TRANSCRIPTION OF ADENOVIRUS TYPE 12 DNA

PHYSICAL STUDIES OF THE ORGANIZATION AND TRANSCRIPTION OF THE GENOME OF HUMAN ADENOVIRUS TYPE 12

By

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ABSTRACT

The adenovirus type 12-specific RNA synthesized during productive infection of human cells and in oncogenically transformed rodent cells was studied using the techniques of nucleic acid hybridization. Before the onset of viral DNA replication during productive infection (i.e., early), RNA derived from about 30% of the viral genome was detected in the cytoplasm, while RNA complementary to at least 80% of the genome was detected in the cytoplasm after the onset of DNA replication (late). At both early and late stages of productive infection RNA complementary to a larger fraction of the viral genome was found in the nucleus than in the corresponding cytoplasm, implying the existence of sequence - specific RNA processing and/or transport mechanisms.

The segments of the viral genome encoding early and late cytoplasmic RNA were mapped using subgenomic DNA fragments generated by bacterial restriction endonucleases. A minimum of four separate regions encoding early and late cytoplasmic RNA were positioned by this method.

Two cloned lines of oncogenically transformed rodent cells were found to contain virus specific RNA mapping within the leftmost 17% of the viral genome. Both "early" and "late" classes of cytoplasmic RNA were found in both cell lines. One line contained, in addition, small amounts of RNA encoded within the extreme right hand 9% of the viral genome. The other line contained only RNA derived from the left end of the genome.

The map of the sites on the adenovirus type 12 genome expressed as early and late cytoplasmic RNA deduced during this study is similar, if not identical, to those recently obtained by other workers with the distantly related adenoviruses 2 and 5. This similarity suggests that although the DNA of adenoviruses 12 and 2 share few nucleotide sequences in common, the overall organization of the two genomes is similar.

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ABBREVIATIONS USED

Ad	adenovirus
Bam H1	restriction endonuclease from *Bacillus amyloliquefaeciens* RUB 500
cAMP	cyclic adenosine monophosphate
CAP	catabolite activator protein
DNA	deoxyribonucleic acid
Eco RI	restriction endonuclease encoded by plasmid rY of *Escherichia coli*
% G + C	mole percent guanine plus cytosine
gal	galactose operon
Hind III	restriction endonuclease from *Hemophilus influenzae* rd
hut	histidine utilization operons
lac	lactose operon
mRNA	messenger ribonucleic acid
poly(A)	polyadenylic acid
poly (U,G)	poly uridylic-guanylic acid
ppGpp	guanosine tetraphosphate
RNA	ribonucleic acid
rRNA	ribosomal ribonucleic acid
SV40	simian virus 40
tRNA	transfer ribonucleic acid
trp	tryptophan operon

INTRODUCTION

I. Adenovirus

The adenoviruses are a large group of animal viruses which share a common architecture. Over fifty distinct types have been found, isolated from a variety of vertebrate species (Widner, 1969). The virion consists of a DNA-protein core surrounded by an icosahedral arrangement of 252 capsomeres (reviewed by Philipson and Lindberg, 1974). The genome of the adenoviruses is a linear duplex DNA molecule of 20-25 x 10^6 daltons (Green *et al.*, 1967; van der Eb *et al.*, 1969) which is not circularly permuted (Doerfler and Kleinschmidt, 1970), and does not bear classical terminal redundancy (Green *et al.*, 1967). However, the DNA molecule does contain an inverted terminal repetition which allows the two ends of a single DNA strand to base pair, forming a single stranded circle (Wolfsson and Dressler, 1972; Garon *et al.*, 1972). In virions the two ends of the adenovirus genome are joined by a pronase-sensitive linker to form a circular structure (Robinson *et al.*, 1973).

The 31 human adenovirus serotypes have been grouped into three classes based on their DNA homology, as assayed by DNA-DNA hybridization. This classification correlates with groupings based on the G + C content of the viral DNA molecules and viral oncogenicity in newborn hamsters (Green, 1970). Group A viruses (e.g., Ad 12) are highly oncogenic in newborn hamsters and have 48-49% G + C; Group B (e.g., Ad 7) are weakly oncogenic, with 50-52% G + C; and Group C viruses (e.g., Ad 2 and Ad 5) are non-oncogenic with 57-59% G + C. Some of the non-oncogenic human adenoviruses have been placed into a fourth group (D) on immunological grounds (McAllister *et al.*, 1969). The correlation between G + C content and oncogenicity does not extend to the simian adenoviruses (Pina and Green, 1968). The DNA sequences of the group A and group C viruses are dissimilar: Lacy and Green (1964) found about 20% homology between the DNA of Ad 12 and Ad 2, using a crude DNA-DNA hybridization technique. More sophisticated methods of analysis give a value of under 5% homology (see discussion).

The genome of adenovirus is large enough to code for about 20 average sized polypeptides. Eighteen Ad 2 coded polypeptides have been identified (Lewis *et al.*, 1975; 1976), six of which are "early" gene products (see below). Genetic analysis of temperature-

sensitive mutants has identified eighteen complementation groups in Ad 5, two of which are required for viral DNA replication (Williams, *et al.*, 1974). Three Ad 12 complementation groups required for viral DNA replication have been identified (Shiroki and Shimojo, 1974). Since it is probable that not all of the viral complementation groups and virus-coded polypeptides have been identified, there is not necessarily a one-to-one correspondence between the eighteen complementation groups and polypeptides identified to date. It is also possible that some of the complementation observed between pairs of mutants was due to intracistronic complementation, resulting in an over-estimation of the number of cistrons.

Infection by adenovirus is either productive or abortive, depending on the combination of virus and host cell used. For example, infection of human cells with human adenovirus leads to the productive response: cell death and the production of progeny virus. Infection of Syrian hamster cells with Ad 12 leads to an abortive response in which viral DNA is not replicated (Doerfler, 1969) and the late viral genes (see below) are not transcribed (Mak, 1975; Ortin and Doerfler, 1975). Similarly, infection of simian cells with Ad 2 is abortive: viral DNA is replicated but few progeny are produced (Friedman *et al.*, 1970). The molecular defect

in each of these abortive interactions remains unclear. Both translational blocks and decreased amounts of specific viral mRNA species have been reported in Ad 2 infected monkey cells (Hashimoto *et al.*, 1973; Klessig and Anderson, 1975).

In addition to inducing tumours in newborn hamsters (Trentin *et al.*, 1962), adenoviruses can morphologically transform cells *in vitro* (McBride and Wiener, 1964). Cells transformed by Ad 12 are invariably oncogenic when injected into newborn rodents. Both Ad 2 and Ad 5 (members of the so-called "non-oncogenic" group C) are capable of transforming rat cells *in vitro*, and some lines are oncogenic when injected into newborn rats (Chen *et al.*, 1976). Other lines become oncogenic in immunosuppressed animals, and still others are totally non-oncogenic, even when injected into immuno-deficient nude mice (Chen *et al.*, 1976). Hamster cells cannot be transformed by wild-type group C adenovirus, but can be transformed by temperature-sensitive mutants of Ad 5 (Williams, 1973) and by non-infective Ad 5 DNA fragments (Graham *et al.*, 1974). These transformed cells are oncogenic. Since both rat and hamster cells are semi-permissive for the replication of group C viruses, the inability of these viruses to induce tumours when injected directly into animals can probably be accounted

for by virus-induced cell death which eliminates potential transformants.

Transformation by DNA viruses is thought to be a direct consequence of the continuous expression of one or more viral genes. The best evidence for this hypothesis is the finding that the transformed phenotype of cells transformed by SV40 bearing a temperature-sensitive mutation in the A gene is temperature-sensitive (Martin *et al.*, 1974; Osborn and Weber, 1974). Although adenovirus type 5 temperature-sensitive mutants in the "early" N complementation group are unable to transform rodent cells at the non-permissive temperature, cells transformed at the permissive temperature retain the transformed phenotype at the restrictive temperature (Williams *et al.*, 1974). Thus this Ad 5 mutation affects the establishment, but not the maintenance, of the transformed state. Indirect evidence consistent with the hypothesis that adenovirus transformation is induced by an adenovirus gene product necessary for lytic infection was obtained by Harrison *et al.*,(1977) who isolated host range mutants of Ad 5 capable of replicating in Ad 5 transformed human embryonic kidney cells but not in HeLa cells. These mutants fall into two complementation groups. Mutants in one group are restricted to growth in adenovirus-transformed cells, while mutants in the other are restricted to growth in cells of embryonic origin. Both complementation groups

are involved with the transformation process, but the behaviour of the mutants is complex and not understood (F.L. Graham, personal communication).

Studies of the transforming ability of specific viral DNA fragments generated by bacterial restriction endonucleases have shown that the leftmost 7% of the Ad 5 or Ad 2 genome is sufficient for transformation (Graham *et al.*, 1974). This conclusion is reinforced by the finding that the leftmost 14% of the Ad 2 genome is always found in transformed rat cells (Gallimore *et al.*, 1974), and that some lines express only the leftmost 6% of the genome as mRNA (Sharp *et al.*, 1974). This mRNA is also synthesized before the onset of viral DNA replication (i.e., "early") during productive infection. None of nine lines of Ad 2 transformed rat cells retained the entire viral genome, explaining why infectious Ad 2 has never been rescued from transformed cells. In most cell lines transformed by Ad 2 and Ad 5 only "early" mRNA, or a subset of "early" mRNA, is present (Fujinaga and Green, 1970; Sharp *et al.*, 1974; Flint *et al.*, 1975; Flint *et al.*, 1976). It is unclear whether this restricted transcription of the viral genome has any significance other than demonstrating that the synthesis of late viral polypeptides is lethal to the cell.

The infected cell nucleus is the site of adenovirus transcription, replication and maturation.

Similarly, the adenovirus specific RNA found in transformed cells is synthesized in the nucleus. Since viral mRNA is translated in the cytoplasm, the adenovirus-cell interaction provides an opportunity to study the synthesis, processing and transport of specific mRNA species from the nucleus of eukaryotic cells.

II. Transcription

Studies of the transcription of nuclear DNA-containing animal virus genomes have depended heavily on concepts developed during the study of the transcription of bacterial and bacteriophage genomes. These concepts are briefly reviewed below. In addition, some of the more obvious differences between the process of transcription in prokaryotic and eukaryotic cells are indicated.

A. Transcription of bacterial genomes

Genes subject to coordinate control commonly map contiguously in both bacteria and bacteriophages. These blocks of coordinately regulated genes (operons, Jacob *et al.*, 1960) are transcribed into polycistronic mRNA containing multiple translational initiation and termination signals (Kiho and Rich, 1965).

Transcription is accomplished by one molecular form of RNA polymerase. The RNA polymerase of *E. coli* is composed of four distinct polypeptide chains (α, β, β', and σ) combined in the ratio of $\alpha_2\beta\beta'\sigma$ to form the holoenzyme (Travers and Burgess, 1969). Some evidence suggests that two distinct holoenzymes, sharing a common core enzyme ($\alpha_2\beta\beta'$) but differing in their σ factors, co-exist in *E. coli* (Fukada *et al.*, 1974). Sigma factor plays no catalytic role in RNA synthesis, but is required for specific initiation at promotor sites (Burgess *et al.*, 1969; Bautz *et al.*, 1969; Bautz and Bautz, 1970).

The promotor specificity of the holoenzyme is influenced by interaction with several molecules (reviewed by Travers, 1976). The nucleotide effector of the stringent response to amino acid deprivation, ppGpp (Cashel and Gallant, 1969), synthesized by an idling reaction of ribosomes (Haseltine and Block, 1973), in combination with translational elongation factor TuTs, interacts with the holoenzyme to reduce transcription of ribosomal DNA (Travers, 1973). In contrast, TuTs in the absence of ppGpp enhances the transcription of ribosomal DNA (Travers *et al.*, 1970). Similarly, charged, formylated initiator tRNA binds tightly to the holoenzyme, stimulating *in vitro* transcription of

the lactose operon, and depressing transcription of ribosomal DNA (Pongo and Ulbrich, 1976). These interactions presumably serve to regulate the partitioning of the single bacterial RNA polymerase between the synthesis of stable RNA and mRNA.

Superimposed on this gross modulation of the promotor preferences of RNA polymerase are more specific controls. Regulatory proteins which bind to specific DNA sequences influence the transcription of adjacent genes positively, negatively, or in a complex fashion. For example, the lactose repressor binds to the lactose operator in the absence of inducer (Gilbert and Muller-Hill, 1966; 1967), preventing transcription of the adjoining structural genes of the *lac* operon. Inducer binds to the repressor, causing its dissociation from the operator and allowing transcription to proceed. In addition to this negative control element, the controlling region of the *lac* operon contains a site which binds the catabolite activator protein (CAP) -cAMP complex, a positively controlling element which enhances transcription of the operon (Silverstone *et al.*, 1970; De Crombugghe *et al.*, 1971; Beckwith *et al.*, 1972). The order of these sites in the control region is: CAP binding site, RNA polymerase binding site, repressor binding site, and the beginning of the structural genes.

The net effect of the interaction of these elements is to ensure that synthesis of the enzymes required for lactose utilization is maximal only when both lactose and cAMP are abundant. Since the intracellular cAMP levels are low when cells are grown on easily metabolized carbon sources (e.g., glucose, Makman and Sutherland, 1965), this system ensures that cells expend energy on enzyme synthesis only when other more readily metabolized carbon sources are exhausted.

In contrast to the *lac* operon, a catabolic system induced by its substrate, the synthesis of enzymes catalyzing anabolic pathways is generally repressed by the end products of the pathway. For example, tryptophan, or a metabolic derivative, interacts with the product of the Trp R gene to form a functional repressor which interacts with the Trp operator, preventing transcription of the structural genes (Zubay *et al.*, 1972).

A further degree of complexity is introduced by the finding that in several systems regulatory DNA binding proteins which modulate the expression of an operon are encoded within that same operon. This mode of control (termed autoregulation, or autogenous control) was first elucidated by the studies of Magasanik on the histidine utilization (*hut*) operons of *Salmonella typhimurium*. The *hut* region, which codes for enzymes

which degrade histidine to ammonia, glutamic acid and formamide, is composed of two contiguous operons under coordinate control by the *hut* repressor. The *hut* repressor is itself encoded within one of these operons (Smith and Magasanik, 1971; Hagen and Magasanik, 1973). Similarly, the transcription of the λ phage repressor gene from the maintenance promotor is autoregulated in a concentration-dependent fashion by repressor. Low concentrations of repressor stimulate transcription; high levels abolish it (reviewed by Ptashne *et al.*, 1976).

A mechanism of transcription regulation distinct from those described above has recently been found in a number of systems. Yanofsky and coworkers have found that transcription of the tryptophan operon in Trp R mutants lacking repressor, grown in the presence of tryptophan, is efficiently terminated at an attenuator site (transcription termination signal) located about 140 nucleotides from the promotor (Bertrand *et al.*, 1976). On average, in the presence of tryptophan, only one out of eight initiated RNA chains is extended beyond this point into the structural genes. RNA polymerase terminates at this point *in vitro* in the presence of rho factor (Lee *et al.*, 1976). Starvation for tryptophan increases the proportion of RNA chains which continue past the attenuator (Bertrand and Yanofsky, 1976). Enhancement

of termination at the attenuator is mediated either by tryptophan aminoacyl tRNA synthetase, or its metabolic product (Morse and Morse, 1976). Polarity suppressors (mutants mapping to the suA gene, see below) decrease the efficiency of attenuator-mediated termination (Korn and Yanofsky, 1976A). In view of the recent demonstration that the suA gene codes for rho (Das *et al.*, 1976), the transcription termination factor (Roberts, 1969), this result implies that termination at the attenuator is mediated by rho. Supporting this view, Korn and Yanofsky (1976B) found that suppressors selected for their ability to suppress the effects of the attenuator site have an altered rho factor. The picture emerging from these findings is that a system independent of the repressor-operator mediated regulation of the initiation of transcription modulates the fraction of the initiated chains which are extended into the structural genes. Termination at the attenuator is enhanced by a product of tryptophan aminoacyl tRNA synthetase, presumably charged $tRNA^{Trp}$. A similar mechanism, involving charged histidyl tRNA, has been proposed for the regulation of the histidine operon of *Salmonella typhimurium* (Artz and Broach, 1975). Similar antitermination mechanisms have been implicated in the regulation of the delayed early genes and late genes of bacteriophage λ, and the delayed early genes of phage T4 (see below).

Translation of bacterial mRNA begins before transcription is completed (Miller *et al.*, 1970). There is recent and growing evidence for some type of coupling between prokaryotic transcription and translation. First, as described earlier, at least two components of the translational machinery interact directly with RNA polymerase. In addition, mutations in the β subunit of RNA polymerase can complement defects in ribosomal proteins (Chakrabarti and Gorini, 1975). Second, nonsense and frameshift mutations, along with insertion sequences, exert polar effects on the expression of genes to the promotor-distal side of the mutation (Jacob and Monod, 1961; Franklin and Luria, 1961; Fiandt *et al.*, 1972; Hirsch *et al.*, 1972). Reduced translation of these genes is correlated with decreased levels of the corresponding mRNA (Imamoto and Yanofsky, 1967). Polarity suppressors have been isolated which alleviate the polar effects of both nonsense mutations (Richardson *et al.*, 1975; Das *et al.*, 1976), and insertion sequences (Das *et al.*, 1976). Both classes of suppressors have an altered rho factor, suggesting that the polar effects of the mutations may be due to transcription termination. Supporting this view, the N protein of bacteriophage λ, a known rho antagonist (Roberts, 1969, Luzzati, 1970, Portier *et al.*, 1972), suppresses the polar effects of nonsense mutations in the tryptophan operon, provided

that transcription begins from a λ promotor (Segawa and Imamoto, 1974; Franklin, 1974). In addition, bacterial sun mutants which suppress the defect for lytic growth of λ N mutants (Brunel and Davidson, 1975) map very close to suA and suppress nonsense-induced polarity.

B. Transcription of bacteriophage genomes

Not all bacteriophage genes are expressed simultaneously during infection. For example, after T4 infection, specific viral genes are maximally expressed before the onset of viral DNA replication (the early genes), others begin their expression before the onset of viral DNA synthesis but are maximally active after replication has begun (quasi-late genes), while others are expressed only after DNA replication has started (true late genes). The control of the expression of these genes is exerted at the level of transcription (Guha *et al.*, 1971).

The order in which the early genes are expressed is determined by the order in which they are transcribed. Two classes of early genes are distinguishable on the basis of the sensitivity of their transcription to chloramphenicol: immediate early genes, which are

located in the promotor-proximal segment of each early region, are transcribed both in the presence and absence of chloramphenicol, while transcription of delayed early RNA is sensitive to the drug. Transcription of the delayed early genes is initiated from the immediate early promotors, and *in vitro* RNA polymerase synthesizes both classes (Milanesi *et al.*, 1970; Witmer, 1971). When rho is added, however, only the immediate early RNAs are made, suggesting that anti-termination is the mechanism of the immediate early to delayed early transition. Transcription of the quasi-late RNA is initiated from separate promotors (O'Farrel and Gold, 1973A; 1973B).

Transcription of the T4 late genes usually requires ongoing DNA replication (Riva *et al.*, 1970A), and is absolutely dependent on the products of genes 33,45 and 55 (Wu *et al.*, 1975). Gene 45 is also involved in DNA synthesis, but is required for late transcription independent of its role in replication. Other proteins required for replication are not directly involved in transcription (Wu *et al.*, 1975). Replication and transcription are uncoupled when phage deficient in T4-coded ligase and exonuclease infect cells (Riva *et al.*, 1970B), suggesting that DNA bearing single chain scissions is able to support late transcription in the absence of replication.

RNA polymerase extracted from T4 infected cells at late times differs from the uninfected cell's enzyme in several respects. The α chain is phosphorylated, a process controlled by phage genes (Horvitz, 1974A; 1974B), and four new polypeptide chains, encoded by genes 33,45, 55 and alc are found associated with the enzyme (Horvitz, 1973; Ratner, 1974; Sirotkin *et al.*, 1977). Three of these polypeptides (P33, P45 and P55) are required continuously for late transcription, and genetic studies suggest that P45 and P55 act to allow late transcription by interacting with RNA polymerase (Pulizter and Takahashi, 1975A; 1975B). Thus, virus induced modifications of RNA polymerase modulate T4 gene expression, presumably by allowing recognition of the late promotors. The product of the alc gene appears to prevent transcription of *E. coli* DNA (Sirotkin *et al.*, 1977).

Bacteriophage T7 regulates its transcription differently. *E. coli* RNA polymerase transcribes the four or five early genes, one of which encodes the T7 late specific RNA polymerase. The T7 coded RNA polymerase transcribes the late genes (Seigel and Summers, 1970). Late transcription of T7 is not coupled to DNA replication.

Bacteriophage λ does not seem to regulate lytic transcription by altering the promotor specificity of the host cell RNA polymerase, or by encoding its own

RNA polymerase. Rather, anti-termination controls the onset of delayed early transcription and may control late transcription. Cells infected with mutants in gene N, or with wild type λ in the presence of chloramphenicol, transcribe only gene N from the *l* strand and the cro region of the *r* strand, defining the immediate early genes. Similarly these two RNA species are the only ones synthesized by *E. coli* RNA polymerase *in vitro* from a λ DNA template in the presence of rho factor (Toberts, 1969). In the presence of functional N protein, transcription proceeds into the delayed early genes. Transcription of the delayed early regions is initiated at the immediate early promotors, demonstrating that N protein overcomes rho-mediated transcription termination at the immediate-early delayed early junctions (Luzzati, 1970; Portier *et al.*, 1970). Late transcription is not coupled to DNA replication, but depends on the product of gene Q. Gene Q allows late transcription to occur by acting at or near the late promotor (Herskowitz and Signer, 1970). Recently a rho-independent termination site active *in vivo* and *in vitro* has been located 200 nucleotides downstream from the late promotor (Sklar *et al.*, 1975; Roberts, 1975). Roberts has suggested that in the presence of Q protein, initiated RNA chains are propagated past this site into

the late genes. This hypothesis suggests that two sorts of distinguishable transcription termination signals are found in λ DNA, one modulated by N protein and rho, the other modulated by Q protein.

C. Transcription in eukaryotes

Very little is known about the control of gene transcription in eukaryotes. Several of the differences between eukaryotic and prokaryotic cells bear directly on the process of transcription.

Genetic studies of fungi and mammals have shown that generally genes encoding enzymes involved in a common metabolic pathway are not clustered (Mortimer and Hawthorne, 1970; Ruddle and Creagan, 1975). Other studies demonstrated that fungal and mammalian mRNA is monocistronic (Kuff and Roberts, 1967; Petersen and McLaughlin, 1973). Thus it is not likely that the unit of transcription in eukaryotes resembles a bacterial operon.

The mammalian genome is about 750 times larger than that of *E. coli*, capable of encoding 3×10^6 average sized polypeptides if all of this DNA were used to specify proteins. Genetic considerations suggest that mammals have no more than 10^5 loci capable of mutating to lethality (Ohno, 1971). Thus either a large fraction

of the genome serves no genetic function, or genetic units in eukaryotes are large, containing in addition to DNA encoding proteins, DNA of unknown function. Supporting the second view is the finding of Judd *et al.* (1972) that there is a one-to-one correspondence between complementation groups mutating to lethality and the chromomeres of salivary gland chromosomes in *Drosophila melanogaster* (see also Painter, 1934; Muller and Prokofyeva, 1935). This result implies that *Drosophila* has about 5×10^3 loci. The genome of *Drosophila* is one twentieth the size of that of mammals, implying that the genetic units of *Drosophila* and mammals are similar in size, on average 3×10^4 base pairs in length.

Eukaryotic DNA contains, in addition to sequences present only once per haploid genome, families of related nucleotide sequences present in multiple copies (Britten and Kohne, 1968). These repeated sequences can usefully be subdivided into two classes: highly repetitious, tandemly reiterated sequences of low complexity which are localized in regions of constitutive heterochromatin (Yunis and Yasmineh, 1971); and intermediately repeated sequences which are intimately interspersed with unique sequences (Davidson *et al.*, 1973; Graham *et al.*, 1974B). Since most DNA sequences which encode protein are unique (see, for example, Goldberg *et al.*, 1973), and since

cistrons are widely dispersed (Judd *et al.*, 1972) it follows that structural genes are separated by DNA of unknown function, which contains intermediately repetitious and unique sequences.

The physical separation of transcription and translation in eukaryotes (the former occurring in the nucleus, the latter in the cytoplasm) imposes a temporal separation of the two processes not found in prokaryotes, and creates a need for a transport mechanism for mRNA.

In contrast to prokaryotes, eukaryotes contain three distinct RNA polymerases which differ in subcellular location and template specificity (Roeder and Rutter, 1969; 1970). Polymerase A is the nucleolar enzyme, resistant to α amanitan, which synthesizes ribosomal RNA, while polymerase B is the chromatin associated, α amanitan-sensitive enzyme thought to transcribe mRNA. Polymerase C appears to synthesize small RNA species, including tRNA (Weimann and Roeder, 1974). Thus eukaryotic cells have no need to partition a single enzyme between the synthesis of ribosomal RNA, tRNA and mRNA.

The mRNA found in the cytoplasm of eukaryotic cells differs in several respects from that of prokaryotes. As mentioned earlier, it is monocistronic. Most mRNA species contain a stretch of polyadenylic acid (poly A)

at their 3' end (Adesnik *et al.*, 1972). The poly A tract, which varies from 50 to 200 bases in length, is added to the RNA in the nucleus post-transcriptionally (Darnell *et al.*, 1971). Specific messenger RNA species including histone mRNA, lack a poly (A) tract (Adesnik and Darnell, 1972). Eukaryotic mRNA also bears a modified 5' terminus involving an inverted, methylated, dinucleotide. Two distinct termini are found: M^7 $GpppX^mpYp$ (cap I) or M^7 $GpppX^mpY^mp$ (cap II) (Rottman *et al.*, 1974; Perry and Kelly, 1976). Cap II is formed from cap I in the cytoplasm, while cap I is formed in the nucleus. Some mRNA species (e.g., Polio virus mRNA, Hewlett *et al.*, 1976) lack a 5' modification, demonstrating that these structures are not required for translation. Supporting this view, vesicular stomatitis virus mRNA, from which the 7-methylguanosine has been chemically removed, can be efficiently translated *in vitro* (Rose and Lodish, 1976). Roles for both the 3' and 5' terminal modifications of mRNA in both translation and intranuclear processing of pre-mRNA have been proposed.

High molecular weight heterogeneous nuclear RNA (hn RNA), distinct from the ribosomal RNA precursor, is the predominant RNA synthesized during a short pulse with labelled uridine (Penman, 1966; Warner, 1966) in mammalian cells. These molecules reach up to 45,000

nucleotides in length (Greenberg and Perry, 1971). Hn RNA is unstable, turning over rapidly in the nucleus (Penman *et al.*, 1968; Soeiro *et al.*, 1968). Little of the label incorporated into Hn RNA during a short pulse ever appears in the cytoplasm. Because the synthesis of hn RNA and mRNA have similar sensitivities to actinomycin D, and because some hn RNA molecules have poly A tails (Darnell *et al.*, 1970) and capped 5' termini, it has been suggested that hn RNA is the precursor to mRNA.

This hypothesis is exceedingly difficult to test: the size distribution of RNA labelled during even the shortest pulses partially overlaps with that of cytoplasmic mRNA, and only a small fraction of the label incorporated during such a pulse appears in the cytoplasm after a prolonged "chase". Therefore, it is possible that rapid synthesis and complete turnover of hn RNA and slower accumulation of mRNA are entirely separate processes. Specific mRNA sequences have been found in high molecular weight hn RNA (Stevens and Williamson, 1972; Imaizum *et al.*, 1973), but a demonstration of a precursor-product relationship will demand experiments showing that label incorporated into the mRNA segments of hn RNA during a short pulse can be chased into mRNA. Recently, a system for purifying

RNA bearing globin mRNA sequences has been developed, which should allow these experiments to be done (Curtis and Weismann, 1976).

Electron microscopic evidence has been presented that the primary transcripts of non-ribosomal DNA in the mealy bug average 20,000 nucleotides in chain length (Foe *et al.*, 1976), a value consistent with the size of HeLa cell hn RNA obtained by Greenberg and Perry (1971) using gel electrophoresis.

The similarity in size between hn RNA and chromomeres is consistent with the hypothesis that chromomeres are the primary unit of transcription (Beerman, 1967). Since only one complementation group is associated with each chromomere (Judd *et al.*, 1972), this hypothesis implies that hn RNA is monocistronic.

The factors controlling specific gene transcription in eukaryotes are poorly understood. The genetic material of interphase eukaryotic cells is organized into a nucleoprotein complex, chromatin (for a review, see *The Structure and Function of Chromatin*, CIBA Foundation Symposium No. 28, Elsevier, Amsterdam, 1975). Chromatin is a restricted template for RNA synthesis *in vitro*, compared to purified DNA (Paul and Gilmour, 1968; Gilmour and Paul, 1969; Bacheler and Smith, 1976). The RNA species synthesized *in vitro* from chromatin are

similar to those present *in vivo* (Paul and Gilmour, 1968; Gilmour and Paul, 1969; Bacheler and Smith, 1976). Studies of RNA synthesized from reconstituted chromatin *in vitro* have shown that histones nonspecifically depress the template activity of DNA, while non-histone chromatin proteins confer tissue and cell-cycle specificity to the RNA synthesized (Paul and Gilmour, 1968; Gilmour and Paul, 1969; Stein *et al.*, 1976; Park *et al.*, 1976). The role of nonhistone proteins in the control of RNA synthesis suggested by this work is consistent with the finding that steroid hormone-receptor protein complexes interact with nonhistone chromatin proteins, apparently stimulating the transcription of specific genes (reviewed by O'Malley and Means, 1974).

D. Transcription of the adenovirus genome

Infection of permissive cells with adenoviruses does not result in a total inhibition of cellular RNA synthesis. Consequently, studies of adenovirus-specific RNA rely on DNA-RNA hybridization methods to identify and analyze viral RNA. This requirement has tremendously complicated the design of experiments.

Adenovirus DNA is transcribed in the nucleus of infected cells. Most viral RNA species are synthesized

by the α-amanitan sensitive RNA polymerase B (Price and Penman, 1972; Wallace and Kates, 1972), but one viral RNA species, VA RNA (or 5.5 S RNA) is synthesized by polymerase C (Weinman *et al.*, 1976A). All viral mRNA species are polyadenylated (Philipson *et al.*, 1971) and capped (McGuire *et al.*, 1976). Some nuclear viral RNA molecules are polyadenylated (Philipson *et al.*, 1974) and some are capped (McGuire *et al.*, 1976). Interestingly, VA RNA, which does not serve as mRNA, is neither polyadenylated nor capped (McGuire *et al.*, 1976).

The possibility that adenovirus mRNA is derived from a high molecular weight nuclear precursor analogous to cellular hn RNA has been studied. Ad 2 specific RNA accumulates in the nucleus five times faster than in the cytoplasm, and only 20% of the viral RNA labelled in a pulse eventually appears in the cytoplasm (Philipson *et al.*, 1974), a value somewhat larger than that observed with cellular hn RNA. Nuclear virus-specific RNA synthesized during a short pulse either early or late post infection is larger than polysomal viral mRNA (Wall *et al.*, 1972; McGuire *et al.*, 1976; Bachenheimer and Darnell, 1975), and is apparently cleaved during a "chase" period in the presence of actinomycin D. However, since the size distribution

of pulse labelled nuclear viral RNA partially overlaps with that of viral mRNA, it is possible that the high molecular weight nuclear RNA turns over entirely within the nucleus, and is not involved in mRNA production. Hybridization studies have suggested that nuclear early and late RNA is complementary to all of both Ad 2 DNA strands (Philipson *et al.*, 1974; Sharp *et al.*, 1974; Wold *et al.*, 1976). It is not clear what role, if any, processing plays in the biogenesis of adenovirus mRNA.

An "early" and a "late" phase can be distinguished during lytic infection. Before the onset of viral DNA replication no structural components of the virion are synthesized (Gilead and Ginsberg, 1965; Russel and Shekel, 1972) but virus induced "T" antigen(s) can be detected. The synthesis of "T" antigen and early virus induced polypeptides is resistant to cytosine arabinoside, an inhibitor of DNA replication (Feldman and Rapp, 1966; Russel and Shekel, 1972). When infected cells are continuously incubated with cytosine arabinoside, or infected with viral temperature-sensitive mutants defective in viral DNA replication at the nonpermissive temperature, no virion proteins are produced (Russel and Shekel, 1972; Russel *et al.*, 1972; Russel *et al.*, 1974; Ledinko, 1974). These results have suggested that the synthesis of virion polypeptides (late proteins) depends on prior DNA replication.

Green and his co-workers attempted to correlate the lack of synthesis of virion proteins early during infection with restricted transcription of the viral genome: using the nitrocellulose filter hybridization method these authors detected RNA sequences complementary to 8 to 20% of the Ad 2 genome and 50% of the Ad 12 genome in early whole cell RNA (Fujinaga and Green, 1970; Green *et al.*, 1970; Green *et al.*, 1970). In contrast, late during infection, RNA complementary to 80 to 100% of both genomes was found (Mak and Green, 1968; Fujinaga *et al.*, 1968).

Conventional DNA-RNA hybridization methods employing unlabelled denatured DNA immobilized on a nitrocellulose support (Gillespie and Speigelman, 1965) have several limitations for the analysis of viral transcription. First, since the DNA is not in homogeneous solution, interspersed with the RNA, the rate of the reaction is slow, and rare RNA species may not be detected. Second, the hybridized DNA cannot be recovered for further analysis. During adenovirus infection it is not possible to label RNA to a steady state; therefore the specific radioactivity of the virus specific RNA remains unknown. For this reason it is not possible to relate hybridized radioactivity to the amount of RNA hybridized. It is necessary to resort to indirect, competition experiments to ascertain the

degree of complementarity of RNA preparations with viral DNA, and to assess the degree of sequence overlap between RNA populations. This requirement further limits the resolving power of the method.

Because of the insensitivity of the filter hybridization method, the results of Green and co-workers cited above did not demonstrate that only a restricted fraction of the viral genome is transcribed during the early phase of infection. It remained possible that rare RNA species complementary to a large fraction of the viral genome were present but not detected in early RNA.

The sensitivity of RNA-DNA hybridization analyses can be increased by performing the reaction in homogeneous solution (reviewed by Bishop, 1972). When this approach is adopted, the hybridized DNA can be recovered and analyzed. Double stranded DNA and DNA-RNA hybrids can be distinguished from single stranded nucleic acids by their resistance to single strand specific endonucleases (Vogt, 1973) or by their chromatographic behaviour on hydroxylapatite (Miyazawa and Thomas, 1965). If the DNA is labelled, the fraction of the DNA in hybrid form, and, at saturation, the degree of complementarity between the DNA and RNA, can be determined. A complication of this approach is that denatured DNA reassociates spontaneously (Marmur and Doty, 1961), interfering with

the detection of RNA-DNA hybrids. Since both DNA renaturation and RNA-DNA hybridization are second order reactions (Thrower and Peacocke, 1968; Nygaard and Hall, 1964) the contribution of renaturation can be eliminated by adjusting both the DNA concentration and incubation time used. If excess, unlabelled RNA is then added, hybridization can be monitored in the absence of DNA renaturation. Alternatively, DNA renaturation can be eliminated by using only one of the two complementary DNA strands as a probe. Several methods are available for separating the complementary strands of DNA molecules (Szybalski *et al.*, 1971; Hayward, 1972). This approach has the advantage that extended hybridization times can be employed to detect even very rare RNA species. In addition, the results serve to determine the polarity of transcription of the RNA detected.

The use of these extremely sensitive hybridization methods cannot, by itself, provide information about which segments of a viral DNA molecule code for specific RNA species. With bacteriophages, this information has been obtained by hybridizing RNA to the DNA of variant phages bearing deletion and/or substitution mutations, which had previously been mapped by heteroduplex analysis (for example, Szybalski *et al.*, 1970). The lack of

such defined variant genomes precludes such an approach with animal viruses.

Recently, bacterial restriction endonucleases (reviewed by Nathans and Smith, 1975) have become readily available. These site specific endonucleases introduce a limited number of double stranded breaks at specific locations on a given DNA molecule. The resulting set of fragments, which are easily separable by electrophoresis, can be used to separately examine defined segments of a viral genome for any desired property. By using partially overlapping fragments generated by different endonucleases, extremely high resolution analyses are possible.

The method of saturation hybridization in solution of complementary-strand specific sequences of specific subgenomic fragments generated by restriction endonucleases has allowed the physical location of the early and late genes of Papova viruses SV40 and Polyoma to be determined (Khoury *et al.*, 1973; Sambrook *et al.*, 1973; Kamen *et al.*, 1974).

This study was undertaken to characterize the transcription pattern of the genome of the highly oncogenic Ad 12 during productive infection. Specifically, it was hoped that the techniques of liquid phase saturation hybridization of labelled viral DNA, coupled with the availability of bacterial restriction

endonucleases, would allow the construction of a physical transcription map of the viral genome. The DNA sequences encoding the mRNAs found "early" and "late" during infection could then be located on this map, allowing a determination of whether or not the failure to synthesize virion proteins during the early phases of infection is correlated with the absence of specific classes of viral mRNA in the cytoplasm. In addition, the segments of the viral genome which are expressed as mRNA in oncogenically transformed rodent cells could be mapped, providing a maximal estimate of the extent of the regions of the genome involved with transformation. It was also hoped that the analysis of any sequence differences between nuclear and cytoplasmic viral RNA would shed some light on the process of mRNA transport from nuclei. These objectives were, to a large extent, realized.

During the past two years, the intensive efforts of several groups of investigators have established transcription maps for the genomes of adenovirus 2 and 5 (of group C). (For example, Sharp *et al.*, 1974; Tal *et al.*, 1974; Craig *et al.*, 1975A; 1975B; Chinnadurai *et al.*, 1976; Flint *et al.*, 1976; Petersson *et al.*, 1976; Wold *et al.*, 1976.) In brief, these studies demonstrate that only a restricted subset of the viral

mRNA sequences present in the infected cell cytoplasm at late times are detectable "early", and that Ad 2 and Ad 5 transformed cells usually express only early mRNAs, or a subset of the early mRNAs. Inhibitors of protein synthesis and DNA replication are reported to block the appearance of cytoplasmic Ad 2 late mRNA (Craig and Raskas, 1974B; Tibbetts *et al.*, 1974), and cells infected at the nonpermissive temperature with Ad 5 temperature sensitive mutants blocked in DNA replication fail to accumulate late RNA (Carter and Ginsberg, 1975). Late transcription is not coupled to ongoing replication, however, as late RNA continues to be synthesized when cells replicating viral DNA are shifted to the non-permissive temperature (Carter and Ginsberg, 1975).

The physical locations of the genes encoding the early and late polypeptides of Ad 2 have been roughly determined by cell-free translation of viral mRNA selected by hybridization to specific DNA fragments (Lewis *et al.*, 1976) and by examining recombinants between Ad 2 and Ad 5 (Mautner *et al.*, 1975). As these reports appeared while the present work was in progress, and bear directly on the results to be presented here, an exhaustive description of the physical map of the group C genome is left for the discussion.

MATERIALS AND METHODS

I. Tissue Culture

A. Human KB cells

Monolayer cultures were maintained in minimal essential medium (MEM, GIBCO) supplemented with 10% fetal calf serum (FCS, Amour), 1% fungizone (GIBCO), 1.21 mg/ml penicillin G and 100 µg/ml streptomycin. Confluent monolayers were subcultured by gently scraping the cells from the glass surface with a rubber policeman and seeding aliquots of the resulting suspension into fresh 32 oz glass prescription bottles. Cells were maintained at 37°C in a humid atmosphere consisting of 5% CO_2 in air.

Suspension cultures were initiated by seeding subconfluent monolayer cells to a concentration of 3×10^5/ml in prewarmed Jokliks modified MEM (spinner medium, GIBCO) supplemented with 5% horse serum (HS). Cells were kept in suspension by continuous agitation with a sterile teflon-coated magnetic stirring bar.

The cultures were maintained at a cell concentration of between 3 to 5 x 10^5 cells/ml by periodic dilution with fresh, prewarmed spinner medium.

B. Primary hamster embryo cultures

All manipulations were done aseptically. Pregnant hamsters were sacrificed on the 13*th* to 14*th* day of gestation, and the embryos were removed. After decapitation and evisceration, the embryos were partially minced and incubated with 0.125% Bacto-trypsin (Difco) in citrate saline (=1% KCl, 0.4% sodium citrate) with stirring for 15 minutes at 37°C. The cell suspension was then filtered through cheesecloth and pelleted by centrifugation at 1000 rpm for 10 minutes in the International centrifuge. The cell pellet was resuspended in αMEM (Flow Laboratories) supplemented with 10% FCS and antibiotics, and 5 x 10^5 cells were seeded into each 60 mm plastic petri dish (Falcon).

Cells were subcultured by trypsinization. Monolayers were washed with prewarmed citrate saline and incubated with 2 ml of 0.125% trypsin in citrate saline. When the cells had rounded, indicating partial detachment, the dishes were gently agitated. The detached cells were dispersed by gentle pipetting and seeded into fresh culture vessels.

C. Transformed rodent cells

Adenovirus transformed rodent cells were cultured as monolayers in Joklik's MEM containing 10% FCS. Cells were grown either in 250 ml plastic flasks (Falcon or Corning), or 100 mm petri dishes (LUX), and were subcultured by trypsinization.

D. Transformation of primary hamster embryo cells

The procedure of Casto (1968) was used. Confluent hamster embryo fibroblast cultures in 60 mm plastic petri dishes were exposed to purified adenovirus type 12 (Huie strain) (Ad 12) in 0.3 ml of Joklik's MEM containing 1% FCS at 37°C for 3 hours. The inoculum was occasionally redistributed over the cell sheet by rotating the plates. After adsorption, the cells were subcultured by trypsinization, each dish being split into 5 new ones. The cells were allowed to grow for 3 days in αMEM + 10% FCS, and then were switched to Joklik's MEM + 10% FCS. Medium (Joklik's) was changed every three days thereafter. Transformed foci were generally visible within 2 to 3 weeks. Infecting multiplicities were varied, but with a multiplicity of infection (MOI) of 10^4 particles/cell, one cell in 2.5×10^4 was transformed. At this MOI one focus was induced by 2.5×10^8 particles, or about

10^5 to 10^6 plaque forming units (PFU). Increasing the MOI resulted in a marked cell killing effect.

Transformed foci were picked with a sterile pasteur pipette, dispersed by vigorous pipetting and plated into 10 mm petri dishes. After one week the cells could be subcultured again. The efficiency of isolation of lines from foci was about 50%. Lines were designated T1, T2, etc., in order of their isolation. Line T6 was best characterized by these studies. All earlier lines were lost, either by bacterial contamination or during a growth crisis.

II. Virus Infection and Purification

KB cells in suspension culture were pelleted by centrifugation and resuspended in Joklik's MEM + 1% FCS at 10^7 cells/ml. Purified Ad 12, strain Huie or 1131, was added at an MOI of 3×10^2 particles/cell and adsorbed for 90 minutes at 37°C. The cells were then resuspended to 3×10^5/ml in an equal mixture of conditioned and fresh Joklik's MEM + 5% HS. Seventy-two hours post infection the cells were harvested by centrifugation and resuspended with 0.01 M tris pH 8.1. The virus was purified by the method of Green and Pina (1963). Cells were sonicated and homogenized

with freon 113 at 4°C and the virus concentrated from the aqueous phase by sedimentation onto a cesium chloride cushion of a density of 1.44 gm/ml. After two cycles of isopycnic banding in cesium chloride solutions with a starting density of 1.34 mg/ml, the virus band was collected aseptically. Virus concentration was estimated from the suspensions' combined absorbance and scattering at 260 nm. One "absorbance" unit at 260 nm corresponds to 5×10^{11} particles/ml. The virus was diluted at least tenfold with tris-buffered saline containing 30% glycerol and stored at -70°C.

Cells infected for viral RNA extraction received an input MOI of 2×10^3 particles/cell.

Virions containing labelled DNA were prepared by adding ^{3}H-thymidine (New England Nuclear, 20 Ci/mmole) to a final concentration of 5 μCi/ml to the culture medium 19 hours post infection. DNA with a specific activity of 5×10^5 cpm/μg was routinely obtained.

III. Nucleic Acid Extractions

A. Viral DNA extraction

Purified virus was dialyzed for two hours at 4°C against 0.01 M Tris pH 8.1. To each millilitre of

suspension was added 0.15 ml of 1.0 M sodium phosphate, pH 6.0, 0.15 ml of 0.05 M disodium ethylene diamine tetracetate (EDTA) pH 7.0, 0.1 ml of 10% (w/v) sodium dodecyl sulphate (SDS) and 0.05 ml of a 20 mg/ml suspension of pronase B (pre-digested for 2 hours at 37°C, Calbiochem). The mixture was incubated 30 minutes at 37°C, then extracted three times with an equal volume of redistilled phenol saturated with 0.05 M sodium phosphate buffer, pH 6.8 at 0°C. Each extraction was for 5 minutes; vigorous shaking was avoided. Phases were separated by centrifugation (Sorval, 10,000 rpm, 5 minutes, 0°C). The final aqueous phase was removed and dialyzed into either 0.1 x SSC (1 x SSC = standard saline citrate, 0.15 M NaCl, 0.015 M sodium citrate) or 0.01 M Tris pH 7.4, depending on its end use. DNA concentration was estimated from the A_{260} of the solution, assuming that an absorbance of 1 is given by a 50 µg/ml solution of DNA.

B. RNA extraction

RNA was extracted either from whole cells or from separated nuclear and cytoplasmic fractions by a modification of the method of Warner *et al.* (1966).

All glassware used was previously baked at 350°F

for 5 hours. The suspension of cells or nuclei, or the cytoplasmic extract, was made 0.05 M sodium acetate, 0.01 M EDTA, pH 5.1 and SDS was added to 1%. After 5 minutes the lysate was extracted at 60-65°C with an equal volume of phenol-chloroform-isoamyl alcohol (75:24:1) saturated with the same buffer lacking SDS. After each 3 minute extraction the mixture was cooled in a methanol-ice bath until phenol crystals appeared on the surface of the extraction vessel. Phases were separated by centrifugation (Sorval, 12,000 rpm, 5 minutes, 0°C). The aqueous phase was removed and re-extracted twice at 60-65°C, then once at room temperature. The final aqueous phase was made 0.15 M NaCl and nucleic acids were precipitated by the addition of two volumes of 95% ethanol at -20°C. After overnight precipitation at -20°C, the nucleic acid was pelleted (Sorval, 12,000 rpm, 30 minutes, 0°C). The pellet was taken up in 0.01 M Tris pH 7.4 and dialyzed at least 5 hours against the same buffer. After $MgCl_2$ was added to 0.01 M, the solution was digested with 100 μg/ml of pancreatic deoxyribonuclease (DNase, Worthington, electrophoretically pure) for one hour at 37°C. The solution was then reextracted as above, precipitated with ethanol and dialyzed against 0.01 M Tris pH 7.3. RNA concentrations were estimated by

A_{260}, assuming that an absorbance of 1 corresponds to 42 µg/ml of RNA.

C. Preparation of nuclear and cytoplasmic fractions

All operations were at 0°C. Cells were pelleted by centrifugation and washed with isotonic buffer (0.15 M NaCl, 0.01 M EDTA, 0.01 M Tris pH 7.8; Kumar and Lindberg, 1972). The pellets were resuspended to 5 x 10^7 cells/ml in isotonic buffer, and an equal volume of isotonic buffer containing either 1.4 % (for KB cells) or 1% (transformed cells) NP40 (Shell Chemical Corp) was added. The mixture was kept on ice for 10 minutes with occasional shaking, and cell lysis was monitored by phase contrast microscopy. Nuclei were pelleted by centrifugation (International, 2,000 rpm, 10 minutes, 0°C), and the supernatant was withdrawn and used as the cytoplasmic fraction. The nuclear pellet was washed twice with isotonic buffer, and finally resuspended in 0.01 M Tris, 0.01 M EDTA, pH 7.3, with homogenization if necessary.

When cells labelled with ^{3}H-thymidine for one hour were extracted in this fashion, greater than 99.5% of the acid insoluble radioactivity was recovered in the nuclear fraction. By phase contrast microscopy

the cytoplasmic fraction was free of nuclei and unlysed cells, while the nuclear fraction contained some unlysed cells and aggregates which could contain unlysed cells. Thus the method yields a "clean" cytoplasm and "dirty" nuclei. Other methods, such as dounce homogenization, yield clean nuclei at the expense of detectable nuclear breakage. In the author's hands, 10% of the acid insoluble label incorporated into DNA was released into the cytoplasmic fraction by dounce homogenization. As will become clear later, in these studies it was essential to minimize nuclear contamination of the cytoplasmic fraction.

IV. Manipulations with DNA

A. Resection of DNA with exonuclease

Exonuclease III was purchased from Miles Labs. DNA was incubated with nuclease in 0.01 M Tris, 0.002 M $MgCl_2$, pH 7.9 at 37°C. The reaction was terminated by heating the samples to 65°C for 10 minutes. Incubation times and DNA to nuclease ratios are indicated in the text.

B. Cleavage of DNA with restriction endonucleases

Endonucleases Bam Hl and Hind III were purchased from Bethesda Research Laboratories, while endonuclease Eco RI was purified by the method of Petterson and Philipson (1974). Digestion buffers were 0.1 M Tris pH 7.9, 0.01 M $MgCl_2$ for Eco RI, 0.02 M Tris pH 7.4, 0.01 M $MgCl_2$, 0.06 M NaCl for Hind III and 0.02 M Tris pH 7.4, 0.01 M $MgCl_2$, 0.002 M β-mercaptoethanol for Bam Hl. Simultaneous digestions with Bam Hl and Eco RI or Hind III were done in Bam Hl buffer; double digestions with Hind III and Eco RI were done in Hind III buffer. DNA was incubated with sufficient endonuclease to ensure complete digestion within 2 hours (usually 2 to 5 units per microgram of DNA).

DNA fragments were separated by electrophoresis in 0.7% or 1.0% (w/v) agarose gels cast in glass cylinders 15 cm long x 0.6 cm internal diameter. Gels were made by autoclaving agarose powder (BDH) mixed with electrophoresis buffer (0.04 M Tris, 0.005 M sodium acetate, 0.001 M EDTA pH 7.8) and pouring the molten agarose (at 45-55°C) into the gel tubes. After the agarose had set, the gels were partially extruded from one end of the tube and a flat surface was created with a razor. The gel was then replaced in the tube,

and held in place at the opposite end with parafilm punctured by at least 30 pinpricks to allow unimpeded current flow.

Samples containing 1 to 5 μg of DNA in less than 80 μl were made 0.5% SDS, 0.005% bromophenol blue and 5% glycerol, and loaded onto the flat gel surface through the electrophoresis buffer. Electrophoresis was at room temperature at 30 V. The electrophoresis time was varied. After electrophoresis the gels were stained with 0.5 μg/ml ethidium bromide in electrophoresis buffer (Sharp *et al.*, 1973) and the DNA bands were visualized under shortwave ultraviolet illumination.

C. Strand separation

i. Separation of intact complementary strands. The intact complementary strands of adenovirus type 12 DNA were separated by centrifuging denatured viral DNA to equilibrium in neutral cesium chloride density gradients in the presence of poly-uridylic-guanylic acid (poly U,G) (Szybalski *et al.*, 1971; Landgraf-Leurs and Green, 1971). Purified virions labelled with ^{14}C or ^{3}H-thymidine were digested with pronase as described in section III.A. A quantity of the digest containing 50 to 100 μg of viral DNA was made up to 1.35 ml and placed in a siliconized tube. To this was added

0.3 ml 1.0 N NaOH. After 10 minutes at room temperature the solution was neutralized by the addition of 0.3 ml each of 1 M Tris pH 8.1 and 1.0 N HCl. To this mixture 500 μg to 1 mg of poly (U,G) in 0.01 M Tris, 0.01 M EDTA, pH 8.1 was added. (Biogenics lot 111 poly (U,G) with a U to G ratio of 3.03:1 was used with success.) Most of the SDS was removed by precipitation at 0°C for 20 minutes followed by centrifugation at 12,000 rpm for 5 minutes in the Sorval. The supernatant was made to 4.2 ml with 0.1 M Tris pH 8.1 and 5.5 grams solid cesium chloride was added. The solution was placed in a siliconized, polyallomer ultracentrifuge tube, overlayed with mineral oil, and centrifuged 60-72 hours at 33,000 rpm at 15°C in the Beckman 50 ti rotor.

Six drop fractions were collected from the bottom of the tube, and 5 μl aliquots from each fraction were assayed for radioactivity. Fractions containing the heavy (*l*) and light (r) DNA strands were separately pooled. In the early phases of this study the pooled fractions were redenatured with sodium hydroxide, neutralized with HCl, and rebanded in the presence of fresh poly (U,G). The peak fractions from the second run were dialyzed against 0.01 M Tris pH 8.1, and centrifuged through 16.5 ml 5-20% (w/w) alkaline sucrose gradients (containing 0.3 N NaOH, 0.01% SDS,

0.001 M EDTA) for 13 hours at 24,000 rpm in the SW27.1 rotor. The 30S intact single strands were pooled, neutralized, extracted twice with phenol saturated with 0.1 M Tris pH 8.1 and exhaustively dialyzed against 0.01 M Tris pH 7.3 containing 2% formamide.

This method resulted in considerable losses of material, and was time consuming. A second, superior method was developed which takes advantage of the fact that at high salt concentrations the sedimentation coefficient of single stranded DNA is much greater than that of duplex DNA (Tibbetts *et al.*, 1973). The fractions pooled from the poly (U,G)-cesium chloride density gradients were dialyzed for 12 hours against 0.14 M sodium phosphate buffer, pH 6.8 containing 50% purified formamide (see below) at 37°C, conditions favouring DNA renaturation, to drive any contaminating sister strands into duplex structures. Duplex DNA was then separated from the single stranded material by sedimentation through an 11 ml high salt 5-20% (w/w) neutral sucrose gradient containing 1.0 M NaCl, 0.01 M Tris pH 7.4 and 0.001 M EDTA. Sedimentation was at 38,000 rpm for 100 minutes at 20°C in the SW40 rotor. Fractions sedimenting as single strands (70S) were pooled and dialyzed against 2% formamide in 10 mm Tris pH 7.3, aliquoted and stored at -20°C.

Formamide was purified by stirring commerical reagent grade formamide with 20 g Norite A/liter for 1 hour. Charcoal was removed by centrifugation and filtration. The formamide was then extracted twice with an equal volume of diethyl ether, and purged of ether with bubbling air.

ii. Electrophoretic strand separation of DNA fragments. The method of Horwitz (1976) was used. The DNA bands were visualized under ultraviolet illumination after staining with ethidium bromide and gel regions containing the desired duplex DNA fragments were excised with a razor blade. Exposure of the DNA to ultraviolet light was minimized. The gel segments were immersed in 10 ml of 0.2 N NaOH for 2 hours at room temperature to denature the DNA, then incubated for 2.5 hours in electrophoresis buffer at 0°C. These segments were then incorporated into fresh 1% agarose gels by placing them in one end of a cylindrical tube and pouring a column of molten agarose over them. The resulting gels were electrophoresed as described earlier for 8 to 10 hours. In some cases, two bands of equal intensity, corresponding to the complementary strands of the starting fragment, were observed. The complementary strands of other DNA fragments were not resolved by this method.

D. Recovery of DNA from agarose gels

DNA was recovered from agarose gel slices by one of two methods.

i. Sodium perchlorate-hydroxyapatite. Gel slices were broken up in a small amount of distilled water with a glass rod. An equal volume of 8 M sodium perchlorate was added and the mixture was gently homogenized in a dounce homogenizer, dissolving the agarose. The solution was diluted to 2 M sodium perchlorate and 0.5 gm of hydroxyapatite was added. The mixture was kept on ice for 10 minutes with continuous agitation, then the hydroxyapatite was loaded into a small column made from a pasteur pipette blocked with glass wool. The resulting column was washed with ten bed volumes of 2 M sodium perchlorate, followed by 5 bed volumes of 0.01 M sodium phosphate buffer, pH 6.8. The absorbed DNA was then eluted in 3 bed volumes of 0.4 M sodium phosphate buffer, dialyzed against .01 M Tris pH 7.3, made 0.15 M NaCl and ethanol precipitated. Recovery was less than 30%.

ii. Freeze-squeeze (Thuring *et al.*, 1975). Gel slices were placed between parafilm sheets and frozen at -20°C for 5 hours. The frozen slices were then squeezed

between forefinger and thumb, and the extruded liquid was collected. Macroscopic chunks of agarose were removed by passing the solution through glass wool packed in a pasteur pipette. Recovery varied from 30 to 60%.

V. Nucleic Acid Hybridization

i. Conventional filter hybridization. The method of Gillespie and Spiegelman (1965) was used. ^{14}C viral *l* and *r* strands were made to 0.2 µg/ml in 2 x SSC, then slowly filtered onto nitrocellulose filters (Sartorius, 0.45 micron pore size) previously equilibrated with 2 x SSC. Each filter received 1 µg of DNA. The filters were then washed extensively with 2 x SSC, air dried overnight and incubated at 80°C for 4 hours to immobilize the DNA. RNA, labelled with ^{3}H-uridine, in 2 x SSC + 0.1% SDS (1 ml) was incubated with each filter at 65°C for 20 hours in a tightly stoppered vial. After hybridization the filters were washed extensively with 2 x SSC under suction, incubated with 20 µg/ml pancreatic ribonuclease at room temperature for 1 hour, then washed again with 2 x SSC. Finally, the filters were oven dried and assayed for bound radioactivity in a liquid scintillation spectrophotometer using a toluene based scintillation

fluid. In quantitative studies, control filters lacking viral DNA were also incubated with RNA to assess the level of background non-specific binding of RNA.

To preparatively isolate complementary strand-specific RNA, aliquots containing 5 x 10^6 cpm of late cytoplasmic RNA (150 µg RNA) or 2 x 10^7 cpm of early cytoplasmic RNA (600 µg RNA) were hybridized to a series of filters as described. After hybridization, filters were washed with 1 litre of 2 x SSC containing 0.1% SDS at 65°C for 20 minutes with stirring, followed by a further wash with suction at room temperature. The hybridized RNA was eluted by boiling for five minutes in 0.1 x SSC, and yeast RNA (500 µg/ml) was added as carrier. After dialysis into 0.01 M Tris pH 7.3 any contaminating DNA was degraded by digestion with 100 µg/ml DNase in the same buffer containing 0.01 M $MgCl_2$. The RNA was then extracted and precipitated as in section III.B.

ii. Hybridization in solution scored by hydroxyapatite. DNA labelled with ^{3}[H]-thymidine to a specific activity of approximately 10^6 cpm/µg was sheared by 10 minutes sonication at 30% of the maximum output of a Bronson Biosonik III sonifier. The DNA, in 0.01 M phosphate buffer pH 6.8 (P.B.), was kept cool in an ice bath during sonication. The sedimentation profile of this

DNA in an alkaline sucrose gradient is displayed in Figure 1. Using intact Ad 12 DNA as a marker (34S, Doerfler, 1969), the sedimentation coefficient of the material in the peak fraction was calculated as 4.9S, corresponding to about 250 bases in chain length (Studier, 1965). Multivalent cations were removed from the DNA preparation by passing it over Chelex (Biorad), previously equilibrated with 0.01 M P.B.

This DNA was denatured by boiling for 10 minutes in 0.01 M P.B., cooled, and mixed with an appropriate quantity of unlabelled RNA. The solution was made 0.14 M P.B., 0.4% SDS and incubated at 65°C. The DNA and RNA concentrations, and incubation times, were varied and are indicated in the text.

Duplex DNA and RNA-DNA hybrids were scored by chromatography on hydroxyapatite (Kohne, 1969). Samples in 0.14 M P.B. were made to about 1 ml and were applied to a 1 ml column of hydroxyapatite packed in a 10 ml disposable syringe. The columns were maintained at 65°C in a water bath. The column effluent collected during three washes of 3 bed volumes each with 0.14 P.B. at 65°C was scored as the single stranded fraction. Duplex DNA and DNA-RNA hybrids were eluted by three washes of 3 bed volumes each with 0.4 M P.B. These fractions were precipitated with 10% trichloroacetic

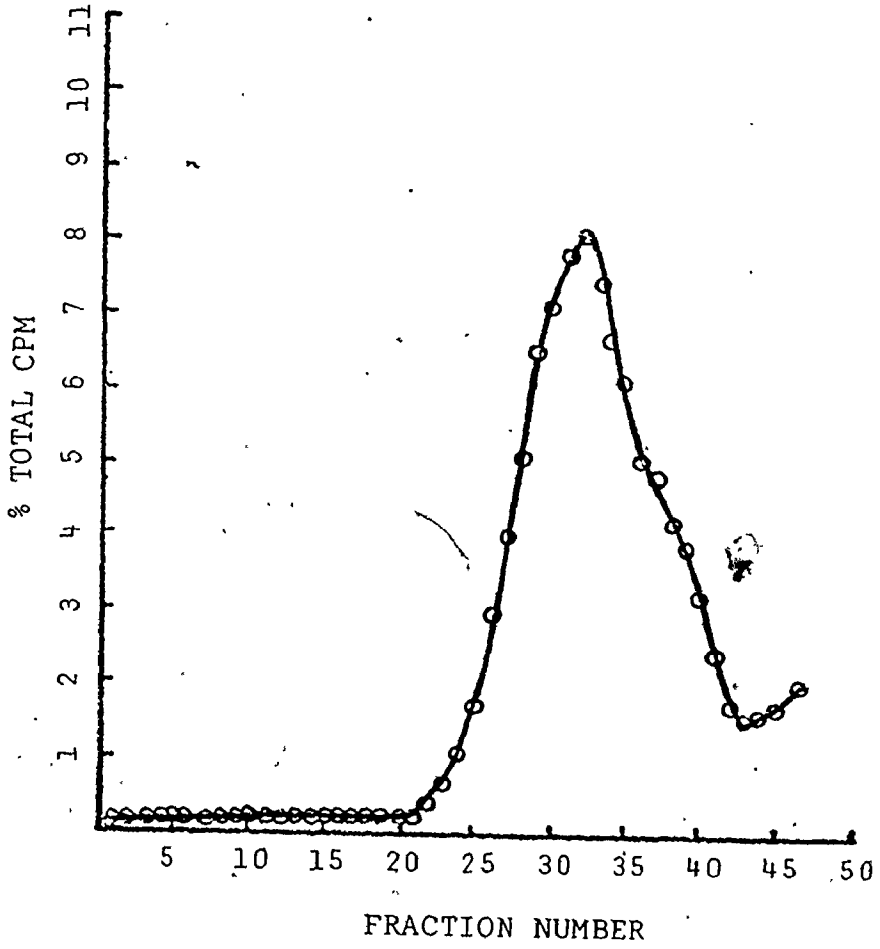


Figure 1: Sedimentation profile of ^{3}H-labelled, sonicated Ad 12 DNA

Sonicated Ad 12 DNA was centrifuged through a 5 ml 5-20% alkaline sucrose gradient for 14 hours at 40,000 rpm at 20°C in the SW50.1 rotor. Fractions were collected, TCA precipitated and counted. Sedimentation is to the left.

acid (TCA) for 20 minutes in the presence of 100 μg carrier calf thymus DNA at 0°C. The precipitates were collected by filtration onto nitrocellulose filters (Sartorius, 0.2 micron pore size). The filters were washed with 10% TCA and oven dried. The precipitated radioactivity was quantitated by counting the filters in 5 ml of toluene containing 0.02 gm omnifluor (New England Nuclear) in a liquid scintillation spectrometer. The results were expressed as the fraction of the label which was eluted at 0.4 M P.B., corrected for the degree or retention of unincubated, denatured DNA at 0.14 M P.B. (zero time background binding). The background binding varied between 4 to 8%.

iii. Hybridization in solution scored by S1 nuclease. Labelled single stranded DNA was incubated alone or with an appropriate quantity of unlabelled RNA in 0.3 M NaCl, 0.01 M Tris pH 7.3 at 65°C in tightly stoppered siliconized glass tubes. After hybridization the solutions were made to 1 ml and 0.3 M NaCl, 0.05 M sodium acetate, 0.0018 M $ZnCl_2$ pH 4.5 and incubated with 10 μg/ml S1 nuclease (Miles Laboratories) at 37°C for one hour.

To overcome quenching effects, the residual RNA was degraded before TCA precipitation by making the solution 0.5 N NaOH and incubating it an additional

2 hours at 37°C. After neutralization the DNA was TCA precipitated and the acid-insoluble radioactivity was determined. The S1 nuclease treatment was sufficient to hydrolyze 97% of denatured, sonicated DNA while degrading less than 5% of intact, native DNA to an acid soluble form.

A typical RNA-DNA hybridization was performed as follows: Duplicate tubes containing a fixed quantity of single stranded DNA and increasing amounts of infected or transformed cell RNA in a total volume of 0.4 ml were incubated at 65°C. Six control tubes containing DNA but lacking the RNA were also incubated. After hybridization, the contents of three of the control tubes, and of all of the experimental tubes were incubated with S1 nuclease. The contents of the three remaining control tubes were directly TCA precipitated. The average TCA precipitable radioactivity in the untreated controls was taken as the input. The zero time resistance to S1 nuclease of the DNA probe used was subtracted from both the input and the experimental values to yield the input cpm initially sensitive to S1, and the decrease in this value observed in each experimental group. The results were then expressed as the fraction of the initially S1 sensitive radioactivity which was protected by the RNA. The

zero time resistance to S1 was 3% for the sonicated, unfractionated DNA used, and varied from 5 to 20% with intact, purified complementary strands, depending on their purity. All complementary strand preparations were exhaustively self annealed before hybridization, removing any contaminating sister strands as duplex (S1 resistant) structures (see results).

When unfractionated DNA was used as the probe, the S1 resistance of the digested control tubes provided an estimate of the amount of DNA renaturation occurring in the absence of hybridization. When complementary strands were used as the probe, this value gave the degree of cross-contamination of the strand preparation (the degree of cross contamination being equal to, or less than, one half of the fraction of the DNA resistant to S1). In every case examined, the S1 resistance of complementary strands was the same before and after hybridization. This was expected because of the prior exhaustive self annealing of the DNA.

When intact complementary strands were used as the probe, the RNA concentration of all tubes, including the controls, was made identical and at least 1.25 mg/ml by the addition of yeast RNA. This step was necessary to eliminate adsorption of the DNA to the hybridization vessel (see results).

iv. Purification of the segments of each DNA strand complementary to late cytoplasmic RNA. ^{3}H-labelled *l* and *r* strands (5 x 10^5 cpm/μg, 0.5 μg/ml) were separately hybridized to unlabelled late cytoplasmic RNA (2 mg/ml) for 24 hours at 67°C in 0.3 M NaCl, 0.01 M Tris pH 7.3, conditions previously determined to be saturating. The material was then treated with S1 nuclease as described earlier, to hydrolyse the unhybridized DNA, then made 0.2 M sodium phosphate pH 6.8, 1% SDS.

Yeast RNA (1 mg/ml) was added as carrier, and the mixture was extracted with phenol saturated with 0.2 M sodium phosphate pH 6.8, dialyzed against 0.01 M Tris pH 8.1 and ethanol precipitated.

The pellets were taken up in 2 ml of 0.3 N NaOH, boiled for 15 minutes, neutralized, buffered at pH 7.3 with 0.1 M Tris and dialyzed against 0.1 x SSC after the addition of 1 mg/ml yeast RNA as carrier.

Control experiments with labelled RNA demonstrated that the alkaline hydrolysis step totally degraded RNA to an acid soluble form. The conditions of hydrolysis at 100°C further served to fragment the DNA to about 250 to 350 bases in chain length (Sharp *et al.*, 1974).

The *l* and *r* strands used for this purpose were initially 5 and 9% resistant to S1 nuclease, respectively.

v. Transfer of denatured DNA from agarose gels to nitrocellulose strips. The blotter method of Southern (1975) was used. One percent agarose gels containing 2 u g of separated DNA fragments generated by restriction endonucleases were stained and the positions of the DNA bands were recorded. The DNA was denatured *in situ* by immersing the gels in 0.5 N NaOH in 1.5 M NaCl for 30 minutes. The gels were then neutralized in 0.5 M Tris pH 7.0 in 3 M NaCl for 90 minutes. Then, as described in detail by Southern (1975), the DNA bands were transferred by capillary action from the gels to nitrocellulose strips pre-equilibrated with 2 x SSC. Briefly, the gels, separated from one another by lucite spacers equal in length to the gels, were placed on a sheet of Whatman no. 1 chromatography paper in a tray. The spacers were not in contact with the gels. The paper and the bottom of the gels were immersed in 20 x SSC. Nitrocellulose strips cut from a sheet (Sartorius, 0.45 micron pore size) presoaked in 2 x SSC, were placed to bridge the gap between adjacent spacers, so that the nitrocellulose partially overlapped with both spacers, and was also in contact with the upper surface of the intervening gel. About 30 sheets of Whatman no. 1 paper were then piled on each spacer, in contact with both overlapping nitrocellulose sheets. Thus,

the nitrocellulose sheets bridged the air gap which separated the gels from the spacers. As liquid was drawn from the nitrocellulose by the Whatman paper, liquid and DNA was drawn from the gel. Since denatured DNA adheres to nitrocellulose, bands of DNA, corresponding in position to those in the gel, were deposited and immobilized on the nitrocellulose sheet.

Transfer was allowed to proceed for at least 24 hours. After transfer, the regions of contact between the gels and the nitrocellulose were cut out as narrow strips, rinsed in 2 x SSC, allowed to dry overnight, and baked for 4 hours at 80°C to immobilize the DNA.

vi. Hybridization of labelled nucleic acids to nitrocellulose strips bearing DNA fragments. The method of Southern (1975) was used. Strips were wetted with 2 x SSC and placed in lucite hybridization vessels which contained a slot 12 cm x 2 cm x 0.1 cm in dimensions. Labelled nucleic acid in 2 x SSC + 0.1% SDS containing 500 μg/ml yeast RNA in 1.5 ml was introduced and the slot was sealed with parafilm. The apparatus was incubated at 65°C for 20 hours.

Unhybridized nucleic acid was removed by one of two methods. In RNA-DNA hybridizations, the strips were incubated for 15 minutes in 500 ml of 2 x SSC + 0.1% SDS at 65°C with stirring, then incubated for

one hour with 20 µg/ml pancreatic ribonuclease in 2 x SSC at room temperature. Finally the strips were rinsed with an additional 15 minute wash of 500 ml of 2 x SSC at room temperature and air dried.

Unhybridized DNA was removed by washing the strips for one hour in 500 ml of 2 x SSC at 65°C with stirring, followed by a one hour wash in 500 ml of 4×10^{-3} M Tris plus 0.1 x SSC pH 9.4 at room temperature (Warnaar and Cohen, 1966). Strips were oven dried.

The hybridized radioactivity was detected and quantitated by fluorography. Dried strips were immersed in 20% (w/v) Omnifluor (New England Nuclear) in toluene and air dried. The omnifluor-impregnated strips were exposed at -70°C to Kodak RP Royal X-omat (RP R14) film previously exposed to a hypersensitizing flash of white light (Laskey and Mills, 1975). Pre-exposure was done to bring the background density of the film to 0.15 D above that of unexposed film, in order to linearise the film's dose-response to tritium (Laskey and Mills, 1975). The resulting negatives were scanned with a Joyce-Loebl microdensitometer and the relative amount of radioactivity hybridized to each band was estimated from the peak areas.

vii. Synthesis of hydroxyapatite. The method of Bernardi (1971) was used. Crystals were prepared by

dripping 1.5 l of 0.5 M $CaCl_2$ into 2 l of 0.5 M P.B. The resulting precipitate was washed twice with 3 l H_2O, then 50 ml NH_3OH in 3.5 l H_2O containing phenolpthalein was added. The slurry was boiled for one hour, with stirring. Ammonium hydroxide was added throughout to maintain a purple colour (alkaline pH). The hydroxyapatite was then washed seven times with 3.5 l of 0.004 M P.B., and stored in the same buffer.

RESULTS

The Ad 12-specific RNA sequences found in productively infected human cells and in stably transformed rodent cells were examined by DNA-RNA hybridization.

I. Hybridization of Total Ad 12 DNA to Unlabelled RNA in Solution

A. Hybridization scored by hydroxyapatite chromatography

1. Late RNA hybridizes 37-50% of Ad 12 DNA. Viral DNA labelled with ^{3}H-thymidine (1 x 10^{6} cpm/µg, 250 nucleotides in chain length) was heat denatured and hybridized for two hours at 65°C to varying amounts of unlabelled whole cell RNA extracted from infected KB cells late (136 hours) post-infection. The DNA concentration of the reaction mixtures was 0.01 µg/ml. Samples were chromatographed on hydroxyapatite (HAP), and the fraction of the total radioactivity retained by the column at 0.14 M PB (duplex DNA and RNA-DNA hybrids) was determined. The data were then corrected for the 8% binding of the same DNA to the column immediately

following denaturation (zero time binding). The corrected data are displayed in Figure 2A. It is clear that the hybridization reaction reached a plateau after about 40% of the DNA sequences reacted. Increasing the RNA concentration from 106 to 423 µg/ml had no effect on the fraction of the DNA hybridized. Only 4.2% of the DNA incubated for two hours in the absence of RNA scored as duplex, demonstrating that virtually all of the duplex material observed after incubation with RNA was present in RNA-DNA hybrid structures. This result implied that about 40% of the Ad 12 DNA sequences, or approximately 80% of one DNA strand equivalent, were capable of reacting with late whole cell RNA.

In the experiments depicted in Figure 2B, the same RNA preparation was incubated at a concentration of 423 µg/ml with 0.01 µg/ml DNA, and the kinetics of the hybridization were followed. In two separate experiments plateau values of 40 to 50% hybridization were observed, in reasonable agreement with the saturation experiment.

The variability (if any) of the sequence complexity of late whole cell RNA was examined by studying the hybridization behaviour of two additional lots of late RNA (both extracted 36 hours post-infection).

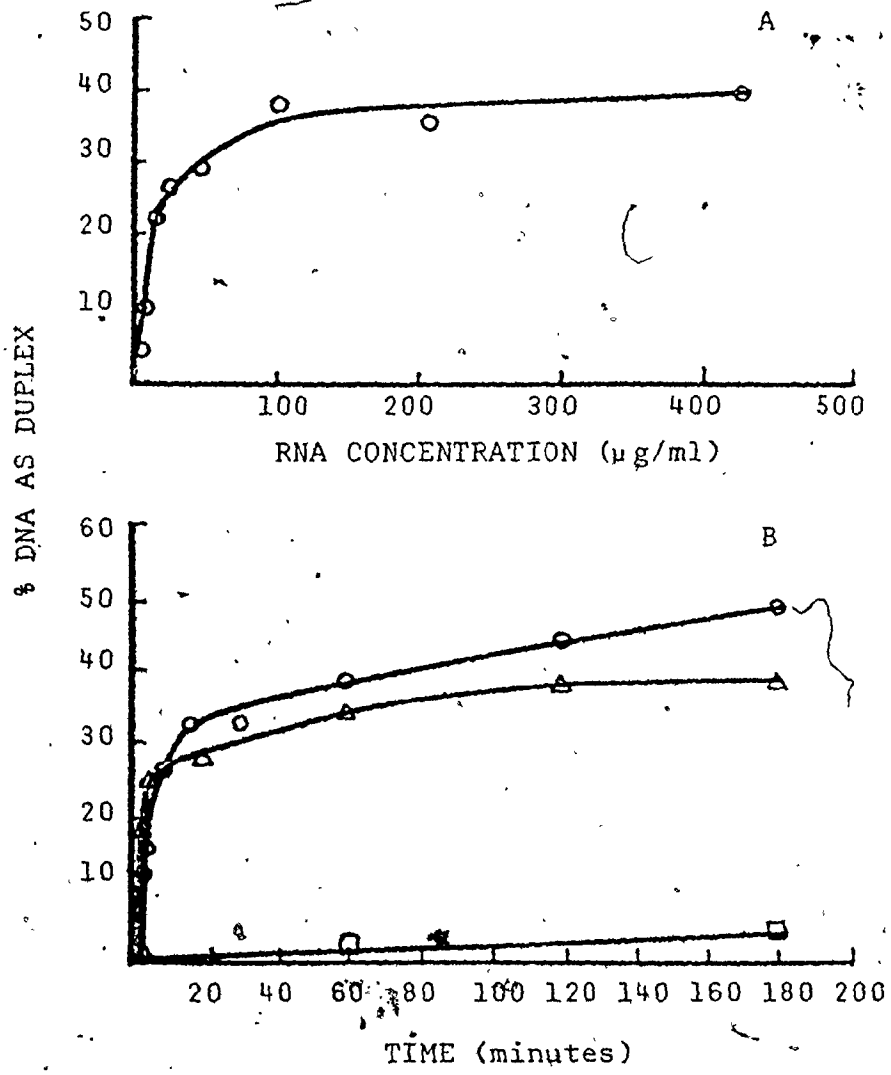


Figure 2: Hybridization of late whole cell RNA to Ad 12 DNA

^{3}H-Ad 12 DNA (0.01 µg/ml) was heat denatured and hybridized to the indicated amounts of unlabelled infected or uninfected cell RNA for the indicated times. Hybridization has assayed by HAP chromatography.

A. hybridization for two hours, with varying amounts infected cell RNA (extracted 36 hours post infection).

B. hybridization for varying times with 423 µg/ml infected cell RNA (o and Δ) and 1700 µg/ml uninfected cell RNA (□).

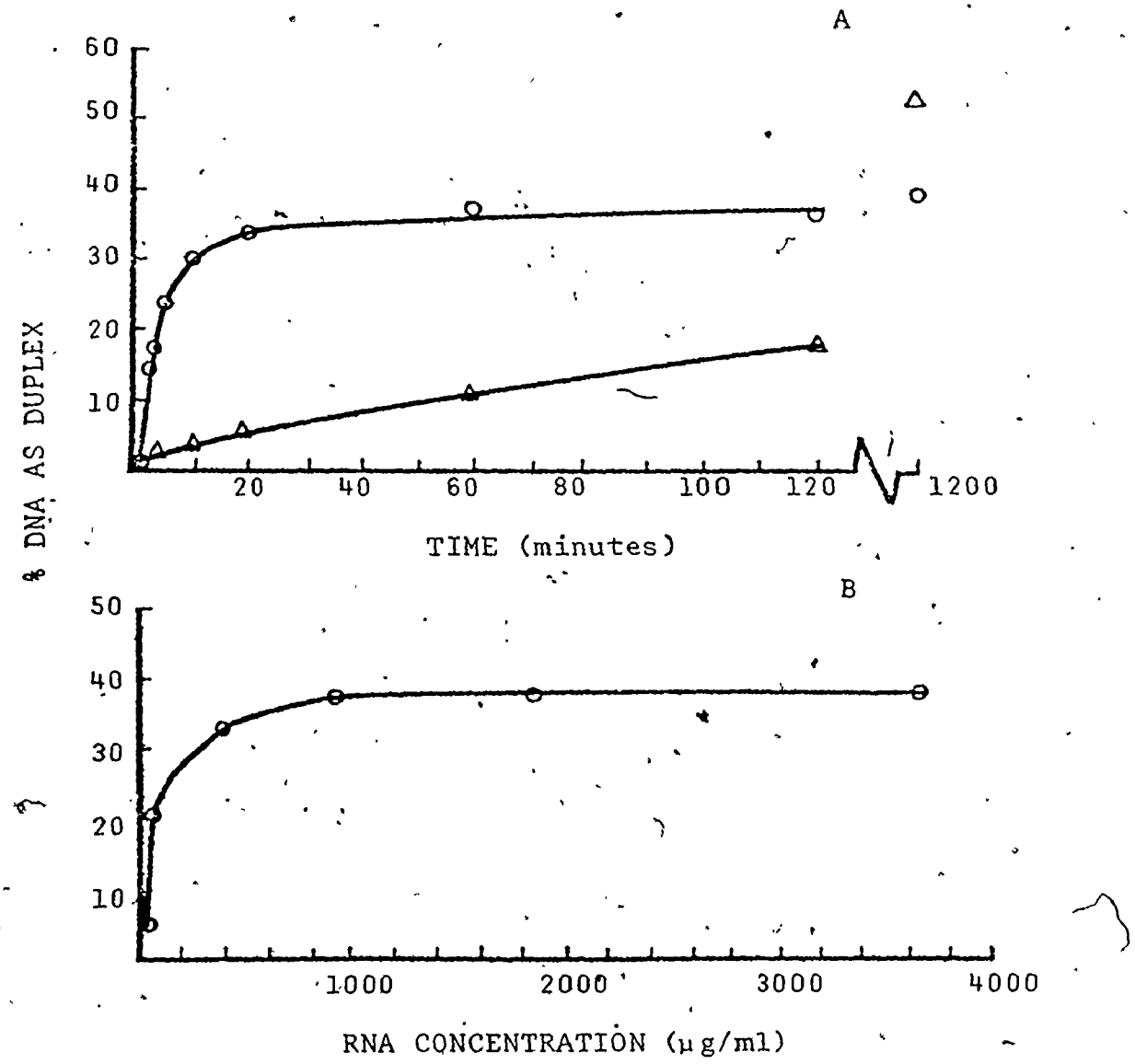


Figure 3: Hybridization of late whole cell RNA to Ad 12 DNA

A. 0.2 µg/ml ^{3}H-DNA was incubated with (o) and without (Δ) 3.5 mg/ml late RNA extracted 36 hours post infection for the indicated times and duplex material was scored on HAP.

B. 0.03 µg/ml ^{3}H-DNA was incubated for 7 hours with the indicated amounts of a new lot of late RNA extracted 36 hours post infection. Hybridization assayed on HAP.

One preparation was hybridized to 0.2 µg/ml DNA at an RNA concentration of 3.5 mg/ml, and the kinetics of hybridization were followed (Figure 3A). The other preparation was used in a saturation-type experiment in which 0.03 µg/ml DNA was hybridized to increasing quantities of RNA (Figure 3B). Both experiments yielded plateau values of 37 to 40% of the viral DNA in hybrid form. Uninfected KB cell RNA did not hybridize to viral DNA.

These results, obtained with three separate lots of RNA, suggested that late whole cell RNA contains sequences capable of hybridizing to 37 to 50% of the viral DNA. This corresponds to the equivalent of 74 to 100% of one DNA strand, provided that the RNA sequences detected are complementary to assymetrically distributed regions of the viral genome. These results agree well with those of Mak and Green (1968), who, using the nitrocellulose-filter hybridization method, estimated that late Ad 12 whole cell RNA is complementary to 80 to 100% of one DNA strand equivalent.

At this point an attempt was made to measure the fraction of the Ad 12 genome expressed as early whole cell RNA. Previous work suggested that about 50% of the Ad 12 genome is transcribed as stable RNA before the onset of viral DNA synthesis (Green *et al.* 1970). Ad 12 DNA replication is first detectable

11 to 12 hours post infection (Mak and Green, 1968). Increasing amounts of unlabelled whole cell RNA extracted 8 hours post infection were hybridized to 0.01 µg/ml Ad 12 DNA for 3 hours (Figure 4). At the highest RNA concentration used (960 µg/ml) only 12.3% of the viral DNA was hybridized. The reaction was probably not saturated, so that the result gave only a minimal estimate of the fraction of the viral genome transcribed at early times (25%). The experiment demonstrated that only small amounts of viral RNA are present in the infected cells at early times. For example, 480 µg/ml of early RNA was required to produce 50% of the *observed* hybridization, while between 7 and 13 µg/ml of late RNA half-saturated the same quantity of DNA in the same reaction time. Therefore, there was at least a 35 to 70 fold difference in the concentration of viral RNA at early and late times post infection.

2. Specificity of the hybridization. The hybridization observed between Ad 12 DNA and infected cell RNA was specific in one sense: uninfected KB cell RNA did not react. Another check on the specificity of the reaction was obtained by separating the viral DNA sequences into two classes: those which bound to

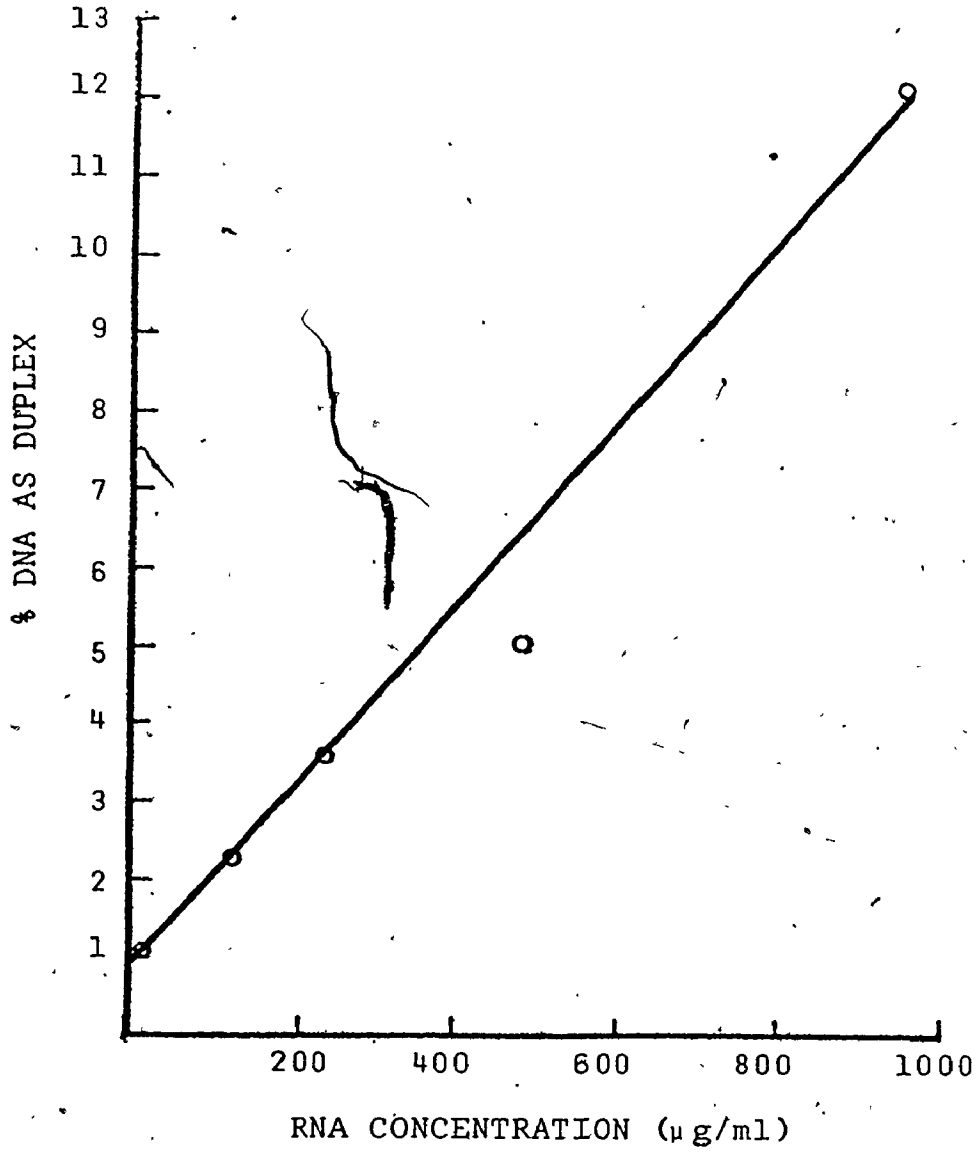


Figure 4: Hybridization of early whole cell RNA to Ad 12 DNA

The indicated amounts of unlabelled RNA extracted 8 hours post infection were hybridized to 0.01 µg ^{3}H-Ad 12 DNA for 3 hours. Hybrids were scored on HAP.

HAP after hybridization, and those which did not. If the reaction was specific, these DNA subpopulations would not overlap. The sequences which bound to HAP during one cycle of hybridization would bind after a second cycle and, conversely, DNA which failed to bind in the first cycle would also fail in the second.

To test these predictions, 0.3 µg/ml DNA was hybridized to 500 µg/ml of a new preparation of late whole cell RNA extracted 36 hours post infection. Aliquots were removed after various periods of incubation and analyzed on HAP (Figure 5). A control reaction, lacking RNA was also tested. Because this was a preparative experiment, the DNA concentration was necessarily high. The reaction with infected cell RNA levelled off within 2 hours, after about 45% of the DNA had reacted. Further incubation, up to 20 hours, had no effect. In contrast, the DNA control reaction continued renaturation. Because RNA-DNA hybridization and DNA-DNA renaturation are competing reactions, and since the DNA concentration used in this experiment was rather high, it was important to estimate what fraction of the duplex material observed was due to DNA renaturation. Renaturation of DNA is a second order reaction. If an appreciable fraction of the observed DNA in a double stranded form was due to renaturation, then varying the DNA concentration during

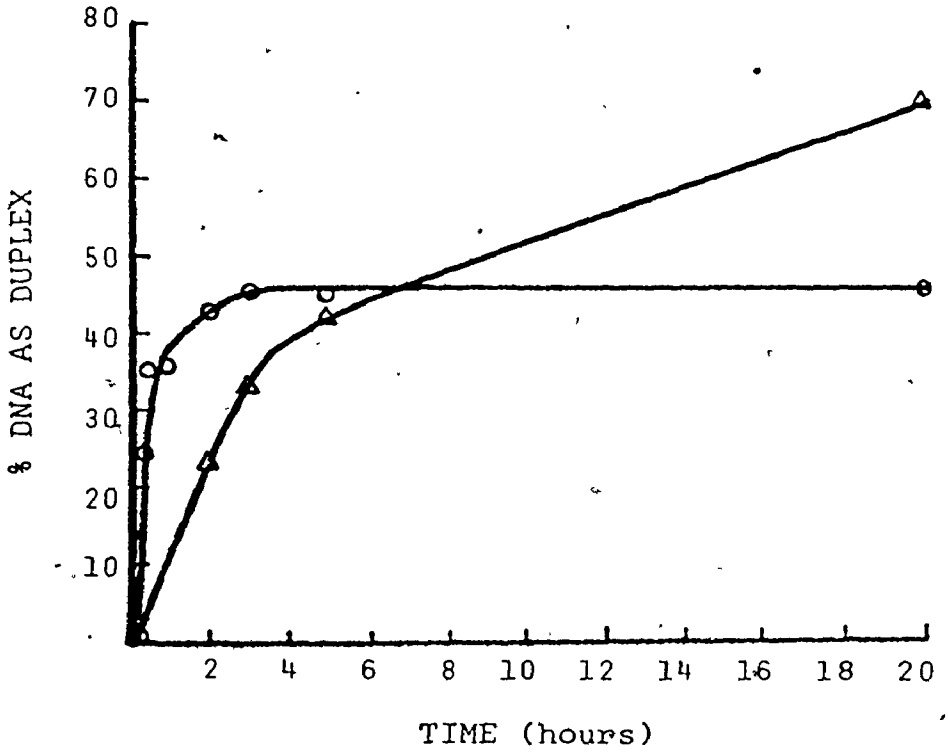


Figure 5: Preparative hybridization of late whole cell RNA to labelled Ad 12 DNA

0.3 µg/ml ^{3}H-DNA was incubated with (o) and without (Δ) 500 µg/ml unlabelled late whole cell RNA for the indicated times. Duplex structures were scored on HAP.

the reaction would alter this fraction, and consequently the final apparent level of hybridization. Table 1 shows that varying the DNA concentration one hundred fold had no detectable effect on this value. Therefore, probably only a small fraction of the double stranded material formed during hybridization was present in DNA-DNA duplexes. This was not an unexpected finding, because the rapid formation of RNA-DNA hybrids should decrease the concentration of single stranded DNA available for renaturation. If the RNA was complementary to about half of the DNA sequences (as appeared to be the case with this preparation), and if these sequences were assymetric (not self-complementary), then RNA-DNA hybridization would remove the equivalent of one DNA strand from the renaturation reaction. The data obtained were consistent with this expectation.

After 24 hours hybridization, the total reaction mixture was chromatographed on HAP, separating the hybridized (positive) and unhybridized (negative) DNA sequences. After alkaline hydrolysis of any RNA present and concentration by dialysis against 50% (w/w) polyethylene glycol 6000, the positive and negative DNA preparations were reincubated with the same lot of late RNA (Figure 6). About 82% of the

Table 1

Effect of Varying DNA Concentrations on the Fraction of the DNA Scoring as Hybrid

DNA concentration (μg/ml)	Fraction hybridized
0.67	.48
0.33	.46
0.17	.45
0.08	.45
0.04	.48
0.01	.44
0.005	.46

Denatured, sonicated ^{3}H-Ad 12 DNA was hybridized at the indicated concentrations to 500 μg/ml late cytoplasmic RNA at 66°C for 24 hours. Hybridization was assayed by HAP chromatography, and the results were corrected for the 7% zero time binding of denatured DNA.

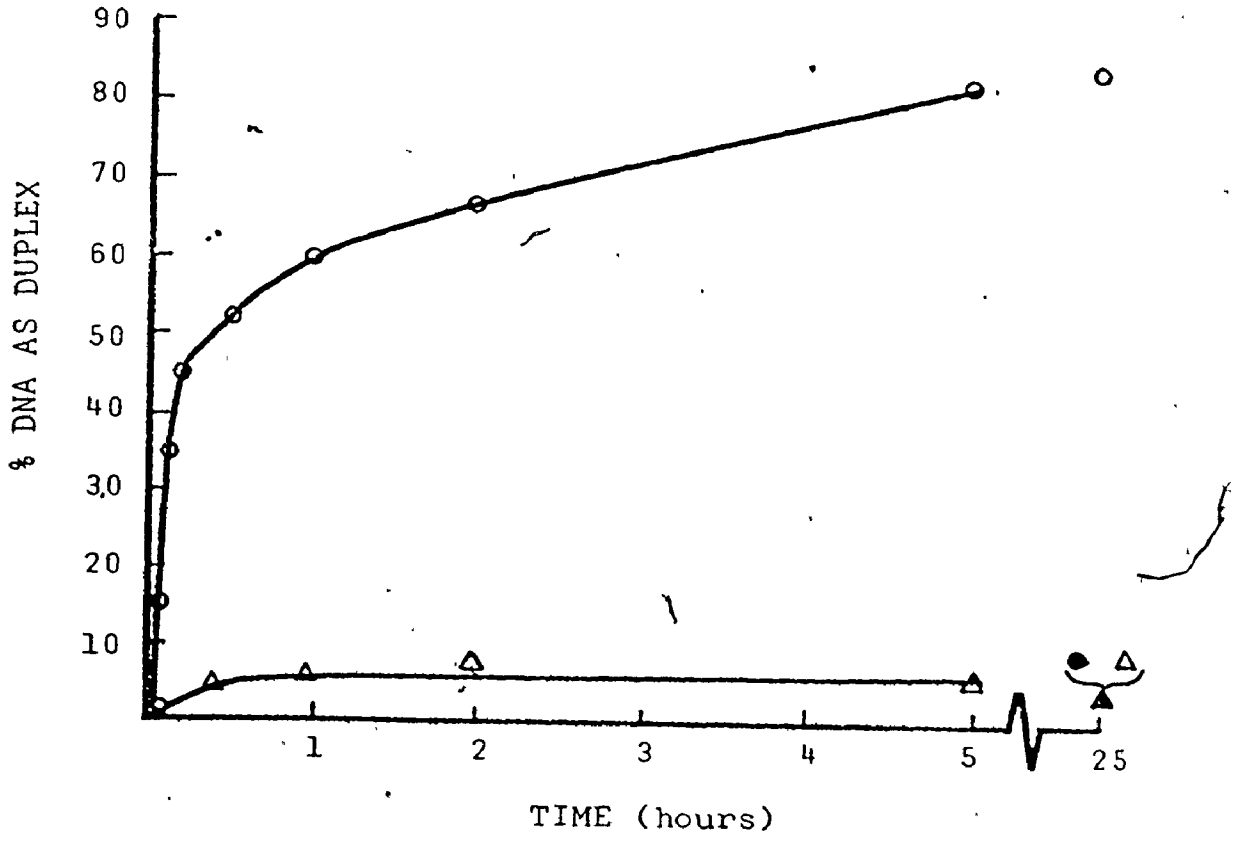


Figure 6: Rehybridization of positive and negative DNA to late whole cell RNA

0.01 µg/ml H-positive (o) and negative (Δ) DNA were incubated with 833 µg/ml late (36 hours post infection) whole cell RNA for the indicated times. Filled symbols denote the corresponding DNA incubated in the absence of RNA. Hybrids were assayed on HAP.

positive sequences rehybridized to late RNA, while only 8% of the negative sequences reacted, indicating that the two DNA sequence populations were largely non-overlapping. There are several possible explanations for the incomplete rehybridization of the positive DNA sequences to late RNA: the DNA may have suffered some chemical damage during the selection process, or the positive and negative sequences could be cross contaminated by some small amount. The prejudice of the author is that both factors contribute to the incomplete rehybridization. The small amount of hybridization of the negative DNA to late RNA suggests that this preparation is partially contaminated with positive sequences.

Two properties of the positive and negative DNA preparations deserve comment. First, the unfractionated starting DNA had a zero-time binding to HAP of 6% while 10% of the positive DNA and 2% of the negative DNA bound at zero time. This suggested that specific DNA fragments contributed to zero time binding, and that these fragments appeared in the positive DNA fraction because of the selection method, irrespective of whether they had hybridized to late RNA. It is possible that these fragments contain

regions of significant self complementarity, enabling them to form hairpin-like structures with duplex regions. The data obtained in all experiments using HAP chromatography were corrected to eliminate zero-time binding of DNA. Thus, if this DNA represents a specific set of sequences, these sequences have been ignored in the hybridization studies. Second, both the positive and negative DNA reassociated up to a plateau level of 20% duplex (excluding zero-time binding) during 100 hours of self-hybridization. This value was not increased by performing the renaturation in 1.0 M Na^+ rather than in 0.14 M Na^+, suggesting that it represented the plateau level. The renaturation of both positive and negative DNA is consistent with partial cross-contamination.

3. Hybridization of early RNA to positive and negative DNA. Because of the effective separation of DNA sequences achieved by this approach, it was of interest to examine the hybridization behaviour of early whole cell RNA with each population of DNA sequences. RNA at a concentration of 1 mg/ml was incubated with 0.01 µg/ml of positive and negative DNA (Figure 7), and hybridization was followed with time. Somewhat surprisingly, both positive and negative DNA hybridized

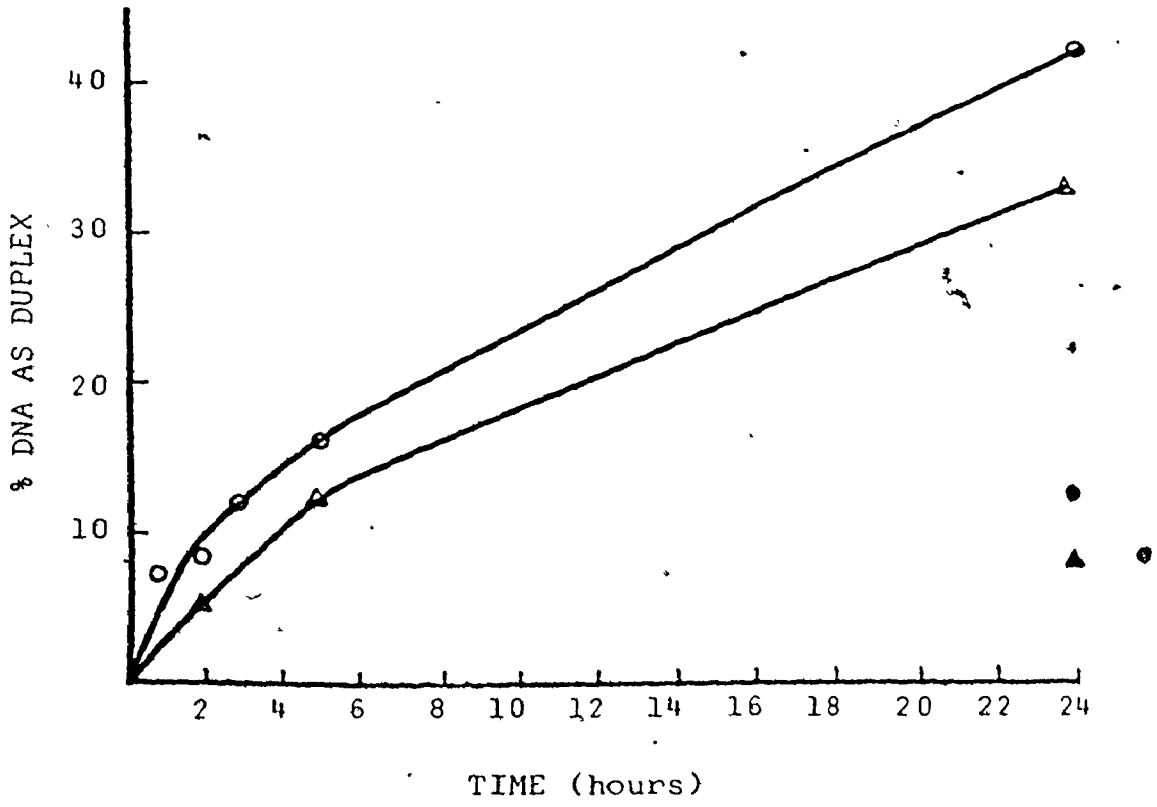


Figure 7: Hybridization of positive and negative DNA to early whole cell RNA (8 hours post infection)

0.01 µg/ml ^{3}H-positive (o) and negative (Δ) DNA were incubated with 1 mg/ml early whole cell RNA for the indicated times. Filled symbols denote the corresponding DNA in the absence of RNA.

significantly with the early RNA, implying that the early whole cell RNA contained sequences which were not detectable in late whole cell RNA. A similar conclusion has been reached in studies of Ad 2 RNA by Lucas and Ginsberg (1971), who found that a specific subset of Ad 2 early RNA sequences is not detectable in late RNA. Although the hybridization reactions depicted in Figure 7 were almost certainly not at saturation, the data permitted a minimal estimate of the fraction of each DNA preparation complementary to early whole cell RNA to be made. This was obtained by subtracting the renaturation observed within 24 hours in the absence of RNA from the hybridization values. RNA complementary to at least 35% of the positive and 20% of the negative DNA sequences were found in early whole cell RNA. If it is assumed that each DNA preparation represented about half of the total Ad 12 DNA, then at least 55% of one DNA strand equivalent is transcribed at early times.

4. Systematic errors associated with hydroxyapatite chromatography. Hydroxyapatite chromatography scores the fraction of the total mass of the DNA present in structures which contain a duplex region of greater than about 50 base pairs in length. The single

stranded regions of such structures are included in the double stranded fraction. The resulting overestimation of the fraction hybridized can be minimized by using small fragments of DNA. For this reason DNA sheared to about 250 bases in length (about .7% of the length of the Ad 12 genome) was used in the hybridization experiments. Even when sheared DNA is used the degree of overestimation cannot be determined without prior knowledge of the topological distribution of the RNA coding regions in the DNA.

Another, counteracting, factor is the requirement for an overlap of about 50 bases between the RNA and the DNA fragment before a hybrid structure can be scored as duplex. The result of this effect is to underscore hybrids. Since DNA fragments less than 50 bases in length are never scored as duplex the magnitude of this error increases with decreasing fragment size. The DNA used in these studies was highly radioactive, so that radiodecay-induced fragmentation could perhaps generate appreciable numbers of fragments less than 50 bases in length. The rather broad distribution of this DNA in alkaline sucrose gradients was consistent with this possibility (Figure 1). To evaluate the magnitude of this effect, denatured DNA was incubated at 0.4 μg/ml

for 24 hours, then chromatographed on hydroxyapatite. The duplex fraction (76% of the total) was collected, and an aliquot was sedimented in alkaline sucrose (Figure 8). Comparison of the sedimentation profiles of the selected and unfractionated DNA showed that selection for fragments capable of entering a structure containing 50 base pairs of duplex DNA resulted in an enrichment for longer fragments. This finding is consistent with the expectation that smaller fragments have a lower probability of contributing to such structures than do large ones. The selected DNA population was then hybridized to late whole cell RNA. At saturation 40% of the DNA scored as hybrid, a value identical to that obtained using the same RNA preparation and unfractionated DNA. This result implied that the underestimation of hybridization due to small DNA fragments was negligible.

Two additional possible sources of systematic error were considered. In the saturation experiments DNA was incubated with increasing amounts of RNA, so that different samples contained varying quantitites of RNA. Depending on how this RNA eluted from hydroxyapatite, the single stranded and double stranded fractions of each sample may have contained widely differing amounts of RNA. If the quantitites present

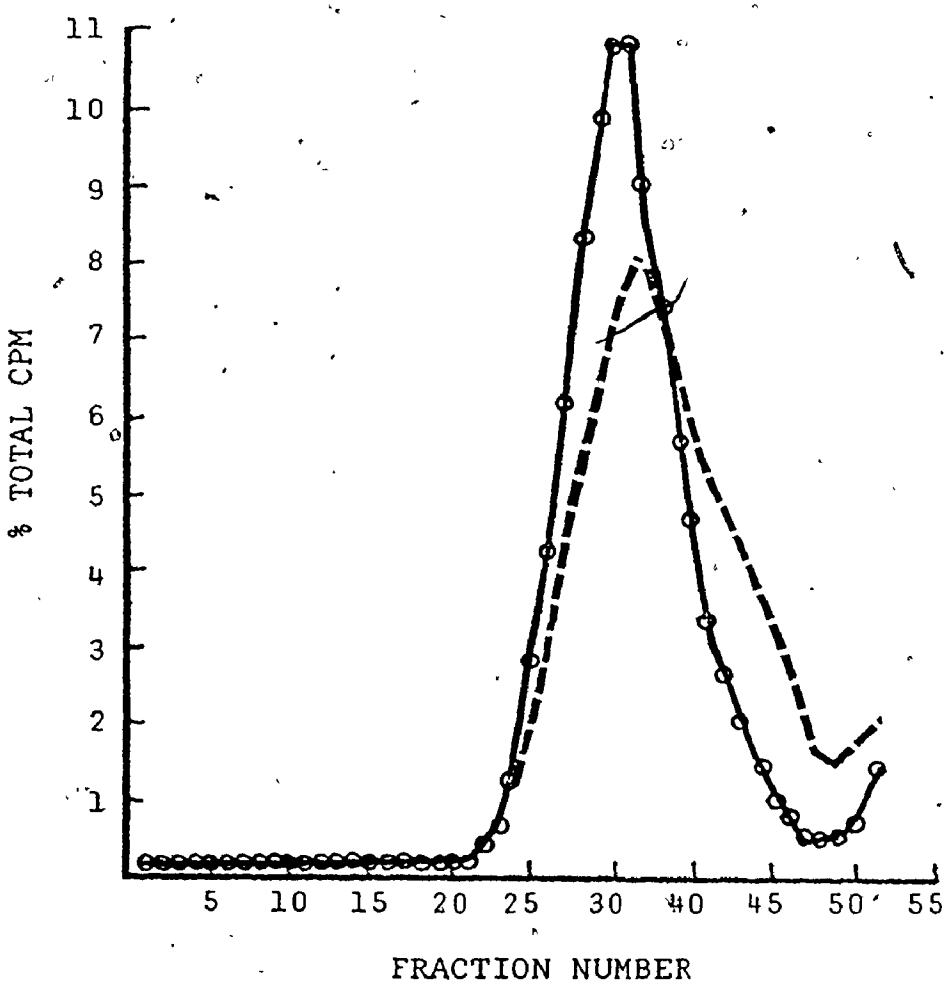


Figure 8: Sedimentation profile of ^{3}H-labelled, sonicated Ad 12 DNA and the subpopulation selected as double stranded after renaturation

Sonicated Ad 12 DNA, and the fraction of this DNA which was selected by renaturation as described in the text, was centrifuged through a 5 ml 5-20% alkaline sucrose gradient for 14 hours at 40,000 rpm at 20°C in the SV50.1 rotor. Fractions were collected, TCA precipitated and counted. o, selected DNA; striped line, unselected DNA.

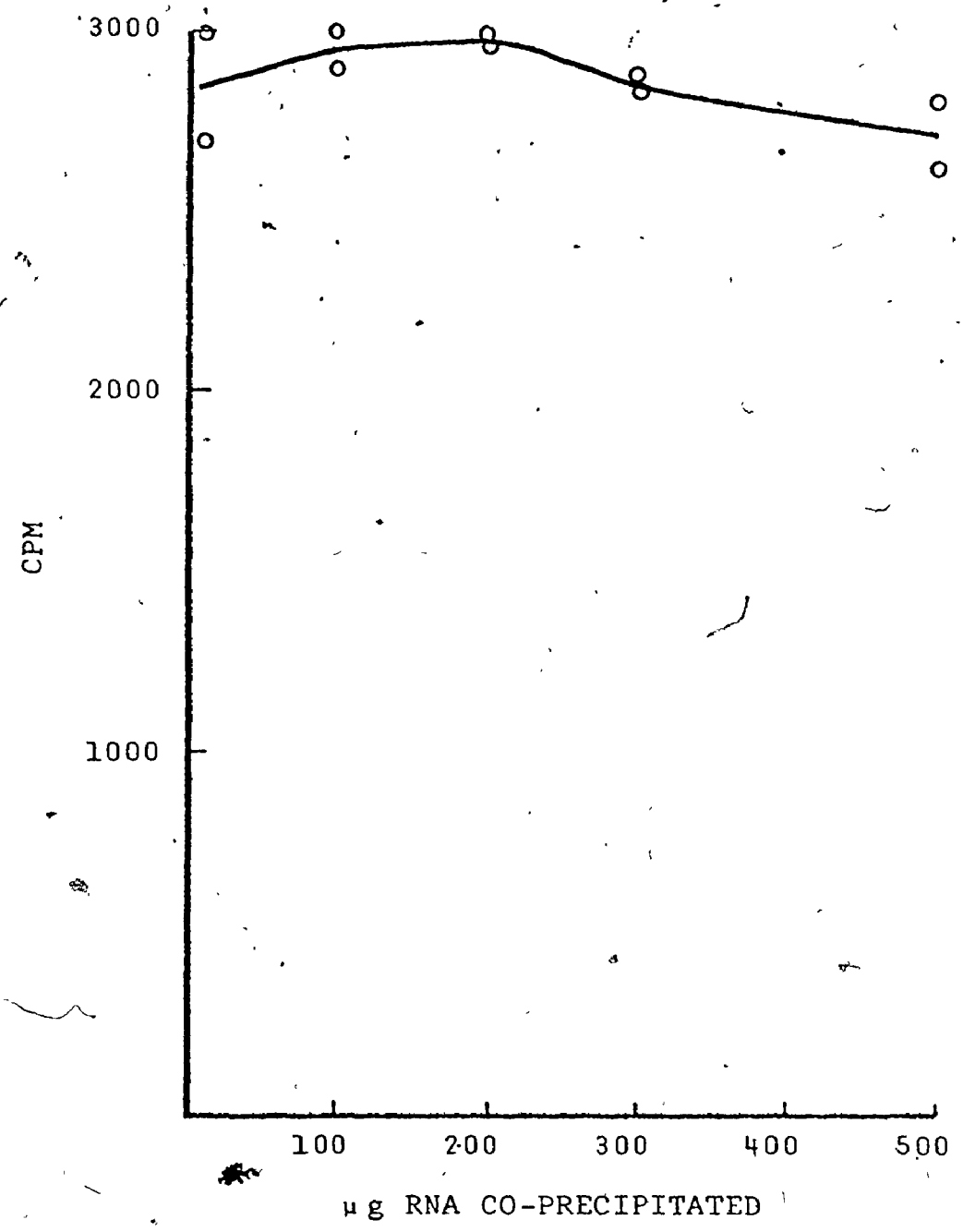


Figure 9: Quenching of tritium counts by increasing quantities of co-precipitated RNA

5×10^3 µg of Ad 12 DNA was mixed with 100 µg of unlabelled salmon sperm DNA and the indicated amounts of uninfected KB cell RNA. The mixtures were TCA precipitated and counted.

were sufficient to differentially interfere with liquid scintillation counting, systematic error would result. The effects of increasing quantitites of RNA on the counting efficiency of ^{3}H-DNA was examined (Figure 9). No effect was observed, up to 500 µg of RNA. The greatest quantity of RNA chromatographed on any analytical column in the experiments described above was 140 µg.

It was conceivable that RNA had an effect on the chromatographic behaviour of DNA. Specifically, large quantities of RNA could interfere with the binding of duplex DNA in 0.14 M PB. To evaluate the magnitude of this effect, 0.01 µg of duplex ^{3}H Ad 12 DNA was mixed with varying amounts of unlabelled infected cell RNA and chromatographed on HAP columns (Table 2). Increasing amounts of RNA did increase the fraction of the DNA eluting at 0.14 M PB, resulting in a slight underestimate of the fraction of duplex DNA. The magnitude of this effect was not large enough to appreciably affect the results presented above. This effect may explain why negative DNA sequences selected above contained a minority (about 8%) of DNA fragments capable of hybridizing to late RNA.

Table 2

Effect of Increasing RNA Concentration on the Chromatographic Behaviour of Duplex DNA on HAP

RNA applied (µg)	Fraction of DNA eluting at 0.14 M P.B.
0	.015
50	.043
100	.050
500	.076

0.01 µg of ^{3}H-Ad 12 DNA was mixed with the indicated amounts of unlabelled, infected KB cell RNA and chromatographed on one ml-HAP columns.

B. Hybridization in solution scored by digestion with endonuclease S1

To provide an independent check on the data obtained using HAP chromatography, several experiments were repeated and the hybridization was assayed by digestion with endonuclease S1 (Sutton, 1971; Vogt, 1973), a single strand specific nuclease obtained from *Aspergillus oryzae*.

1. Specificity of the S1 nuclease. In order to choose assay conditions allowing an adequate discrimination between single and double stranded DNA, the kinetics of hydrolysis of ^{14}C-labelled intact duplex and ^{3}H-labelled sonicated denatured Ad 12 DNA by S1 nuclease were followed in the presence of 1.6 mg/ml unlabelled uninfected KB cell RNA (Figure 10). RNA was included to simulate an assay in a hybridization experiment. At an S1 nuclease concentration of 20 µg/ml, degradation of 97% of the denatured DNA was completed within 1 hour, while 95-100% of duplex DNA remained acid precipitable. Significant hydrolysis of duplex DNA was observed during prolonged incubation. It is possible that the slow hydrolysis of duplex DNA is a consequence of hydrolysis during transient unwinding

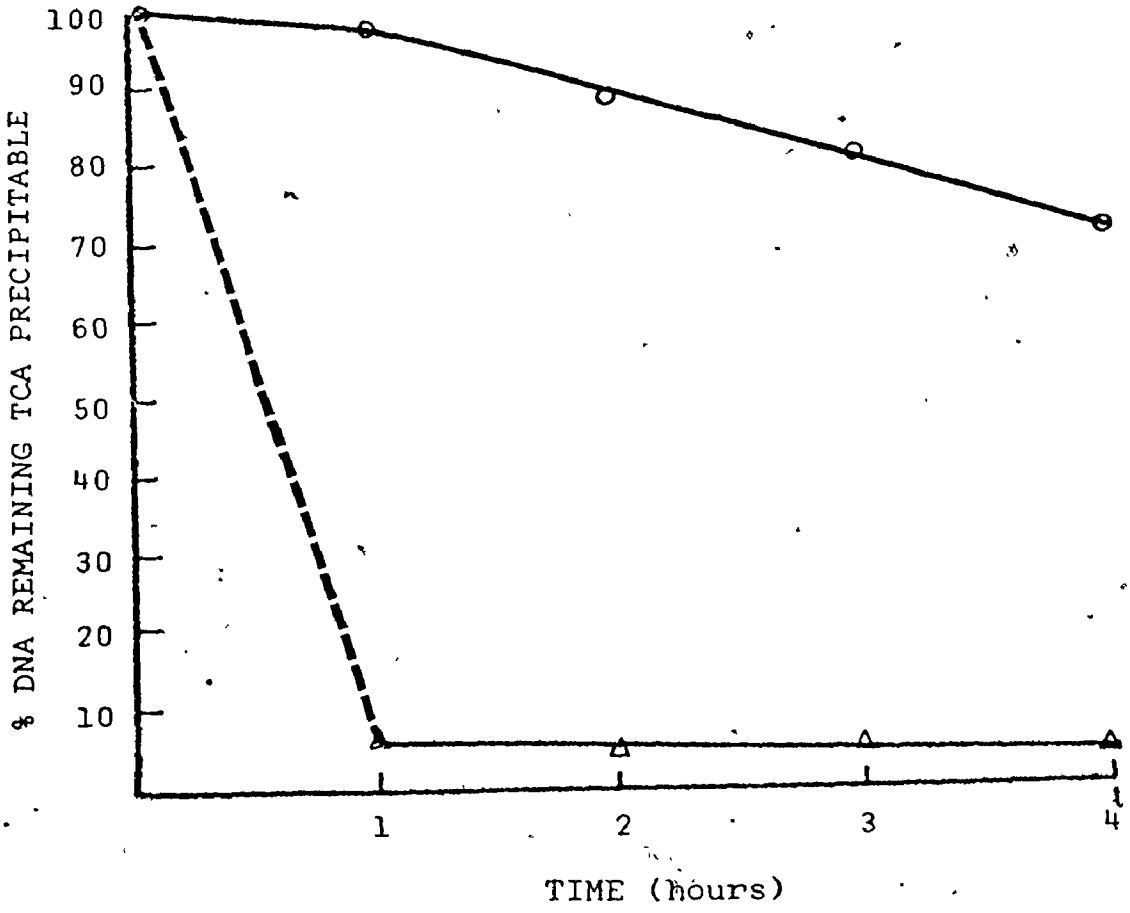


Figure 10: Kinetics of hydrolysis of duplex and denatured Ad 12 DNA by S1 nuclease

20 µg/ml S1 nuclease was incubated with 10 µg/ml denatured calf-thymus DNA, 0.01 µg/ml denatured, sonicated ^{3}H-Ad 12 DNA, 1 µg/ml ^{14}C-labelled intact, duplex Ad 12 DNA and 1.6 mg/ml unlabelled, uninfected KB cell RNA for the indicated times. TCA precipitable ^{3}H and ^{14}C radioactivity was determined. o, ^{14}C, intact native DNA; Δ, ^{3}H, sonicated denatured DNA.

of the duplex structure, particularly at the molecular ends ("fraying of the ends"). Alternatively, it is possible that the nuclease used was partially contaminated with other nucleases capable of hydrolysing duplex DNA. For routine assays of duplex material, 10 µg/ml S1 was incubated with samples for 1 hour, resulting in about 5% hydrolysis of duplex DNA and 97% hydrolysis of sonicated, denatured DNA. All assays were carried out in the presence of 1.6 mg/ml RNA, yeast RNA being added to equalize RNA concentrations. RNA was degraded by alkaline hydrolysis after the nuclease digestion, as described in Materials and Methods.

2. Late whole cell RNA hybridizes 37 to 40% of Ad 12 DNA. Three independent lots of unlabelled late whole cell RNA, extracted 36 hours post infection, were hybridized to sonicated denatured Ad 12 DNA and hybrids were assayed by their resistance to S1 nuclease (Figure 11). Preparation 1 was hybridized for 7 hours, while preparations 2 and 3 were hybridized 20 hours. Preparations 2 and 3 hybridized 37 to 40% of the labelled DNA at apparent saturation, while preparation 1 hybridized about 32%. It is not possible to decide whether this represents a

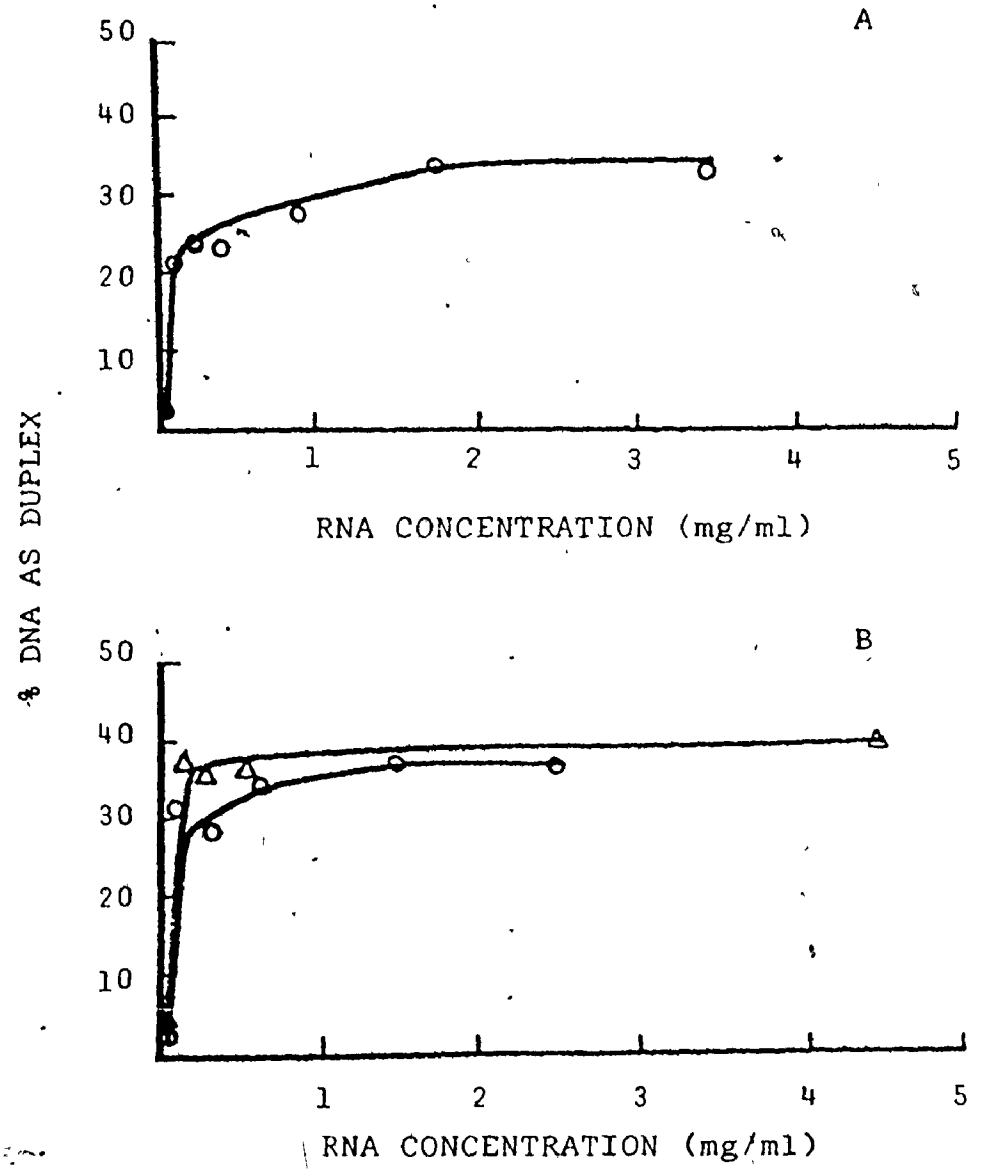


Figure 11: Hybridization of late whole cell RNA to Ad 12 DNA (S1 assay)

Denatured, sonicated ^{3}H-Ad 12 was hybridized to the indicated quantities of unlabelled late whole cell RNA (36 hours) in 0.3 M NaCl, 0.01 M Tris pH 7.3. Hybrids were assayed by S1 nuclease. The data were corrected for the 3% zero time resistance of the DNA as described in Materials and Methods.

A. RNA preparation 1 hybridized to 0.025 µg/ml DNA for 7 hours.

B. preparation 2 (Δ) and preparation 3 (o) hybridized to 0.003 µg/ml DNA for 20 hours.

real difference, or whether a longer hybridization time would have allowed the detection of more RNA sequences in preparation 1. These results are in reasonable agreement with those obtained by HAP chromatography, and suggest that about 80% of one DNA strand equivalent reacts with late whole cell RNA.

3. Early whole cell RNA hybridizes at least 25 to 30% of Ad 12 DNA. Two independent lots of whole cell RNA extracted 7.5 hours post infection were hybridized to 0.003 µg/ml DNA for 20 hours (Figure 12). The reactions were not saturated, so that the maximum levels of hybridization observed (25 and 30%) can only serve as minimal estimates of the true levels. These values correspond to 50 to 60% of one DNA strand equivalent.

Two independent attempts were made to measure the degree of overlap between RNA sequences present at early and late times post infection. Saturating amounts of late RNA were mixed with early RNA and the fraction of Ad 12 DNA hybridized by the mixtures was determined (Table 3). A mixture of lot 1 early and lot 4 late RNA did not hybridize more DNA than lot 4 late RNA alone. However, a mixture of lot 2 early

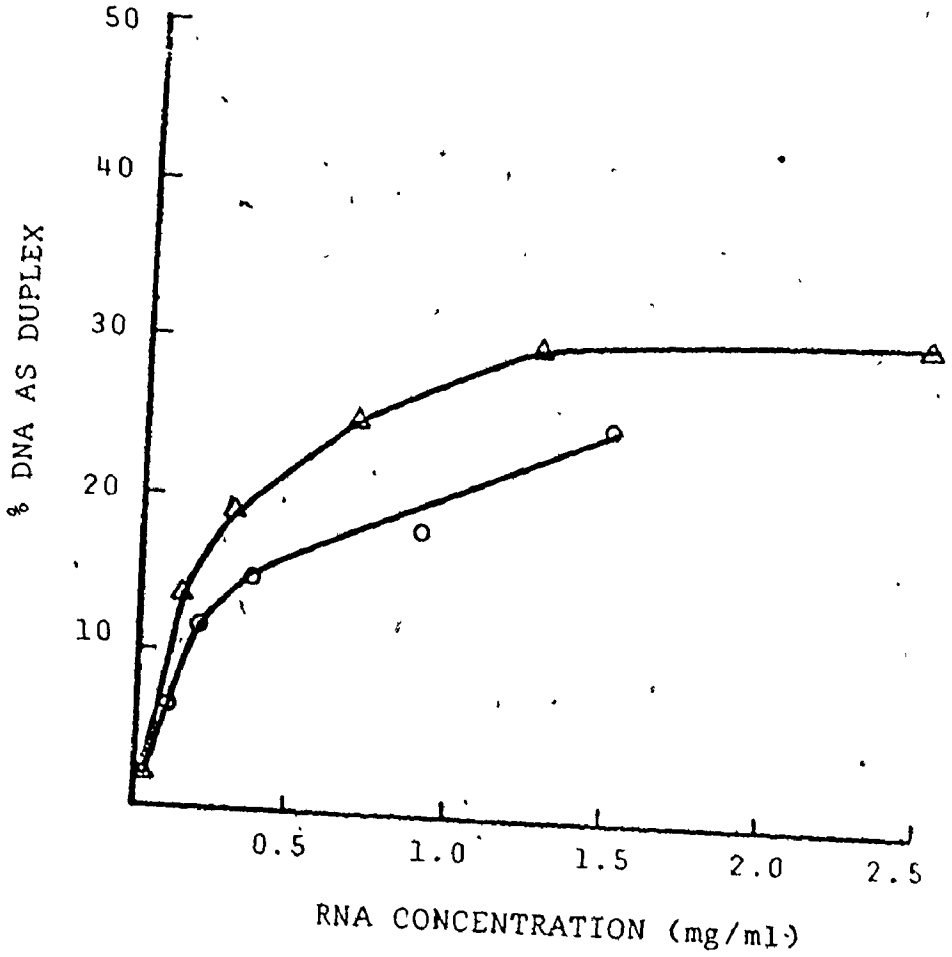


Figure 12: Hybridization of early whole cell RNA to Ad 12 DNA (S1 assay)

Denatured, sonicated ^{3}H-Ad 12 DNA (0.003 µg/ml) incubated for 20 hours with the indicated amounts of unlabelled early whole cell RNA. o, preparation 1; Δ, preparation 2.

Table 3

Sequence Relationship of Early and Late Whole Cell RNA

RNA	Concentration (μg/ml)	Fraction DNA hybridized
Early #1	750	.182
Late #4	2800	.383
Early #1 + Late #4	750 + 2800	.351
Early #2	1250	.301
Late #3	4500	.391
Early #2 + Late #3	1250 + 4500	.470,.465

0.003 μg/ml denatured ^{3}H-Ad 12 DNA was incubated with the indicated RNA at 67°C in 0.3 M NaCl, 0.01 M Tris pH 7.3. Hybridization was assayed by S1 nuclease, and the data was corrected for the 3% zero time resistance of the DNA. DNA incubated in the absence of RNA for 25 hours was 4.5% resistant to the nuclease (zero time resistance subtracted). Early RNA was prepared 8 hours, and late RNA 36 hours, post infection. Hybridization was for 25 hours with RNA preparation 1 and 4, and for 20 hours for preparations 2 and 3.

and lot 3 late RNA hybridized 47% of the DNA, significantly more than lot 3 late RNA alone (40%). The results obtained with positive and negative DNA presented above strongly suggested that early RNA contains sequences not detectable late, consistent with the latter result. The reaons for the disagreement between the results obtained with lots 1 and 2 early RNA remain unclear, and will be dealt with at length in the Discussion.

II. Relationship of Infected KB Cell RNA to the Separated Complementary Strands of Ad 12 DNA

The necessity for short hybridization times and low DNA concentrations in the experiments described above reduced the sensitivity of the analysis. DNA renaturation can be totally eliminated by using the separated complementary strands of DNA as probes in hybridization experiments. In addition, experiments employing the complementary DNA strands determine the transcriptional polarity of the RNA sequences found. For these reasons, labelled complementary strands of Ad 12 DNA were physically separated and used as hybridization probes.

A. Separation of the complementary DNA strands

1. Poly (U,G) cesium chloride density gradient centrifugation. Figure 13 shows a typical profile obtained by centrifuging ^{3}H-labelled, denatured, Ad 12 DNA to equilibrium in a neutral cesium chloride density gradient containing poly (U,G). Two major peaks were observed, which correspond to the two intact complementary DNA strands. The lighter band was poorly resolved from a less dense component which varied widely in quantity between experiments. This band may be analogous to the U band observed by Tibbetts *et al.* (1974), in similar gradients containing Ad 2 DNA. Its nature was not investigated. Figure 14 shows that the bulk of the material recovered from these two peaks co-sediments with intact adenovirus DNA in alkaline sucrose.

Ortin *et al.* (1976) have determined the 3'→5' polarity of the Ad 12 DNA strands with respect to the Eco RI cleavage map of the viral genome (see below). The 3' terminus of the strand which binds the most poly (U,G) is at the right end of the genome, while the 3' end of the other strand is at the left end. Thus, the poly (U,G) heavy strand is transcribed from right to left, and the light strand is transcribed

Figure 13: Separation of the Ad 12 complementary DNA strands

Denatured, ^{3}H-labelled Ad 12 DNA was centrifuged to equilibrium in a cesium chloride density gradient containing poly (U,G), as described in Materials and Methods. The starting density was 1.76 gm/ml. Fractions (6 drops) were collected from the bottom of the tube and 5 µl aliquots were assayed for radioactivity. The indicated fractions were pooled.

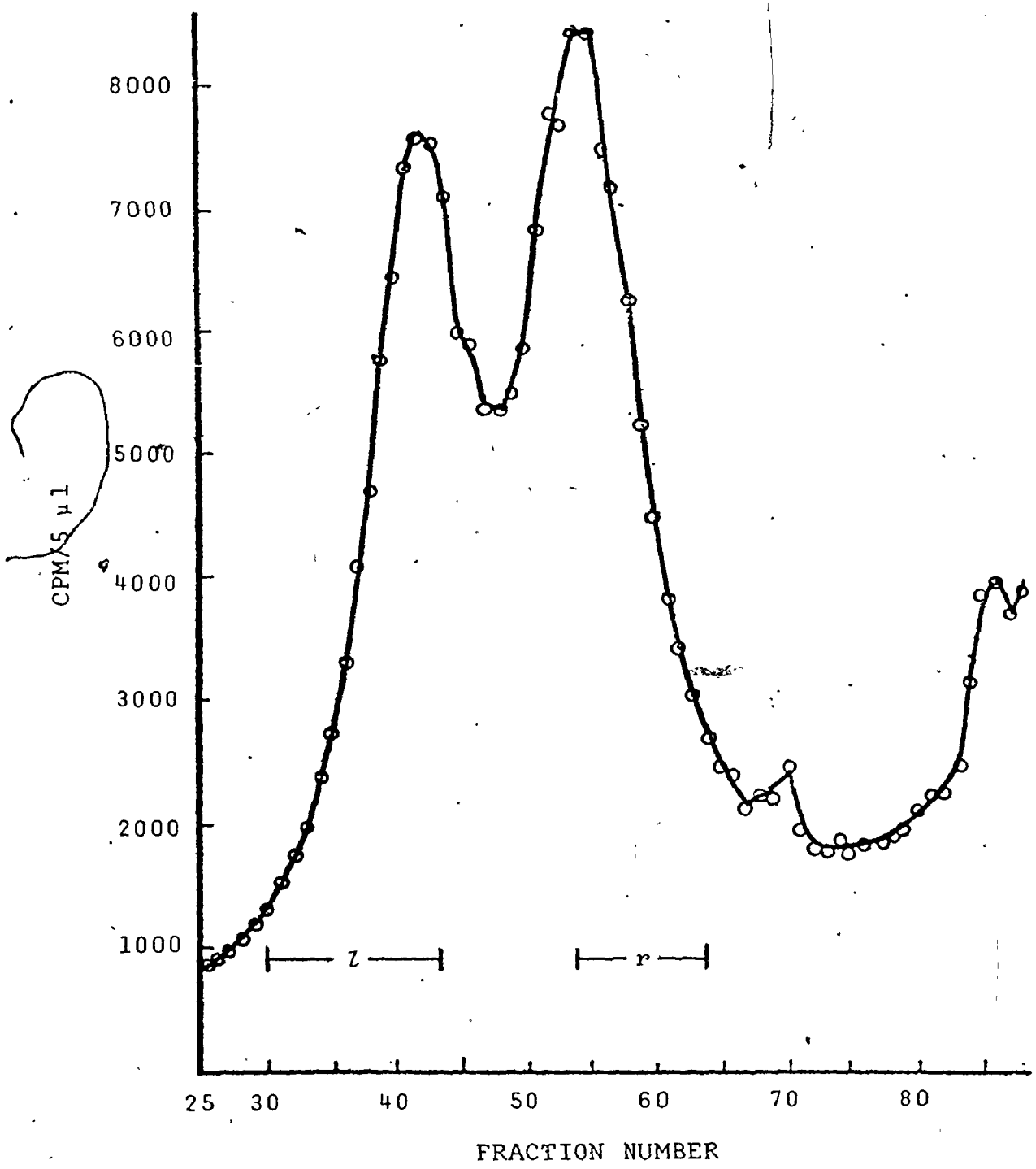

8000
7000
6000
5000
4000
3000
2000
1000
CPM/5 µl
l
r
25
30
40
50
60
70
80
FRACTION NUMBER

Figure 14: The *l* and *r* strands are largely intact

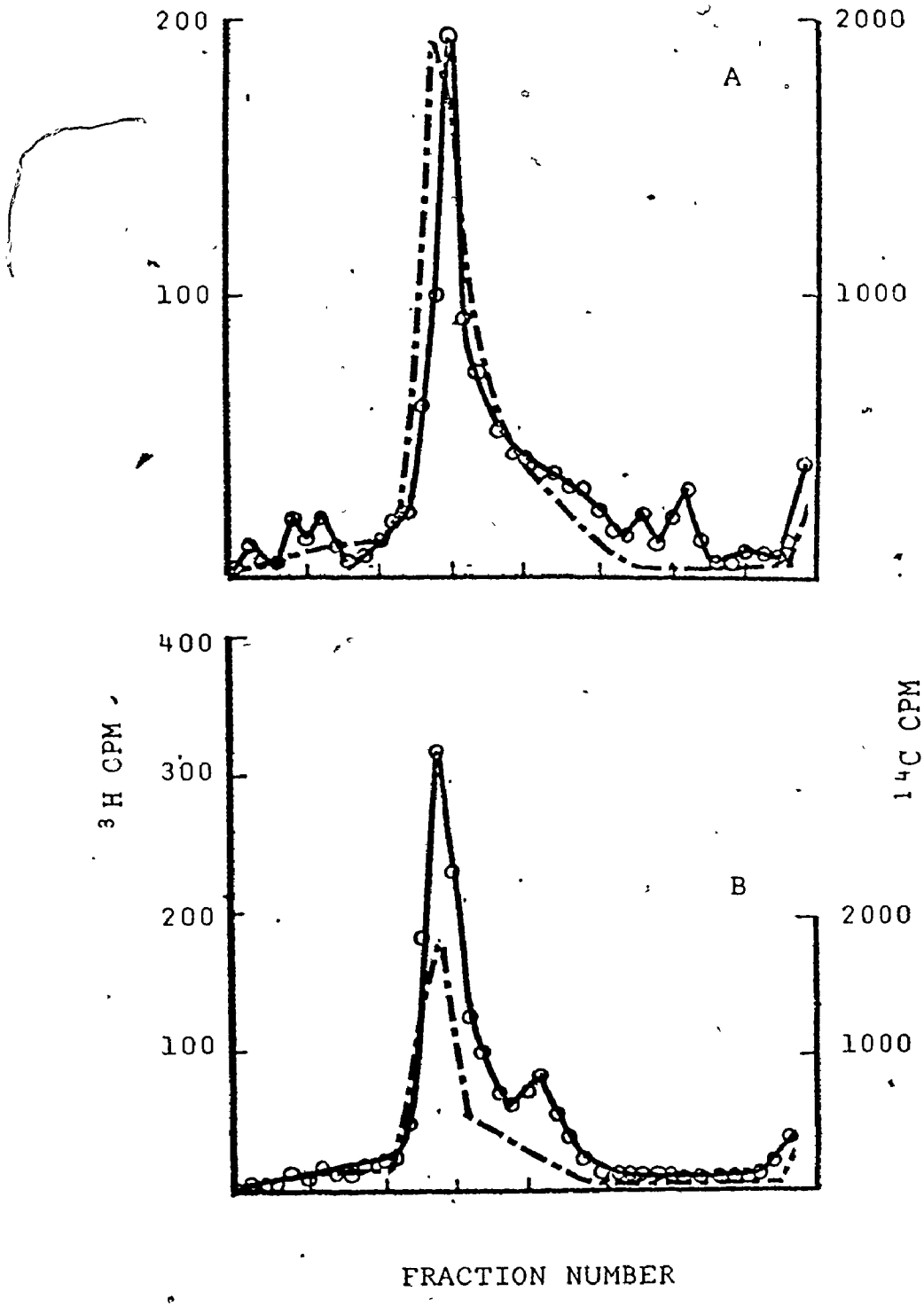
Aliquots of fractions pooled from a poly (U,G) cesium chloride density gradient were dialyzed into 0.01 M Tris pH 7.3, mixed with Ad 2 virions labelled with ^{14}C-thymidine, lysed 10 minutes in 0.5 N NaOH, 0.2% SDS, .01 M EDTA and centrifuged for 90 minutes at 48,000 rpm through a 5-20% alkaline sucrose gradient at 20°C in the SW50.1 rotor. Fractions were collected, TCA precipitated, and ^{14}C and ^{3}H radioactivity was determined.

A. *l* strand;

B. *r* strand;

o ^{3}H CPM;

-- ^{14}C CPM.


200
100
2000
1000
A
400
300
200
100
2000
1000
B
^{3}H CPM
^{14}C CPM
FRACTION NUMBER

from left to right. Conforming to conventions established with bacteriophages (Szybalski *et al.*, 1970) the complementary strands of Ad 12 DNA are referred to below as the *l* and *r* strands, denoting leftward and rightward trancription, respectively. Thus, the poly (U,G) heavy strand is the *l* strand, and the light strand is the *r* strand.

To obtain a greater purification of the complements, one of two procedures was followed:

1. The peak fractions were pooled and rebanded in cesium chloride in the presence of fresh poly (U,G) (Figure 15). The peak fractions from the second gradient were then fractionated according to size by alkaline sucrose density gradient centrifugation, and the DNA co-sedimenting with intact Ad 12 DNA was used for hybridization.

2. Alternatively, the peak fractions were pooled and exhaustively renatured at 37°C in 50% formamide. The complement present in excess was then separated from renatured DNA by centrifugation through high salt neutral sucrose gradients (Figure 16). Intact, single stranded DNA, sedimenting at 70S was recovered and used for hybridization.

2. Prevention of adsorption of ^{3}H-DNA during hybridization. During the characterization of the

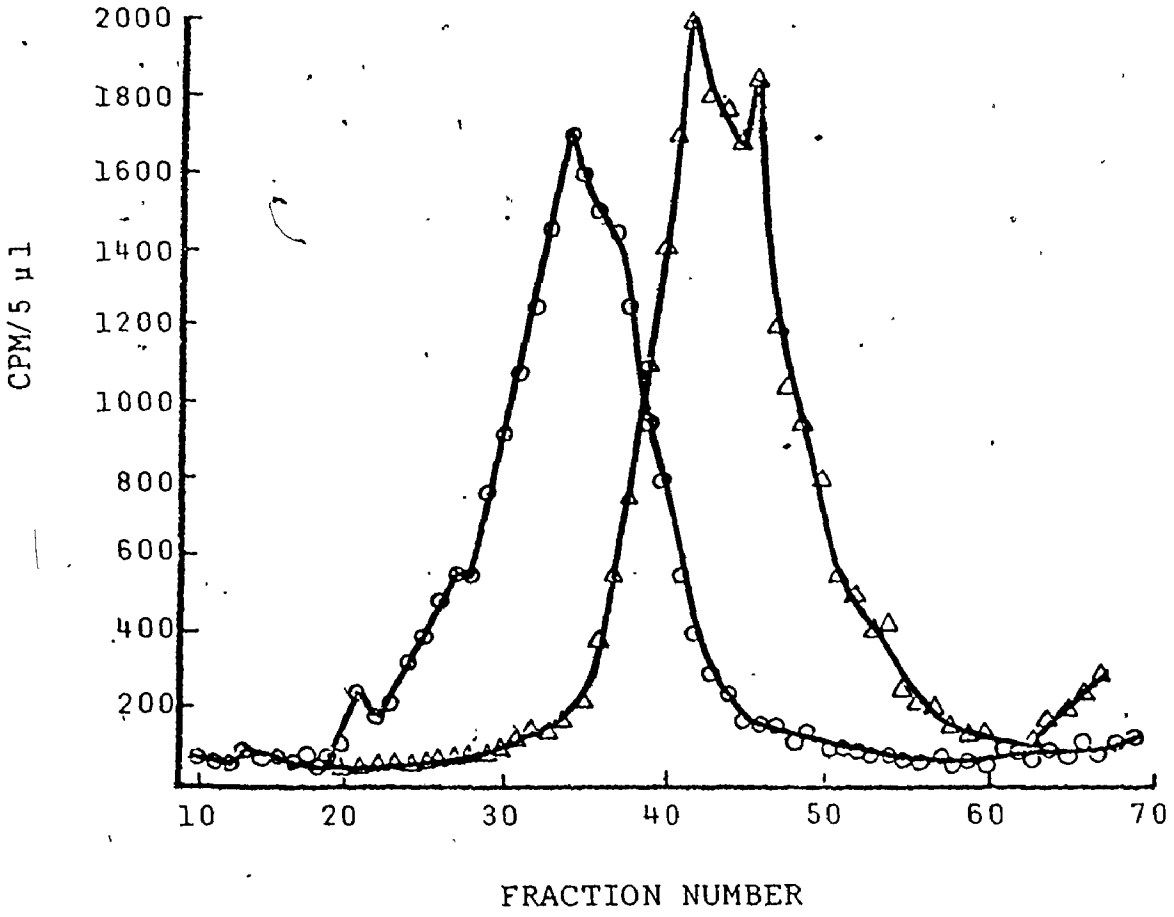


Figure 15: Rebanding profile of partially purified *l* and *r* strands

Material pooled from a poly (U,G) cesium chloride density gradient was dialyzed into 0.01 M Tris pH 8.1, denatured in alkali, neutralized, and rebanded in the presence of fresh poly (U,G) as described in Materials and Methods. o, *l* strand DNA; Δ, *r* strand DNA.

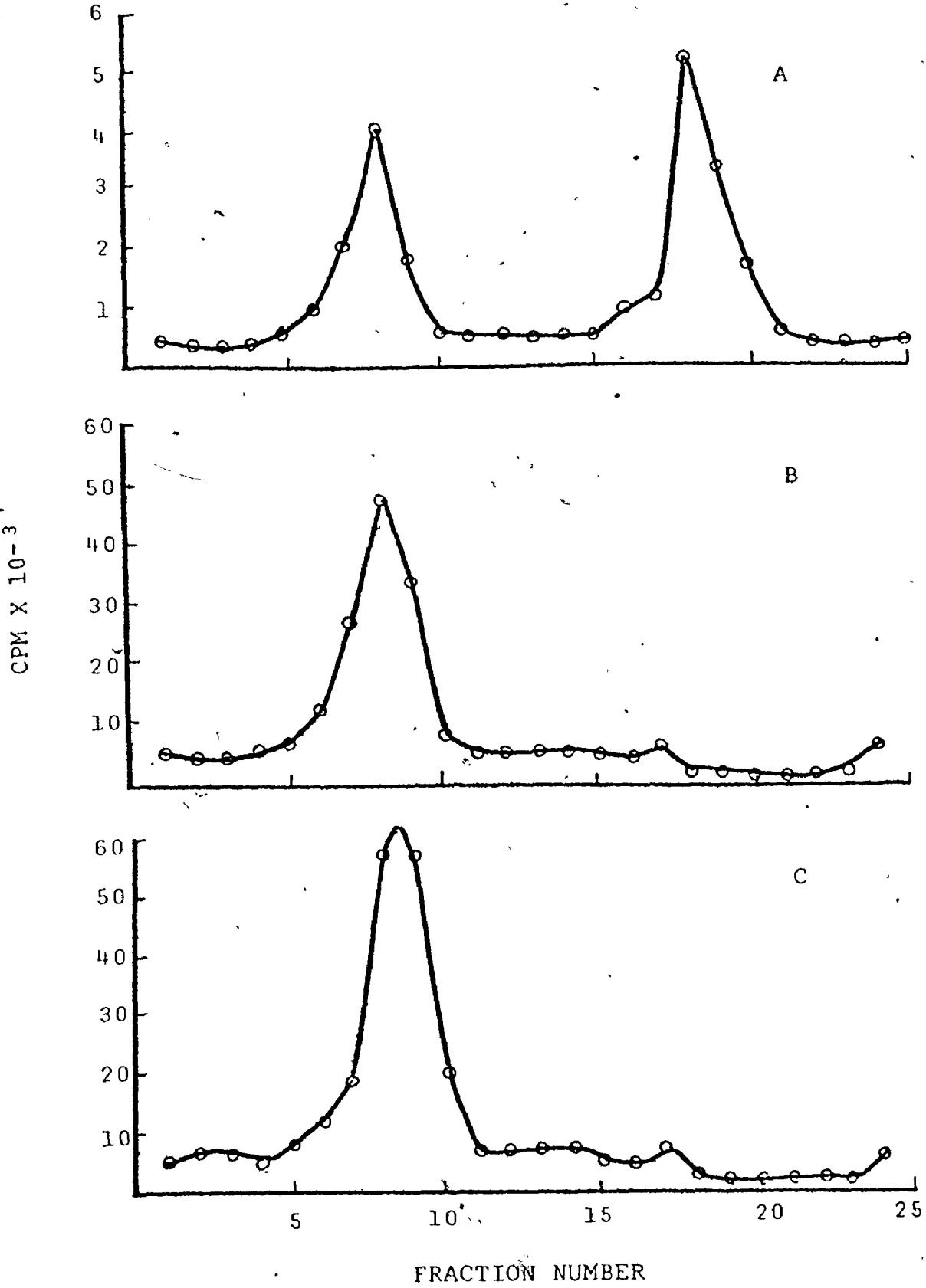
Figure 16: Sedimentation of exhaustively self-annealed *l* and *r* strand DNA in high salt neutral sucrose gradients

Partially purified *l* and *r* DNA was exhaustively self-annealed at 37°C as described in Materials and Methods. This DNA was then sedimented at 38,000 rpm for 100 minutes through 11 ml high salt 5-20% neutral sucrose gradients at 20°C. Fractions were collected, and aliquots were assayed for radioactivity.

A. an equal mixture of native and alkali-denatured intact, ^{3}H-Ad 12 DNA;

B. *l* strand DNA;

C. *r* strand DNA.


A
B
C
CPM X 10-3
FRACTION NUMBER
6
5
4
3
2
1
60
50
40
30
20
10
5
10
15
20
25

separated DNA strands it was found that ^{3}H-DNA was lost during RNA-DNA hybridization, and that this loss was not observed in samples containing large quantities of RNA. It seemed possible that RNA prevented the loss by competing with the DNA for sites on the surface of the hybridization tube. Therefore, an attempt was made to prevent such loss by adding unlabelled yeast RNA to the solution. ^{3}H-labelled *r* strand DNA was incubated alone or with 1.25 mg/ml yeast RNA for 48 hours under the standard hybridization conditions. Samples were removed and counted directly in Aquasol (New England Nuclear) (Table 4). The loss of ^{3}H-DNA from solution in the absence of RNA was both severe (60 to 80%) and variable, whereas virtually no loss occurred in the presence of RNA. Consequently, in all subsequent incubations, the RNA concentration of each sample was made identical, and at least 1.25 mg/ml by the addition of yeast RNA.

Such loss of ^{3}H-DNA was not observed in previous experiments employing fragmented DNA. This is presumably due to the large size differences in the DNA used. The use of intact DNA may increase the loss of material per adsorption event.

Table 4

Prevention of Adsorption of Intact, Denatured ^{3}H DNA to Glassware

Sample	Incubation time (hr)		
	0	24	48
lacking RNA	2528,2599	510,1609	1116,444
containing 1.25 mg/ml yeast RNA	2152,2279	2133,2031	2099,2008

0.001 µg/ml ^{3}H-r strand was incubated with or without 1.25 mg/ml yeast RNA in 0.4 ml of 3 M NaCl, 0.01 M Tris pH 7.3 at 66°C in siliconized glass test tubes for the indicated times. The radioactivity remaining in solution was determined by direct counting of the solution in Aquasol (New England Nuclear).

3. Purity of *l* and *r* DNA. The purity of the complementary DNA strand preparations was assayed by incubating the strands separately and together under annealing conditions (Table 5). The purity of the strands varied from preparation to preparation. With one preparation, no renaturation of the DNA occurred after 100 hours incubation at a concentration of 0.1 μg/ml. When equimolar amounts of both strands were incubated, almost complete renaturation was observed. With the other preparation described in Table 5, almost 25% of the label of each strand preparation became resistant to S1 nuclease after incubation, while an equimolar mixture of the strands became 84% resistant. Since denatured intact strands were 5% resistant to the nuclease, about 20% of the mass of the latter *l* and *r* preparations became duplex during self-annealing, indicating that each strand preparation was cross-contaminated with the other complement by 10%. Both of these lots were prepared by the rebanding-alkaline sucrose method. Strands prepared by the renaturation-neutral sucrose method consistently renatured to give about 5% S1 resistance for the *l* strand and 10% for the *r* strand (including zero time resistance).

Both purification methods select for intact DNA strands. Consequently the material remaining

Table 5

Purity of *l* and *r* Strands

Incubation time (hr)	Fraction of radioactivity resistant to S1 nuclease					
	lot 1			lot 2		
	l	*r*	*l* + *r*	*l*	*r*	*l* + *r*
0 (denatured)	.075	.050	-	.056	.056	-
4	.247	.318	-	-	-	-
9	.256	.286	-	-	-	-
24	.244	.306	-	-	-	-
100	.275	.257	.840	.058	.065	.842

Partially purified ^{3}H-*l* and *r* strands were incubated at 66°C in 0.3 M NaCl, 0.01 M Tris for the indicated times, then assayed for secondary structure, with S1 nuclease. Data was not adjusted. Lot 1 strands were incubated at an unknown DNA concentration; Lot 2 strands were incubated at 0.1 µg/ml.

single stranded after exhaustive self renaturation should be derived from only the complement initially present in excess. To test this prediction, 1.2 µg/ml of ^{14}C *l* or *r* strand DNA, prepared by the renaturation-neutral sucrose method, was hybridized to 0.015 µg/ml ^{3}H-labelled *l* and *r* strand DNA after prior self-annealing (Table 6). The result clearly demonstrated that only the strand initially present in excess remained available for hybridization. Self-annealed complementary strand DNA preparations were therefore suitable for use in RNA-DNA hybridization experiments.

B. Hybridization of infected KB cell RNA to the complementary strands

1. Whole cell RNA. Three separate preparations of early and late whole cell RNA were hybridized to the complementary DNA strands. RNA extracted at both 24 and 36 hours post infection hybridized to about 75 to 80% of the *r* strand and 10 to 15% of the *l* strand of the viral genome (Figure 17). Provided that the RNA sequences detected were complementary to assymetrically distributed DNA sequences, late RNA hybridizes to 85 to 95% of one DNA strand equivalent. Hybridization to the *r* strand was

Table 6

Only the Complement in Excess Remains Single Stranded After Self Annealing

^{3}H-DNA	^{14}C-DNA	Fraction ^{3}H-DNA resistant to S1 nuclease
l	-	.075
l	*l*	.117, .133
l	*r*	.945, 1.058
r	-	.130
r	*l*	.945, .941
r	*r*	.130

^{3}H-DNA (0.015 µg/ml) and ^{14}C-DNA (1.2 µg/ml), both previously exhaustively self annealed, were mixed and hybridized for 20 hours at 67°C. Secondary structure was assayed by S1 nuclease. Data were not adjusted.

Figure 17: Hybridization of late whole cell RNA to the complementary DNA strands

0.01 µg/ml of *l* and *r* strand DNA were hybridized to the indicated amounts of unlabelled, late whole cell RNA. Hybrids were assayed by S1 nuclease.

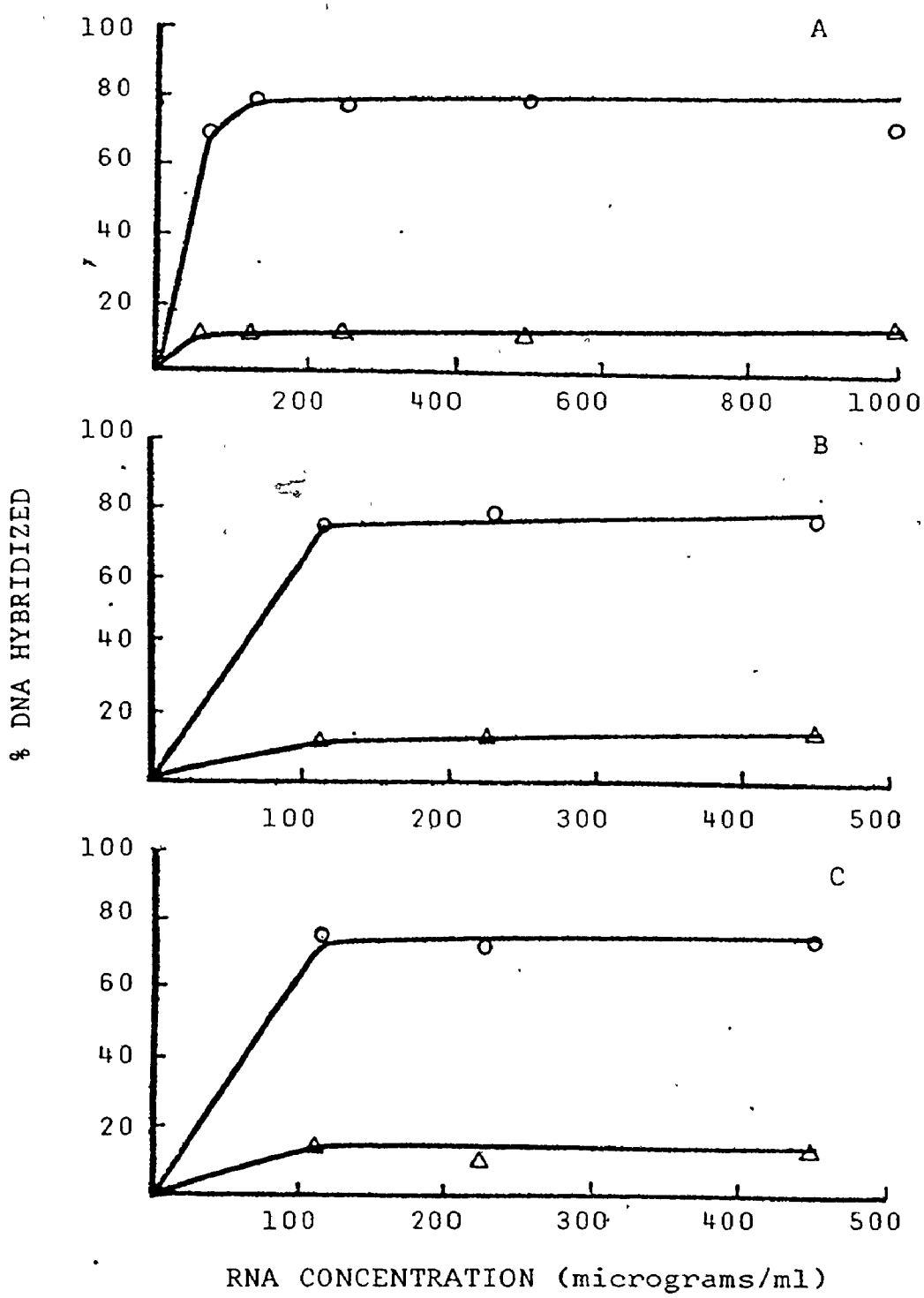
A. lot 1 RNA (36 hours post infection) hybridized for 24 hours;

B. lot 2 RNA (24 hours post infection) hybridized for 72 hours;

C. lot 3 RNA (36 hours post infection) hybridized for 72 hours;

o *r* strand hybrids;

Δ *l* strand hybrids.


A
B
C
100
80
60
40
20
200
400
600
800
1000
100
200
300
400
500
% DNA HYBRIDIZED
RNA CONCENTRATION (micrograms/ml)

abolished by previously treating 100 µg aliquots of the RNA with 0.5 N NaOH at 37°C for 24 hours, demonstrating that the RNA preparations used were free of contaminating viral DNA. The observed saturation levels were close to, but somewhat higher than those obtained using total, fragmented DNA in section I.B.2. above. The discrepancy may be related to the large size difference between the DNA probes used: if hydrolysis of duplex nucleic acids by S1 nuclease proceeds in part by "fraying" of the ends of duplex regions then the use of small fragments would underestimate the true fraction hybridized to a greater extent than the use of large DNA fragments. The complementary strands used were not deliberately fragmented.

RNA extracted early (7.5 hours post infection) hybridized up to 50% of the *r* strand and 15% of the *l* strand (Figure 18). The sequence relationships of the early and late whole cell RNA species were examined by mixing experiments (Table 7). A mixture of early and late RNA hybridized no more DNA than late RNA alone, suggesting either that the sequences found in early whole cell RNA are a subset of those found late, or that late RNA interferes with the detection of any early RNA sequences which are not

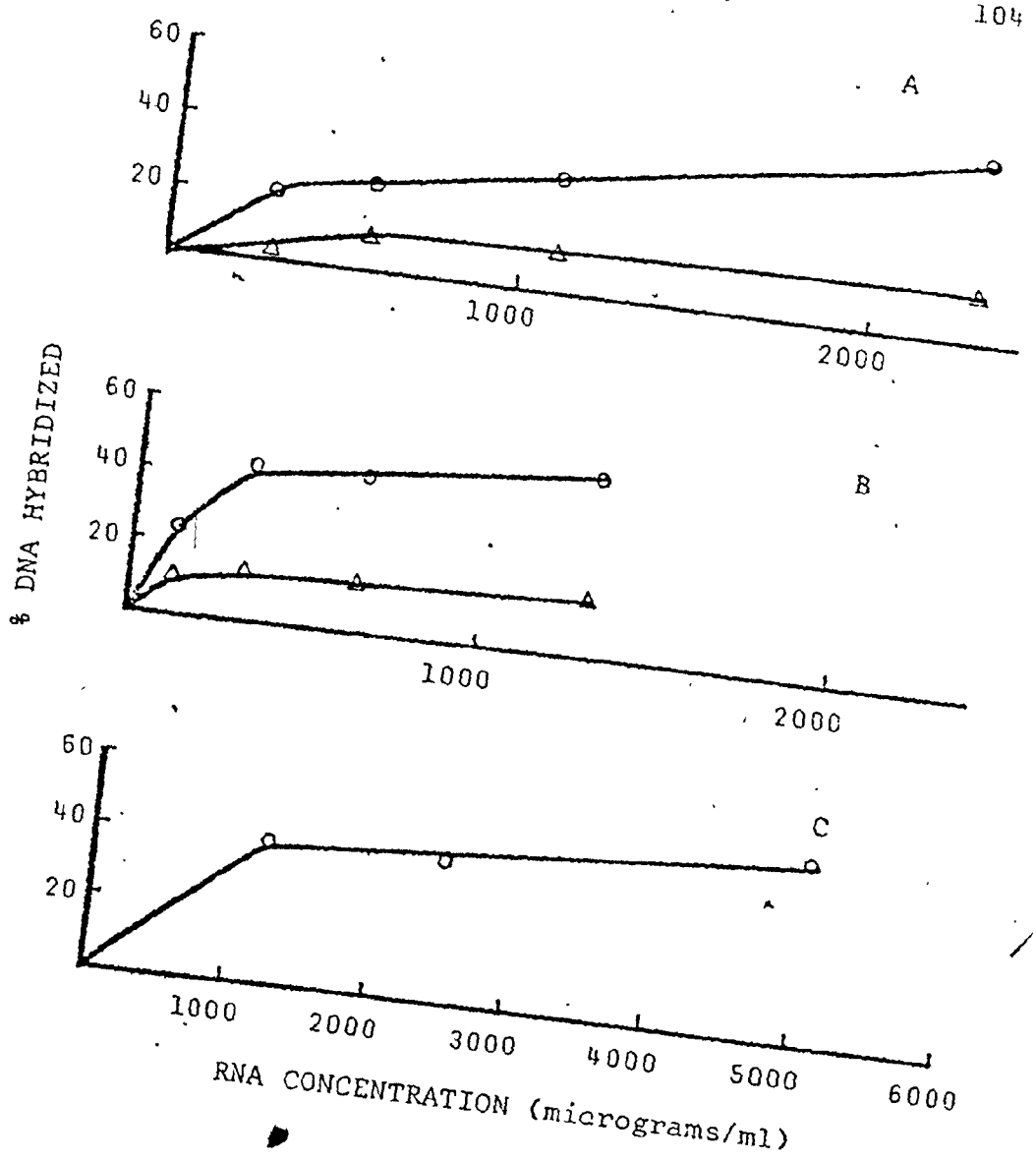


Figure 18: Hybridization of early whole cell RNA to the complementary DNA strands

0.01 µg/ml of *l* and *r* strand DNA was hybridized to the indicated amounts of early whole cell RNA (7.5 hours post infection) for 72 hours. Hybrids were assayed by S1 nuclease. o, *r* strand hybrids; Δ, *l* strand hybrids. Panels A,B,C show the results obtained with three different lots of RNA.

Table 7

Sequence Relationship of Early and Late Whole Cell RNA

RNA	Hybridization time (hr)	Fraction of DNA hybridized	
		r	*l*
late, 42.5 µg/ml	48	0.736	0.113
early, 662.5 µg/ml	72	0.463	0.183
late, 42.5 µg/ml + early, 662.5 µg/ml	72	0.715	0.124
KB cell, 2250 µg/ml	72	0.000	0.000

The indicated amounts of RNA were hybridized to 0.01 µg/ml ^{3}H-*l* and *r* strand DNA for the indicated times. Late RNA was extracted 24 hours post infection, early RNA was extracted 7.5 hours post infection. Hybridization was assayed by S1 nuclease digestion.

present late. The apparent contradiction between these results and those presented in sections I.A.3. and I.B.3. above, is dealt with in the Discussion.

2. Late nuclear and cytoplasmic RNA. Adenovirus DNA is transcribed within the nucleus of infected cells, while translation of viral mRNA occurs in the cytoplasm. It was of interest to determine whether all viral RNA sequences synthesized in the nucleus were detectable in the cytoplasm. At 24 hours post infection, cells were harvested and nuclear and cytoplasmic fractions were prepared by NP40-induced cell lysis. The RNA extracted from these fractions was hybridized to the complementary DNA strands (Figure 19). The nuclear RNA hybridized to about 90% of the *r* strand and 35% of the *l* strand, while the cytoplasmic RNA of the same cells hybridized to 65% of the *r* strand and 15% of the *l* strand. These data demonstrated that nuclei contain RNA sequences which are not detectable in the cytoplasm.

Since at least 125% of one DNA strand equivalent is transcribed in the infected cell late post infection, at least 25% of the viral genome must be transcribed from both DNA strands. It should be possible to isolate duplex RNA complementary to at least 25% of the genome from self-annealed late

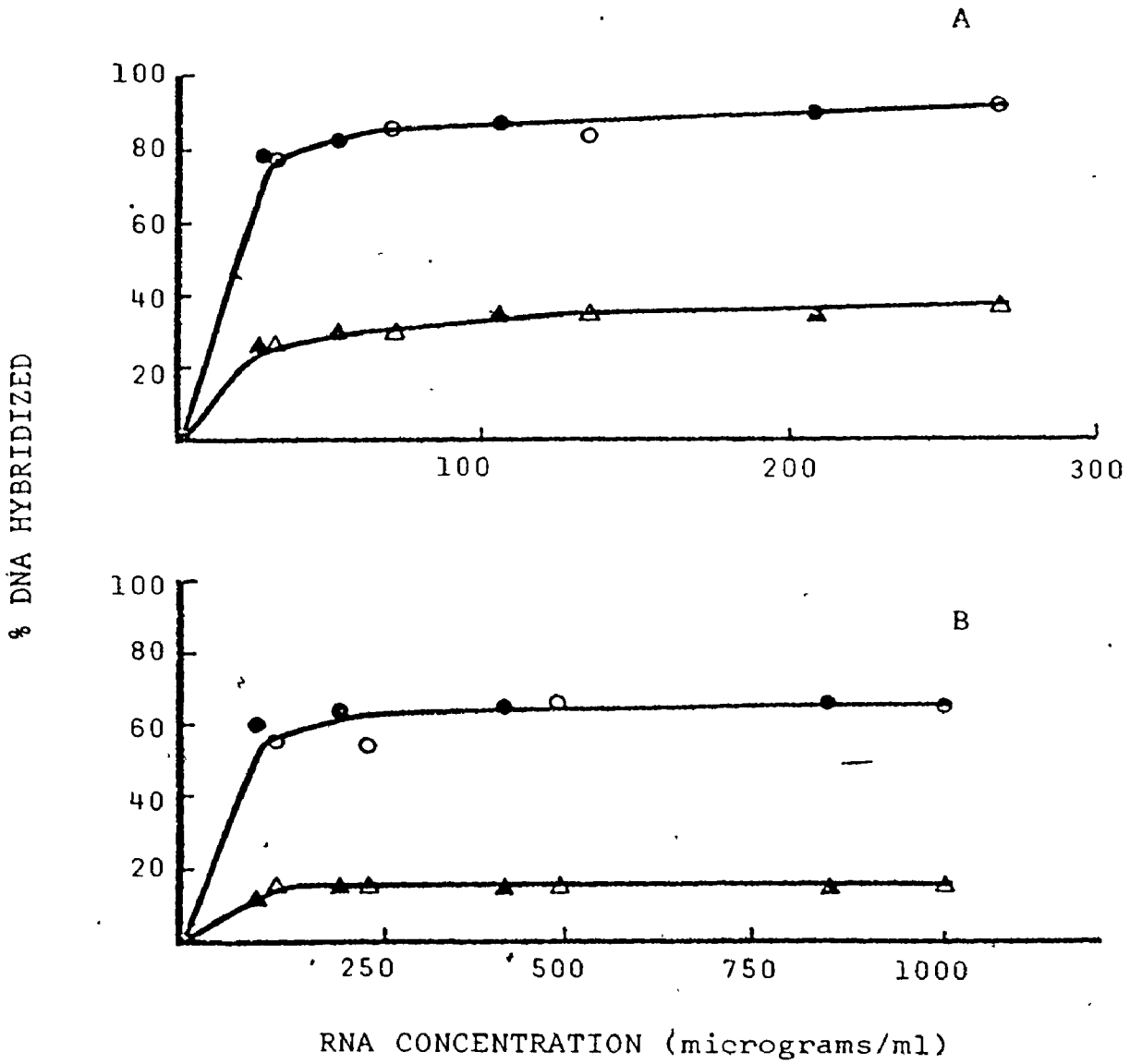


Figure 19: Hybridization of late nuclear and cytoplasmic RNA to the complementary DNA strands

0.01 µg/ml of *l* and *r* strand DNA was hybridized to the indicated amounts of late (24 hours post infection) nuclear and cytoplasmic RNA for 48 hours. Hybrids were assayed by S1 nuclease.

A. nuclear RNA;

B. cytoplasmic RNA;

o *r* strand hybrids;

Δ *l* strand hybrids;

open symbols, preparation 1 RNA;

closed symbols, preparation 2 RNA.

whole cell RNA. This prediction was verified experimentally (Figure 20). Ribonuclease-resistant RNA complementary to greater than 30% of both strands was isolated. The hybridization of this material to DNA was dependent on prior heat denaturation, and was totally eliminated by alkaline hydrolysis, demonstrating that the active material was double stranded and was not DNA. The hybridization reactions between the ribonuclease-resistant RNA (double stranded RNA) and the complementary DNA strands may not have reached saturation, allowing only a minimal estimate of its sequence complexity. At least 30% of the Ad 12 genome is transcribed from both DNA strands.

3. Early cytoplasmic and nuclear RNA. Nuclear and cytoplasmic RNA extracted 7.5 hours post infection was hybridized to the complementary DNA strands (Figure 21). Early cytoplasmic RNA hybridized to about 17% of the *r* strand and 14% of the *l* strand, accounting for about 30% of the assymetric coding capacity of the viral genome. Saturation was difficult to obtain with early nuclear RNA, presumably because of the small quantities of virus-specific RNA. Since early whole cell RNA consistently hybridized to greater than 40% of the *r* strand, while cytoplasmic

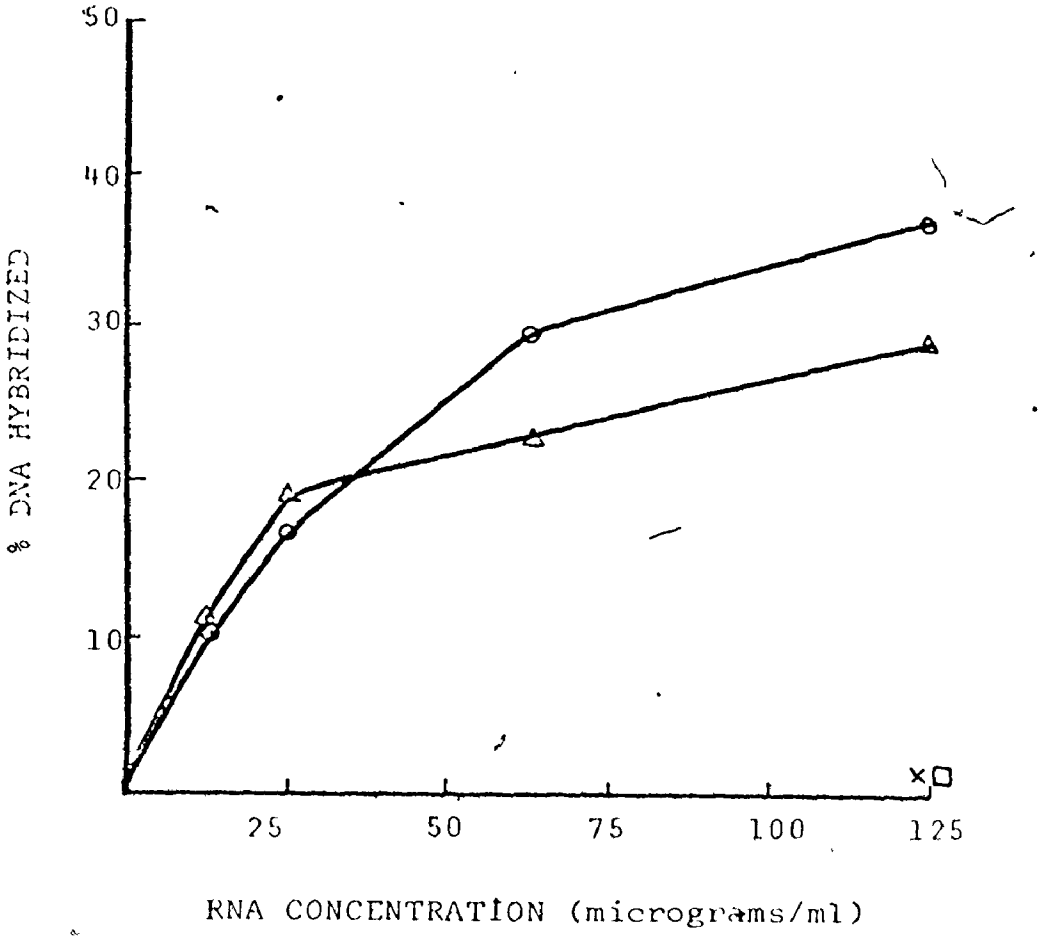


Figure 20: Hybridization of ribonuclease resistant RNA isolated from late whole cell RNA to the DNA strands

Whole cell RNA (extracted 24 hours post infection) was self annealed in 2 x SSC at 67°C for 24 hours, incubated with 40 µg/ml pancreatic ribonuclease at 37°C for 1 hour in 2 x SSC, then phenol extracted and ethanol precipitated. This RNA was then incubated with 0.01 µg/ml *l* (Δ) and *r* (o) DNA after heat denaturation for 48 hours. In addition, two aliquots of 50 µg of RNA, one not heat denatured (□) and one subjected to 24 hours alkali hybrolysis (0.5 N, 37°C) (X) were also incubated with *r* strand DNA. Hybrids were assayed by S1 nuclease.

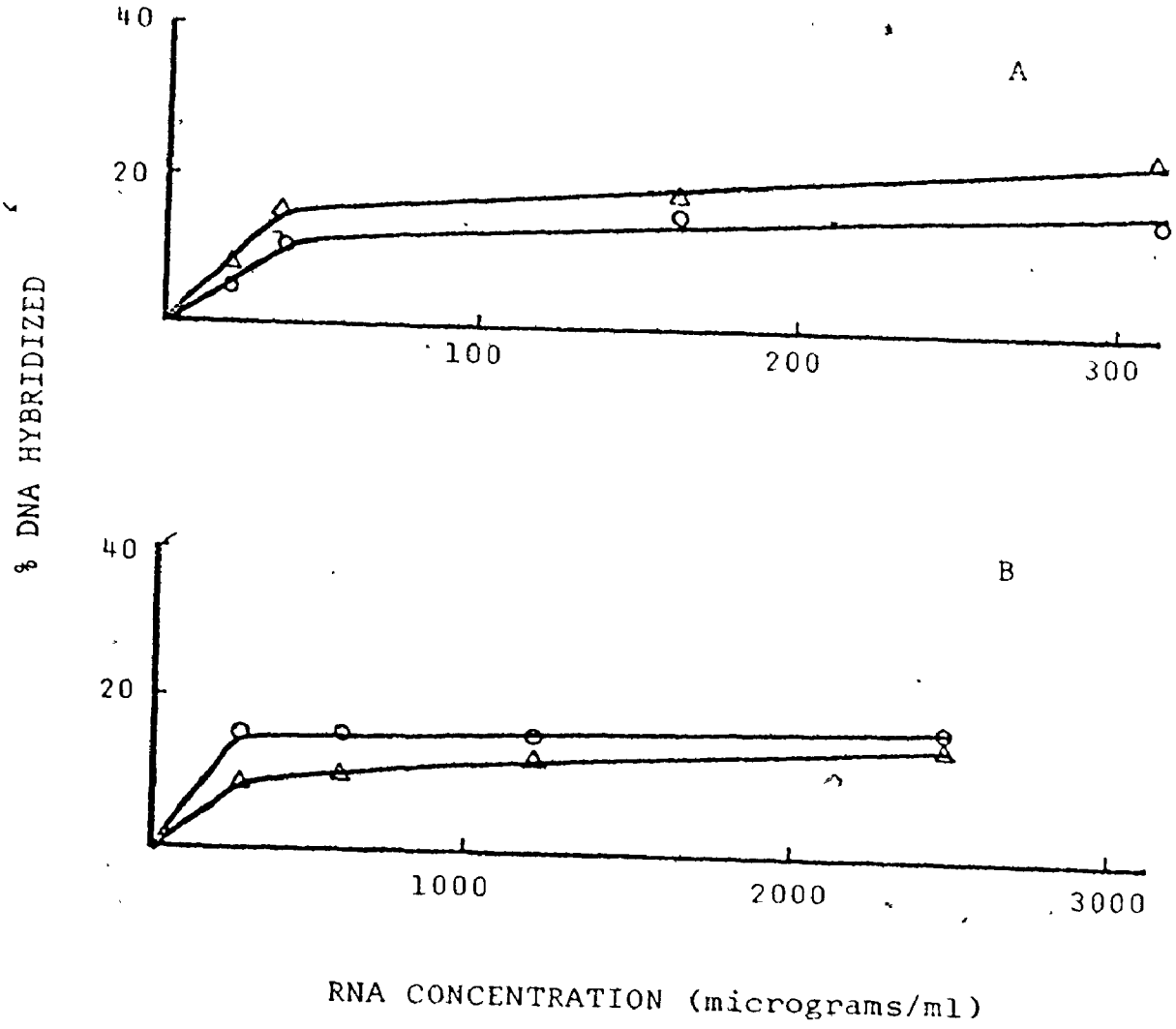


Figure 21: Hybridization of early nuclear and cytoplasmic RNA to the complementary DNA strands

0.01 µg/ml of *l* and *r* strand DNA was hybridized to the indicated amounts of early (7.5 hours post infection) nuclear and cytoplasmic RNA for 48 hours. Hybrids were assayed by S1 nuclease.

A. nuclear RNA;

B. cytoplasmic RNA;

o *r* strand hybrids;

Δ *l* strand hybrids.

RNA hybridized to about 20%, RNA derived from at least 20% of the *r* strand must be confined to the nucleus. In the experiment shown in Figure 21, early nuclear RNA hybridized up to 24% of the *l* strand, while the cytoplasmic RNA hybridized to less than 15%. This may indicate that some *l* strand sequences are confined to the nucleus. Early whole cell RNA consistently hybridized 15% of the *l* strand, the same level as early cytoplasmic RNA, suggesting that any nuclear-specific *l* strand RNA is not detectable in whole cell RNA and is perhaps complementary to a more abundant cytoplasmic RNA. Because of the tremendous labor involved in obtaining even sub-saturating quantities of early nuclear RNA, the experiments with nuclear RNA were not repeated, and therefore the above suggestions must remain speculative.

It was clear that early cytoplasmic RNA contains fewer viral sequences than does late cytoplasmic RNA. The degree of sequence overlap between these two RNA populations was examined by mixing experiments (Table 8). A mixture hybridized the same fraction of each strand as did late RNA alone, suggesting that all early cytoplasmic sequences are present in late cytoplasmic RNA.

Table 8

Sequence Relationship of Early and Late Cytoplasmic RNA

RNA	Fraction DNA hybridized	
	r	*l*
Late (500 µg/ml)	.663	.149
Early (2500 µg/ml)	.174	.131
Early (2500 µg/ml) + late (500 µg/ml)	.641	.152

The indicated amounts of RNA were hybridized to 0.01 µg/ml ^{3}H-*l*, and *r* strand DNA for 48 hours at 66°C. Hybridization was assayed by S1 nuclease digestion.

4. Ad 12 strain 1131. All of the data presented above were obtained using the Huie strain of Ad 12. The substrain of Ad 12 Huie used in this laboratory contained an appreciable quantity of defective particles which gave rise to anomalous DNA fragments when cleaved with restriction endonucleases (see section III.3., below). In addition, the DNA of plaque-purified virus of this strain has an unusual feature at the right end (see below). These features could render mapping experiments with DNA fragments ambiguous. Another independent isolate of Ad 12, the 1131 strain (Pereira and MacCallum, 1964) was available. This strain did not have these undesired properties. As shown below, the DNA of the 1131 is similar, but not identical to the DNA of Ad 12 Huie. It was of interest to determine whether similar fractions of each of the complementary DNA strands of the two strains were expressed as early and late cytoplasmic RNA. Strain 1131 early and late cytoplasmic RNA was hybridized to the complementary strands of 1131 DNA (Figure 22). Late RNA hybridized 65% of the *r* strand and 15-18% of the *l* strand and early RNA hybridized 17% of the *r* strand and 15% of the *l* strand, values similar to those observed with Ad 12 Huie. In addition, 500 µg/ml of Huie late cytoplasmic RNA

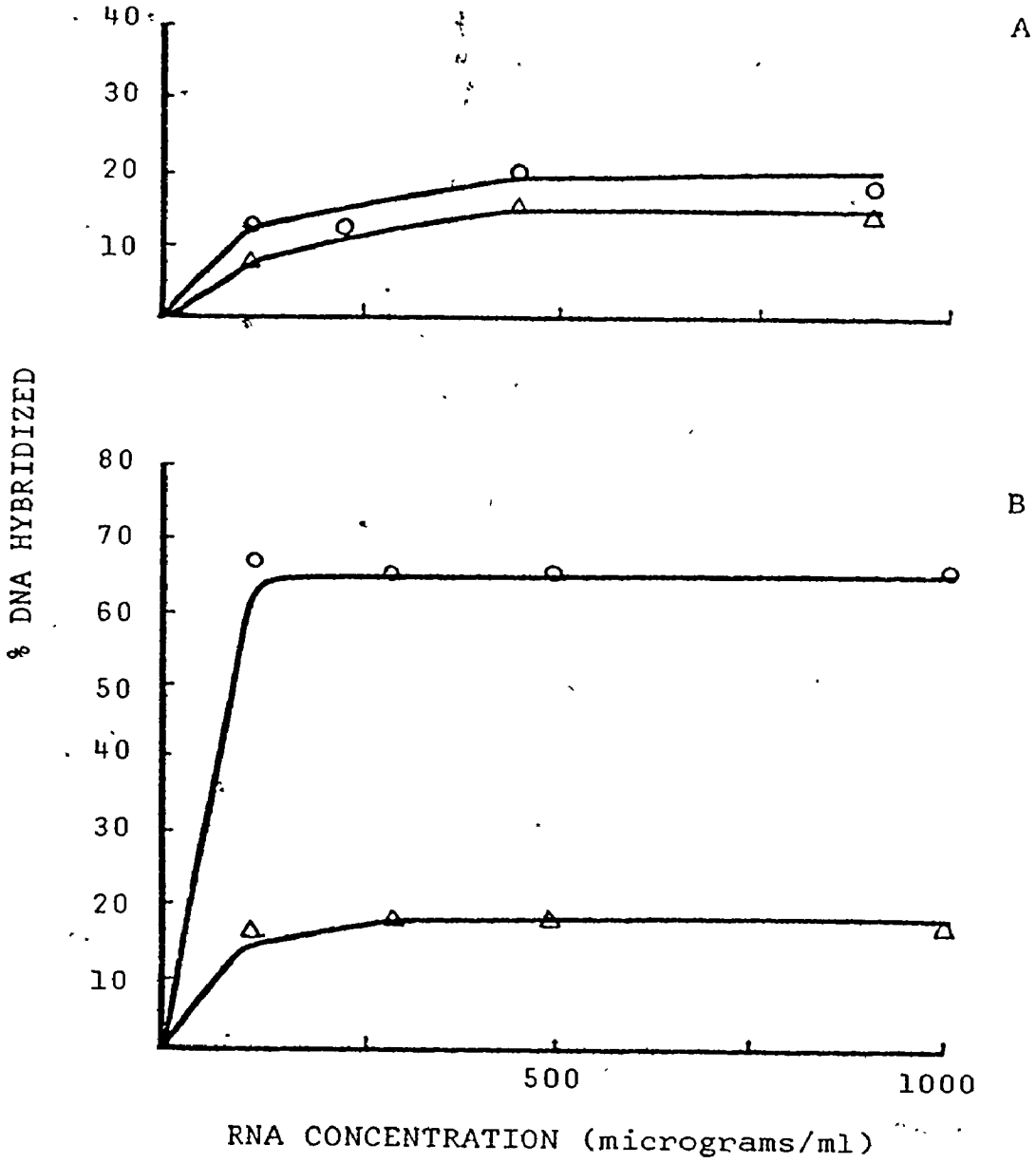


Figure 22: Hybridization of 1131 strain early and late cytoplasmic RNA to the 1131 complementary DNA strands

0.01 µg *l* and *r* strand Ad 12 1131 DNA was incubated with the indicated quantities of unlabelled early (7.5 hours) and late (24 hours) cytoplasmic RNA for the indicated times. Hybrids were assayed by S1 nuclease.

A. early RNA;

B. late RNA;

o *r* strand hybrids;

(lot used in Figure 19B) was hybridized to the 1131 DNA strands for 48 hours. This RNA hybridized to 60% of the 1131 *r* strand and 16% of the 1131 *l* strand, indicating a high degree of homology between the DNA of the two virus strains.

III. Restriction Endonuclease Cleavage of Ad 12 DNA

Bacterial restriction endonucleases which cleave DNA at a limited number of sites have proven to be valuable aids for physically mapping the coding locations of RNA on viral genomes. In order to use fragments of Ad 12 DNA for this purpose it was necessary to characterize the fragment sets produced by cleavage with several endonucleases.

1. Viral strains used. Restriction endonuclease cleavage of the DNA of two separate isolates of Ad 12 were examined. The two strains used were Huie (obtained from Dr. M. Green, St. Louis Mo.) and 1131 (Pereira and McCallum, 1964). The stocks of Huie used had been passaged in mass culture in KB cells, without plaque purification, for at least ten years. In 1976, several plaque purified stocks of Huie were initiated by Dr. S. Mak. One of these plaques (# 8)

was selected for study. It was found that both the mass cultured and plaque purified Huie differed from the stocks of Huie used by C. Mulder (University of Massachusetts) in his studies of restriction endonuclease fragmentation of Ad 12 DNA (personal communication, see below). Mulder's stock of Huie was originally obtained from A.J. van der Eb, University of Leiden. It is therefore necessary to distinguish between three strains of Ad 12: Huie of van der Eb (Huie V), Huie of Mak (Huie M) and 1131. In the following sections, Huie M refers to mass passaged virus, while Huie M8 refers to plaque purified Huie M.

2. Nomenclature of DNA fragments. Following convention, DNA fragments produced by digestion with one restriction endonuclease were designated by capital Roman letters in order of their increasing electrophoretic mobility. For example, Eco RI A refers to the slowest migrating (largest) fragment produced by Eco RI. For certain purposes it was desirable to examine fragments produced by a double digestion of DNA with two restriction endonucleases. Two conventions were used for designating these fragments. Distinguishable bands observed after electrophoresis were indicated by Roman numerals in order of increasing mobility. For example Eco RI + Bam Hl I refers to the largest size

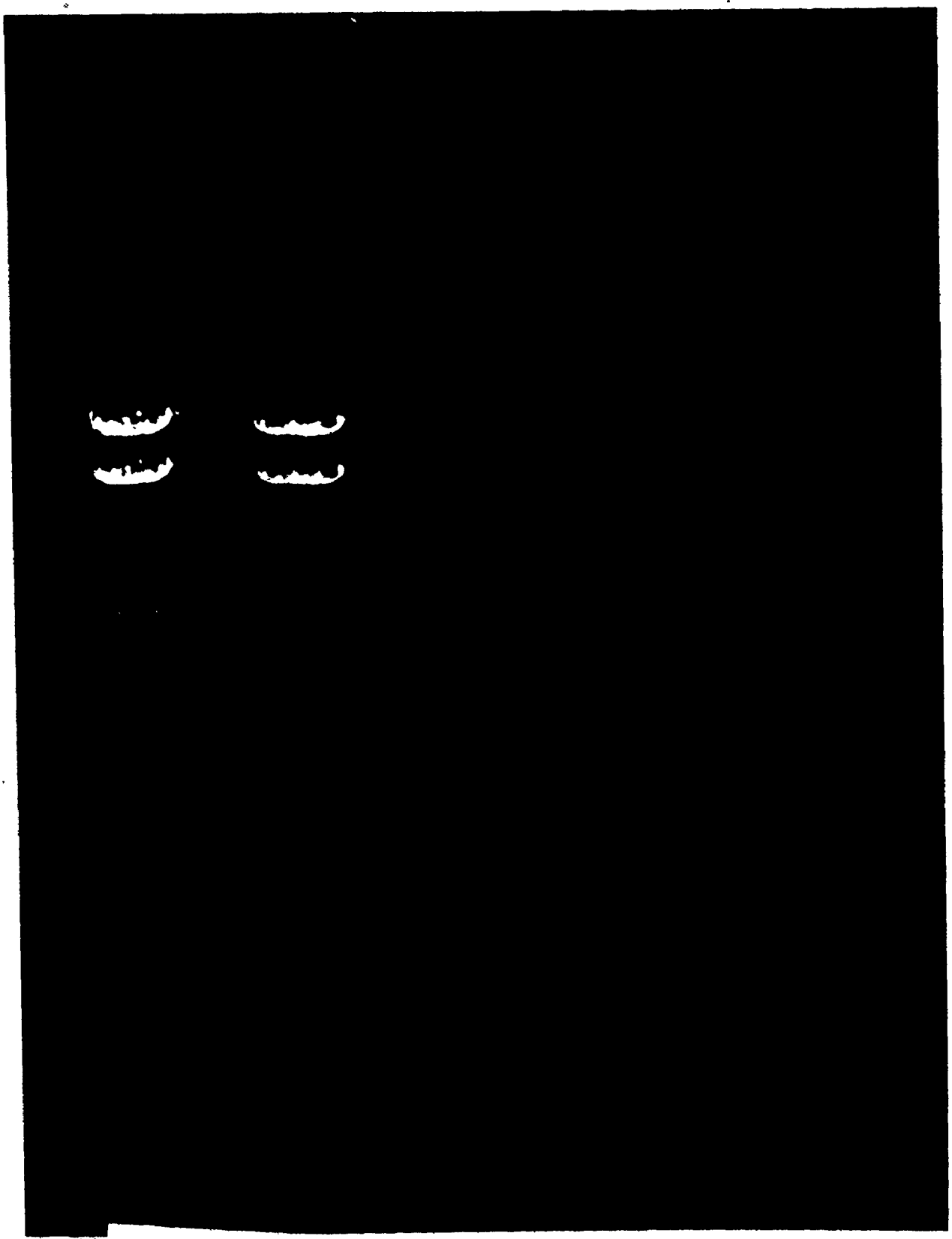
class of DNA produced by combined cleavage with Eco RI and Bam Hl. When the origin of fragments was clear, another designation was used. Fragments identical to those produced by single digestion were referred to as such, while new fragments generated by internal cleavage of fragments by the other enzyme have a composite designation. For example, the Bam Hl C fragment of Ad 12 is cleaved twice by Eco RI, generating three fragments: Eco RI F, and two fragments not found in either single digest, designated Bam Hl C/ Eco RI-α and Bam Hl C/Eco RI-β in order of decreasing molecular weight (see Figure 28). The same fragment can have two alternative designations: Bam Hl C/ Eco RI-α is equivalent to Eco RI A/Bam Hl-α.

3. Eco RI and Bam Hl cleavage of Ad 12 DNA. Mulder *et al.* (1974) reported that endonuclease Eco RI cleaves Ad 12 (Huie V) DNA to produce six fragments. Delius and Mulder (personal communication) determined the order of these fragments to be CDBEFA. The map is oriented so that the GC rich end of the Ad 12 genome is placed to the left, following convention (Doerfler and Kleinschmidt, 1970).

Figure 23 shows the electrophoretic separation of the fragments produced by Eco RI digestion of

Figure 23: Electrophoretic separation of the Ad 12 1131 and Huie M Eco RI fragments

Unlabelled Ad 12 DNA was cleaved to completion with Eco RI. Aliquots of the digestion mix were made 0.5% SDS, 5% glycerol and electrophoresed 16 hours at 30V through a 2.2% polyacrylamide - 0.7% agarose gel slab at room temperature. The gel was stained with 0.5 µg/ml ethidium bromide, and the DNA bands were visualized and photographed under ultraviolet illumination. From left to right the sample wells contained: 1131 DNA (2 µg); 1131 DNA (2 µg); 1131 DNA (0.5 µg); Huie M DNA (1 µg); and Huie M DNA (0.5 µg).



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DNA extracted from the Huie M and 1131 stocks used in the experiments described above. The 1131 digest contained fragments with electrophoretic mobilities similar to those reported by Mulder *et al.* (1974) for the Huie V Eco RI fragments. The smallest fragment (F) was run out of the gels displayed. However, the enzyme generated more than six fragments, varying in molar yield, from the Huie M DNA. This finding suggested that the Huie M stocks were heterogeneous, containing in addition to "normal" Ad 12, rearranged genomes producing fragments of altered electrophoretic mobility. The presence of extra fragments was correlated with the presence of particles banding at a slightly lower density in cesium chloride density gradients than infective virus. These particles are defective, capable of inducing T antigen synthesis but incapable of forming a plaque (Mak, 1969). The defectives contain deleted genomes lacking about 8% of the viral genome (I. Mak, H. Ezoe and S. Mak, manuscript in preparation). The defective population is heterogeneous, but contains several discrete classes of deletions and/or rearrangements which result in an altered cleavage pattern by Eco RI (I. Mak, H. Ezoe and S. Mak, manuscript in preparation).

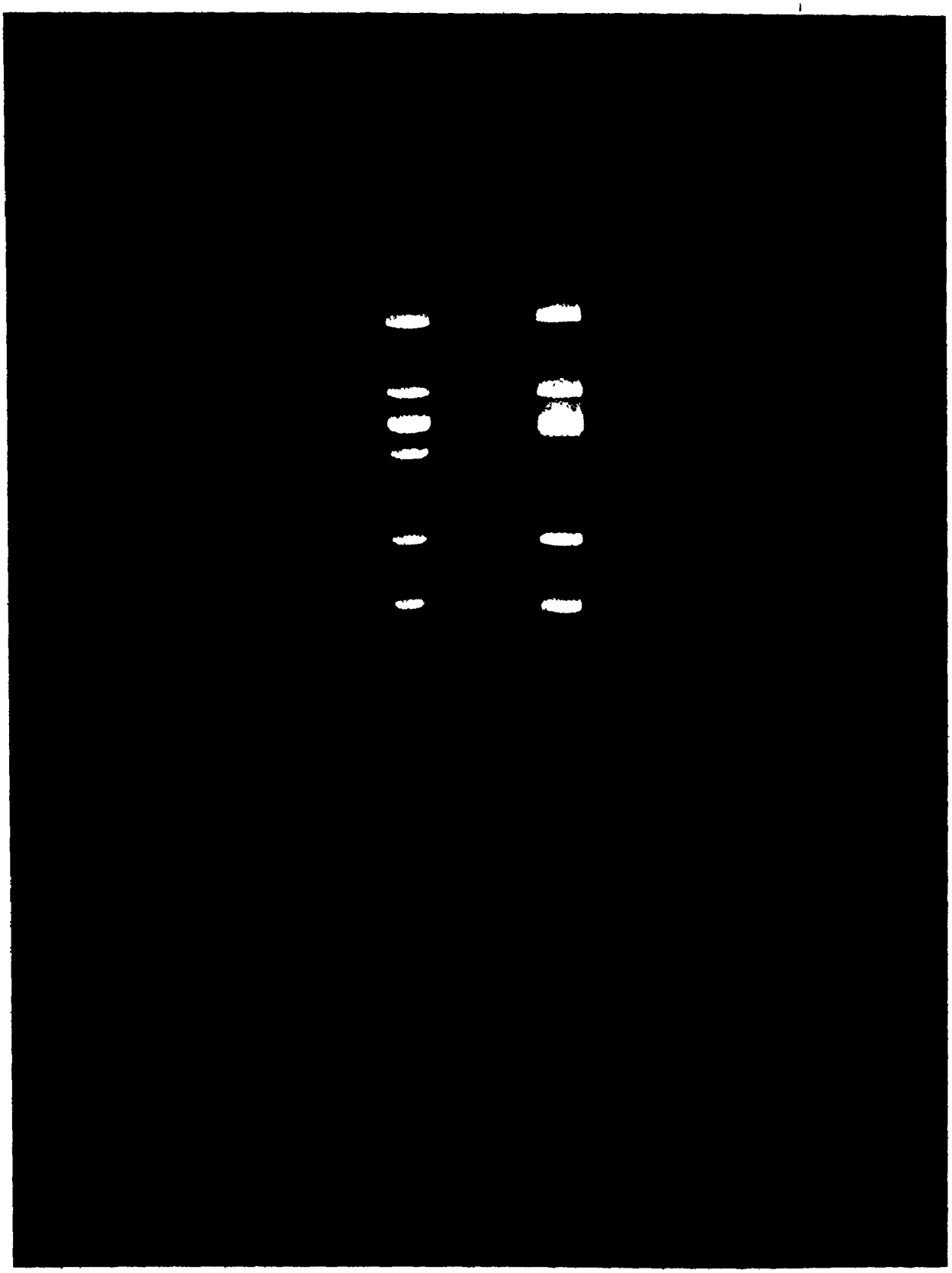
The DNA extracted from plaque purified Huie M virus (plaque 8, provided by S. Mak) produced only

six visible Eco RI fragments, identical in electrophoretic mobility to those obtained with 1131 DNA (not shown). This observation supported the suggestion that the altered fragments present in digests of the Huie M DNA used in Figure 23 originated from defective particles in the population.

Delius and Mulder (personal communication) have found that endonuclease Bam Hl produces nine fragments of eight discrete size classes from Huie V DNA (fragments C and D are of identical molecular weight). Figure 24 shows the electrophoretic separation in 1% agarose gels of the Bam Hl fragments of 1131 and Huie M8 DNA. The cleavage pattern observed with 1131 DNA is apparently identical to that observed by Delius and Mulder with Huie V DNA (personal communication). However, only seven size classes were produced with Huie M8 DNA, which lacked a fragment corresponding to 1131 Bam Hl E. The other fragments produced were identical in mobility to the 1131 fragments. The DNA extracted from mass cultured Huie M also lacked a fragment comigrating with 1131 Bam Hl E. Delius and Mulder found the order of the Huie V Bam Hl fragments to be AG(D,H,I)FCBE, where the relative order of fragments D,H and I remains unknown. Since Huie M8 DNA lacked the E fragment present in both

Figure 24: Electrophoretic separation of the Bam H1 fragments of Ad 12 1131 and Huie M8 DNA

2 µg of Ad 12 DNA fragments were electrophoresed for 11 hours at 30V in 1% agarose gels at room temperature. The gel on the left contained 1131 DNA, while that on the right contained Huie M8 DNA.



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1131 and Huie V DNA, it was of interest to examine the right end Eco RI A fragment of both Ad 12 strains. This fragment contains the segment of the Huie V and 1131 genomes which gives rise to Bam Hl E.

Figure 25 shows that the purified Eco RI A fragment of Huie M DNA gave rise to only two of the expected fragments when cleaved with Bam Hl, corresponding in size to Bam Hl B and Bam Hl C/Eco RI α, In addition, at least 5 additional bands, present in sub-equimolar quantities, were detected, with molecular weights of about 31,25,14,13, and 12% of Ad 12. Purified 1131 Eco RI A fragment gave rise to the three expected fragments (Bam Hl B, Bam Hl E and Bam Hl C/Eco RI α) when cleaved with Bam Hl. Several of the sub-equimolar bands observed in the Bam Hl digest of Huie M Eco RI A were also identifiable in Bam Hl digests of total Ad 12 DNA: the 31 and 25% fragments were clearly visible in both the 1131 and M8 digests. These fragments correspond in size to those predicted to result from incomplete digestion of Eco RI A by Bam Hl. The 14,13 and 12% fragments cannot be accounted for by incomplete digestion, and demonstrate that the Huie M Eco RI A fragment is heterogeneous. These three fragments would be difficult to identify in digests of total Huie M8 DNA

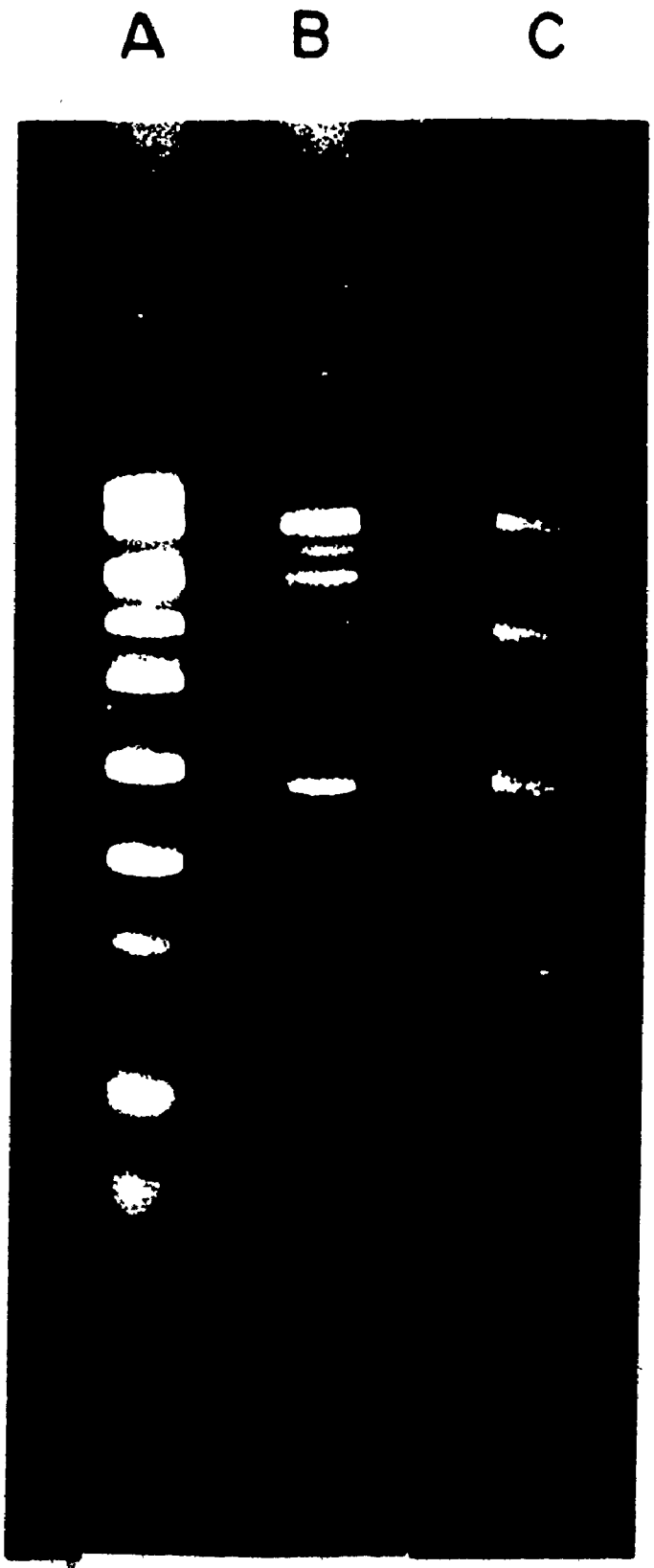
Figure 25: Electrophoretic separation of the Bam H1 cleavage products of the 1131 and Huie M Eco RI A fragments

Approximately 0.5 µg of 1131 and Huie M Eco RI A fragment DNA, purified by the sodium perchlorate-hydroxyapatite method was cleaved with Bam H1, and electrophoresed as in Figure 24. For purposes of comparison, 2 µg of 1131 DNA cleaved to completion with a mixture of Eco RI and Bam H1 was also run.

A. 1131 DNA cleaved with Eco RI + Bam H1;

B. Huie M Eco RI A DNA cleaved with Bam H1;

c. 1131 Eco RI A DNA cleaved with Bam H1.



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because they migrate close to the broad Bam Hl C/D doublet band. Close inspection of Figure 24 reveals that Huie M8 Bam Hl digests contain sub-equimolar fragments migrating slightly faster and slower than the C/D doublet, perhaps corresponding to two of those fragments seen in Figure 25.

These results suggest that Huie M DNA has a heterogeneous right end. The simplest interpretation of the data is that the vast majority of the molecules have unaltered Bam Hl B and Bam Hl C/Eco RI α regions, and have one of at least three distinct classes of termini, all of which are larger than the right terminal fragments of both Huie V and 1131 DNA. According to this explanation, the Eco RI A fragments of strains Huie M and 1131 are indistinguishable because of the difficulty of resolving large DNA fragments of similar size. The three or more discrete Huie M Eco RI A fragments, differing slightly from one another in size, may comigrate.

Regardless of whether this explanation of the anomalous behaviour of Huie M DNA is correct, the fact that plaque purified Huie M8 DNA also lacks a fragment corresponding in size to 1131 and Huie V Bam Hl E demonstrates that this altered genome is viable (not defective). The purified Eco RI A

fragment of Huie M8 DNA was not directly tested for heterogeneity, and it is possible that only one of the 3 classes of ends found in mass passaged virus is found in plaque 8 DNA.

A simple model accounting for these results is suggested by the finding that the three sub-equimolar fragments form a series of size classes of fragments differing from the normal Bam Hl E fragment by 1,2 and 3% of the genome. It is tempting to propose that these fragments differ from E by bearing a duplication, triplication and quadruplication, respectively, of 1% of the viral genome. If the reiterated segments were tandemly arranged, unequal crossovers could generate the whole spectrum of ends, starting from any reiteration class.

Because of the difficulty of preparing stocks of even plaque purified Huie M lacking defectives, and the unusual behaviour of Huie M8 DNA, strain 1131 was used in most of the hybridization-mapping studies using DNA fragments generated by restriction endonucleases.

4. Restriction endonuclease cleavage maps of Ad 12 (1131) DNA. The Eco RI fragments of 1131 DNA are identical to those previously described for Ad 12 Huie V DNA (Mulder *et al.*, 1974). In addition, the

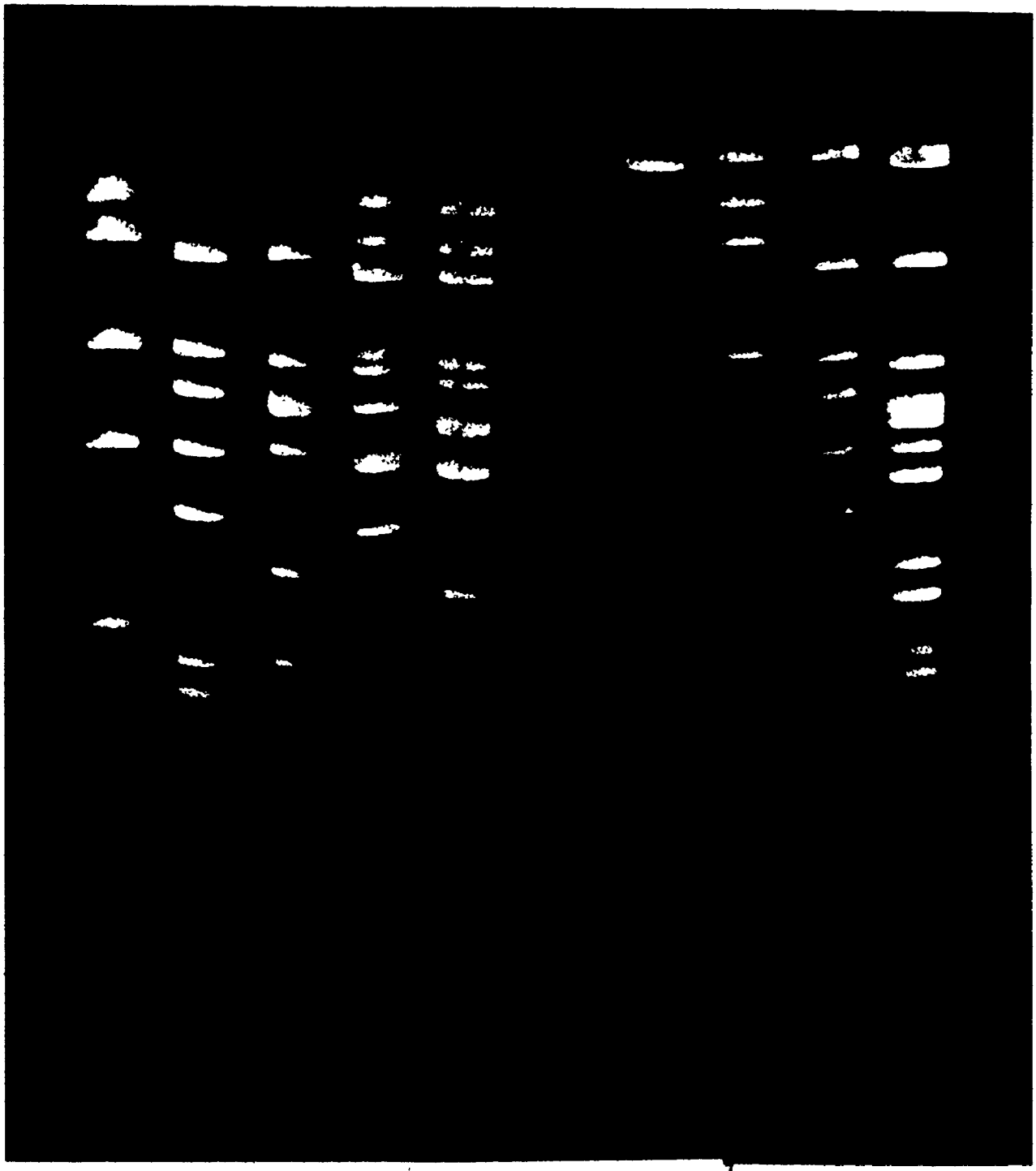
1131 Bam H1 fragments are also identical to the Huie V fragments (Delius and Mulder, personal communication). Consequently, it was assumed that the order of the Eco RI and Bam H1 fragments of 1131 DNA is the same as that determined for Huie V DNA (Delius and Mulder, personal communication). In order to map more precisely within some Eco RI and Bam H1 fragments, the fragments of 1131 DNA produced by endonuclease Hind III were also examined. Figure 26 shows the electrophoretic separation in 1% agarose gels of the Ad 12 1131 Eco RI, Bam H1 and Hind III fragments. The Eco RI fragments of Ad 2 DNA were included as molecular weight standards. Using the published molecular weights of the Ad 2 (Petersson *et al.*, 1973) and Ad 12 (Mulder *et al.*, 1974) Eco RI fragments, a correlation between electrophoretic mobility and molecular weight was established (Figure 27). Using this correlation the molecular weights of the Ad 12 (1131) Bam H1 and Hind III fragments were estimated (Table 9). Note that fragments Hind III J to L were run out of the gels displayed in Figure 26. Their relative mobilities were determined on gels run for 8.5 hours (see, for example, Figure 31). The sum of the molecular weights of both the Bam H1 and Hind III

Figure 26: Electrophoretic separation of the Ad 12 1131 Eco RI, Bam H1 and Hind III fragments

2 µg of the indicated cleaved DNA was electrophoresed in 1% agarose gels as in Figure 24.

A. Ad 12 Eco RI;
B. Ad 12 Hind III;
C. Ad 12 Bam H1;
D. Ad 12 Eco RI + Ad 12 Hind III;
E. Ad 12 Eco RI + Ad 12 Bam H1;
F. Ad 2 Eco RI;
G. Ad 2 Eco RI + Ad 12 Eco RI;
H. Ad 2 Eco RI + Ad 12 Hind III;
I. Ad 2 Eco RI + Ad 12 Bam H1.

A B C D E F G H I



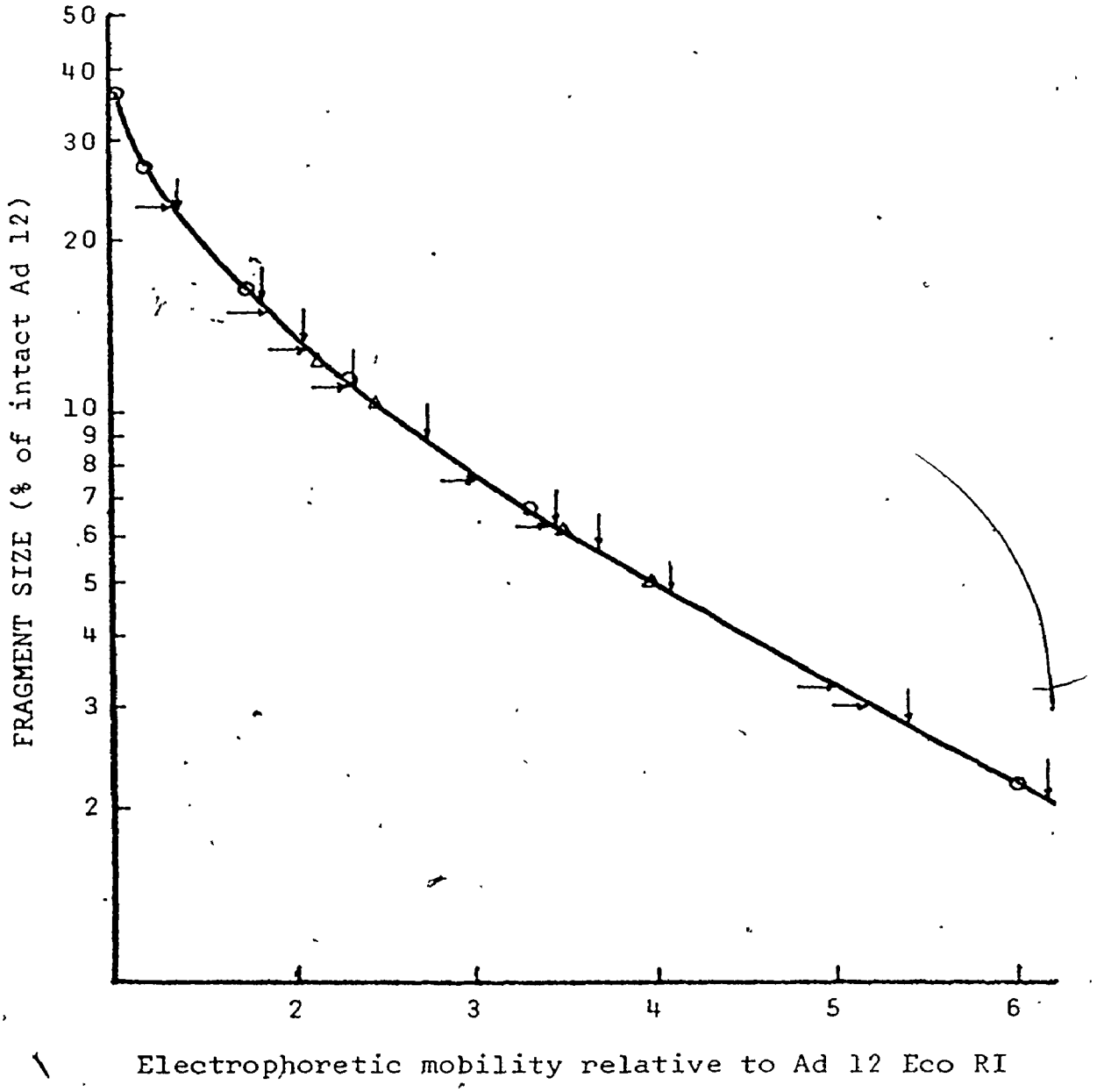


Figure 27: Correlation between electrophoretic mobility and size of DNA fragments

The electrophoretic mobilities of the Ad 2 and Ad 12 Eco RI fragments, relative to Ad 12 Eco RI A, are plotted against fragment size (as a fraction of the intact adenovirus genome). The electrophoretic mobilities of the Ad 12 1131 Hind III fragments are indicated by vertical arrows, while those of the Ad 12 1131 Bam Hl fragments are shown by the horizontal arrows. o, Ad 12 Eco RI fragments; Δ, Ad 2 Eco RI fragments.

Table 9

Electrophoretic Mobilities and Molecular Weights of Ad 12 DNA Fragments

Fragment		Mobility (relative to Eco RI A)	Molecular weight (fraction of total Ad 12)
Eco RI	A	1.00	.36
	B	1.175	.27
	C	1.75	.165
	D	2.3	.115
	E	3.3	.07
	F	5.98	.02
Bam Hl	A	1.35	.24
	B	1.90	.15
	C/D	2.10	.13
	E	2.35	.115
	F	3.00	.075
	G	3.50	.063
	H	5.02	.034
	I	5.20	.031
Hind III	A	1.36	.23
	B	1.83	.155
	C	2.05	.13
	D	2.32	.115
	E	2.72	.087
	F	3.46	.063
	G	3.60	.060
	H	4.10	.049
	I	5.40	.029
	J	6.15	.021
	K	6.55	.018
	L	7.46	.013

The Eco RI, Bam Hl and Hind III fragments of Ad 12 (1131) DNA, and the Ad 2 Eco RI fragments were separated by electrophoresis in 1% agarose gels. The mobilities of the Ad 12 DNA fragments relative to the Ad 12 Eco RI A fragment were determined. The molecular weights of the fragments were estimated from the correlation between mobility and fragment size shown in Figure 27.

fragments closely approximates that of intact Ad 12 DNA, indicating that none of the larger Hind III size classes contain more than one fragment. It is possible that some of the faster migrating bands (J to L) contain two comigrating fragments. Evidence is presented below suggesting that the L band may contain two separate fragments. The order and molecular weights of the Eco RI and Bam Hl fragments were combined in a cleavage map of the 1131 genome (Figure 28).

Several complementary approaches were adopted to order the 1131 Hind III fragments. First, ^{3}[H]-labelled Hind III fragments A to J were purified and separately hybridized to unlabelled Eco RI and Bam Hl fragments to determine which of the ordered, unlabelled fragments contained regions of sequence overlap with the labelled fragments. The unlabelled DNA fragments were transferred from agarose gels to nitrocellose strips by the blotter method (Southern, 1975) prior to hybridization. Hybridization was detected by fluorography (Figure 29). Each labelled Hind III fragment hybridized predominantly to one or two Eco RI and Bam Hl fragments. Hind III D and F each hybridized to Eco RI C. Hind III F also hybridized to a lesser degree to Eco RI A,D and E. The

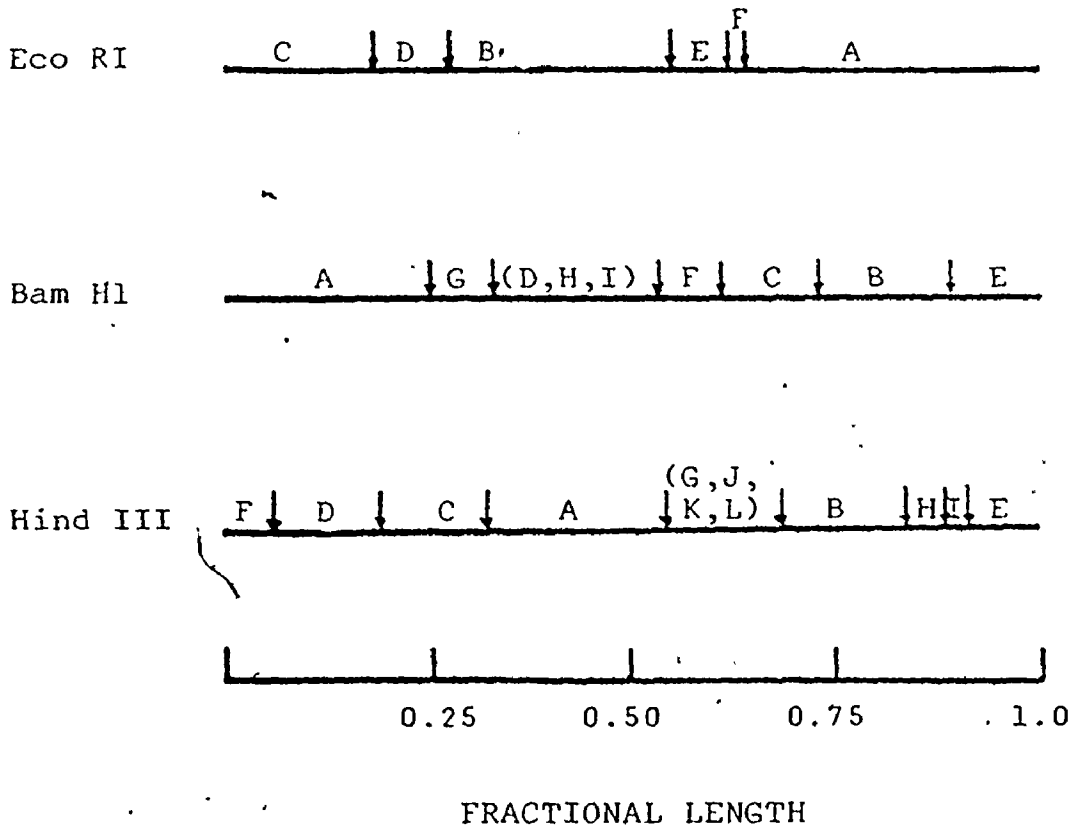
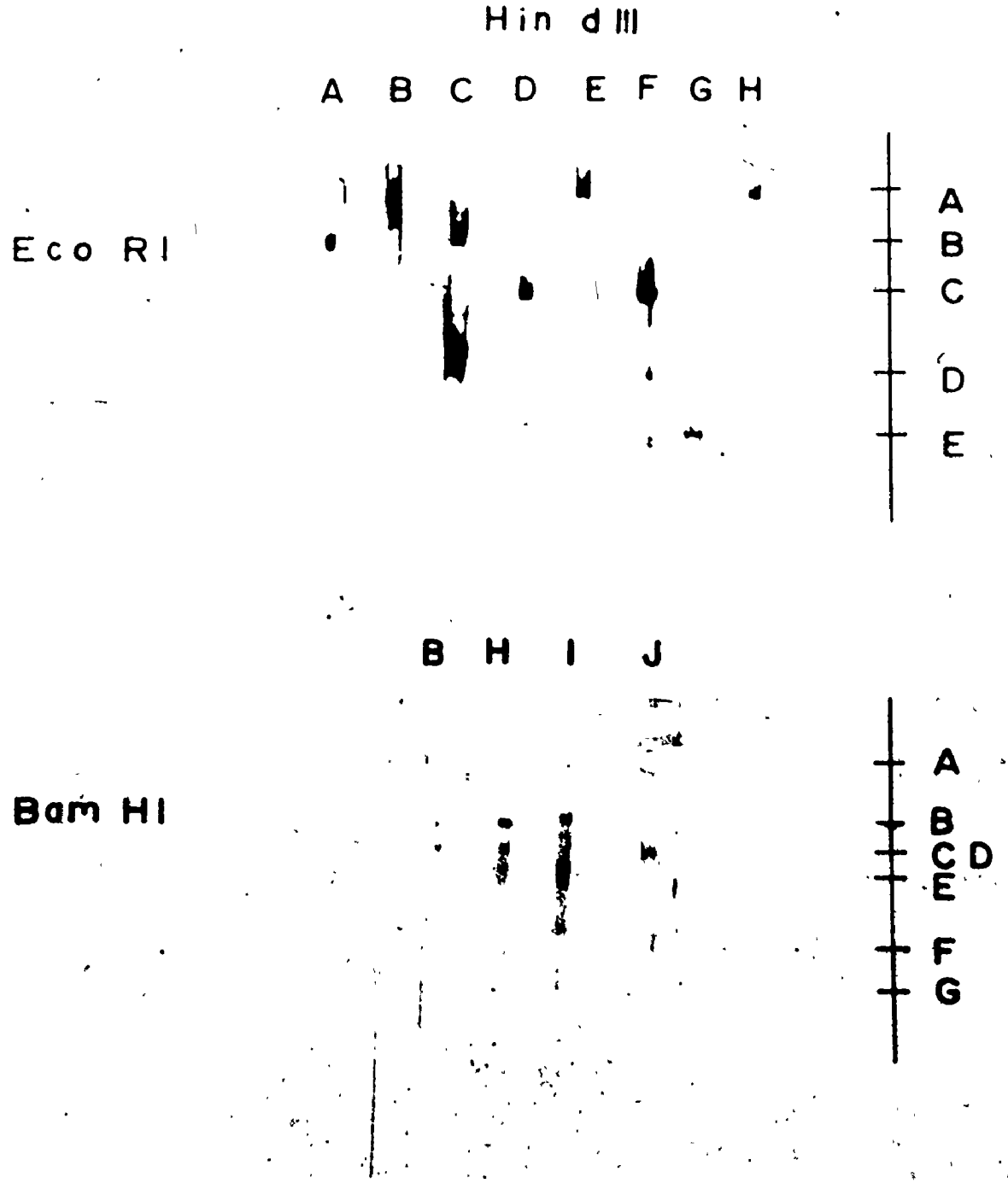


Figure 28: Restriction endonuclease cleavage maps of the Ad 12 (1131) genome

The Eco RI and Bam Hl maps are those of Delius and Mulder. (personal communication). Details of how the Hind III map was deduced are given in the text.

Figure 29: Hybridization of ^{3}[H]-labelled Hind III fragments to Eco RI and Bam Hl fragments

^{3}[H]-labelled Hind III fragments of Ad 12 (1131) DNA (5×10^{5} cpm/µg) were purified by the freeze-squeeze method, heat denatured, and hybridized for 20 hours to nitrocellulose strips bearing denatured electrophoretically separated Eco RI and Bam Hl fragments of Ad 12 (1131) DNA. Strips were prepared from 1% agarose gels containing 2 µg of unlabelled, separated DNA fragments by the blotter method of Southern (1975). An unknown quantity of the indicated labelled Hind III fragment was hybridized to each strip. Hybridization was detected by fluorography. The fluorograms shown resulted from a 20 day exposure.


Hin d III
A B C D E F G H
Eco RI
A
B
C
D
E
B H I J
Bam HI
A
B
C
D
E
F
G

hybridization to Eco RI A and E may be a result of contamination of the Hind III F DNA with Hind III E and G. The hybridization to Eco RI D could result if Hind III F partially overlapped with Eco RI D, or may be due to some other factor. Hind III C clearly hybridized to Eco RI B and D, and therefore, overlaps with both these fragments. Hind III A hybridized to only Eco RI B, and must be entirely contained within this fragment. Similarly, Hind III G is either contained entirely within Eco RI E, or overlaps Eco RI F (which was not included in the analysis).

Hind III B,E,H and I all hybridized only to Eco RI A (the fluorograph showing the hybridization of Hind III I to Eco RI A was too faint to be photographed).

These results obtained with the Eco RI fragments showed that the Hind III fragments A to I are arranged in the relative order of (D,F)C,A,G, (B,E,H,I) where the order of fragments enclosed by brackets remains unknown.

The order of some of the Hind III fragments mapping within Eco RI A was investigated by hybridization to unlabelled fragments produced by Bam Hl. Hind III B hybridized to both Bam Hl B

and C/D, Hind III H reacted primarily with Bam Hl B, (and to a lesser extent with Bam Hl C/D and E), and Hind III I hybridized primarily to Bam Hl B and E (and to a lesser degree with Bam C/D and F). Since Hind III J hybridized to Bam C/D and F, the hybridization of I to these bands may be due to contamination of the Hind III I DNA with some Hind III J. Hind III E had previously been assigned as terminal by exonuclease resection (see below), and was not hybridized to the Bam Hl fragments.

These data suggest that the Hind III fragments mapping within Eco RI A are arranged in the order BHI(E). The hybridization of Hind III J to Bam Hl C/D and, to a lesser extent, to Bam Hl F, does not allow an unambiguous positioning of this fragment to be made. Attempts were made to hybridize this fragment to the Eco RI fragments, and to hybridize Hind III L and M to both the Eco RI and Bam Hl fragments, with negative results. The small size of these fragments made it difficult to obtain reasonable quantities of material to work with.

To resolve some of the ambiguities in the map, and to provide an independent check on the hybridization data, another approach was adopted. Exonuclease III hydrolyzes duplex DNA at 3' hydroxyl

termini (Richardson *et al.*, 1964). Consequently, the enzyme hydrolyzes opposite DNA strands, beginning at both ends of a DNA molecule. If intact DNA is resected to varying degrees with exonuclease III and then cleaved with a restriction endonuclease, the electrophoretic mobility of fragments derived from the resected segments will be changed (Figure 30). Figure 31 shows the results of three such experiments. At a low exonuclease to DNA ratio, resection affected the electrophoretic mobility of only fragments E and F, which are therefore terminal. These data allowed the assignment of F as the terminal and D as the penultimate fragment at the left end of the genome, and E as the right end terminal fragment.

At a higher exonuclease to DNA ratio, additional fragments were affected. The order in which the other fragments were affected by resection was consistent with the order deduced from the DNA-DNA hybridization data: fragments E and F were affected first, followed by D and I, then B_2C and H, and finally A. Fragments G,J,K and L remained unaffected during a 40 minute incubation.

The data show that fragments Hind III A to L are arranged on the genome in the relative order of FDCA(G,J,K,L)BHIE. This information was combined

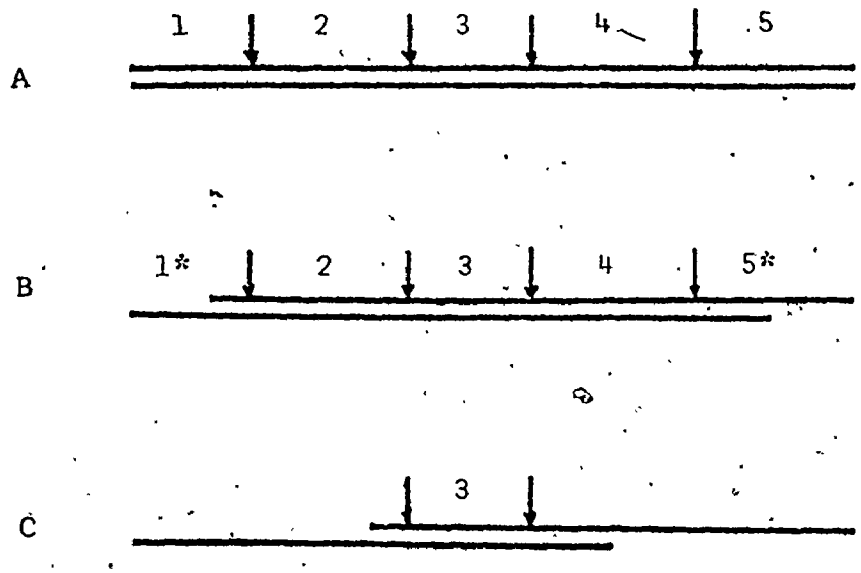


Figure 30: The use of exonuclease III to aid the positioning of restriction endonuclease sites

A hypothetical DNA molecule is cleaved by a restriction endonuclease at four sites, generating five fragments (A). If the DNA is incubated with exonuclease prior to cleavage with the endonuclease, the fragments produced will be altered. Initially, the molecular weights of terminal fragments 1 and 5 are reduced (B). As resection proceeds, crossing cleavage sites for the restriction enzyme, two cleavage sites are rendered single stranded, and therefore resistant to the endonuclease (C). Consequently, the penultimate fragments 2 and 4 disappear.

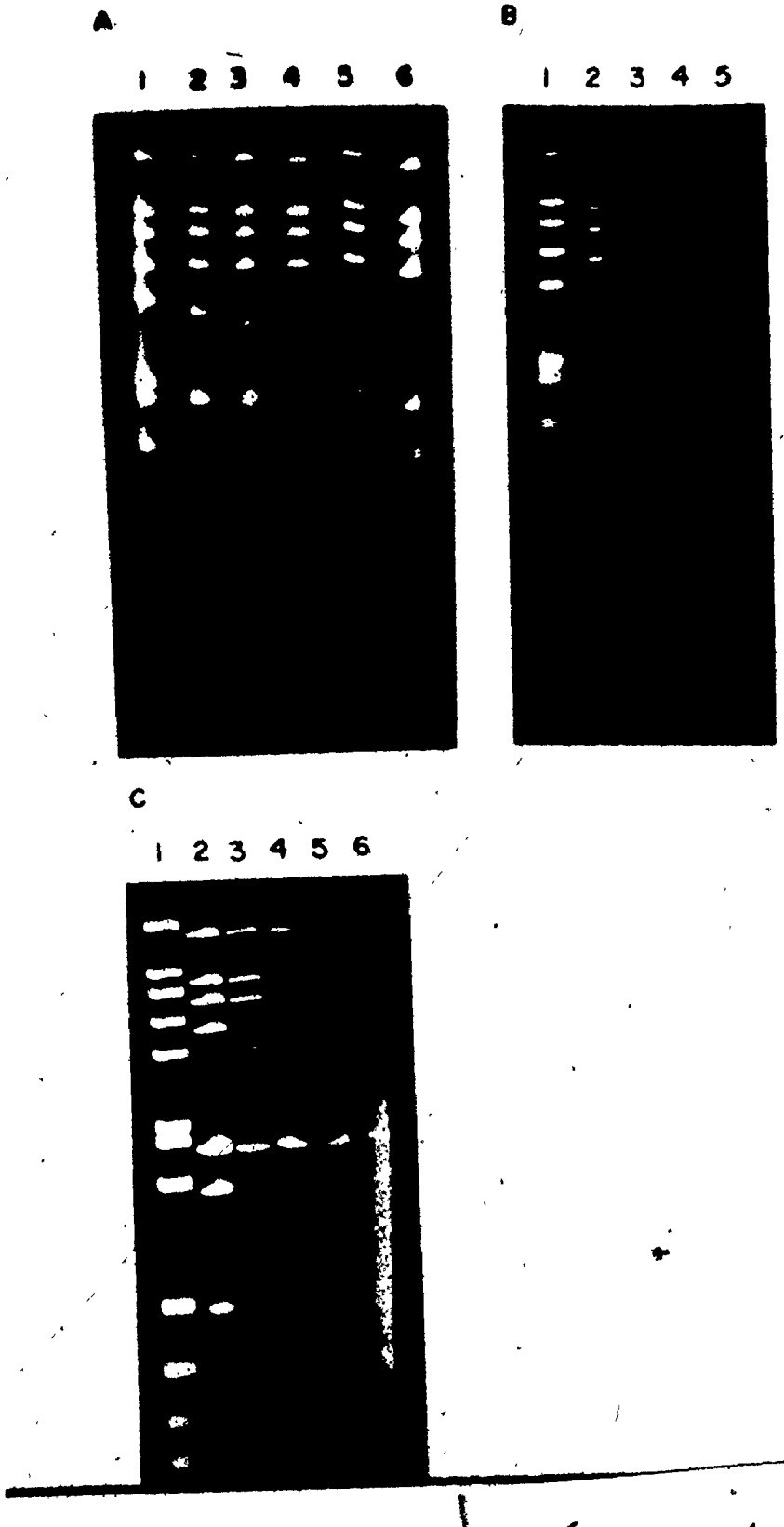
Figure 31: Hind III cleavage of partially resected Ad 12 (1131) DNA

Ad 12 DNA was incubated with exonuclease III in 0.01 M Tris, 0.002 M $MgCl_2$ for the indicated times. Aliquots containing 1 to 2 µg of DNA were removed, and the exonuclease was inactived for 10 minutes at 65°C. Samples were made 0.06 M NaCl, 0.02 M Tris, 0.006 M $MgCl_2$, pH 7.4, and were incubated with 5 units Hind III for 2 hours at 37°C. The digestion mixtures were then electrophoresed in 1% agarose gels at 30V, at room temperature.

A. 20 µg of Ad 12 DNA was incubated with 4 units exonuclease III in a total volume of 0.5 ml. Electrophoresis was for 11 hours. 1, control, unresected DNA; 2, 5 minutes resection; 3, 10 minutes; 4, 15 minutes; 5, 30 minutes; 6, 60 minutes.

B. 5 µg of DNA was incubated with 30 units of exonuclease III in a total volume of 0.1 ml. Electrophoresis was for 11 hours. 1, control, unresected DNA; 2, 5 minutes of resection; 3, 10 minutes; 4, 20 minutes, 5, 40 minutes.

C. 15 µg of DNA was incubated with 50 units exonuclease III in a total volume of 0.1 ml. Electrophoresis was for 8 hours. 1, control, unresected DNA; 2, 5 minutes of resection; 3, 10 minutes; 4, 20 minutes; 5, 40 minutes; 6, 80 minutes.


A
1 2 3 4 5 6
B
1 2 3 4 5
C
1 2 3 4 5 6

with the molecular weights of the fragments to form a Hind III cleavage map of the Ad 12 (1131) genome (Figure 28).

The Hind III map deduced above allowed a number of predictions to be made about the fragments which would be produced by double cleavage of Ad 12 DNA with a mixture of Hind III and either Eco RI or Bam Hl. Because of the uncertainty about the relative order of fragments Hind III G,J,K and L, it was not possible to predict which fragments would be produced from this region of the genome by double digestion. Figure 32 shows the electrophoretic separation of the fragments produced by such double digestions, and in Table 10 these observed size classes are correlated with those predicted on the basis of cleavage maps shown in Figure 28. The excellent agreement between the predicted and observed size classes (particularly with the Hind III + Eco RI fragments) provides strong support for the cleavage map shown in Figure 28.

The results shown in Figure 32 can be considered in another fashion: several fragments present in a single digest were absent from double digests, a finding which served to map one or more cleavage sites of the second enzyme to these fragments. For example, neither Bam Hl A nor Hind III A survived

Figure 32: Electrophoretic separation of the fragments produced by double digestion of Ad 12 (1131) DNA with Hind III and either Eco RI or Bam Hl

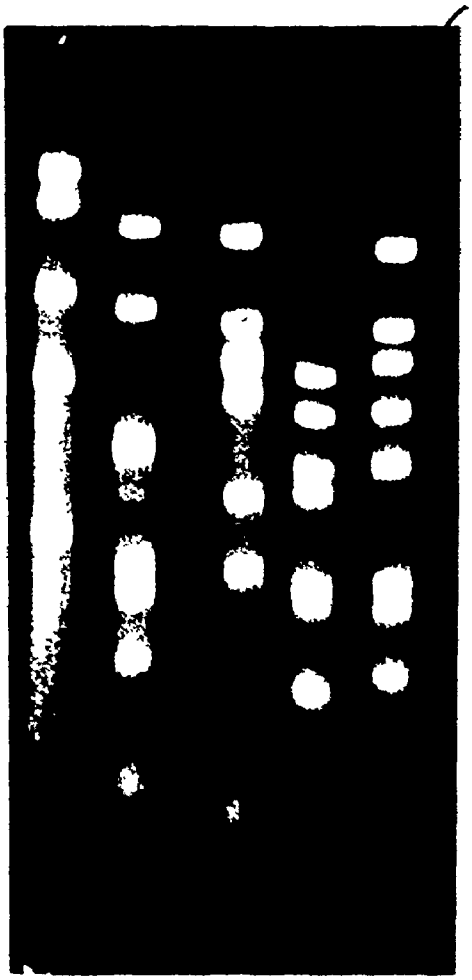
The fragments produced by complete digestion of Ad 12 (1131) DNA with a mixture of Hind III and Eco RI and Bam Hl were resolved electrophoretically in 1% agarose gels.

A. Electrophoresis was for 11 hours. 1, Eco RI fragments; 2, Eco RI + Hind III double digest; 3, Bam Hl fragments; 4, Bam Hl + Hind III double digest; 5, Hind III fragments.

B. Electrophoresis was for 8 hours. The photograph shown is a composite of two separate exposures, in which the ultraviolet light was concentrated on the upper and lower portions of the gels, respectively. The upper exposure was 15 seconds; the lower exposure was 45 seconds. 1, Eco RI fragments; 2, Eco RI + Hind III double digest; 3, Hind III fragments.

A

1 2 3 4 5



B

1 2 3

Very Dark Copy.

Table 10

Fragments Produced by Double Cleavage of Ad 12 (1131) DNA with Hind III and Eco RI or Bam H1

	Predicted sizes	Observed sizes
Hind III +	0.23 (Hin A)	0.25
	0.155 (Hin B)	0.16
Eco RI	0.105 (Hin D/Eco RI α)	
	0.095 (Hin C/Eco RI α)	0.09 (at least two comigrating fragments)
	.09 (Hin E)	
	0.063 (Hin F)	0.064
	(0.06, Hin G?)*	0.06
	0.049 (Hin H)	0.05
	0.035 (Hin C/Eco RI β)	0.037
	0.029 (Hin I)	0.029
		0.022 (Hin J?)
		0.015 (Hin L?)
		0.014
		0.012
Hind III +		
Bam H1	0.13 (Bam D)	0.14
	0.115 (Hin D)	0.12
	0.10 (Hin B/Bam H1 α)	0.092
	0.09 (Hin E)	0.098
	0.07 (Hin C/Bam H1 α)	
	0.063 (Hin F)	0.063
	0.06 (Hin C/Bam H1 β)	
	(0.06, Hin G?)*	0.060
	0.049 (Hin H)	0.050
	0.034 (Bam H)	0.034
		0.030
	0.031 (Bam I)	0.025

The molecular weights of the size classes larger than Hind III I produced by double cleavage with Hind III and Bam H1, and of all the size classes produced by double cleavage with Hind III and Eco RI, as estimated from their electrophoretic mobilities, are given. These size classes are correlated with those larger than Hind III I from the cleavage maps shown in Figure 28. Data from Figure 32. *From the map shown in Figure 28, it is not clear whether or not Hind III G is cleaved by Eco RI and Bam H1.

cross-cleavage by the other endonuclease, demonstrating that Hind III A contains one or more Bam Hl sites, and vice versa. It must be stressed that the presence of a fragment in the double digest corresponding in size to a fragment present in either single digest does not demonstrate that this fragment has survived cross-cleavage. It is possible that the fragment present in the single digest was cleaved by the second enzyme and that the double digestion generated a new fragment of identical size. Table 11 presents the unambiguous cases of cross-cleavage observed; no cases inconsistent with the maps given in Figure 28 were observed.

Evidence suggesting that the Hind III L band contains at least two distinct fragments was obtained. Figure 32 shows that Eco RI reduced, but did not eliminate, the fluorescence of the L band, suggesting that the L band contains two fragments, one of which is cleaved by Eco RI. It is conceivable that one of these L fragments maps outside the region between Hind III A and B. Because of the small size of this fragment, this ambiguity did not detract from the utility of the Hind III fragments for transcription mapping.

Table 11

Unambiguous Cases of Cross Cleavage of DNA Fragments by Double Digestion with Hind III and Eco RI or Bam Hl

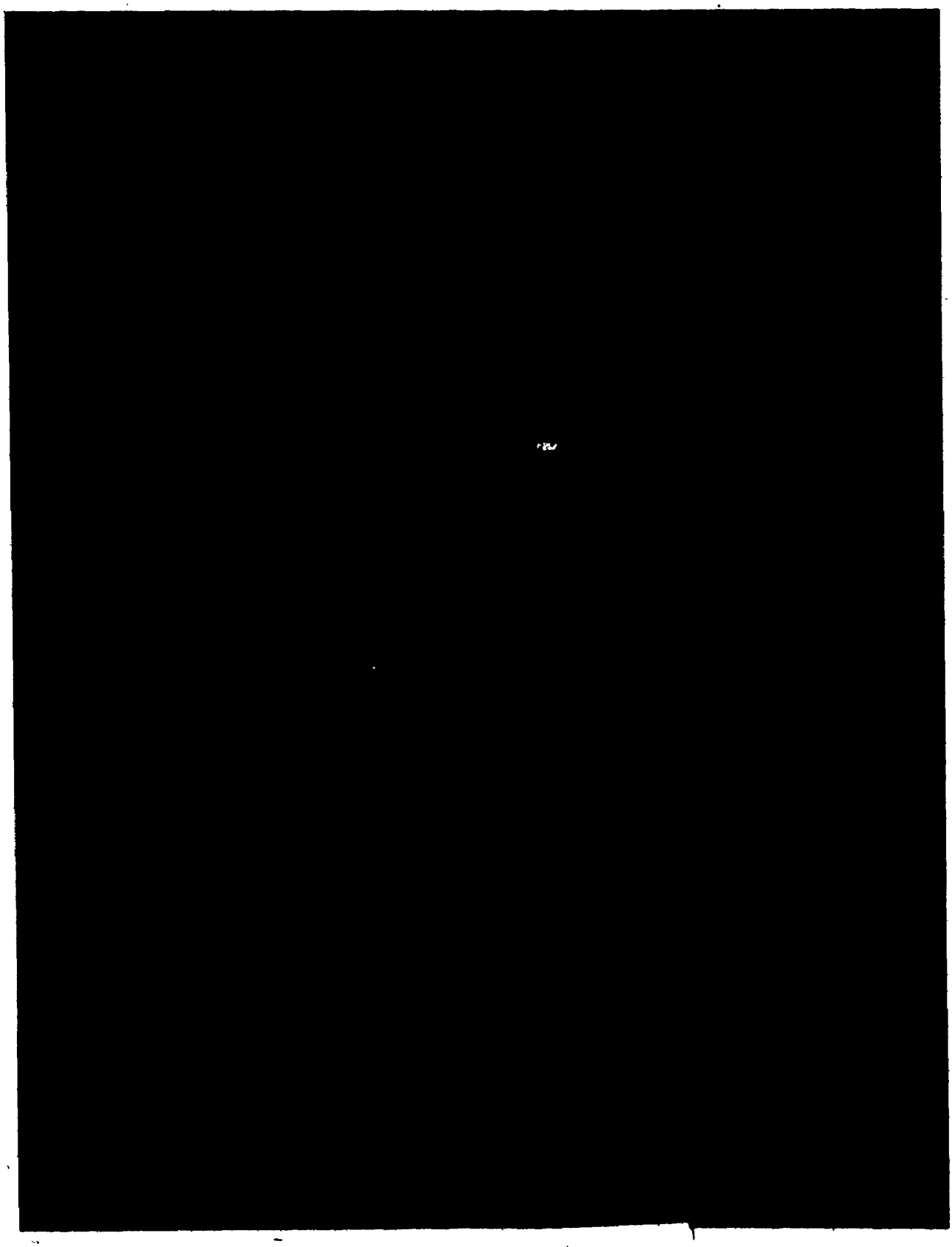
Fragment	Cleaved by	Predicted by cleavage map?
Eco RI A	Hind III	+
Eco RI B	Hind III	+
Eco RI C	Hind III	+
Eco RI D	Hind III	+
Eco RI E	Hind III	+
Hind III A	Bam Hl	+
Hind III B	Bam Hl	+
Hind III C	Eco RI	+
Hind III D	Eco RI	+
Hind III I	Bam Hl	+
Hind III K	Eco RI	?
Hind III L*	Eco RI	?

See text for an explanation of how cross-cleavage was determined.

* As shown in Figure 32, Eco RI reduced, but did not eliminate the fluorescence of the Hind III L band, suggesting that the L band contains at least two fragments, one of which is cleaved by Eco RI.

Figure 33: Comparison of the Hind III fragments of strain 1131 and Huie M8 DNA

1 µg of the indicated fragments were electrophoresed for 11 hours at room temperature at 30V. From left to right the gels contained: 1131 Eco RI, 1131 Hind III, Huie M8 Hind III, 1131 Hind III + Huie M8 Hind III, and Huie M8 (Hind III + Eco RI) double digest.



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5. Hind III cleavage of AD 12 (Huie) DNA. The fragments produced by Hind III cleavage of Ad 12, strain Huie M8 DNA were compared to those of the 1131 strain (Figure 33). The molecular weights and correspondences between the two fragment sets are presented in Table 12. Several differences were noted between the fragments generated from the DNA of the two virus strains. Because of the high degree of homology of the DNA of the two virus strains demonstrated by RNA-DNA hybridization and cleavage with Eco RI and Bam H1, it is reasonable to presume that fragments of identical molecular weight are derived from common map positions.

Data presented in section IV.B. below, demonstrated that fragments Hind III, F,H and one of C or D of Huie M8 DNA map at least partially within Eco RI C. It is probable that Hind III F is the leftmost fragment of Huie M8 DNA.

IV. Hybridization Maps of Early, Late and Transformed Cell RNA.

A. Cytoplasmic RNA synthesized during productive infection of KB cells.

1. Separation of the complementary strands of DNA fragments. Hybridization maps have been obtained

Table 12

Comparison of the Hind III Fragments of Ad 12 1131 and Huie M8 DNA Larger Than 1131 Hind III I

DNA fragment		Fraction size
1131	Huie M8	(fraction of total Ad 12 DNA)
A	-	0.23
B	A	0.155
C	B	0.13
D	-	0.115
-	C = D	0.095
E	-	0.087
-	E	0.074
F	F	0.063
G	-	0.060
H	G	0.049
-	H	0.040
I	I	0.029

Molecular weights and relationships of the Hind III fragments of 1131 and Huie M8 DNA are given. Molecular weights were determined from Figure 35; fragments of identical size are placed on the same line.

for the genomes of several DNA containing animal viruses (SV40, Sambrook *et al.*, 1972; 1973; Khoury *et al.*, 1973B; Polyoma, Kamen *et al.*, 1974; and adenovirus type 2, Petersson *et al.*, 1976).

The experimental design of all these studies was the same: radioactive complementary strands of DNA fragments produced by digestion with restriction endonucleases were hybridized to saturating amounts of unlabelled RNA. The arrangement of RNA coding regions on the genome both consistent with the data and requiring the fewest ad hoc assumptions was deduced.

There are two major technical problems associated with this approach. First, a means of separating the complementary strands of individual restriction endonuclease fragments must be designed. Secondly, large quantities of RNA are required to saturate detectable amounts of small restriction fragments. The viral RNA not complementary to the particular probe used is wasted.

The complementary strands of subgenomic DNA fragments have been separated by several methods. Khoury *et al.* (1973A) showed that *E. coli* RNA polymerase transcribes only one strand of SV40 DNA *in vitro*. This complementary RNA has been used to

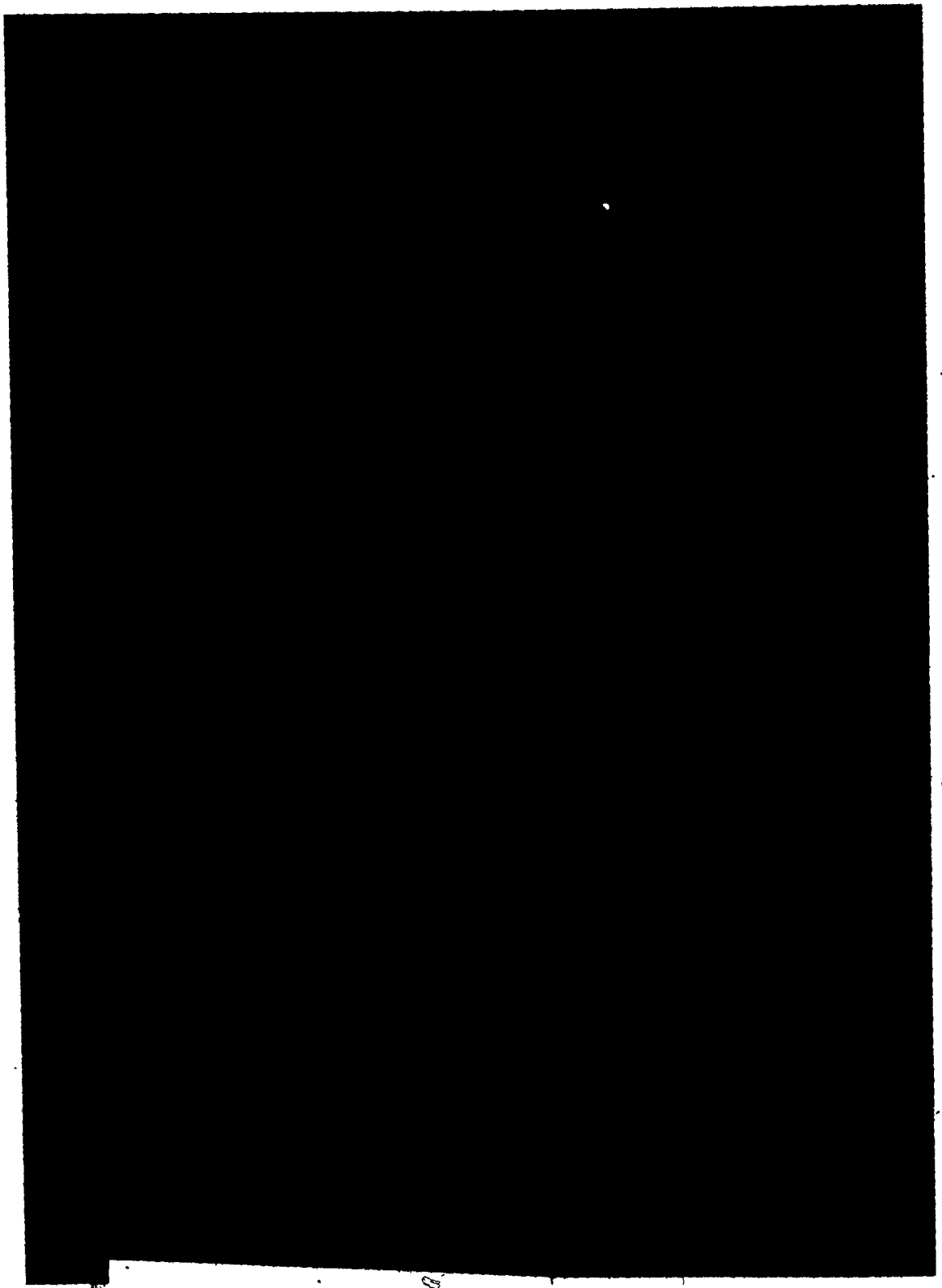
strand separate SV40 DNA fragments (Khoury *et al.*, 1973B). A similar approach has proven successful with polyoma virus DNA (Kamen *et al.*, 1974). *E. coli* RNA polymerase transcribes regions of both adenovirus DNA strands *in vitro* (Petersson *et al.*, 1974) so that this method cannot easily be applied to adenovirus DNA fragments.

Tibbetts *et al.* (1974) have strand separated labelled Ad 2 DNA fragments by hybridizing them to excess, unlabelled intact Ad 2 complementary DNA strands. After the hybridization reaction had reached completion, the single stranded DNA (derived from the complement initially present in excess) was purified. This approach requires large quantities of intact complementary strands and suffers from the additional disadvantage that the specific activity of the DNA obtained is reduced by the unlabelled complement added. For these reasons, this method of strand separation was not adopted.

The complementary strands of some DNA molecules differ sufficiently in electrophoretic mobility to allow their separation (Hayward, 1972). This method has been used with Ad 2 and Ad 5 DNA fragments (Sharp *et al.*, 1974; Flint *et al.*, 1976). Figure 34 shows that the complementary strands of the Ad 12 (1131)

Figure 34: Electrophoretic DNA strand separation

4 µg of unlabelled Ad 12 (1131) DNA, cleaved with Eco RI, was electrophoresed as in Figure 33. After staining with ethidium bromide, gel slices containing fragments A to E were visualized and cut out with a razor. The DNA was denatured in situ and re-electrophoresed for 8 hours as described in Materials and Methods. From left to right the gels contained Eco RI fragments A,B,C,D, and E.



Very Dark Co. 4.

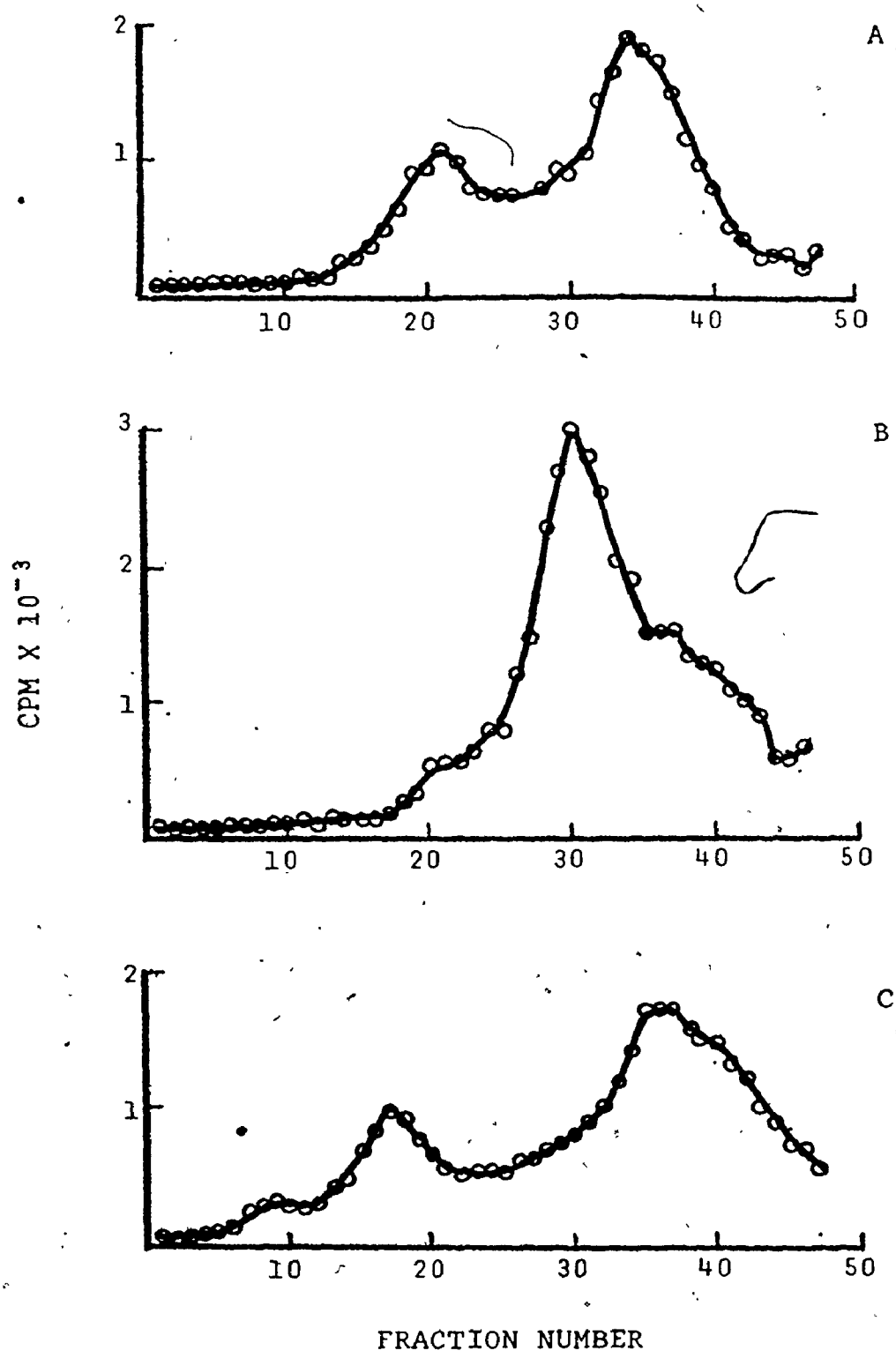
Eco RI A and C fragments were well resolved by this method, but that little or no separation was obtained with Eco RI B and E DNA. This method of strand separation was therefore useful only with some Ad 12 DNA fragments.

An alternative method was tested. Fragments Eco RI A,B and C were purified, denatured and centrifuged to equilibrium in cesium chloride density gradients in the presence of poly (U,G) (Figure 35). A suggestion of strand separation was observed with Eco RI A and C, while only one peak was observed with Eco RI B DNA. Similar results have been reported recently by Ortin *et al.* (1976).

2. A novel approach to hybridization mapping. As outlined above, strand separation of all Ad 12 DNA fragments was difficult. Consequently, an alternative mapping strategy, which bypassed the requirement for strand separation of individual DNA fragments, was adopted. In brief, the segments of each DNA strand complementary to late cytoplasmic RNA were purified by hybridizing saturating amounts of unlabelled RNA to each DNA strand, and degrading the unhybridized DNA with S1 nuclease. The DNA sequences recovered by this procedure were referred to as $r+$ and $l+$ DNA. The arrangement of these sequences on the viral DNA

Figure 35: Separation of the complementary strands of Eco RI A,B and C DNA in poly (U,G) cesium chloride gradients

An unknown amount of ^{3}[H] Eco RI A,B and C DNA, purified by the sodium perchlorate method from gels containing a total of 20 µg of Ad 12 DNA, was denatured and centrifuged to equilibrium in a cesium chloride density gradient in the presence of 500 µg of poly (U,G). Six drop fractions were collected and 10 µl aliquots were assayed for radioactivity.


A
B
C
2
1
3
10
20
30
40
50
CPM X 10^{-3}
FRACTION NUMBER

molecule was examined by hybridizing the *l*+ and *r*+ DNA to excess (2 μg) unlabelled fragments generated by restriction endonucleases - Eco RI, Bam Hl and Hind III - which had previously been transferred from agarose gels to nitrocellulose strips by the blotter method. Hybridization was detected by fluorography (Figure 36). As a control, total Ad 12 DNA degraded to the same extent as *r*+ and *l*+ DNA in alkali was hybridized to unlabelled Eco RI fragments.

Since the *r*+ and *l*+ probes were derived from 0.65 of the intact *r* strand and 0.15 of the *l* strand, respectively, it was possible to calculate the fraction of each intact strand transcribed as stable cytoplasmic RNA within any region of the genome defined by a DNA fragment (Table 13). This quantity was obtained by multiplying the fraction of the probe hybridizing to each fragment by the sequence complexity of the probe (i.e., 0.65 or 0.15). When the Hind III fragments were used, fragments J to L were run out of the gels. This resulted in the exclusion of a specific 8% of Ad 12 DNA from the analysis, necessitating corrections to be applied to the complexities of the DNA probes (see below). Similarly, Eco RI F (2% of Ad 12) was also excluded. Because of the small size of this fragment, its

Figure 36: Hybridization of *l*+ and *r*+ DNA to unlabelled DNA fragments generated by Eco RI, Bam Hl and Hind III

Nitrocellulose strips bearing 2 µg of unlabelled DNA fragments produced by Eco RI, Bam Hl and Hind III were incubated with 0.02 µg (10^4 cpm) of *r* strand probe or 0.005 µg (2.5×10^3 cpm) of *l* strand probe in 2 x SSC plus 0.1% SDS at 65° C for 20 hours. The hybridized radioactivity was detected by fluorography to flash hypersensitized x-ray film for 1 week. Microdensitometer scans of the resulting negative are shown.

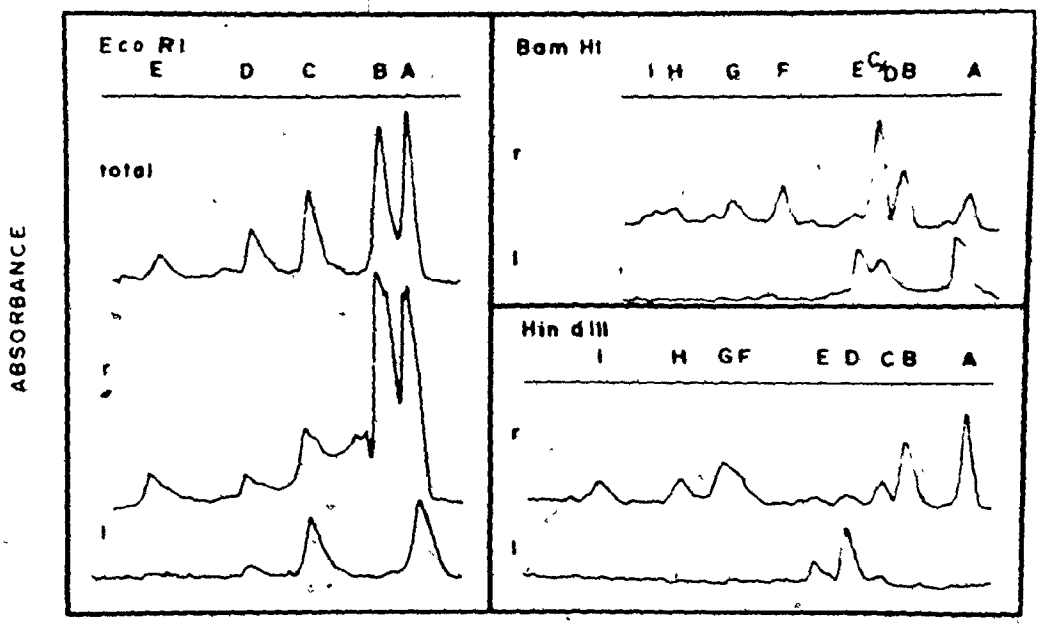

Eco RI
E D C B A
total
r
l
Bam HI
I H G F E C D B A
r
l
Hin d III
I H GF E D CB A
r
l
ABSORBANCE
MIGRATION

Figure 13

Distribution of the *l* and *r* Strand Sequences Complementary to Late Cytoplasmic RNA Among Specific DNA fragments[1]

Probe	Fragment	Fragment size (fraction)	Fraction of probe hybridized	Fraction of genome represented in probe within fragment[2]
r	Eco RI A	.36	.35	.23
r	Eco RI B	.27	.36	.24
r	Eco RI C	.165	.17	.11
r	Eco RI D	.115	.06	.04
r	Eco RI E	.07	.06	.04
r	Eco RI F	.02	ND	ND
r	Bam Hl A	.25	.13	.08
r	Bam Hl B	.16	.16	.11
r	Bam Hl C	.13	(.32)[3]	(.08)[3]
r	Bam Hl D	.13	(.32)[3]	(.13)[3]
r	Bam Hl E	.115	.04	.02
r	Bam Hl F	.075	.12	.08
r	Bam Hl G	.063	.10	.07
r	Bam Hl H+I	.065	.12	.08
r	Hind III A	.23	.29	.18
r	Hind III B	.155	.19	.12
r	Hind III C	.13	.06	.04
r	Hind III D	.115	.02	.01
r	Hind III E	.09	.01	.006
r	Hind III F	.063	.13	.08
r	Hind III G	.060	.12	.07
r	Hind III H	.049	.10	.06
r	Hind III I	.029	.07	.04
r	Hind III J-L	.071	ND	ND
l	Eco RI A	.36	.56	.084
l	Eco RI C	.165	.36	.053
l	Eco RI D	.115	.08	.012
l	Bam Hl A	.25	.39	.058
l	Bam Hl C	.13	.31	.046
l	Bam Hl E	.115	.30	.046
l	Hind III C	.13	.09	.009
l	Hind III D	.115	.67	.07
l	Hind III E	.09	.24	.025

Probe	Fragment	Fragment size (fraction)	Fraction of probe hybridized	Fraction of genome represented in probe within fragment[2]
Total DNA	Eco RI A	.36	.39	.39
Total DNA	Eco RI B	.27	.26	.26
Total DNA	Eco RI C	.165	.21	.21
Total DNA	Eco RI D	.115	.10	.10
Total DNA	Eco RI E	.07	.04	.04

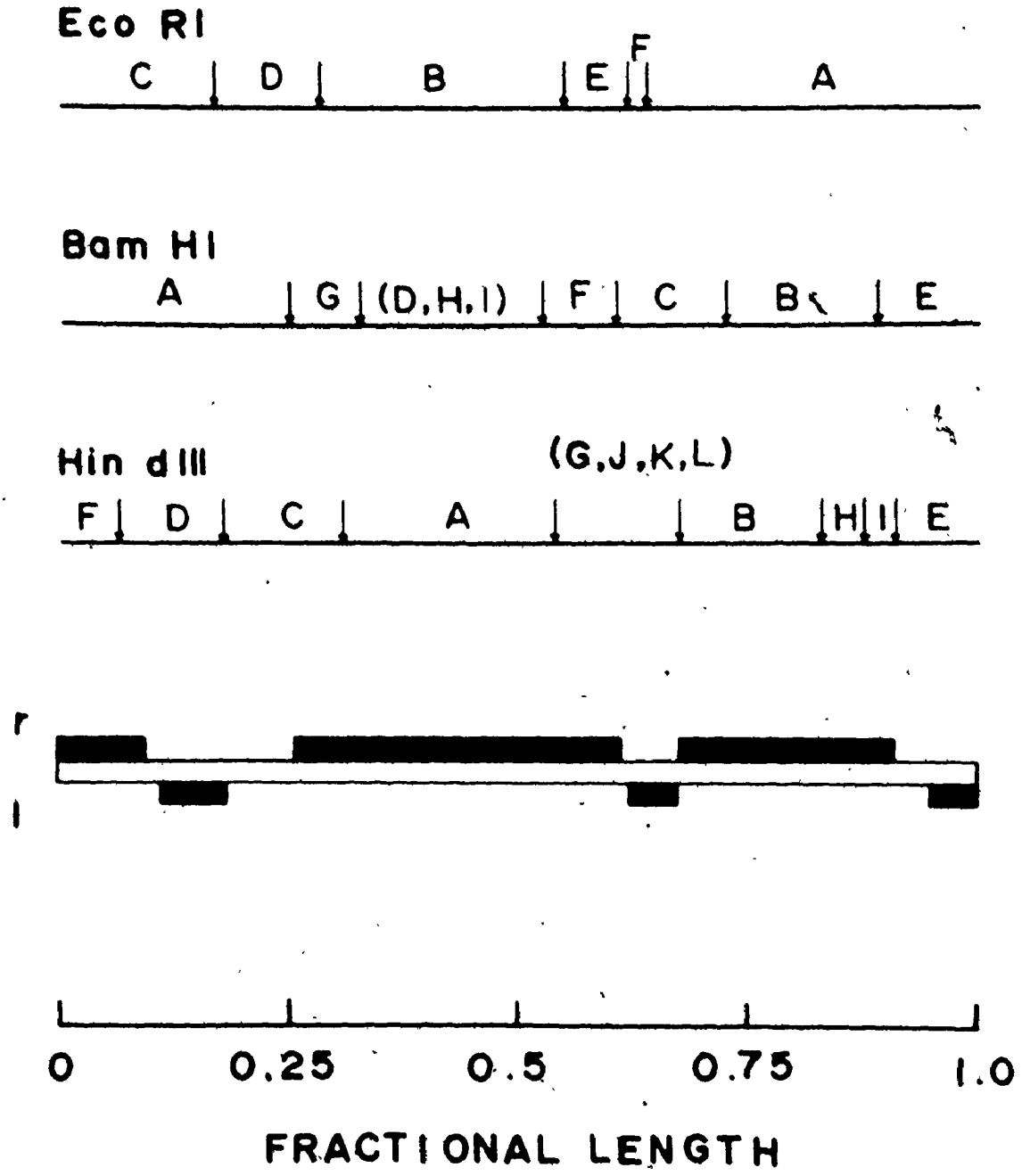
[1] ^{3}[H] *l* and *r* strand probes complementary to late cytoplasmic RNA were hybridized to nitrocellulose strips bearing electrophoretically separated DNA fragments, and the hybridized radioactivity was quantitated by fluorography (Figure 38).

[2] Calculated as the product of the effective sequence complexity of the probe used and the fraction of the total hybridized radioactivity annealed to each fragment. The effective complexity of the *r* strand probe was 0.65 when hybridized to Eco RI and Bam Hl fragments, 0.61 when annealed to the Hind II fragments A to I., Effective complexities of 0.15 and .106 were assumed for the *l* strand probe (see text).

[3] Fragments Bam Hl C and D comigrate. Together they contain sequences homologous to 32% of the *r* strand probe (=21% of the total *r* strand). The rationale for apportioning these sequences between the two fragments is given in the text.

Figure 37: Tentative map of the sites on the Ad 12 (1131) genome expressed as late cytoplasmic RNA

Details of construction of the map are given in the text.


Eco RI
C
D
B
E
F
A
Bam HI
A
G
(D,H,I)
F
C
B
E
Hin dIII
(G,J,K,L)
F
D
C
A
B
H
I
E
0
0.25
0.5
0.75
1.0
FRACTIONAL LENGTH

exclusion had a negligible effect on the interpretation of the data, and was ignored.

The results obtained with the three sets of DNA fragments were in good quantitative agreement, and were used to construct a tentative map of the regions of the viral genome which are expressed as late cytoplasmic RNA (Figure 37). The map shown was deduced from the data given in Table 13 by assuming that cytoplasmic RNA is assymetric (not self-complementary), and by reducing the number of separate, contiguously transcribed regions to the minimum demanded by the data. Details about the construction of this map are given below.

3. *l* strand specific RNA. A minimum of two separate regions of transcribed *l* strand DNA were detected within the Eco RI fragments A to E. One region about 7 map units in length, mapped at the junction of fragments Eco RI C and D, and the other, comprising about 8 map units, was found within Eco RI A. The data obtained with the Bam Hl fragments allowed the splitting of the block within Eco RI A into two regions of approximately 4 map units each, one within Bam Hl C and the other in Bam Hl E. (Although Bam Hl C and D were not separable electrophoretically,

the lack of hybridization of the *l*+ probe to Eco RI B, which contains Bam Hl D, allowed the assignment to Bam Hl C.) The fragments produced by Hind III allowed a more precise localization of these three regions. Hybridization of the *l*+ probe was observed to Hind III C,D and E (Hind III J,K and L were run out of the gel used in the analysis). The left hand block was placed from 10.8 to 18.5 map units, using the Hind III data, in good agreement with the assignment of from 11.2 to 17.7 map units based on the Eco RI data. The extreme right hand region mapped entirely within Hind III E, and consequently the rightmost end of this segment must be located no more than 4 to 5 map units from the end of the genome. The region detected within Bam Hl C was not detectable within fragments Hind III A to I, and must, therefore, be contained within one or more of the fragments excluded from the analysis (J to L). The combined data obtained with the three sets of fragments allowed the placing of this region within Bam Hl C, outside the regions of overlap with Eco RI E and Hind III B.

It is clear that a transcribed segment of the *l* strand is excluded from analysis by running fragments Hind III J to L out of the gels used. The size of this region is estimated as 4.6 map units

from the Bam H1 data. Consequently, the effective complexity of the *l+* probe when hybridized to Hind III A to I is about 0.15-0.046 = 0.104. This value was used to calculate the sizes of the other *l* strand regions detected within Hind III A to I.

4. *r* strand specific RNA. Using similar reasoning, the regions of the *r* strand complementary to late cytoplasmic RNA were deduced. The combined data obtained with the three fragment sets indicated that late cytoplasmic RNA is derived from a minimum of three separate segments of the viral *r* strand, whose sizes were estimated as approximately 10,32 and 22 map units in length, from left to right. All three sets of data indicated that a region of the Ad 12 genome extending from about 17 to 25 map units is not transcribed into stable cytoplasmic RNA. A similar region of non-transcribed DNA apparently exists within Hind III and Bam H1 E (about 5 map units in size).

Several difficulties were encountered in the interpretation of the data obtained with the *r+* probe. First, because Bam H1 C and D were not separable electrophoretically, the data gave an estimate of the fraction of the *r* strand transcribed from within both these fragments. The data obtained

with the Eco RI and Hind III fragments indicated that virtually all of the *r* strand of Eco RI B and Hind III A is transcribed as cytoplasmic RNA. The Bam Hl D fragment maps entirely within this region, and consequently it was assumed that all of the *r* strand of Bam Hl D is transcribed. The excess hybridization to the Bam Hl C/D doublet was assigned to Bam Hl C.

Second, the method of calculating the fraction of each strand transcribed within each region assumes that all of the DNA sequences complementary to the *r*+ and *l*+ probes used are present in the analysis. Fragments Hind III J to L were excluded because of their small size. This exclusion of 8% of Ad 12 complicates matters. However, as argued above, a region comprising 4.6 map units, located entirely within fragments Hind III J to L is transcribed from the *l* strand, and can be excluded from the analysis of *r* strand specific RNA. Effectively only about 3.4% of Ad 12 is then excluded by not examining these fragments. Assuming that all of this DNA is represented as *r* strand specific cytoplasmic RNA, the effective complexity of the *r* strand probe becomes 0.61. The effects of this alteration in complexity are small. The values given in Table 13 for the Hind III fragments were calculated using 0.61 as the complexity of the *r* strand probe.

5. RNA actively synthesized and transported to the cytoplasm at late times. The results obtained above served to map the positions and strand specificities of the DNA sequences complementary to late cytoplasmic RNA, but gave no indication which segments of the genome were actively transcribed at late times. It has been reported that late Ad 2 RNA is transcribed exclusively from the *r* strand of the genome (Philipson *et al.*, 1974). To determine whether this was the case during Ad 12 infection, cells were pulse labelled from 21 to 24 hours post infection with 10 μCi per millilitre of ^{3}H-uridine, and the cytoplasmic RNA was purified. Aliquots of this RNA (10 μg) were hybridized to membrane filters bearing 1 μg of ^{14}C-labelled *l* and *r* strand DNA. The results (Table 14) clearly demonstrated that RNA derived from both DNA strands was synthesized and transported to the cytoplasm during the labelling interval. In order to examine which segments of each DNA strand were actively transcribed, RNA selected by hybridization to filters bearing ^{14}C-labelled *l* and *r* strand DNA was rehybridized to the Eco RI fragments A to E (Figure 38). Most RNA species which are present in the cytoplasm at late times were actively synthesized during the labelling interval. Since the strand

Table 14

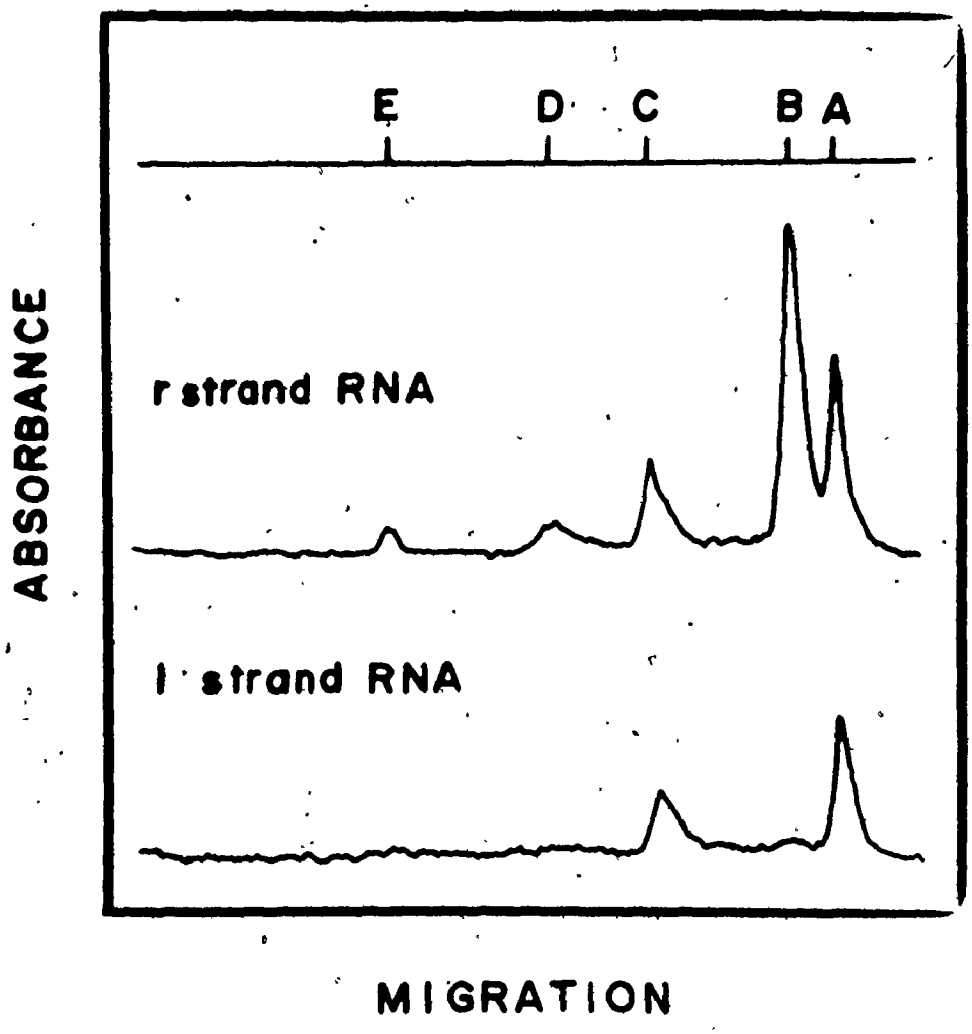
Hybridization of ^3H Late Cytoplasmic RNA to the *l* and *r* Strands of Ad 12 DNA[a]

CPM hybridized to	
l strand	*r* strand
3694	24646
3351	23690

[a] 5×10^5 cpm of cytoplasmic RNA (10 µg) labelled 21 to 24 hours post infection with 20 µCi/ml ^3H-uridine (20 Ci/mmole) was hybridized to membrane filters bearing 2 µg ^{14}C-labelled *l* or *r* strand DNA as described in Materials and Methods.

Figure 38: Hybridization of labelled late complementary strand-specific RNA to unlabelled Eco RI fragments

Cytoplasmic RNA labelled from 21 to 24 hours post infection was strand-selected by hybridization to pure *l* and *r* strands as described in Materials and Methods. This RNA was hybridized to nitrocellulose strips bearing 2 µg of denatured separated Ad 12 (1131) Eco RI fragments as described in Materials and Methods. Hybridized radioactivity was detected as in Figure 36. Exposure was for one week.


E
D
C
B
A
r strand RNA
l strand RNA
ABSORBANCE
MIGRATION

specific RNA used in this experiment was isolated by hybridizing near saturating amounts of RNA to each strand, the results shown in Figure 38 cannot be used to estimate the relative amounts of label incorporated into RNA derived from different regions of the genome.

To obtain this type of estimate, cytoplasmic RNA labelled 24 to 26 hours post infection was degraded in alkali to about 500 nucleotides in chain length (.25 N NaOH, 5 minutes, 0°C, Bachenheimer and Darnell, 1975) and 2 µg aliquots of this RNA (specific activity of 3.4×10^4 cpm per microgram) were hybridized to excess (2 µg) fragments produced by Eco RI and Bam H1. The relative amount of radioactivity hybridizing to each band was taken as a measure of the relative rate of accumulation of cytoplasmic RNA complementary to each fragment. The data obtained (Table 15) indicated that RNA species derived from different regions of the viral genome accumulate at widely differing rates. During the labelling period used, RNA derived from DNA extending from about 25 to 50 map units on the genome (Eco RI B and D, Bam H1 D,G,H,I) accumulated at a greater rate than any other class.

6. Mapping of the DNA expressed as early cytoplasmic RNA. Data presented in section II.3. above, demonstrated that only a limited subset of the viral

Table 15

Distribution of Labelled Late Cytoplasmic RNA Sequences Among DNA Fragments[1]

Fragment	Fraction of total hybridized RNA annealed to fragment	Specific accumulation rate[2]
Eco RI A	0.07	0.19
Eco RI B	0.60	2.15
Eco RI C	0.07	0.40
Eco RI D	0.20	1.91
Eco RI E	0.06	0.86
Bam Hl A	0.06	0.22
Bam Hl B	0.03	0.18
Bam Hl C	(0.55)[3]	(0.42)[4]
Bam Hl D	(0.55)[3]	(3.78)[4]
Bam Hl E	0.02	0.17
Bam Hl F	0.07	0.87
Bam Hl G	0.16	2.55
Bam Hl H + I	0.12	1.80

[1] Two micrograms (6.8×10^4 cpm) of late cytoplasmic RNA labelled 24 to 26 hours post infection with ^{3}H-uridine degraded to 500 nucleotides in chain length were hybridized to excess(2 µg) unlabelled DNA fragments, and the hybridized radioactivity was quantitiated by fluorography.

[2] Defined as the fraction of the total hybridized radioactivity annealing to each fragment divided by the fractional length of that fragment.

[3] Bam Hl C and D are not separable electrophoretically. This figure represents the sum of the radioactivity hybridizing to each fragment.

[4] The relative distribution of hybridized radioactivity between Bam Hl C and D was estimated by measuring hybridization to fragments produced by simultaneous cleavage with Eco RI and Bam Hl.

Table 15 (cont'd)

The ratio of hybridized radioactivity between Bam C/ Eco RI α and Bam Hl D was 0.07. Bam Hl C/Eco RI represents 0.71 of Bam Hl C. Assuming that accumulation within Bam Hl C is uniform, this yeilds a specific accumulation rate of 0.42 for Bam Hl C.

RNA sequences present late were found in the cytoplasm at 7.5 hours post infection (early). All sequences found in early cytoplasmic RNA persisted until late post infection. Consequently the map of the sites on the viral genome expressed as late cytoplasmic RNA deduced above included those sites expressed as early cytoplasmic RNA. Before the onset of viral DNA synthesis, viral RNA form an extremely small fraction of the total cytoplasmic RNA (see section I.A.1., above). The method used to map late RNA required sufficient viral RNA to saturate substantial quantities of viral DNA, and was therefore difficult to apply to mapping early cytoplasmic RNA.

Some information about the DNA sequences encoding early cytoplasmic RNA was obtained by hybridizing cytoplasmic RNA labelled 5.5 to 7.5 hours post infection with ^{3}[H]-uridine to DNA fragments produced by Eco RI, Bam H1 and Hind III (Figure 39). Quantitation of the hybridization is given in Table 16.

Hybridization was detected with Eco RI A,B and C, Bam H1 A,B,C and E and Hind III B,C,E,F and H. (Hybridization to Eco RI B and Bam H1 G was too weak to be photographed, but was clearly visible on the original fluorogram.) The lack of hybridization to Bam H1 D in double digests with Eco RI and Bam H1

Figure 39: Hybridization of early RNA to DNA fragments generated by restriction endonucleases.

2 x 10^6 cpm (60 µg) of early RNA (labelled 5.5 to 7.5 hours post infection) of early RNA was incubated with nitrocellulose strips bearing 2 µg of denatured, separated Ad 12 (1131) Eco RI, Bam H1, and Hind III, as described in Materials and Methods. Strip labelled Eco RI A was hybridized to early nuclear RNA, all others were hybridized to early cytoplasmic RNA. The Eco RI and Bam H1 strips were exposed 18 days and the Hind III strips were exposed 8 days.

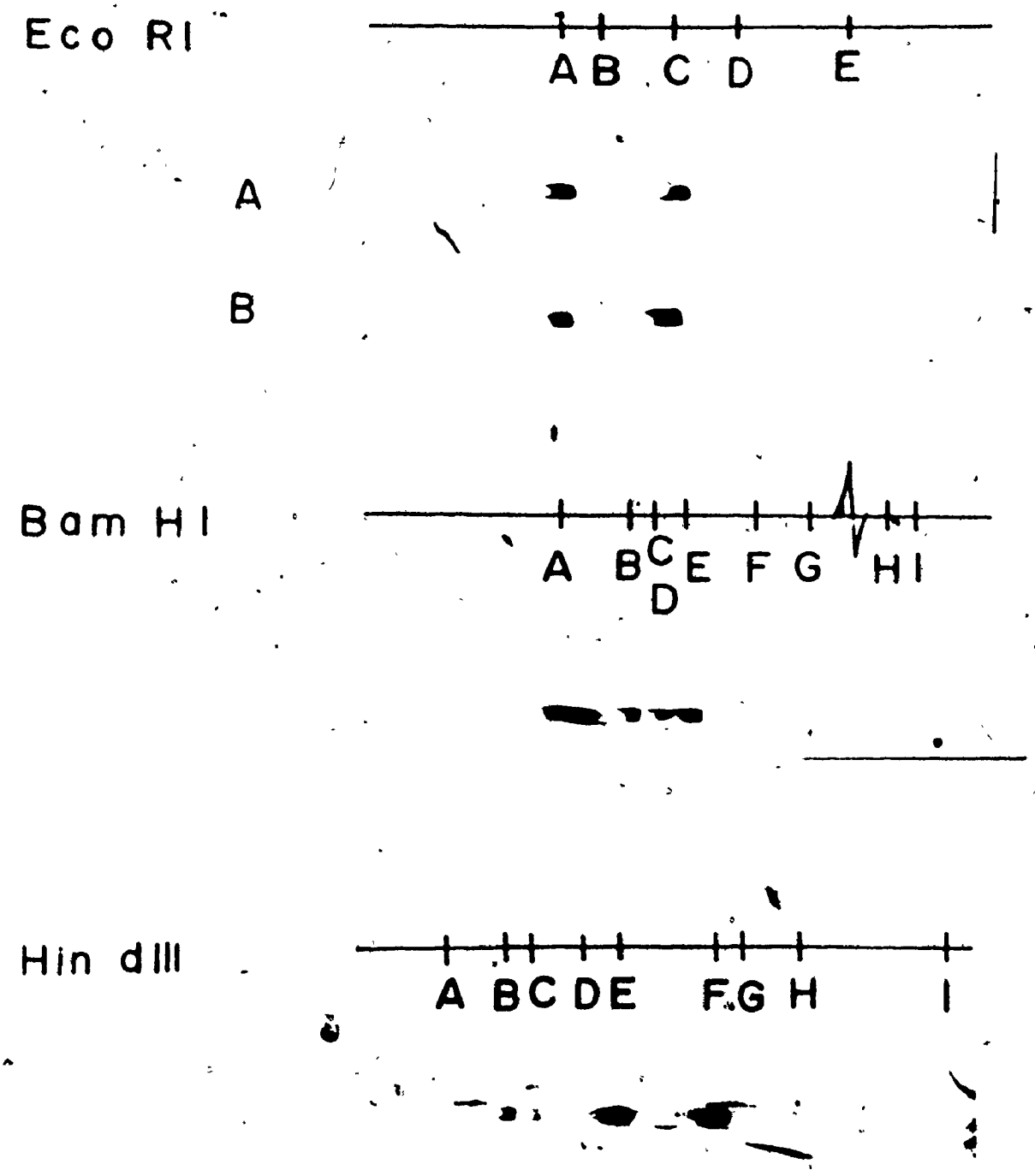

Eco RI
A B C D E
A
B
Bam HI
A B C D E F G HI
Hin dIII
A B C D E F G H I

Table 16

Distribution of Labelled Early RNA Sequences Among DNA Fragments.[1]

Fragment	Fraction of total hybridized radioactivity annealed to fragment
Eco RI A	0.47
Eco RI B	0.06
Eco RI C	0.47
Bam Hl A	0.60
Bam Hl B	0.11
Bam Hl C + D	0.09
Bam Hl E	0.21
Bam Hl G	<0.01
Hind III B	0.05
Hind III C	0.01
Hind III E	0.40
Hind III F	0.50
Hind III H	0.13
Bam Hl + Eco RI[2] I + II	0.63
Bam Hl + Eco RI III	0.00
Bam Hl + Eco RI IV	0.28
Bam Hl + Eco RI VI	.083

[1] Sixty micrograms (2×10^6 cpm) cytoplasmic RNA labelled 5.5 to 7.5 hours post infection were hybridized to 2 µg of unlabelled DNA fragments. The analysis with Eco RI and Bam Hl fragments were done on the same RNA preparation, while another lot was used for the experiments with the Hind III fragments.

[2] The identity of fragments produced by the double digestion are: I = Eco RI C, II = Bam Hl B, III = Bam Hl D, IV = Bam Hl E and VI = Bam Hl C/Eco RI α.

(Table 16) allowed the assignment of the RNA hybridizing to the Bam Hl C/D doublet to Bam Hl C. These data demonstrated that early cytoplasmic RNA is derived from at least three separate regions of the viral genome, one contained entirely within Hind III F, one within Hind III E, at least one within Bam Hl B and C. (The lack of hybridization to Hind III I allows the assignment of the RNA coding sequences within Bam Hl B and Hind III E to separate regions.) The observed hybridization of early cytoplasmic RNA to Eco RI B (6% of the total) does not agree with the data obtained with Bam Hl, where no hybridization was seen with fragments D,H,I and F, and only a very small amount (under 1% of the total) was observed with G. Similarly, no hybridization was observed to Hind III A and G, and only a small amount was observed to Hind III C (1%). The rather high levels of hybridization of early RNA to Eco RI B can perhaps be explained by postulating the existence of a minority class of rearranged viral genomes bearing sequences derived from the normal Eco RI A and/or C fragments on fragments of about the size of Eco RI B. Such genomes have been observed in Huie M populations (I. Mak, H. Ezoe and S. Mak, manuscript in preparation).

The small amounts of hybridization of early RNA to both Bam Hl G and Hind C (≤ 1% of the total) but not to Eco RI D serves to map the location of the DNA encoding this RNA to the region of overlap of these fragments, outside the region of overlap with Eco RI D. The region so defined extends from 28.5 to 30 map units. It is of interest to note that the Ad 2 specific 5.5S RNA (VA-RNA) maps to this position of Ad 2 DNA (Mathews, 1975; Petersson and Philipson, 1975) and that Ad 12 encodes an analogous RNA which is synthesized early in productive infection (Varrichio and Raska, 1976). It is apparent that the different early RNA species accumulate at widely differing rates, with RNA encoded by the molecular ends of the genome accumulating most rapidly. These RNA species together comprise from 75 to 90% of the total viral RNA detected.

7. Strand derivations of early cytoplasmic RNA. From the map of the late coding regions of the Ad 12 genome deduced above (Figure 37), it can be inferred that the early RNA species derived from Hind III F, Bam Hl B, and Hind III B and H are transcribed from the *r* strand of the genome. The strand derivation of the RNA derived from Bam Hl C and Hind III E was ambiguous.

Early RNA selected by hybridization to 2 μg each of ^{14}C-labelled *l* and *r* strands were rehybridized to unlabelled Eco RI fragments (Figure 40). RNA hybridizing to the *r* strand was derived primarily from the Eco RI A and C fragments, and to a lesser extent from Eco RI B and D, while *l* strand specific RNA hybridized to only Eco RI A and B. It is of interest to note that RNA hybridizing to Eco RI D was detectable in strand selected RNA, but not in unfractionated RNA. This is probably a result of the large quantities (600 μg) of early RNA which were hybridized to each filter bearing 1 μg of DNA to prepare the complementary strand specific RNA. If the amount of RNA used was close to saturating, concentration differences between RNA species would be reduced. Therefore, rare RNA species would be over-represented in the strand-selected RNA preparation.

Several attempts were made to rehybridize strand selected early RNA to the Bam Hl fragments. In all cases, no hybridization was observed to Bam Hl B and C. This failure is presumably a consequence of both the small amounts of early RNA synthesized complementary to these regions, and the losses of material inherent in the strand selection procedure.

The complementary strands of Eco RI A and C were resolvable electrophoretically (Figure 34). For

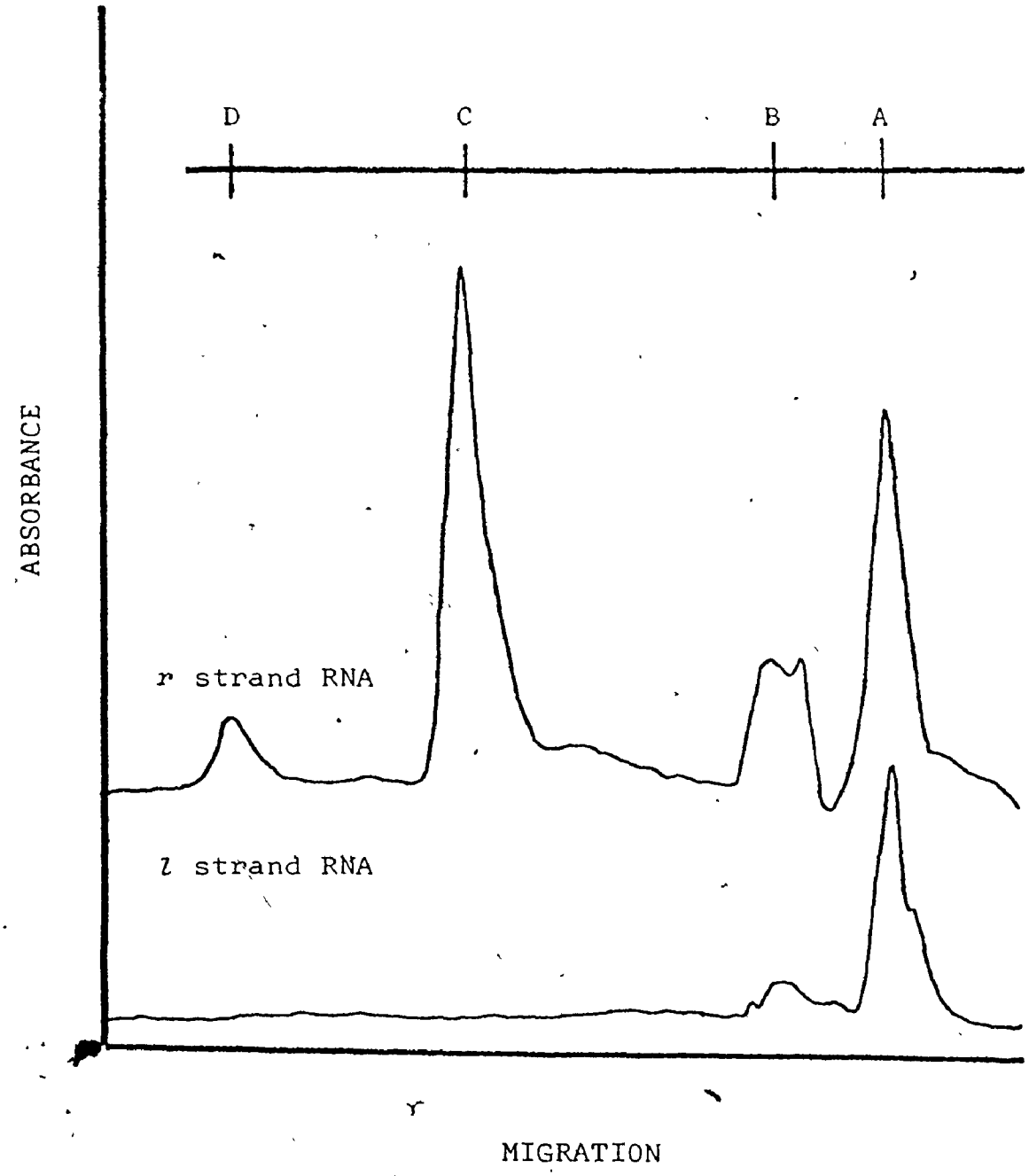


Figure 40: Hybridization of labelled early strand specific RNA to unlabelled Eco RI fragments

Cytoplasmic RNA labelled from 5.5 to 7.5 hours post infection was strand selected by hybridization to pure *l* and *r* strands as described in Materials and Methods. This RNA was hybridized to nitrocellulose strips bearing 2 µg of denatured, separated, Ad 12 (1131) Eco RI fragments, as described in Materials and Methods. Hybridization was detected as in Figure 36. Exposure was for 1 month.

this reason electrophoretic strand separation of Bam Hl C,B and E was attempted. If successful, this approach would eliminate the need for an RNA strand selection step. In order to resolve the segment of Bam Hl C bearing the early coding segment from Bam Hl D, which comigrates electrophoretically, viral DNA was cleaved simultaneously with Eco RI and Bam Hl and the resulting fragments were separated electrophoretically (Figure 41A). Fragment Bam Hl C is cleaved twice by Eco RI, generating three fragments, the largest of which (Bam Hl C/Eco RI α) bears the early coding region (see Table 16). Fragments Eco RI C, Bam Hl B and Bam Hl E are all resistant to cross cleavage, and can be directly recovered from the double digest. Gel slices containing Eco RI C (the largest fragment in the double digest, or band I), Bam Hl B (II), Bam Hl E (IV) and Bam Hl C/Eco RI α (VI) were isolated and the DNA was denatured *in situ* and re-electrophoresed as described by Horwitz (1976) (Figure 41B). Fragments Eco RI C, Bam Hl B and Bam Hl E were resolved into doublets, while denatured Bam Hl C/Eco RI α migrated as a single band.

The strand derivation of the fast and slow migrating bands of Eco RI C, Bam Hl B and Bam Hl E were determined. The relevant portion of each gel

Figure 41: Electrophoretic strand separation of some Ad 12 DNA fragments

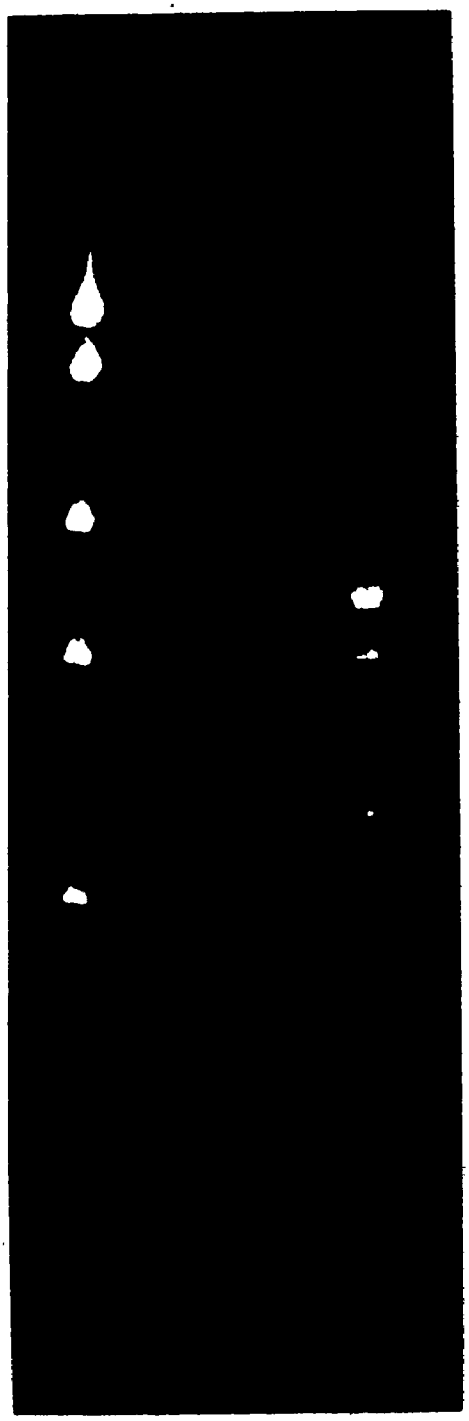
Ad 12 DNA was cleaved simultaneously with Bam Hl and Eco RI, and the resulting fragments were separated in 1% agarose gels. Fragments Eco RI C, Bam Hl B, Bam Hl E, and Bam Hl C/Eco RI α were isolated in gel slices, denatured in situ, and re-electrophoresed.

A. Electrophoretic separation of fragments produced by Eco RI (1), Eco RI + Bam Hl (2,3) and Bam Hl (4) in 1% agarose gels, allowing the identification of the origin of some of the fragments produced by double digestion.

B. Electrophoresis of denatured DNA fragments in 1% agarose gels. 1, Eco RI C DNA; 2, Bam Hl B DNA; 3, Bam Hl E DNA; 4, Bam Hl C/Eco RI α DNA. Electrophoresis was for 8 hours at 2V/cm.

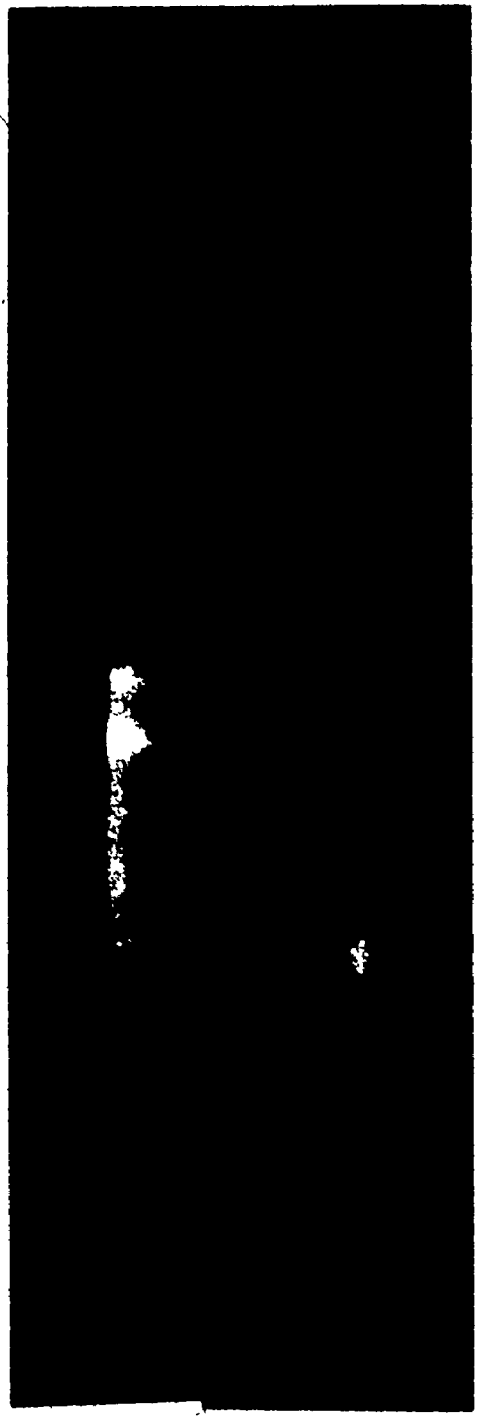
A

1 2 3 4



B

1 2 3 4



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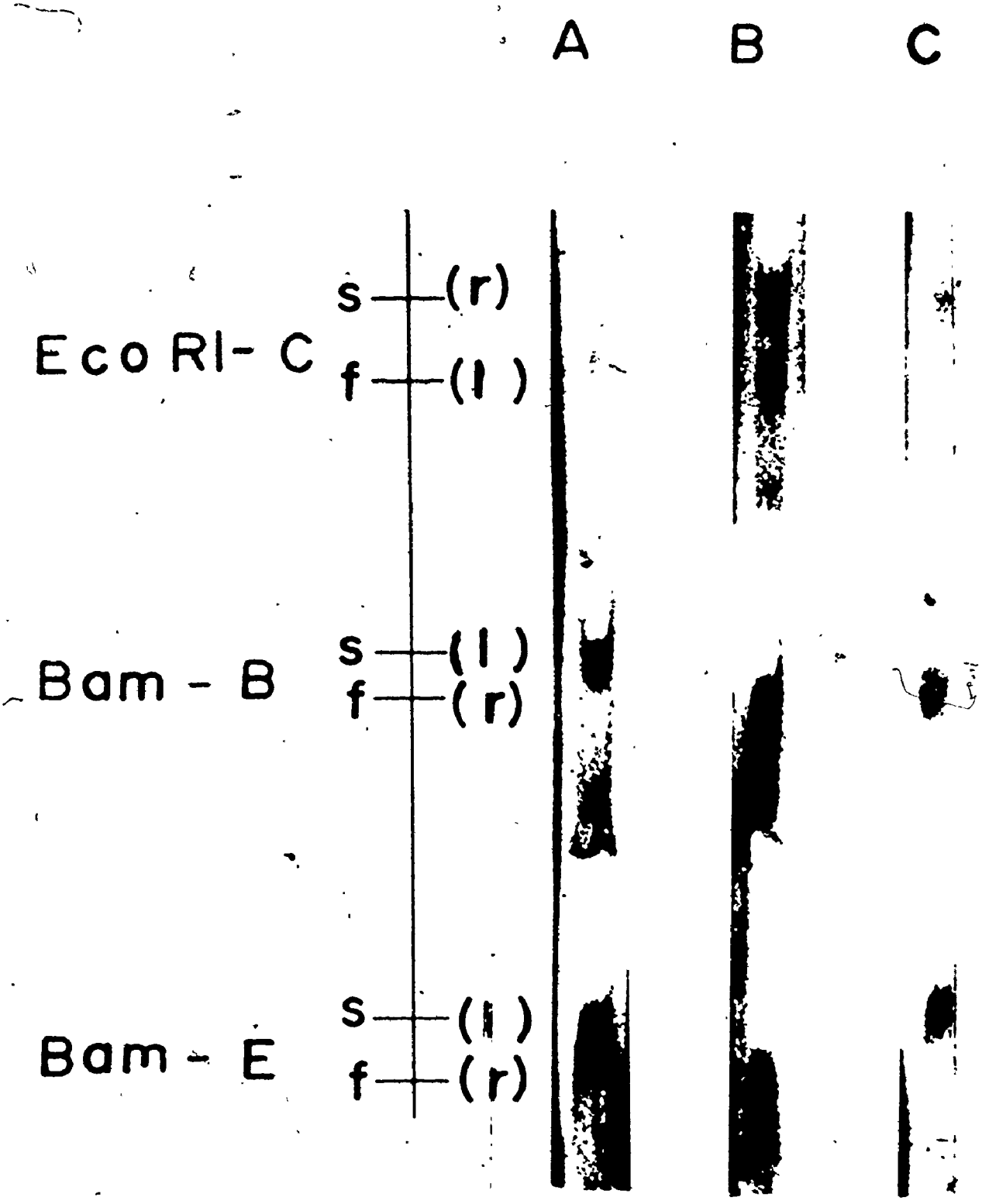
was excised and composites were made. The DNA present in the composite gels was transferred to nitrocellulose strips without further denaturation. These strips were then hybridized to purified, intact, ^{3}H-labelled *l* and *r* strands, and fluorographed (Figure 42). The intact *r* strand hybridized to the fast strand of Eco RI C and the slow strands of Bam Hl B and E, and conversely, the intact *l* strand hybridized predominantly to the slow strand of Eco RI C and the fast strands of Bam Hl B and E. The reason for the smaller amount of hybridization of the *l* strand to the fast strand of Eco RI C is not understood. These data suggest that the fast migrating band of Eco RI C is derived from the intact *l* strand, while the fast bands of Bam Hl B and E are part of the intact *r* strand.

The strand derivation of early cytoplasmic RNA transcribed from these fragments was then directly determined by hybridizing labelled RNA to such strips (Figure 42). As predicted, RNA derived from Eco RI C and Bam Hl B hybridized exclusively to DNA derived from the intact *r* strand. The RNA encoded by Bam E hybridized only to the viral *l* strand. The strand derivation of early RNA complementary to Bam Hl C was not directly determined.

Figure 42: Hybridization of ^{3}H-labelled intact *l* and *r* strand DNA, and early cytoplasmic RNA to the complementary strands of Eco RI C, Bam HI B and Bam HI E DNA.

Denatured DNA fragments were electrophoresed in agarose gels as in Figure 41, and the resulting bands were visualized under ultraviolet illumination after staining with 0.5 µg/ml ethidium bromide. The region of each gel containing the double bands was excised, and composite gels were created by end to end alignment of the excised segments. The DNA in the composites was then transferred to nitrocellulose and hybridized to 10^{4}cpm of H-labelled *r* (A) or *l* (B) DNA and to 5 x 10^{6} cpm of H-early cytoplasmic RNA (C). Hybridization was detected by fluorography. Exposure was for 2 weeks.


A
B
C
s
(r)
EcoRI- C
f
(l)
s
(l)
Bam - B
f
(r)
s
(l)
Bam - E
f
(r)

B. Viral RNA synthesized in oncogenically transformed rodent cells

As reviewed in Introduction, two separate approaches have demonstrated that transformation by group C adenoviruses (Ad 2 and Ad 5) is induced and maintained by a small segment of the viral genome mapping at the extreme left end. In addition, the collaborative efforts of F.L. Graham and S. Mak have demonstrated that the extreme left end Eco RI C fragment of Ad 12 (Huie M) DNA is sufficient for oncogenic transformation of rat kidney cells (personal communication).

The techniques of hybridization mapping used above were applied to determine which segments of the Ad 12 genome were expressed in two transformed rodent cell lines: T6, a hamster embryo cell line transformed by Ad 12 (Huie M) virus, and 702.C2, a rat kidney line transformed by purified Ad 12 (Huie M) Eco RI C fragment. 702.C2 cells were provided by Dr. F.L. Graham.

1. Viral RNA in T6 cells. Unlabelled T6 whole cell RNA, extracted at passage 40, was hybridized to purified, ^{3}H-labelled Ad 12 (1131) *l* and *r* strands

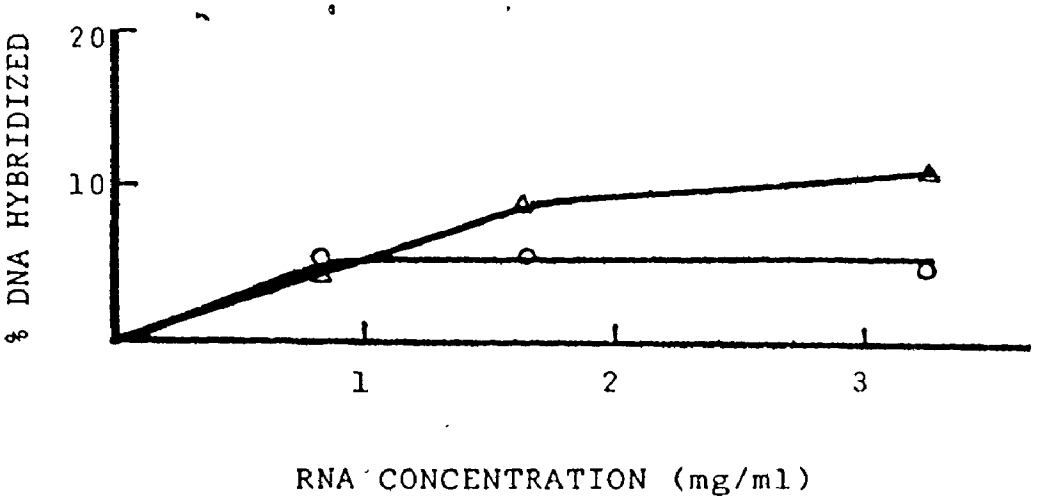


Figure 43: Hybridization of T6 RNA to the *l* and *r* DNA strands

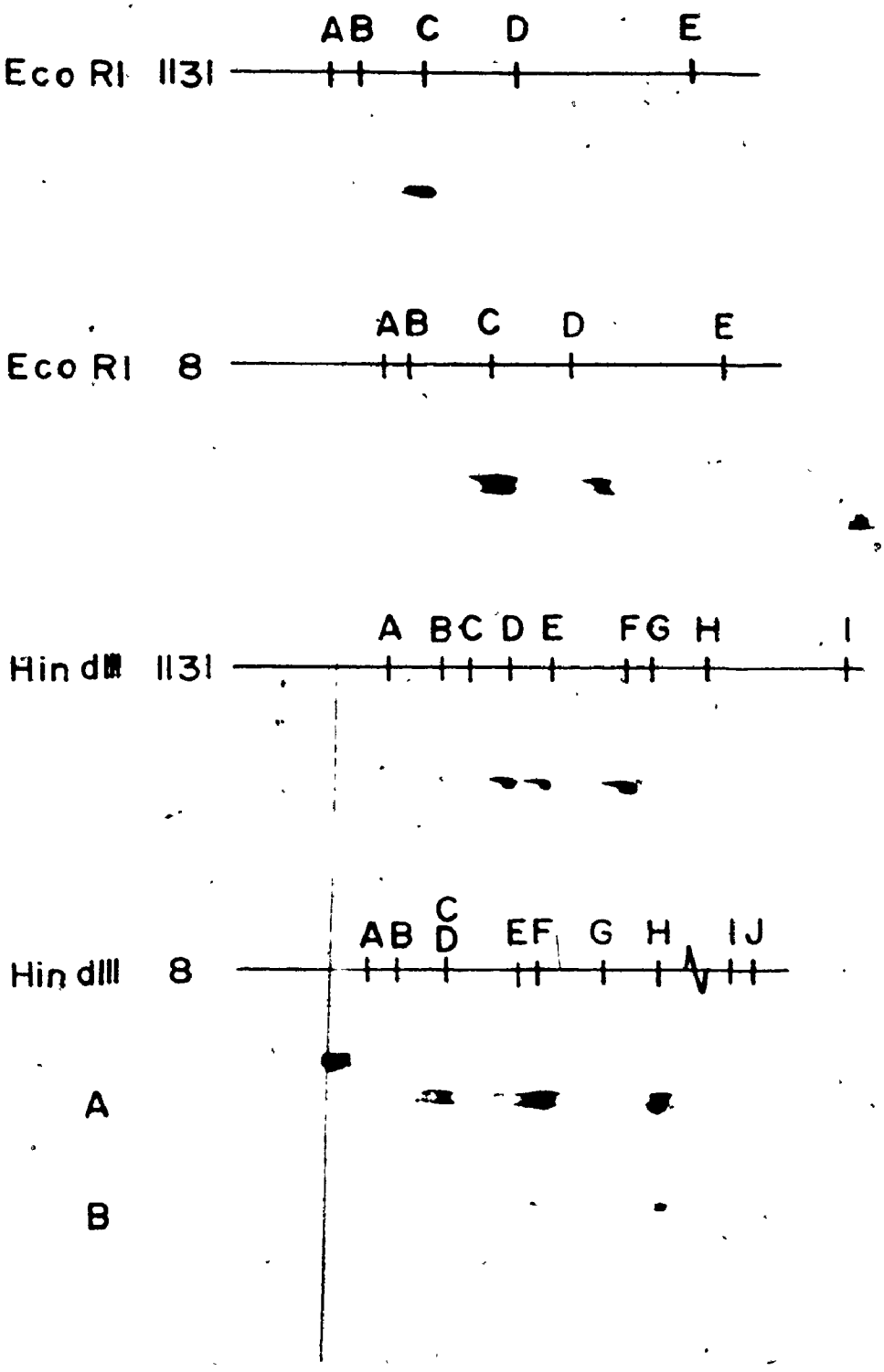
The indicated amounts of unlabelled, whole cell RNA, extracted from T6 cells at passage 40, were hybridized to purified 1131 *l* and *r* DNA (0.005 µg/ml) for 72 hours. Hybridization was assayed using S1 nuclease.

(Figure 43). At apparent saturation, about 5% of the *r* strand and 10% of the *l* strand were hybridized.

To map the regions of the viral genome expressed, passage 45 cells were labelled with 100 μCi/ml of ^{3}H-uridine for 2 hours, and the total cellular RNA was extracted and hybridized to unlabelled DNA fragments, using the blotter method (Figure 44). The Eco RI and Hind III fragments of both Huie M8 and 1131 DNA were used. As a control, primary hamster embryo whole cell RNA was hybridized to Huie M8 Eco RI fragments. No hybridization was observed. T6 cell RNA hybridized to the 1131 and Huie Eco RI C fragment, and in much smaller amounts, to the Eco RI A fragment. (Not visible in Figure 44, but clearly discernable on the original.) Hybridization was also observed to a Huie M8 Eco RI fragment migrating faster than Eco RI D. This fragment was not visible in the gels used to make the strip after ethidium bromide staining and therefore was present in small amounts. This fragment contains sequences derived from Eco RI C (I. Mak and S. Mak, personal communication). The RNA derived from the left end of the genome (Eco RI C) hybridized to both the 1131 Hind III F and D fragments, and the Huie M8, Hind III F (≡1131 F) H, and C/D fragments. Only a small fraction of the

Figure 44: Hybridization of labelled T6 RNA to DNA fragments generated by restriction endonucleases

$5. \times 10^7$ cpm of T6 RNA (labelled for 2 hours with ^3H-uridine) was hybridized to nitrocellulose strips bearing 2 µg of denatured, separated DNA fragments. The Hind III 8 A strip was hybridized to nuclear RNA, while the Hind III 8 B strip was incubated with cytoplasmic RNA. All other strips were incubated with whole cell RNA. Exposures were 30 days for the Eco RI strips, 14 days for the 1131 Hind III strips and 40 days for the Huie M8 Hind III strips.


AB C D E
Eco RI 1131
AB C D E
Eco RI 8
A BC D E FG H I
Hin dIII 1131
C
AB D EF G H IJ
Hin dIII 8
A
B

total viral RNA hybridized to the Huie M8 Hind III C/D fragment. Correlating the results obtained with the Hind III fragments of 1131 and Huie M8 DNA, it is apparent that most of the RNA which hybridized to the 1131 D fragment was complementary to the Huie M8 H fragment. This result implies that the Huie M8 H fragment is contained within Eco RI C. These results demonstrated that T6 whole cell RNA contained sequences derived from at least part of the region from 6.3 to 17 map units. Note that this region is not expressed as early cytoplasmic RNA during productive infection.

At least some of the small amounts of viral RNA detected within Eco RI A maps within the 1131 Hind III E fragment. It is possible that the region defined by 1131 Hind III J,K and L, which were excluded from the analysis, is also transcribed. The use of Bam Hl fragments could resolve this point (not done). The extremely small amounts of RNA complementary to Eco RI A (≤5% of the total viral RNA) made study of this RNA extremely difficult.

Since T6 whole cell RNA contained large amounts of RNA derived from a segment of the Ad 12 genome which is not expressed as early cytoplasmic RNA (1131 Hind III D, Huie M8 Hind III H and C/D),

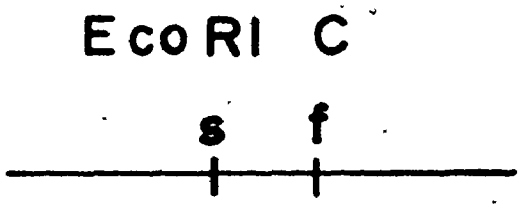
it was of interest to determine whether these RNA sequences were exported to the T6 cell cytoplasm. Figure 44 shows that both nuclear and cytoplasmic RNA hybridized to Huie M8 Hind III H fragment.

The strand derivation of the T6 whole cell RNA complementary to Eco RI C was determined by hybridizing labelled T6 RNA to the complementary strands of Huie M8 Eco RI C fragment, separated electrophoretically (Figure 45). The RNA hybridized to only the slower migrating (r) strand. The strand derivation of the rare, Eco RI A specific RNA remains unknown.

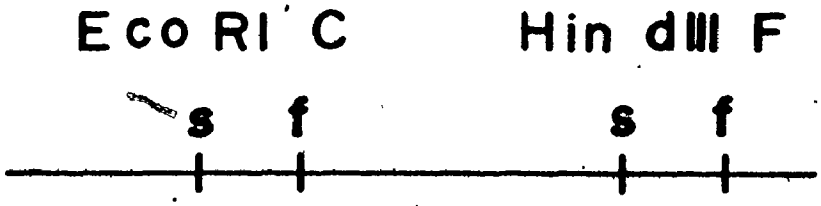
2. Viral RNA in 702.C2 cells. Labelled 702.C2 cell RNA (passage 15) was extracted. Whole cell RNA was hybridized to 1131 and Huie M8 Hind III fragments and Huie M8 Eco RI fragments; nuclear and cytoplasmic RNA was hybridized to Huie M8 Hind III fragments (Figure 46). Hybridization was observed to 1131 Hind III F and D, Huie M8 F,H and C/D, and Huie M8 Eco RI C. Nuclear RNA also appeared to hybridize to Huie M8 Hind III E, and to a high molecular weight fragment not visible in the gel by eye. Because the background radioactivity on this strip was quite high, it is not clear whether the apparent hybridization of nuclear RNA to Huie M8 Hind III E

Figure 45: Hybridization of labelled T6 and 702.C2 cell RNA to the separated strands of left end DNA fragments

The complementary strands of Ad 12 Huie M8 Eco RI C and Hind III F DNA were separated electrophoretically, as detailed in Materials and Methods. The gels were cut between the two bands, and the separation between the bands was artificially enhanced by the insertion of 1 cm spacers of 1% agarose, to facilitate alignment of the fluorograms. The DNA was then transferred to nitrocellulose and hybridized to 5×10^7 cpm of 702.C2 (passage 15) and T6 (passage 45) cell RNA. Exposure was for 40 days.



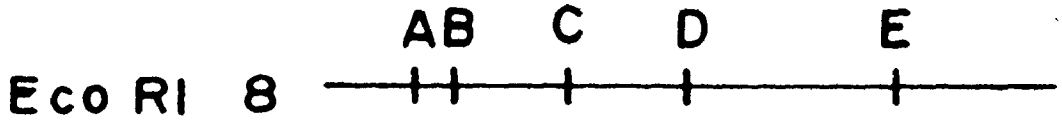
T6

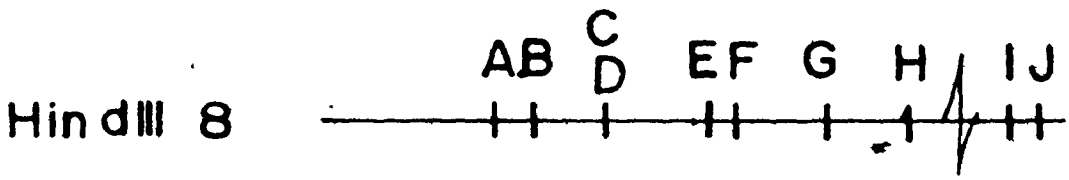


702.C2

Figure 46: Hybridization of labelled 702.C2 cell RNA to DNA fragments generated by restriction endonucleases

5×10^7 cpm of 702.C2 RNA (passage 15) was hybridized to nitrocellulose strips bearing 2 µg of denatured, separated DNA fragments. The Hind III 8 A strip was hybridized to nuclear RNA while the Hind III 8 B strip was hybridized to cytoplasmic RNA. The other strips were hybridized to whole cell RNA. Exposures were 40 days for the Eco RI and Hüie M8 Hind III strips and 7 days for the 1131 Hind III strips.





A

B

is real or an artefact. In another experiment, 702.C2 whole cell RNA was not observed to hybridize to Huie M8 Hind III E. In addition, the RNA hybridized to the minority Huie M8 Eco RI fragment which migrates faster than Eco RI D. These results are identical to those observed with T6 RNA, with the exception that no RNA hybridizing to Eco RI A or 1131 Hind III E was observed. This difference was expected as this cell line was transformed by purified Huie M8 Eco RI C fragment. As was observed with T6 RNA, all viral RNA sequences detected were found in both the nucleus and cytoplasm. The viral RNA detected in 702.C2 cells was derived exclusively from the slow (r) strand of Huie M8 Eco RI C DNA, and the slow strand of the Huie M8 Hind III F fragment (Figure 45). This result demonstrated that the slow strand of Hind III F is part of the intact r strand.

The sequence complexity of cytoplasmic 702.C2 RNA was estimated by saturation hybridization of unlabelled RNA to labelled slow (r) strand of Huie M8 Eco RI C DNA (Figure 47). At saturation, 50 to 60% of the DNA, corresponding to 8 to 10% of the intact r strand, was hybridized.

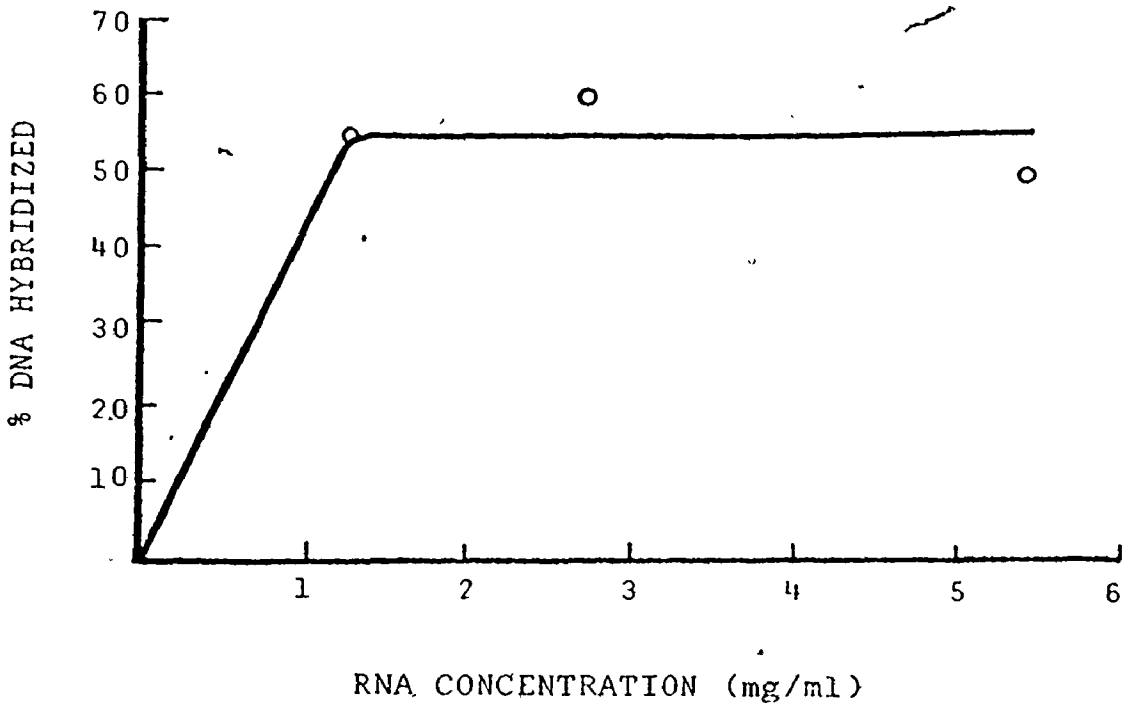


Figure 47: Hybridization of 702.C2 RNA to the *r* strand of Ad 12 Huie M8 Eco RI C DNA

The indicated quantities of 702.C2 cell cytoplasmic RNA (extracted at passage 15) were hybridized to 0.005 µg/ml of ^{3}H *r* strand of Huie M8 Eco RI C DNA, for 72 hours. Hybridization was assayed by S1 nuclease. The *r* strand DNA was recovered from 1% agarose gels by the freeze-squeeze method.

DISCUSSION

I. The Sites on the Ad 12 (1131) Genome Expressed as Cytoplasmic RNA During Productive Infection

A. Positions of the early and late genes

Saturation hybridization of labelled, complementary DNA strands with unlabelled late cytoplasmic RNA demonstrated that this RNA is derived from about 0.65 of the *r* strand and 0.15 of the *l* strand of the viral genome (Figures 19 and 22), values similar to those determined for Ad 2 RNA by other authors (Tibbetts *et al.*, 1974; Wold *et al.*, 1976).

The methods used to map these DNA sequences on the viral genome (section IV.2.) have several advantages over the more conventional ones. First, the requirement for strand separating the individual DNA fragments generated by restriction endonucleases is bypassed. Second, the transcribed segments of each strand can be hybridized to any set of ordered DNA fragments to refine the map with a minimum of additional effort. The method also has several drawbacks. The

results give no information concerning the relative abundance of the RNA species detected, and rather large quantities of virus specific RNA are required to prepare the DNA probes used. Because the probes are annealed to a large excess of unlabelled DNA fragments to deduce the map, any unusual fragments generated by cleavage of minority rearranged genomes could confuse the analysis. In addition, since the fraction of the total hybridized radioactivity which has bound to each fragment is used to calculate the degree of complementarity of each probe with each fragment, these estimates are not independent. For example, if the degree of complementarity of the probe to Eco RI A is underestimated, the degree of complementarity to the other Eco RI fragments (all other parameters being equal) will be proportionally overestimated. Thus it is difficult to assess the reliability of the method solely by determining the reproducibility of an experiment.

The validity of the approach was more rigorously tested by hybridizing the *l*+ and *r*+ DNA probes to three distinct sets of DNA fragments in which identical DNA sequences were borne on fragments of widely differing size. The close quantitative agreement between the three data sets strongly suggests that the method is reliable. In particular, the possibility

that the efficiency of hybridization of the labelled probe to the separated fragments varies in a systematic way with fragment size is eliminated.

Despite the fact that Ad 12 DNA shares few nucleotide sequences with that of Ad 2 and 5, the map of the sites on the Ad 12 genome expressed as late cytoplasmic RNA is similar, if not identical, to those recently obtained by other workers with the group C adenoviruses (Pettersson *et al.*, 1976; Sharp *et al.*, 1974; Flint *et al.*, 1976). A possible exception is the region of Ad 12 DNA extending from approximately 17 to 25 map units which is apparently not transcribed into stable cytoplasmic RNA (compared in Figure 48).

Early cytoplasmic RNA is complementary to 17 to 20% of the *r* strand and about 15% of the *l* strand, again similar to values obtained with Ad 2 (Sharp *et al.*, 1974; Wold *et al.*, 1976) (Figures 21 and 22). Mixing experiments suggested that early cytoplasmic RNA forms a subset of late cytoplasmic RNA, and further, that most or all of the late-specific cytoplasmic RNA sequences are transcribed from the *r* strand of the viral genome (Table 8). The map positions of these early coding regions were determined by hybridizing labelled early cytoplasmic RNA to unlabelled DNA fragments (section IV.6). It must be emphasized that

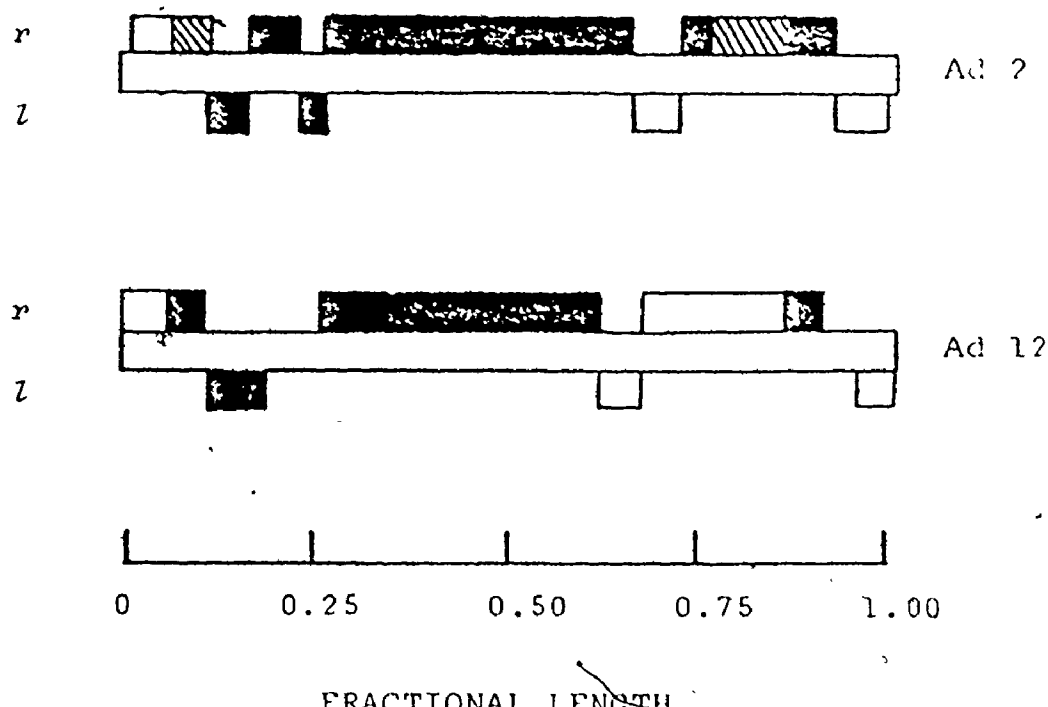


Figure 48: Comparison of the sites on the Ad 2 and Ad 12 genomes expressed as cytoplasmic RNA

The Ad 12 map is that deduced by correlating the data obtained in this study with that of Ortin *et al.* (1976), while the Ad 2 map is based on a correlation of the data of Pettersson *et al.* (1976) and Craig *et al.* (1975A; 1975B). Open bars represent Ad 2 class I early RNA and Ad 12 early RNA coding sequences; stipled bars denote Ad 2 class II early regions; while filled bars designate the Ad 2 and Ad 12 late regions.

this approach does not yield a direct estimate of the size of each early coding region. Indirect inferences can be made on the basis of the sizes of the DNA fragments to which the RNA hybridizes. Early cytoplasmic RNA is derived from the *r* strand of Hind III F and Bam Hl B, and the *l* strand of Hind III E. A segment of Bam Hl C, outside the region of overlap with Eco RI E, is also represented in early RNA. Because Bam Hl C could not be electrophoretically strand-separated the polarity of this early RNA was not directly determined. Very recently, Ortin *et al.* (1976) have examined the Ad 12 (Huie V) early cytoplasmic RNA by saturation hybridization of the complementary strands of DNA fragments generated by Eco RI and Bam Hl, separated by the poly (U,G) method. These authors detected four separate early coding regions on the genome, on the *r* strand of Eco RI C and Bam Hl B, and the *l* strand of Bam Hl C and E. These assignments are entirely in agreement with the data obtained in this study, and further demonstrate that the early segment within Bam Hl C is transcribed separately from that within Bam Hl B.

A correlation between the data of Ortin *et al.* (1976) and that obtained in the present study allows a quite precise positioning of these four early regions.

The leftmost region, transcribed from the *r* strand, maps between 0 and 6.3 map units (entirely within Hind III F). Ortin *et al.* have estimated the size of this region as about 7.5% of Ad 12, implying that most or all of the DNA between 0 and 6.3 map units encodes early RNA. The early region within Bam Hl C, transcribed from the *l* strand, maps between the Eco RI E - F junction (62 map units) and the leftmost end of Hind III B (68 map units). This RNA is presumably identical to the *l* strand-specific RNA detected late which maps to this position, which is derived from 4 to 5% of the genome. Ortin *et al.* have estimated the size of this region as about 4% of Ad 12.

The early region within Bam Hl B does not extend leftward into Bam Hl C (Ortin *et al.*, 1976), or past the Hind III H - I junction to the right (early RNA hybridized to Hind III H, but not to I). The maximum possible size of this region is then 15 map units, identical to the estimate of Ortin *et al.* The right-most early region, transcribed from the *l* strand, comprising 4 to 5 map units, maps entirely to the right of 91 map units.

These considerations imply that about 0.21 of the *r* strand and 0.08 to 0.10 of the *l* strand encodes early cytoplasmic RNA. The value for the *r* strand

agrees well with that obtained by saturation hybridization (Figures 21 and 22), but saturation hybridization of the *l* strand consistently yielded 0.15 as the fraction transcribed early. This value was also obtained with late RNA, and a mixture of early and late cytoplasmic RNA hybridized the same fraction of the *l* strand as did early RNA alone. It is clear, however, that the *l* strand specific RNA derived from Eco RI C and D, found late, is not detectable in the early cytoplasm by the filter hybridization method. RNA comprising under 1% of the total virus-specific radioactivity would have been detected. This apparent disagreement may merely reflect the differing sensitivities of the two hybridization methods used: close inspection of Figure 21 reveals that 0.15 of the *l* strand was hybridized at only the highest input RNA concentration, while the *r* strand was saturated by even the lowest RNA concentration used. This result is consistent with the presence of a rare RNA complementary to the *l* strand of Eco RI C. This hypothetical RNA was not directly detected in the early cytoplasm, and is not considered to be an "early" class in the discussion below.

As argued in the Results (section IV.A.6.), small amounts of RNA ($\leq$1% of the total viral RNA detected), derived from 28.5 to 30 map units, were

detected in the cytoplasm early post infection. The Ad 2 5.5 S RNA (VA RNA) is encoded by the analogous segment of the Ad 2 genome (Mathews, 1975; Pettersson and Philipson, 1975). Since Ad 12 encodes a VA RNA which is synthesized both early and late during productive infection (Varrichio and Raska, 1976) it is reasonable to presume that this result maps the coding location of Ad 12 VA RNA.

Knowledge of the positons of the early coding segments allows the identification of the coding regions for the exclusively late cytoplasmic RNA. The positions of these two classes of coding sequences are indicated in Figure 48. The majority of the exclusively late RNA sequences are encoded by a contiguous segment of the *r* strand mapping between about 25 to 60 map units. The extensive homology between the transcription maps of Ad 2 and Ad 12 is obvious, and will be discussed below. A difference of particular interest is the smaller size of the left end, *r* strand-specific early region of Ad 12. As will be argued below, it is possible that the assignment of the right end of this block as an "early" class in Ad 2 is due to a drug-induced artefact. All studies published to date purporting to map Ad 2 "early" RNA have used RNA synthesized in the presence of either cycloheximide or cytosine arabinoside.

The lack of synthesis of late polypeptides before the onset of viral DNA synthesis (reviewed in Introduction) can be accounted for by the absence of late mRNA (in particular, see Lewis *et al.*, 1975;1976).

B. Rates of accumulation of specific classes of viral RNA

The relative degree of hybridization of labelled cytoplasmic RNA to excess, unlabelled DNA fragments (Tables 15 and 16) can be taken as an estimate of the relative rates of accumulation of the RNA derived from each of these fragments. If the fraction of each fragment serving as template for the synthesis of these RNAs is known, then the specific accumulation rate of the RNA can be calculated (defined as the radioactivity incorporated into an RNA species divided by the size of the DNA template encoding that RNA). This value is simple to obtain for early RNA: the fraction of the total hybridized virus specific radioactivity which annealed to a given fragment is divided by the maximum possible size of the segment of that fragment encoding the RNA. Since precise estimates of the sizes of the DNA segments *actively transcribed* at late times are not available, the data for late RNA are normalized to total fragment sizes. When the data

obtained with early and late RNA are compared in this fashion, it is apparent that the RNAs actively synthesized and transported to the cytoplasm at both early and late times differ widely in their rates of accumulation (Figure 49). The major RNA species synthesized and transported to the cytoplasm early are derived from the molecular ends of the genome, from opposite strands (Hind III E and F). Smaller amounts are detected from Bam Hl B and C. By 24 to 26 hours post infection the pattern has drastically changed, with RNA derived from about 25 to 60 map units being by far the most rapidly accumulating class. This RNA is derived from the largest region of the genome encoding exclusively late RNA. The corresponding region of Ad 2 encodes the major virion proteins (Lewis *et al.*, 1975).

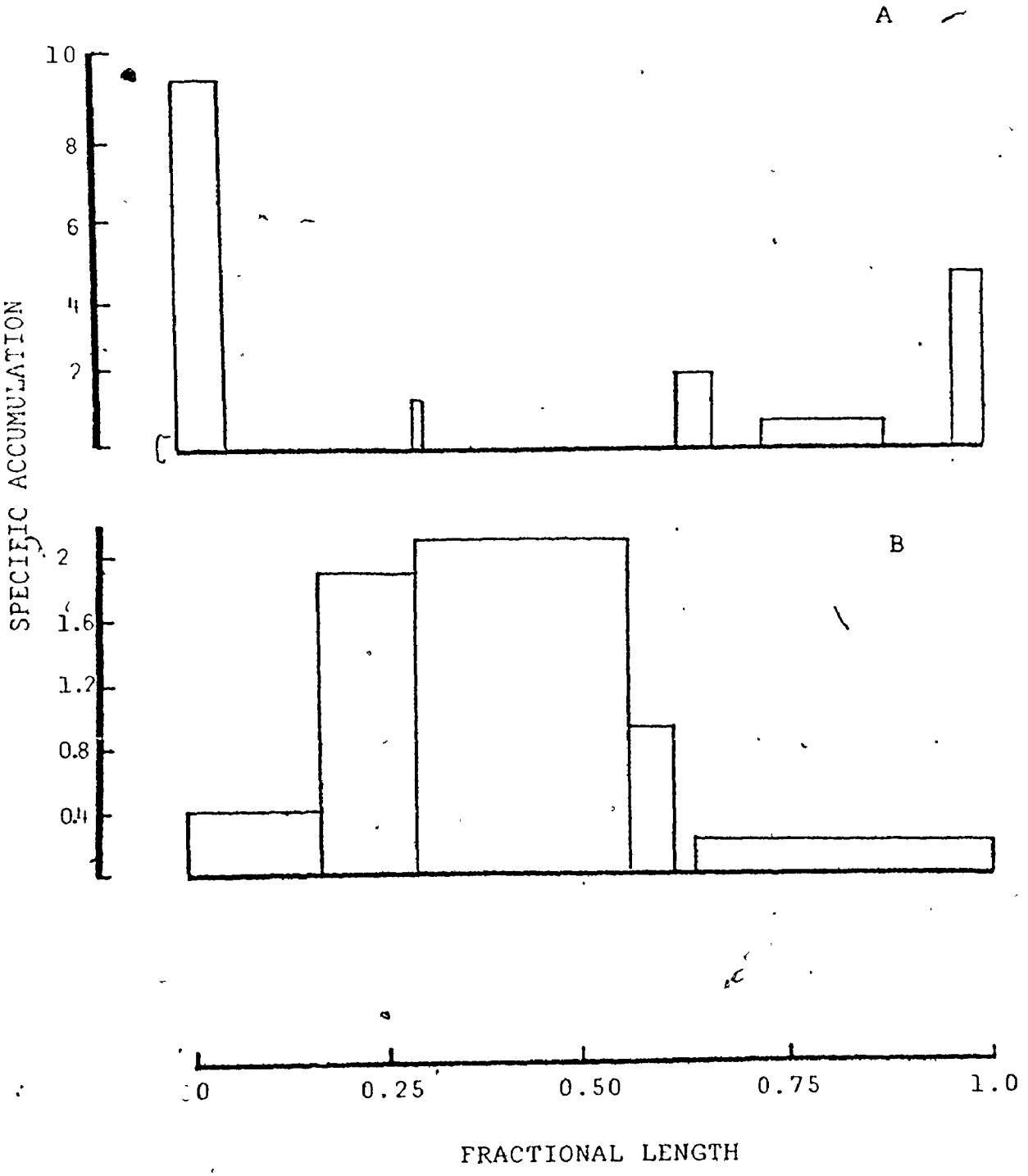
These results show that both the "early" and "late" cytoplasmic RNAs can be subdivided according to their rate of accumulation. By examining only two periods post infection four classes of RNA were distinguishable on this basis: 1, RNA which accumulates rapidly early (derived from Hind III E and F); 2, those early RNA species which accumulate more slowly (Bam Hl B and C); 3, RNA not detectable early which accumulates rapidly late (derived from Eco RI B,D and E); 4, late-specific RNA which accumulates more slowly (for example, from the *l* and *r* strands of Eco RI C).

Figure 49: Correlation of the rates of accumulation of cytoplasmic RNA complementary to DNA fragments with map position

The data obtained with the Bam Hl fragments and early RNA, and with Eco RI fragments and late RNA, shown in Tables 15 and 16, are displayed. Specific accumulation rates for early RNA species were calculated by dividing the fraction of the total early RNA found in each class by the maximum possible fractional size of the corresponding early coding region. Specific accumulation rates for late cytoplasmic RNA were obtained by dividing the fraction of the total radioactivity hybridized to each fragment by the fractional size of that fragment.

A. Cytoplasmic RNA labelled 5.5 to 7.5 hours post infection.

B. Cytoplasmic RNA labelled 24 to 26 hours post infection.


A
10
8
6
4
2
B
2
1.6
1.2
0.8
0.4
SPECIFIC ACCUMULATION
0
0.25
0.50
0.75
1.0
FRACTIONAL LENGTH

It is interesting to compare the findings to those of workers examining Ad 2 RNA. Craig and Raskas (1974A) have identified two classes of Ad 2 early RNA, distinguished on the basis of their relative abundance late in infection. Class I early RNA is not synthesized late, and is present in reduced amounts at late times compared to class II early RNA, which is synthesized throughout lytic infection. These studies used RNA synthesized in the presence of cycloheximide, and must be interpretated with caution; cycloheximide has a drastic and poorly understood stimulatory effect on viral RNA synthesis (Craig and Raskas, 1974B; Wold *et al.*, 1976). Using restriction endonuclease fragments of Ad 2 DNA the coding regions of class I and II early RNA have been localized on the viral genome (Craig *et al.*, 1975A; 1975B). Class I early RNA maps to between 0 and 7.5 map units, 58.5 and 70.7 map units and 89.7 and 100 map units. Class II early RNA is derived from DNA between 7.5 and 17.0 map units and 75.9 and 83.4 map units (see Figure 48). Consistent with these data is the finding that Ad 2 RNA derived from the leftmost 7.3% of the genome is not transcribed late (Chinnadurai *et al.*, 1976). The present data do not indicate whether the analogous leftmost Ad 12 RNA (derived from Hind III F) is transcribed late. It is interesting to note that no

Ad 12 cytoplasmic RNA derived from 6.3 to about 17 map units (analogous to the left end, class II Ad 2 early RNA) is detectable early. Such *r* strand specific RNA is found both late during Ad 12 productive infection and in both lines of Ad 12 transformed rodent cells examined. Therefore the RNAs encoded by the left and right portions of this region (0 to 17 map units) are differentially regulated in Ad 12.

The Ad 2 early RNA transcribed from Eco RI D is subject to class II control. The analogous RNA of Ad 12 (from the *r* strand of Bam Hl B) may also be transcribed late. It clearly does not accumulate at late times at a rate comparable to the major late-specific class.

Both Ad 2 *l* strand specific early RNA sequences are subject to class I control (Craig *et al.*, 1975A), and consistent with this finding, at late times post-infection no newly synthesized *l* strand specific cytoplasmic RNA can be detected (Philipson *et al.*, 1974), while large quantities of *r* strand specific RNA are synthesized. At least one of the early Ad 12 *l* strand specific RNA's is synthesized late, demonstrated by the fact that *l* strand specific RNA labelled 21 to 24 hours post infection hybridized to both Eco RI A and C (Figure 38). It is possible that the labelling

time used in this study are not comparable to the 16 to 18 hours period used by Philipson *et al.* (1974) with Ad 2, and that an analysis of RNA synthesized later would reveal only *r* strand transcription. It is worth noting, however, that the maximal rates of viral DNA and RNA synthesis occur at 24 hours post infection with Ad 12 (Mak and Green, 1968), while this occurs at 18 hours post infection with Ad 2 (Thomas and Green, 1969).

The finding that only the *r* strand of Ad 2 DNA is transcribed to yield cytoplasmic RNA at late times (Philipson *et al.*, 1974) demonstrates that the accumulation of the *late specific l* strand RNA of Ad 2 is regulated differently from that of the late *r* strand RNA. This *l* strand RNA may be synthesized at an intermediate time, beginning after the onset of DNA replication. The corresponding Ad 12 late specific *l* strand RNA may also be maximally synthesized at intermediate times; by 24 to 26 hours post infection RNA derived from between positions 25 and 60 of the *r* strand accumulates far more rapidly.

Several considerations suggest that the Ad 2 early *l* strand specific RNA derived from about 60 to 65 map units (and its Ad 12 homologue, encoded by Bam Hl C) is a quasi-late class. This RNA encodes the

adenovirus-specific nuclear single stranded DNA binding protein (Lewis *et al.*, 1976) which is required for viral DNA replication (van der Vliet *et al.*, 1975). This protein is detectable before the onset of viral DNA replication, and increases in concentration dramatically, concurrent with the onset of replication, reaching a constant level at an intermediate time post infection (Gilead *et al.*, 1976). Correlated with these findings, the level of the mRNA for this protein increases from 350 copies per cell at 8 hours post infection to 2600 copies per cell by 18 hours (Flint and Sharp, 1976), while the levels of mRNA derived from the left end *r* strand region do not increase. Thus both the synthesis of this mRNA and its coded protein occur maximally after DNA replication has begun. The low rate of accumulation of the analogous Ad 12 RNA observed well before the onset of DNA replication is consistent with a similar pattern of regulation.

Flint and Sharp (1976) have quantitated the levels of Ad 2 nuclear and cytoplasmic RNA derived from different segments of the viral genome at various times post infection. At both 18 and 32 hours, RNA derived from 65 to 80 map units, encoded by the *r* strand, is the most abundant class in both the nucleus and cytoplasm. At 32 hours post infection this RNA

class comprises over half of the total viral cytoplasmic RNA. The present data indicate that during the interval from 24 to 26 hours post infection with Ad 12, RNA derived from 25 to 60 map units accumulate most rapidly. Since the labelling time chosen corresponds to the time at which the maximal rate of viral RNA synthesis occurs this RNA will presumably eventually constitute the most abundant cytoplasmic class. This may indicate a real difference in the fine control of Ad 2 and Ad 12 RNA accumulation.

A correlation of the findings reviewed above reveals that different classes of Ad 2 RNA are differentially regulated, and further, that counterparts of all four classes of Ad 12 RNA distinguished above have been observed by various workers with Ad 2. RNA accumulating maximally early is encoded by the left end of the leftmost *r* strand region of both genomes (this study, Craig *et al.*, 1975A; Flint and Sharp, 1976). The DNA binding protein is encoded by RNA which accumulates slowly before the onset of DNA replication and more rapidly thereafter (Flint and Sharp, 1976; Gilead *et al.*, 1976); RNA accumulating only after the onset of DNA replication, but at a reduced rate later in infection is encoded by the late specific *l* strand region(s) (Pettersson *et al.*, 1976; Sharp *et al.*, 1974; Philipson *et al.*, 1974); and RNA which

accumulates maximally very late is encoded by the *r* strand (Philipson *et al.*, 1974; Flint and Sharp, 1975). Other classes of viral RNA may exist.

II. Nuclear Viral RNA, and the Precursor of mRNA

The nuclei of cells infected with Ad 12 contain RNA complementary to greater than 90% of the *r* strand and at least 35% of the *l* strand late during infection (Figure 19). Consistent with these findings, it was possible to isolate duplex RNA complementary to about 30% of both DNA strands from self-annealed late RNA (Figure 20). Late cytoplasmic RNA annealed to only 65% of the *r* strand and 15% of the *l* strand, and there is no evidence in the mapping data to suggest that any of these sequences are self-complemenatry. It is clear that RNA sequences complementary to at least 25% of the *r* strand and 20% of the *l* strand are confined to the nucleus. Since RNA hybridizing to 35% of the *l* strand was detected in the presence of RNA complementary to greater than 90% of the *r* strand, the complementary nuclear RNA sequences must be present in near equimolar concentrations. Otherwise the presence of the more abundant class would prevent the detection of its complement.

Late whole cell RNA hybridized to only 75% of the *r* strand and 15% of the *l* strand, implying that some component of whole RNA interfered with the detection of nuclear RNA complementary to at least 20% of the *l* strand and about 15% of the *r* strand. This finding may be explained by hypothesizing that some rare nucleus-specific RNA sequences are complementary to more abundant cytoplasmic RNA. Rapid formation of RNA-RNA duplexes would render the rarer nuclear RNA unavailable for the DNA-RNA reaction. One of several possible models for the distribution of the regions coding for nuclear-specific RNA, consistent with these findings, is presented in Figure 50.

Regardless of the precise distribution of these sites (particularly the *l* strand sites), it is clear that hybridization experiments employing whole cell RNA can give widely varying results, depending on the ratio of the concentrations of cytoplasmic and nuclear-specific RNA. One can envision a continuum of possible results, ranging from detection of 100% or more of one DNA strand equivalent, to detection of only cytoplasmic RNA sequences, depending both on this ratio and on the sensitivity of the hybridization analysis. For this reason, the interpretation of the early experiments using only whole cell RNA, and in particular those not using the separated DNA strands as probes, is

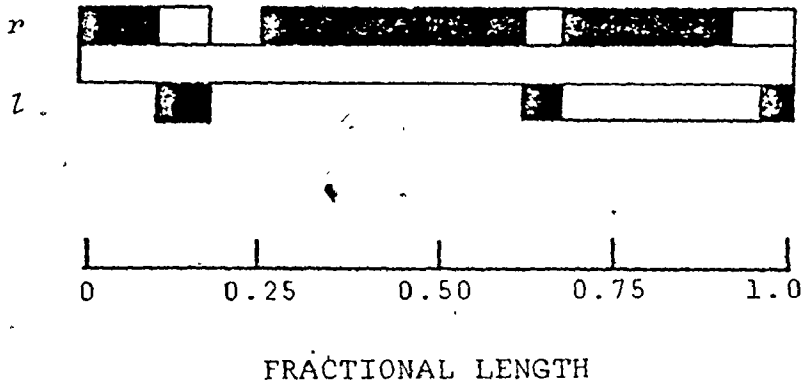


Figure 50: Possible distribution of DNA segments encoding late nucleus-specific RNA

A distribution of late nucleus-specific RNA coding regions consistent with the data presented in section II.B. is shown. Filled regions denote segments encoding late cytoplasmic RNA (from Figure 37) while open blocks represent those encoding nucleus specific RNA.

difficult. Investigators working with Ad 2 RNA have found late nuclear RNA complementary to 100% of both DNA strands (Sharp *et al.*, 1974).

Early whole cell RNA consistently hybridized to 45% of the *r* strand, and 15% of the *l* strand, while early cytoplasmic RNA hybridized to only about 20% of the *r* strand and 15% of the *l* strand. Experiments with early nuclear RNA were inconclusive. The results with whole cell RNA suggest that early nuclei contain sequences complementary to 25% of the *r* strand which are not found in the cytoplasm. Nuclear RNA labelled from 5.5 to 7.5 hours post infection did not hybridize to Eco RI D and E, and the degree of hybridization to the other fragments was not noticeably different than was observed with the corresponding cytoplasmic RNA (Figure 39). This result suggested that nucleus-specific early RNA is either not detectable by this method, or is transcribed only from Eco RI A,B and C. The recent results of Ortin *et al.* (1976) suggest that some Ad 12 nucleus-specific early RNA is derived from Eco RI D and E, favouring the former explanation. It should be noted, however, that these authors found that nuclear early RNA hybridizes to 55% of the *l* strand and 20% of the *r* strand, values strikingly different from those consistently obtained with whole cell RNA in this

study (15% *l*, 45% *r*). The reasons for such disagreement are not clear. Workers dealing with Ad 2 RNA have claimed to detect early nuclear RNA complementary to all of both DNA strands (Sharp *et al.*, 1974). These studies are of dubious value, however, as "early" RNA was obtained 8 hours post infection from cells incubated with cytosine arabinoside. Beard *et al.* (1976) have clearly shown that neither 5-fluorodeoxyuridine nor cytosine arabinoside block the onset of late transcription in polyoma virus infected cells (Ad 2 DNA replication begins 4 to 5 hours post infection in the absence of drugs). It should be apparent from the above discussion that the fraction of the adenovirus genome transcribed in the nucleus of infected cells at early times is not known. The results of the present study suggest that RNA derived from at least 60% of one DNA strand equivalent is present, provided that the RNA detected is derived from assymetrically distributed DNA sequences.

Regardless of the precise sequence complexity of early and late nuclear RNA, it is clear that some RNA sequences found in the nucleus are not detectable in the cytoplasm. Perhaps they are not recognized by a specific RNA processing and/or transport mechanism, or perhaps they are degraded in the cytoplasm.

The existence of nuclear-specific RNA sequences, and the complementarity between cytoplasmic RNA and

some of the nuclear-specific RNA sequence helps to explain the seemingly contradictory results obtained in the experiments designed to examine the sequence relationships between early and late whole cell RNA (Sections I.A.3.; I.B.3.; and II.B.1.). Early RNA hybridized to some of the DNA sequences which did not react with late whole cell RNA (Figure 7). However, when early and late whole cell RNA were mixed and hybridized to purified *l* and *r* strands (Table 7) the mixture hybridized the same fraction as late whole cell RNA alone. Note that the experiment depicted in Figure 7 was carried out in the *absence* of late whole cell RNA. The late RNA preparation used to isolate the positive and negative DNA sequences hybridized about 45% of the viral DNA, or about 90% of one DNA strand equivalent. As argued above, the DNA complementary to rare, nuclear-specific RNA sequences, which themselves are complementary to a more abundant cytoplasmic RNA, would not be expected to hybridize to late whole cell RNA, and would therefore be included in the negative DNA fraction. If early nuclear RNA contained some of the nuclear-specific sequences found in late nuclear RNA, which are complementary to late, but not early, cytoplasmic RNA, the early whole cell RNA would hybridize to negative DNA, while late whole

cell RNA would not. However, a *mixture* of early and late whole cell RNA would be expected to yield varying results in summation-hybridization experiments, depending on the ratio of early to late RNA, the ratio of nuclear specific to cytoplasmic RNA in each preparation, and the sensitivity of the hybridization analysis. Mixing experiments between early and late whole cell RNA were found to yield varying results.

Adenovirus polysomal mRNA is monocistronic (Persson *et al.*, 1977). As reviewed in the Introduction, there is good evidence that the bulk of the nuclear viral RNA synthesized in a short pulse is considerably larger than mRNA. It is clear from data obtained in this study, and that of other workers, that adenovirus infected cell nuclei contain RNA sequences not detectable in the cytoplasm. These findings are consistent with the hypothesis that adenovirus mRNA is derived from transient, high molecular weight transcripts which contain, in addition to mRNA sequences, "antisense" which is retained in the nucleus.

At most, 20% of the label incorporated into nuclear viral RNA during a short pulse reaches the cytoplasm (Philipson *et al.*, 1974), and the size distribution of the nuclear RNA partially overlaps with that of mRNA (Philipson *et al.*, 1974; McGuire

et al., 1976). Therefore it is possible that the rapidly labelled high molecular weight RNA turns over entirely within the nucleus and is irrelevant to the synthesis of mRNA. Thus studies examining the properties of viral RNA labelled during a short pulse cannot by themselves elucidate the pathways to mRNA synthesis. Before these studies can have any direct relevance to the problem it must first be demonstrated that this pulse labelled high molecular RNA is the precursor to mRNA.

A more productive approach is to ask how many promotors for viral *mRNA* synthesis exist. Three general models can be envisioned. The first proposes that the *l* and *r* strands have one promotor each, located at their 3' termini; the primary transcript is a full length copy of each strand; the transition from the early to late phase of infection is accomplished exclusively by the changing specificities of a mRNA processing and transport system. The second model proposes that each mRNA has its own promotor, and that high molecular weight RNA, bearing some mRNA sequences, is transcribed independently and irrelevantly from its own promotor(s). The third model suggests that each DNA strand has several promotor sites for mRNA synthesis, and that mRNA is derived from several discrete, but perhaps

partially overlapping, high molecular weight precursors. According to the third model it is conceivable that transcription of a mRNA is initiated at one of several alternative promotors. The available evidence is sufficient to rule out the first model as stated: pulse labelled Ad 2 late nuclear RNA is 99% assymetric (Pettersson and Philipson, 1974), and pulse labelled early Ad 2 RNA is 90% assymetric (Zimmer and Raskas, 1976). Thus if total transcripts exist, the complementary RNA sequences are not present in equimolar amounts. This differential could be generated by unequal initiation frequencies, or by differential termination. It is apparent that some degree of transcription control exists. It remains possible, however, that each DNA strand has only one promotor site for mRNA synthesis.

The mechanisms which delay the appearance of late-specific mRNA in the cytoplasm remain obscure. Transcription control, differential processing and/or transport, or both, may occur. It is unlikely that the mechanisms controlling the early to late transition will be understood until the fundamental problem of the pathway to mRNA synthesis is resolved, and the promotors of mRNA synthesis are enumerated and mapped.

One promotor site has been definitively mapped on the adenovirus genome: that for Ad 2 VA RNA.

VA RNA made *in vitro* bears a guanosine tetraphosphate at its 5' terminus (McGuire *et al.*, 1976), and VA RNA synthesized in isolated nuclei can be labelled with γ-^{32}P GTP (L. Philipson, personal communication to Dr. S. Mak), demonstrating that synthesis of VA RNA is initiated at its own promotor site (by RNA polymerase C (Weinmann *et al.*, 1976)). The promotor for VA RNA synthesis thus maps very close to the 5' end of the major late *r* strand region. In some respects the synthesis of VA RNA resembles that of the 6 S RNA of bacteriophage λ, thought to be the leader sequence for λ late RNA (see Introduction). It is tempting to speculate that transcription of the immediately adjacent late region is modulated by an anti-termination mechanism. Thus at early times, transcription initiated at the VA promotor terminates with 100% efficiency at a terminator site, generating VA RNA. Later, termination antagonists allow transcription to proceed beyond the terminator, resulting in the synthesis of late RNA. VA RNA could be derived both from large primary transcripts by processing and by incomplete suppression of termination. Ad 12 VA RNA is synthesized early in productive infection, but not during abortive infection of hamster cells (Varrichio and Raska, 1976). Since Ad 12 DNA is not replicated, and Ad 12 late RNA is not

synthesized during abortive infection, this observation may indicate a role for VA RNA is both transcription and replication.

If VA RNA is involved in a termination control mechanism, such a mechanism must be more complex than stated above: at least four separate, non-overlapping small viral RNA species, mapping to the VA region, have been detected in Ad 2 infected cells (Wienmann *et al.*, 1976A). These RNA species are transcribed by RNA polymerase C *in vitro*, but it is not clear whether this merely reflects the ability of this polymerase to initiate new chains *in vitro*. (Synthesis of the bulk of the late viral RNA species *in vitro* is accomplished by polymerase B (Price and Penman, 1972; Wallace and Kates, 1972) which initiates poorly, if at all, *in vitro*.) It is possible that these small RNA species are transcribed by both polymerase B and C *in vivo*. Regardless of whether the analogy with the λ 6 S RNA is a valid one, the map position of VA RNA strongly suggests that it has some role in the control of the transcription of the adjacent genes.

In addition to the promotor(s) of VA-like RNA synthesis, promotors for mRNA synthesis must exist at or near the 3' termini of the *l* and *r* strands.

A most powerful approach to the identification of additional promotor sites is to study mutants

with altered cis-dominant regulatory sites controlling the expression of adjacent genes. Most promotor lesions with drastic effects would be lethal, so that mutants would have to be selected in the presence of complementing wild-type virus. A potentially valuable approach is to use restriction endonucleases to construct deletion, substitution and rearrangement mutants *in vitro*. Any such change involving a promotor site would have drastic, cis-dominant effects. Berg and co-workers have successfully created deletion mutants of SV40 enzymatically *in vitro* (Shenk *et al.*, 1976).

The maps of the adenovirus early and late mRNA coding regions obtained in this and other studies will assist in the rational design and interpretation of experiments studying cis-dominant sites.

III. Viral RNA in Transformed Cells

Both 702.C2 cells and T6 cells contain appreciable quantities of virus specific RNA transcribed from the *r* strand of the Huie M8 Hind III F and H fragments, and smaller amounts of RNA from Hind III C/D (Section IV.B.). Saturation hybridization revealed that 702.C2 cytoplasmic RNA is derived from about 10% of the intact *r* strand. Both cell lines thus contain

cytoplasmic RNA derived from a "late" region of the viral genome (between 6.3 and 17 map units). The analogous RNA species have also been observed in Ad 2 transformed cells (Sharp *et al.*, 1974), but in marked contrast to the present results, RNA encoded by the leftmost 11% of the *r* strand was also found in the cytoplasm of productively infected cells "early" post infection. However, all studies of Ad 2 "early" RNA which have detected transcription of DNA between 7.3 and 17 map units have been done in the presence of either cycloheximide or cytosine arabinoside. It is possible that the widely accepted generalization that adenovirus transformed cells contain only "early" mRNA is entirely fallacious, being based on a drug-induced artefact. This possibility obviously deserves consideration.

In addition to RNA derived from the left end of the viral genome, T6 cells contain small quantities encoded by the sequences borne on the right hand 1131 Hind III E fragment. Since T6 cells were transformed by Huie M virus, the interpretation of this RNA is difficult, as the nature of the right end anomaly of this strain remains unclear.

Renaturation kinetic analyses have demonstrated that 702.C2 cells contain only a partial copy of the

Eco RI C fragment (Dr. S. Mak, personal communication), while T6 cells contain fragments Eco RI C,D,B,E, but no detectable Eco RI A sequences (K.C. Lee, personal communication). The results obtained in this study demonstrate that the leftmost portion of Eco RI C is present in both cell lines, and that a segment of the rightmost end of Eco RI A must be present in T6 cells. This segment is presumably too small to be detected by renaturation kinetic analysis. If a viral gene product maintains the adenovirus type 12 transformed cell phenotype, this gene is transcribed from the leftmost 10% of the r strand. This conclusion is in agreement with results obtained in studies of Ad 2 and Ad 5 transformed cells (Graham *et al.*, 1974; Sharp *et al.*, 1974).

The restricted transcription of the Ad 12 genome present in T6 cells is informative: an early and a late region at the left end of the genome are transcribed into stable, cytoplasmic RNA, while the late regions of Eco RI D,B and E are silent. There are innumerable possible explanations for this differential expression, one of which being that all of the silent late regions have been separated from their promotors by the integration event. Ortin *et al.* (1976) have found that during abortive infection of hamster cells by Ad 12, all of the early regions are

expressed, but that the late segments of Eco RI D,B and E are silent. Their analysis could not reveal whether the late region within Huie M8 Hind III H was expressed. It is of great interest to determine whether this segment is transcribed during abortive infection: such a finding would demonstrate that the synthesis of this late RNA is not coupled to DNA replication. This could explain why the analogous Ad 2 RNA has been considered an "early" class.

IV. The Functional Organization of the Adenovirus Genome

A. Comparison of the genomes of Ad 12 Huie M, Huie V, and 1131

The results of section III and those of C. Mulder (personal communication) strongly suggest that Huie V can be considered as the parental strain from which Huie M evolved during years of passage through KB cells. The Huie V and 1131 genomes are identical as assayed by Eco RI and Bam HI, and have clearly related Hind III cleavage patterns, while the Huie M genome differs from that of Huie V only at the right end. Since Huie M8 plaques on KB cells, the alteration at the right end of the Huie M8 genome does not affect

the ability of the virus to be propagated in tissue culture. It will be of interest to determine which, if any, viral genes are involved in the alteration (whether reiteration or some more complex alteration), and what effect this has on their expression. The alteration may involve part of the rightmost, *l* strand specific early coding region.

B. Comparison of the organization of the group A and C genomes

Early work by Lacey and Green (1964) using a crude DNA-DNA hybridization technique suggested that Ad 2 and Ad 12 DNA share about 15 to 20% of their nucleotide sequences in common. The method almost certainly grossly overestimated the true degree of homology, as the single stranded portions of partially paired structures were scored as duplex. The homology of Ad 2 and Ad 12 DNA was re-examined using DNA-DNA hybridization in solution and an S1 nuclease assay for duplexed segments. Seven μg/ml of unlabelled Ad 2 DNA was heat-denatured at incubated with 0.01 μg/ml ^{3}H-Ad 12 DNA for 5 hours at 67°C in 0.3 M NaCl. The $Cot_{\frac{1}{2}}$ of adenovirus DNA in this system is 3.2 μg h ml^{-1}, so that this incubation corresponds to greater

than 10 times the $Cot_{\frac{1}{2}}$. If Ad 12 DNA were totally homologous to Ad 2 DNA, 92% of the label would be in a duplex form. Ad 12 DNA incubated alone was 5.4% resistant to S1, while that incubated with Ad 2 DNA was 8.2% resistant. Thus Ad 2 and Ad 12 share about 3% of their DNA sequences in common.

Figure 48 documents the striking similarities between the transcription maps of Ad 12 and Ad 2. Another property of the viral genome also appears to be common to the two adenovirus groups. Sharp *et al.* (1974) and Flint *et al.* (1976) have shown that the property which allows electrophoretic DNA strand separation is distributed assymetrically throughout the Ad 2 and Ad 5 genomes, such that the faster migrating strands of DNA fragments containing the left end of the genome are derived from the intact *l* strand, while the reverse is true of most other fragments. The data obtained in this study similarly demonstrated that the fast strands of the left end Ad 12 Eco RI C and Hind III F fragments are part of the intact *l* strand, while the fast strands of the right end Bam Hl B and E fragments are part of the intact *r* strand.

The structural differences between complementary DNA strands which allow electrophoretic strand separation have not been identified, but a correlation

between electrophoretic mobility and ribopolymer binding sites has been observed. Studies with bacteriophage DNA demonstrated that the DNA strand which binds more ribopolymer migrates more slowly in agarose gels than does its complement (Hayward, 1972). This correlation can be extended to the DNA of adenovirus type 12: Ortin *et al.* (1976) demonstrated that there is a reversal of "heaviness" in poly (U,G) cesium chloride density gradients with Ad 12 DNA fragments derived from the left end of the genome, such that the heavy strands of these fragments are part of the intact light (=r) strand, while the reverse is true of other DNA fragments. Thus the Ad 12 DNA strands which bind more poly (U,G) migrate more slowly in agarose gels than do their complements.

The degree of strand separation of Ad 12 DNA varies from fragment to fragment, but the extent of separation obtained with one method correlates with that obtained with the other. For example, Ortin *et al.* (1976), using the poly (U,G) binding method, obtained the best separation with Eco RI C, followed by A, then by D. Little, if any, separation was obtained with Eco RI B and E. Figure 36 shows that this variation correlates with the degree of separation obtained by electrophoresis.

The obvious homology between the Ad 12 and group C transcription maps, and the similar assymetric distribution of the property allowing electrophoretic DNA strand separation in the two genomes implies that the overall layout of the viral genome has been preserved during the evolutionary divergence of the group A and C adenoviruses. These features have been preserved while the base sequences of the individual genes have diverged to the point where virtually no sequence homology can be detected. If this supposition is correct, genes with similar functions probably share common map positions. Supporting this suggestion, the genes responsible for the maintainence of *in vitro* transformation in both groups map to the left (GC rich) end of the viral genome. In addition, Williams *et al.* (1974) found that wild type Ad 12 (1131) can complement the defect of some, but not all, Ad 5 temperature sensitive mutants, demonstrating that some Ad 12 genes share sufficient functional homology with their Ad 5 counterparts to serve in their stead. The situation is reminiscent of the relationships between the enteric bacteriophages λ, P22 and P2, which have very similar genetic maps, but very limited base sequence homology (Dove, 1971).

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