 5—5 molecules without 2—5 amplification, since only the input circular DNA would be of the type 2—5. The isolation of plaques exclusively containing 2—2 viruses (1/65 or 1.5%) (Table 3.7) could be attributed to an occasional but rare initiation of replication at the Ad2 (mac) termini suggesting an almost perfect ITR recognition mechanism. Also presumably rare, nicking of the circular templates at the ITR junction would produce 2—5 viruses and account for the low frequency of 2—5 viruses observed (1.5%) (Table 3.7). Alternatively, 2—5 molecules could be produced by subsequent rounds of replication without nicking of the input DNA (in which case concatemers would also be produced; see previous paragraph). Those 2—5 viruses presumably produce, upon replication and repair of their ends, large amounts of 2—2 virions, as described for molecules with non identical termini. In contrast, replication of the covalently closed circles primarily produces 5—5 viruses. The abundance of 2—2 or 5—5 molecules would then depend on how early in the infection cycle 2—5 viruses are produced. If produced early then 2—2 molecules should predominate, whereas if produced late large quantities of 5—5 viruses would already have been produced by the circular template. The 5—2 viruses observed could arise as a result of a double template switch, once following replication of the Ad5 ITR (beginning of the new strand) and once prior to synthesis of the 3' ITR (Fig. 4.7). The higher

Figure 4.7:   Generation of 5—2 Molecules by pFG154 via Double

Template Switching.  Replication is initiated using the L

strand for template as before.  Switching of parental

templates immediately after replication of the 5' ITR

would result in the use of the R strand for template.

Replication would proceed as usual all the way around the

template up to the 3' ITR where a second template switch

may occur such that the parental L strand is used again

for template.  Subsequent rounds of replication would

produce linear 5—2 molecules.  As for 2—5 molecules,

concatemers may also be produced.  See Fig. 4.6 for

symbols used.
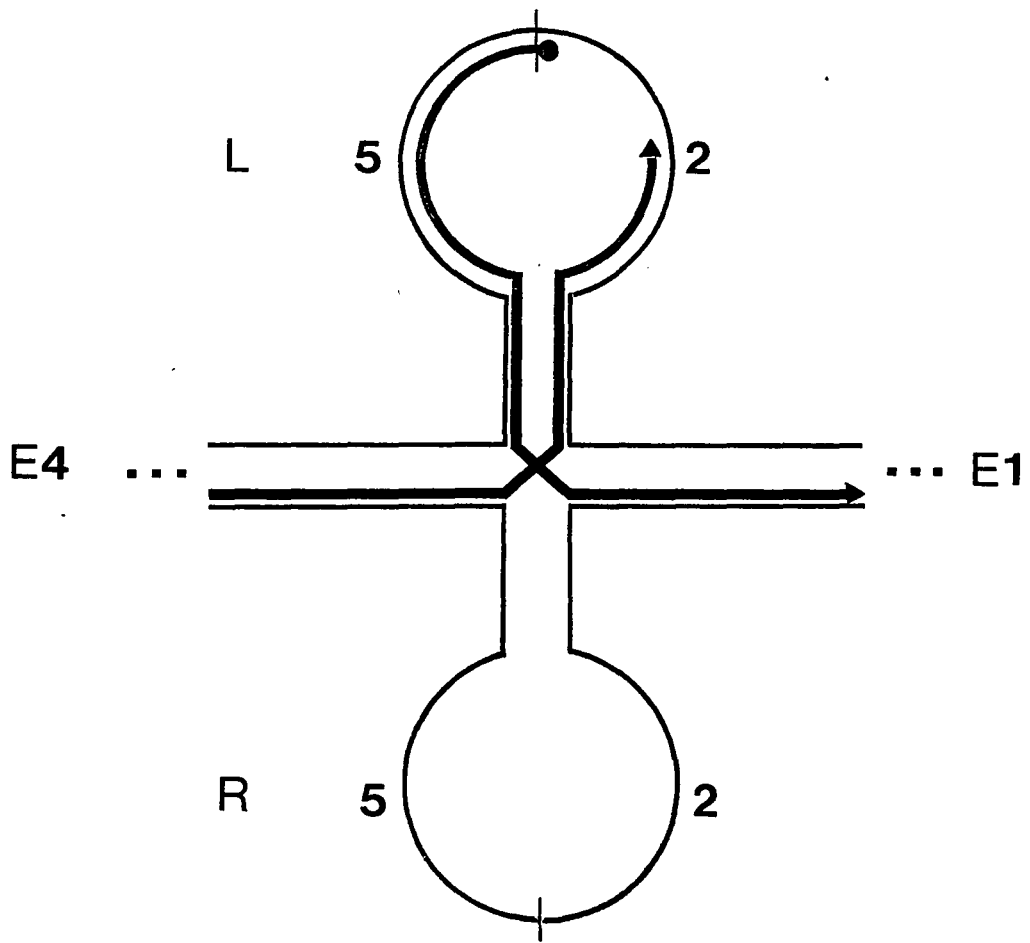
frequency of isolation of 5—2 viruses (≈ 8%) compare to 2—2 or 2—5 would suggest that template switching is an efficient and frequent phenomenon. As described above, the relative proportion of 2—2 and 5—5 viruses in plaques containing 5—2 viruses may depend on when, in the infection cycle, 5—2 molecules are produced. Alternatively 2—5 and 5—2 viruses could arise by recombination between 2—2 and 5—5 viruses but this is unlikely since recombination would yield equivalent amounts of 2—5 and 5—2 molecules, which was clearly not the case (Table 3.7).

The replication and repair model proposed for pFG154 would allow infectious adenovirus circles to generate unit length molecules, bypassing the need for virus or cellular encoded enzymes which would specifically recognize ITR junctions and cleave them. The mechanism by which the preferential replication of Ad5 ITRs may occur in circles is unknown but it may well be common to both linear and circular genomes. The extra three base pairs present at the junction of the two ITRs (Fig. 3.10) may play a role in the preference for Ad5 termini since the sequence of those extra base pairs is not the same on both strands (5'-TTC-3' in the L strand and 5'-GAA-3' in the R strand). It is not clear how that could facilitate initiation of replication in the L loop (Fig. 4.6). The additional 'G' in the R loop may be used to initiate replication and somehow block replication at that loop or result in a non viable progeny.

It would be of interest to see whether 5—2 circles obtained from sub54 also produce a majority of 5—5 viruses or if this orientation of the ITRs would primarily result in the production of 2—2 virions. Similarly, analysis of the progeny of circles containing a complete Ad5

replication machinery and non identical ends might also shed some light on the mechanisms of both the replication of circles and of the repair of the ITRs.


4.6 Summary

I have shown that viruses with non identical termini could be isolated and replicated, observations which argue against an absolute need for identical ITRs for viral viability. An interconversion of the ITRs was observed with 2—5 and 5—2 viruses, as they readily repaired their ends. The restoration of the ends presumably occurs during replication and suggests that the panhandle is an important and active replicative intermediate. Analysis of the coinfection data suggested that two conversion mechanisms existed, one in favour of Ad2 (mac) ITRs (possibly repair via the panhandle) and one in favour of Ad5 ITRs (possibly replication) and that the balance between the two interconversion mechanisms determined the net conversion of the termini. The results obtained with pFG154 seemed to contradict the ones obtained from the plaque purification of linear genomes with non identical termini. This contradiction may only be apparent and may be related to the particular structure of pFG154 (covalently closed circles) and its mode of replication (template switching). The possible preference for the Ad5 ITRs during replication by both linear and circular templates and for Ad2 (mac) ITRs during repair by sub54 suggested that, although internal viral sequences do not rigidly specify the type of termini present in a particular serotype, they may preferentially use ITRs of that same serotype.

APPENDIX:  COMPUTER PROGRAM

```
/*****************************************************************/
/*                                                             */
/*      PROGRAM TO ANALYZE DENSITOMETRY SCANNING DATA FROM      */
/*                                                             */
/*      AUTORADIOGRAMS USING QUICK C version 1.00 (MICROSOFT    */
/*                                                             */
/*      SOFTWARE).                                             */
/*                                                             */
/*                                                             */
/*          WRITTEN BY ROGER LIPPE  (MAY 1988)                */
/*                                                             */
/*                                                             */
/*      N.B.:Set the stack at 4000 bytes or more (via the "runtime */
/*                                                             */
/*          options").                                        */
/*                                                             */
/*****************************************************************/
#include <stdio.h>

#include <stdlib.h>

FILE *stream;                        /*      POINTER TO DATA FILE     */


main ()

{


/*   MARKER VARIABLES (AD2 AND AD5)                            */
/*   THERE ARE 2 SCANS (1 AND 2) AND THEIR AVERAGE (X) FOR     */
/*   EACH BAND                                                 */
    float ad5g_1, ad5g_2, ad5gx;   /* AD5 MARKER "G" FRAGMENTS- AB335 */
    float ad5i_1, ad5i_2, ad5ix;   /* AD5 MARKER "I" FRAGMENTS -AB335 */
```

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```
float ad5bg_1, ad5bg_2, ad5bgx;      /*    BACKGROUND HYBRIDIZATION     */

float ad5bi_1, ad5bi_2, ad5bix;      /*    BACKGROUND HYBRIDIZATION     */

float ad2g_1, ad2g_2, ad2gx;    /*  AD2 MARKER "G" FRAGMENTS - AB12   */

float ad2i_1, ad2i_2, ad2ix;    /*  AD2 MARKER "I" FRAGMENTS - AB12   */

float ad2bg_1, ad2bg_2, ad2bgx;      /*    BACKGROUND HYBRIDIZATION     */

float ad2bi_1, ad2bi_2, ad2bix;      /*    BACKGROUND HYBRIDIZATION     */

float ad5, ad2;


/*   SCANNED SAMPLE VARIABLES                                          */

int samples;

float s5g[59];    /*    ARRAY FOR "G" FRAGMENT SIGNALS WITH AB 335    */

float s5i[59];    /*    ARRAY FOR "I" FRAGMENT SIGNALS WITH AB 335    */

float s2g[59];    /*    ARRAY FOR "G" FRAGMENT SIGNALS WITH AB 12     */

float s2i[59];    /*    ARRAY FOR "I" FRAGMENT SIGNALS WITH AB 12     */

float s5gx[29];   /*     AVERAGES OF "G" SIGNALS WITH AB 335          */

float s5ix[29];   /*     AVERAGES OF "I" SIGNALS WITH AB 335          */

float s2gx[29];   /*     AVERAGES OF "G" SIGNALS WITH AB 12           */

float s2ix[29];   /*     AVERAGES OF "I" SIGNALS WITH AB 12           */

float s5gg[29];                 /*    ARRAY FOR NORMALIZED DATA        */

float s5ii[29];                 /*            IBID                     */

float s2gg[29];                 /*            IBID                     */

float s2ii[29];                 /*            IBID                     */


/*   OTHER DECLARATORS REQUIRED TO RUN THE PROGRAM                     */

int c, x;
```

```c
float _22a, _22b, _22min, _22max;

float _55a, _55b, _55min, _55max;

float _25a, _25b, _25min, _25max;

float _52a, _52b, _52min, _52max;

float factor2, factor5, factor25;

int _22percentmin, _22percentmax;

int _55percentmin, _55percentmax;

int _25percentmin, _25percentmax;

int _52percentmin, _52percentmax;


/*     LOADING THE DATA FROM A FILE                                  */
    stream = fopen("data.dat", "r");


    if (stream == 0) {
        printf("Data file not opened/inexistant\n");
        printf("Verify data file name and restart program\n");
        exit(0);
    }
    else
        printf("Data file opened\n");


    fscanf(stream, "%d", &samples);
    fscanf(stream, "%f %f %f %f", &ad5g_1, &ad5i_1, &ad5g_2,&ad5i_2);
    fscanf(stream, "%f %f %f %f", &ad2bg_1, &ad2bi_1,&ad2bg_2, &ad2bi_2);
    fscanf(stream, "%f %f %f %f", &ad5bg_1, &ad5bi_1,&ad5bg_2, &ad5bi_2);
    fscanf(stream, "%f %f %f %f", &ad2g_1, &ad2i_1, &ad2g_2,&ad2i_2);
```

```
for (c=0; c<samples*2; c++)

        fscanf(stream, "%f %f", &s5g[c], &s5i[c]);


for (c=0; c<samples*2; c++)

        fscanf(stream, "%f %f", &s2g[c], &s2i[c]);


fclose(stream);


/*    AVERAGE OF THE 2 SCANS FOR EACH LANE                              */
  ad5gx = (ad5g_1 + ad5g_2) / 2.0;

  ad5ix = (ad5i_1 + ad5i_2) / 2.0;

  ad2gx = (ad2g_1 + ad2g_2) / 2.0;

  ad2ix = (ad2i_1 + ad2i_2) / 2.0;

  ad5bgx = (ad5bg_1 + ad5bg_2) /2.0;

  ad5bix = (ad5bi_1 + ad5bi_2) /2.0;

  ad2bgx = (ad2bg_1 + ad2bg_2) /2.0;

  ad2bix = (ad2bi_1 + ad2bi_2) /2.0;


for (x=0, c=0; c<samples*2; x++, c+=2) {

        s5gx[x] = (s5g[c] + s5g[c+1]) / 2.0;

        s5ix[x] = (s5i[c] + s5i[c+1]) / 2.0;

        s2gx[x] = (s2g[c] + s2g[c+1]) / 2.0;

        s2ix[x] = (s2i[c] + s2i[c+1]) / 2.0;

}
```

```
/*      BACKGROUND SUBSTRACTION                                          */

/*      N.B.:  Values cannot be negative                                 */

   for(c=0; c< samples; c++) {

         s5gg[c] = ((s5gx[c] - ad2bgx) > 0.0) ? (s5gx[c]-ad2bgx):0.0;

         s5ii[c] = ((s5ix[c] - ad2bix) > 0.0) ? (s5ix[c]-ad2bix):0.0;

         s2gg[c] = ((s2gx[c] - ad5bgx) > 0.0) ? (s2gx[c]-ad5bgx):0.0;

         s2ii[c] = ((s2ix[c] - ad5bix) > 0.0) ? (s2ix[c]-ad5bix):0.0;




/*      NORMALIZATION OF THE SIGNALS FOR THE HINDIII "G" AND "I"         */

/*      FRAGMENTS (POSITIVE CONTROLS)                                    */

         factor5 = ad5gx / ad5ix;

         factor2 = ad2gx / ad2ix;

         ad5 = ad5ix * factor5;

         ad2 = ad2ix * factor2;

         s5ii[c] = s5ii[c] * factor5;

         s2ii[c] = s2ii[c] * factor2;




/*      NORMALIZATION TO GET THE SAME SIGNALS WITH AD5 AND AD2           */

/*      MARKERS                                                          */

         factor25 = ad5 / ad2;

         s2gg[c] = s2gg[c] * factor25;

         s2ii[c] = s2ii[c] * factor25;




/*      NORMALIZATION TO GET EQUAL SIGNALS FOR THE HINDIII G AND I       */

/*      fragments for each sample (as opposed to the markers).  This is  */
```

```
/*   required to satisfy the equations below.                              */

        if (s5ii[c]+s2ii[c] != 0) {

                factor25 = (s5gg[c] + s2gg[c]) / (s5ii[c] + s2ii[c]);

                s5ii[c] = s5ii[c] * factor25;

                s2ii[c] = s2ii[c] * factor25;

        }

    }   /*   END OF 'FOR' LOOP (SEE SUBSTRACTION OF BACKGROUND SECTION)   */
```

---

```
/*       END OF PART ONE:   NORMALIZATION OF THE DATA                      */


/*       SEE NEXT PAGE FOR PART TWO:   ANALYSIS OF THE DATA                */
```

---

```
/****************************************************************/
/*                                                            */
/*                   ANALYSIS OF THE DATA                     */
/*                                                            */
/*                                                            */
/*                                                            */
/*        This analysis is derived from the following         */
/*                                                            */
/*      "non-independant" equations:  1) s2gg = sum of 22 and 25 */
/*                                                            */
/*      molecules  2) s2ii = sum of 22 and 52 molecules        */
/*                                                            */
/*      3) s5gg = sum of 55 and 52 molecules  and             */
/*                                                            */
/*      4) s5ii = sum of 55 and 25 molecules (NB: There are four */
/*                                                            */
/*      equations and four unknowns (22, 25, 52, 55).  Because  */
/*                                                            */
/*      these equations aren't independant one cannot resolve them. */
/*                                                            */
/*      A number of different combinations can satisfy them.   */
/*                                                            */
/*      Another important constraint is that none of the values can */
/*                                                            */
/*      be negative.  This enables one to determine a range of */
/*                                                            */
/*      values that satisfy the above equations.               */
/*                                                            */
/*            In order to resolve all four equations, one of the */
/*                                                            */
/*      variables must first be determined.  The following section */
/*                                                            */
/*      does this four times (by determining each one of the    */
/*                                                            */
/*      variables first).  In all four cases the end results are */
/*                                                            */
/*      always the same since the equations are not independent. */
/*                                                            */
/*      Each time the output is a table with the results stored in */
/*                                                            */
/*      a separate file on floppy disk that can latter be retrieved */
/*                                                            */
/*      by Word Perfect.                                       */
/*                                                            */
/****************************************************************/
```

```
/* FIRST TIME */

  stream = fopen("data.52","a");

  fprintf(stream,"ANALYSIS BY DETERMINING '52'\n\n\n\n");

  fprintf(stream,"FILE #    :            progeny\n\n\n\n");

  fprintf(stream,"    SAMPLE              AREA (normalized)
            PLAQUE COMPOSITION \n");

  fprintf(stream,"
                  (percent)\n");

  fprintf(stream,"                        SAM                    AB
      22        25        52        55\n");

  fprintf(stream,"                    G       I        G           I
      min   max   min   max   min   max   min   max\n\n");


  for (c=0;c<samples;c++) {

      _52min = s2ii[c]-s2gg[c];

      _52min = (s5gg[c]-s5ii[c] > _52min) ? s5gg[c]-s5ii[c]:_52min;

      _52min = (0 > _52min) ? 0:_52min;

      _52max = s2ii[c]-s2gg[c]+s5ii[c];

      _52max = (s2ii[c] < _52max) ? s2ii[c]:_52max;

      _52max = (s5gg[c] < _52max) ? s5gg[c]:_52max;

      _52max = (s5gg[c]-s5ii[c]+s2gg[c] < _52max) ?
               s5gg[c]-s5ii[c]+s2gg[c]:_52max;


      _22a = s2ii[c]-_52min;

      _55a = s5gg[c]-_52min;

      _25a = s5ii[c]-_55a;

      _22b = s2ii[c]-_52max;

      _55b = s5gg[c]-_52max;
```

```
_25b = s5ii[c]-_55b;


_22min = _22b;

_22max = _22a;

_55min = _55b;

_55max = _55a;

_25min = _25a;

_25max = _25b;


if (s5gg[c]+s5ii[c]+s2gg[c]+s2ii[c] != 0) {

    _22percentmin = ( _22min /
     ((s5gg[c]+s5ii[c]+s2gg[c]+s2ii[c])* 0.5) ) * 100;

    _25percentmin = ( _25min /
     ((s5gg[c]+s5ii[c]+s2gg[c]+s2ii[c])* 0.5) ) * 100;

    _52percentmin = ( _52min /
     ((s5gg[c]+s5ii[c]+s2gg[c]+s2ii[c])* 0.5) ) * 100;

    _55percentmin = ( _55min /
     ((s5gg[c]+s5ii[c]+s2gg[c]+s2ii[c])* 0.5) ) * 100;


    _22percentmax = ( _22max /
     ((s5gg[c]+s5ii[c]+s2gg[c]+s2ii[c])* 0.5) ) * 100;

    _25percentmax = ( _25max /
     ((s5gg[c]+s5ii[c]+s2gg[c]+s2ii[c])* 0.5) ) * 100;

    _52percentmax = ( _52max /
     ((s5gg[c]+s5ii[c]+s2gg[c]+s2ii[c])* 0.5) ) * 100;

    _55percentmax = ( _55max /
     ((s5gg[c]+s5ii[c]+s2gg[c]+s2ii[c])* 0.5) ) * 100;


    fprintf(stream,"%2d)          %10.1f %10.1f %10.1f
     %10.1f",c+1,s5gg[c],s5ii[c],s2gg[c],s2ii[c]);
```

```
            fprintf(stream,"%6d %5d %5d %5d %5d %5d %5d
            5d\n",_22percentmin,_22percentmax,
            _25percentmin,_25percentmax,_52percentmin,
            _52percentmax,_55percentmin,_55percentmax);

    }

    else

            fprintf(stream,"%2d)          %10.1f %10.1f %10.1f
            %10.1f   ND   ND   ND   ND   ND   ND   ND
            ND\n",c+1,s5gg[c],s5ii[c],s2gg[c],s2ii[c]);

}

fclose(stream);



/* SECOND TIME */

stream = fopen("data.25","a");

fprintf(stream,"ANALYSIS BY DETERMINING '25'\n\n\n\n");

fprintf(stream,"FILE #   :          progeny\n\n\n\n");

fprintf(stream,"   SAMPLE                    AREA (normalized)
            PLAQUE COMPOSITION \n");

fprintf(stream,"
            (percent)\n");

fprintf(stream,"                    SAM                 AB
    22          25          52          55\n");

fprintf(stream,"                 G        I        G          I
    min  max  min  max  min  max  min  max\n\n");


for (c=0;c<samples;c++) {

    _25min = 0;

    _25min = (s2gg[c]-s2ii[c] > _25min) ? s2gg[c]-s2ii[c]:_25min;

    _25min = (s5ii[c]-s5gg[c] > _25min) ? s5ii[c]-s5gg[c]:_25min;

    _25max = s2gg[c];
```

```
_25max = (s2gg[c]-s2ii[c]+s5gg[c] < _25max) ?
  s2gg[c]-s2ii[c]+s5gg[c]:_25max;

_25max = (s5ii[c] < _25max) ? s5ii[c]:_25max;

_25max = (s5ii[c]-s5gg[c]+s2ii[c] < _25max) ?
  s5ii[c]-s5gg[c]+s2ii[c]:_25max;



_22a = s2gg[c]-_25min;

_55a = s5ii[c]-_25min;

_52a = s2ii[c]-_22a;

_22b = s2gg[c]-_25max;

_55b = s5ii[c]-_25max;

_52b = s2ii[c]-_22b;



_22min = _22b;

_22max = _22a;

_55min = _55b;

_55max = _55a;

_52min = _52a;

_52max = _52b;



if (s5gg[c]+s5ii[c]+s2gg[c]+s2ii[c] != 0) {

    _22percentmin = ( _22min /
        ((s5gg[c]+s5ii[c]+s2gg[c]+s2ii[c])* 0.5) ) * 100;

    _25percentmin = ( _25min /
        ((s5gg[c]+s5ii[c]+s2gg[c]+s2ii[c])* 0.5) ) * 100;

    _52percentmin = ( _52min /
        ((s5gg[c]+s5ii[c]+s2gg[c]+s2ii[c])* 0.5) ) * 100;

    _55percentmin = ( _55min /
        ((s5gg[c]+s5ii[c]+s2gg[c]+s2ii[c])* 0.5) ) * 100;
```

```c
        _22percentmax = ( _22max /
            ((s5gg[c]+s5ii[c]+s2gg[c]+s2ii[c])* 0.5) ) * 100;

        _25percentmax = ( _25max /
            ((s5gg[c]+s5ii[c]+s2gg[c]+s2ii[c])* 0.5) ) * 100;

        _52percentmax = ( _52max /
            ((s5gg[c]+s5ii[c]+s2gg[c]+s2ii[c])* 0.5) ) * 100;

        _55percentmax = ( _55max /
            ((s5gg[c]+s5ii[c]+s2gg[c]+s2ii[c])* 0.5) ) * 100;



        fprintf(stream,"%2d)          %10.1f %10.1f %10.1f
            %10.1f",c+1,s5gg[c],s5ii[c],s2gg[c],s2ii[c]);

        fprintf(stream,"%6d %5d %5d %5d %5d %5d %5d
            %5d\n",_22percentmin,_22percentmax,
            _25percentmin,_25percentmax,_52percentmin,
            _52percentmax,_55percentmin,_55percentmax);

    }

    else

        fprintf(stream,"%2d)          %10.1f %10.1f %10.1f
            %10.1f    ND    ND    ND    ND    ND    ND    ND
            ND\n",c+1,s5gg[c],s5ii[c],s2gg[c],s2ii[c]);

}

fclose(stream);


/* THIRD TIME */

stream = fopen("data.55","a");

fprintf(stream,"ANALYSIS BY DETERMINING '55'\n\n\n\n");

fprintf(stream,"FILE #   :          progeny\n\n\n\n");

fprintf(stream,"   SAMPLE              AREA (normalized)
            PLAQUE COMPOSITION \n");
```

```
fprintf(stream,"
                (percent)\n");

fprintf(stream,"                              SAM                    AB
     22         25         52          55\n");

fprintf(stream,"                      G         I         G            I
     min    max    min    max    min    max    min    max\n\n");


for (c=0;c<samples;c++) {

    _55min = s5gg[c]-s2ii[c];

    _55min = (0 > _55min) ? 0:_55min;

    _55min = (s5ii[c]-s2gg[c] > _55min) ? s5ii[c]-s2gg[c]:_55min;

    _55max = s5gg[c];

    _55max = (s5gg[c]-s2ii[c]+s2gg[c] < _55max) ?
      s5gg[c]-s2ii[c]+s2gg[c]:_55max;

    _55max = (s5ii[c] < _55max) ? s5ii[c]:_55max;

    _55max = (s5ii[c]-s2gg[c]+s2ii[c] < _55max) ?
      s5ii[c]-s2gg[c]+s2ii[c]:_55max;


    _25a = s5ii[c]-_55min;

    _52a = s5gg[c]-_55min;

    _22a = s2ii[c]-_52a;

    _25b = s5ii[c]-_55max;

    _52b = s5gg[c]-_55max;

    _22b = s2ii[c]-_52b;


    _22min = _22a;

    _22max = _22b;

    _25min = _25b;
```

```
_25max = _25a;

_52min = _52b;

_52max = _52a;



if (s5gg[c]+s5ii[c]+s2gg[c]+s2ii[c] != 0) {

        _22percentmin = ( _22min /
          ((s5gg[c]+s5ii[c]+s2gg[c]+s2ii[c])* 0.5) ) * 100;

        _25percentmin = ( _25min /
          ((s5gg[c]+s5ii[c]+s2gg[c]+s2ii[c])* 0.5) ) * 100;

        _52percentmin = ( _52min /
          ((s5gg[c]+s5ii[c]+s2gg[c]+s2ii[c])* 0.5) ) * 100;

        _55percentmin = ( _55min /
          ((s5gg[c]+s5ii[c]+s2gg[c]+s2ii[c])* 0.5) ) * 100;



        _22percentmax = ( _22max /
          ((s5gg[c]+s5ii[c]+s2gg[c]+s2ii[c])* 0.5) ) * 100;

        _25percentmax = ( _25max /
          ((s5gg[c]+s5ii[c]+s2gg[c]+s2ii[c])* 0.5) ) * 100;

        _52percentmax = ( _52max /
          ((s5gg[c]+s5ii[c]+s2gg[c]+s2ii[c])* 0.5) ) * 100;

        _55percentmax = ( _55max /
          ((s5gg[c]+s5ii[c]+s2gg[c]+s2ii[c])* 0.5) ) * 100;



        fprintf(stream,"%2d)          %10.1f %10.1f %10.1f
          %10.1f",c+1,s5gg[c],s5ii[c],s2gg[c],s2ii[c]);

        fprintf(stream,"%6d %5d %5d %5d %5d %5d %5d
          %5d\n",_22percentmin,_22percentmax,
          _25percentmin,_25percentmax,_52percentmin,
          _52percentmax,_55percentmin,_55percentmax);

}

else

        fprintf(stream,"%2d)          %10.1f %10.1f %10.1f
```

```
                    %10.1f   ND   ND   ND   ND   ND   ND   ND
                    ND\n",c+1,s5gg[c],s5ii[c],s2gg[c],s2ii[c]);

    }

    fclose(stream);


/* FOURTH TIME */

    stream = fopen("data.22","a");

    fprintf(stream,"ANALYSIS BY DETERMINING '22'\n\n\n\n");

    fprintf(stream,"FILE #   :           progeny\n\n\n\n");

    fprintf(stream,"    SAMPLE                    AREA (normalized)
            PLAQUE COMPOSITION \n");

    fprintf(stream,"
                    (percent)\n");

    fprintf(stream,"                            SAM                  AB
        22          25          52          55\n");

    fprintf(stream,"                        G          I          G          I
        min    max   min    max   min    max   min    max\n\n");


    for (c=0;c<samples;c++) {

        _22min = s2gg[c]-s5ii[c];

        _22min = (0 > _22min) ? 0:_22min;

        _22min = (s2ii[c]-s5gg[c] > _22min) ? s2ii[c]-s5gg[c]:_22min;

        _22max = s2gg[c];

        _22max = (s2gg[c]-s5ii[c]+s5gg[c] < _22max) ?
          s2gg[c]-s5ii[c]+s5gg[c]:_22max;

        _22max = (s2ii[c] < _22max) ? s2ii[c]:_22max;

        _22max = (s2ii[c]-s5gg[c]+s5ii[c] < _22max) ?
          s2ii[c]-s5gg[c]+s5ii[c]:_22max;
```

```
_25a = s2gg[c]-_22min;

_52a = s2ii[c]-_22min;

_55a = s5ii[c]-_25a;

_25b = s2gg[c]-_22max;

_52b = s2ii[c]-_22max;

_55b = s5ii[c]-_25b;


_25min = _25b;

_25max = _25a;

_52min = _52b;

_52max = _52a;

_55min = _55a;

_55max = _55b;


if (s5gg[c]+s5ii[c]+s2gg[c]+s2ii[c] != 0) {

    _22percentmin = ( _22min /
        ((s5gg[c]+s5ii[c]+s2gg[c]+s2ii[c])* 0.5) ) * 100;

    _25percentmin = ( _25min /
        ((s5gg[c]+s5ii[c]+s2gg[c]+s2ii[c])* 0.5) ) * 100;

    _52percentmin = ( _52min /
        ((s5gg[c]+s5ii[c]+s2gg[c]+s2ii[c])* 0.5) ) * 100;

    _55percentmin = ( _55min /
        ((s5gg[c]+s5ii[c]+s2gg[c]+s2ii[c])* 0.5) ) * 100;


    _22percentmax = ( _22max /
        ((s5gg[c]+s5ii[c]+s2gg[c]+s2ii[c])* 0.5) ) * 100;

    _25percentmax = ( _25max /
        ((s5gg[c]+s5ii[c]+s2gg[c]+s2ii[c])* 0.5) ) * 100;

    _52percentmax = ( _52max /
```

```
                ((s5gg[c]+s5ii[c]+s2gg[c]+s2ii[c])* 0.5) ) * 100;

        _55percentmax = ( _55max /
                ((s5gg[c]+s5ii[c]+s2gg[c]+s2ii[c])* 0.5) ) * 100;



        fprintf(stream,"%2d)           %10.1f %10.1f %10.1f
            %10.1f",c+1,s5gg[c],s5ii[c],s2gg[c],s2ii[c]);

        fprintf(stream,"%6d %5d %5d %5d %5d %5d %5d
            %5d\n",_22percentmin,_22percentmax,
            _25percentmin,_25percentmax,_52percentmin,
            _52percentmax, _55percentmin, _55percentmax);

    }

    else

        fprintf(stream,"%2d)           %10.1f %10.1f %10.1f
            %10.1f    ND    ND    ND    ND    ND    ND    ND
            ND\n",c+1,s5gg[c],s5ii[c],s2gg[c],s2ii[c]);

}

fclose(stream);



}
```

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