Ultra-Violet

Transcription Unit Mapping

of Adenovirus Type 2

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By

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A Thesis

Submitted to the School of Graduate Studies

in Partial Fulfilment of the Requirements

for the Degree

Master of Science

McMaster University

March, 1984

Master of Science
(Biology)

MCMASTER UNIVERSITY
Hamilton, Ontario

TITLE: Ultra-Violet Transcription Unit Mapping of Adenovirus Type 2

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SUPERVISOR: Professor A.J. Rainbow

NUMBER OF PAGES: 176

ABSTRACT

At late times during the infection of human KB cells with Adenovirus type 2 (Ad 2), the ultraviolet (uv) sensitivity of transcription was assayed by DNA-RNA hybridization across the length of the genome. Assuming that the majority of the nuclear transcripts at this time of infection are read from the rightward transcribing DNA strand, the resulting plot of the surviving fraction of viral transcription vs. genome position indicates two transcription units are responsible for the expression of late Ad 2 genes. Dose response curves for the transcription from selected DNA restriction fragments of the genome were also obtained. The uv inactivation cross sections generated from such curves identified a long, uv sensitive transcript originating from the major late promotor at approximately 17 map units, and a shorter, less uv sensitive from approximately 63 map units on the genome. The shorter transcription unit accounts for about one third to a half of the viral nuclear RNA synthesized from the right hand 30 to 40% of the genome. The majority of the late viral nuclear transcripts, however, originated at approximately 17 map units and terminated at around 60-70 map units.

Similar experiments examining the uv sensitivity of cytoplasmic poly A RNA production at various sites across the length of the genome are consistent with two rightward transcribing transcription units expressed during late Ad 2 infection.

The transcriptional organization of late Ad 2 gene expression was also approached through uv transcription unit mapping experiments by examining the uv sensitivities of the synthesis of late Ad 2 proteins for which the approximate gene locations are known. The effect of uv on Ad 2 nuclear transcription was also reflected at the polypeptide level indicating two transcription units are responsible for the synthesis of mRNA coding for late viral proteins. The differential radiosensitivities of late protein synthesis confirmed the relative gene positions on the Ad 2 genome.

ACKNOWLEDGEMENTS

The author wishes to express his sincere thanks to Dr. A.J. Rainbow for his assistance, supervision, encouragement, and patience throughtout the course of this investigation. I also thank my supervisory committee, Drs. S. Mak and D. McCalla for their help and advice. Special thanks are due to Drs. F. Graham and S. Bacchetti for their help and encouragement. I wish to thank D. Rowe, R. Lai Fatt, R. Redfield and Drs. J. Smiley, N. Lassam, P. Jeeves, and J. Campione for many helpful discussions. I also thank R. McKinnon for his help with the molecular cloning and M. Howe for her technical assistance. I am grateful to Miss S. Hawkes, Miss L. McKnight and Mr. C. Bilinski for their help in the preparation of this manuscript, and special thanks to Ms. Josie Maljar for the excellent job of typing the final copy of this manuscript.

The financial support of the National Cancer Institute of Canada and McMaster University is gratefully acknowledged.

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LIST OF ABBREVIATIONS

Ad	adenovirus
A-T	adenosine-thymidine
BamHI	restriction endonuclease from *Bacillus amyloliquefaeciens* RUB500
BglII	restriction endonuclease from *Bacillus globiggi*
BSA	Bovine serum albumin
DNA	deoxyribonucleic acid
DRB	5,6 dichloro-1-β-diribofuranosyl-benzimidazole
EcoRi	restriction endonuclease encoded by plasmid rY of *Escherichia coli*
EtBr	ethidium bromide
%G+C	mole percent guanine plus cytosine
HindIII	restriction endonuclease from *Hemophilus influenzae* rd
hnRNA	heterogeneous nuclear ribonucleic acid
mRNA	messenger ribonucliec acid
poly A	polyadenylic acid
PR	promotor
RNA	ribonucleic acid
ts	temperature sensitive
uv	ultra-violet

INTRODUCTION

Proteins are the structural and functional building blocks of living cells and represent the end products of the process of genetic expression. Proteins are synthesized from amino acids through the process of translation. The specificity of protein synthesis is due to large polymeric molecules of ribonucleic acid (RNA), which are intermediate templates providing the information to specify each amino acid in a defined order for a given polypeptide. The RNA molecules are synthesized by the process of transcription, reproducing specific information coded in the self replicating genetic material, deoxyribonucleic acid (DNA). This process of the transfer of genetic information from DNA to RNA through transcription and from RNA to protein through translation is known as the central dogma of molecular biology and provides the framework of our understanding of the molecular basis of life.

The particular state of differentiation of any given eukaryotic cell is believed to be a result of the expression of a limited number of specific genes, which differentially specify the cell type, despite the fact that each cell contains a full genetic complement. There are at least two levels at which gene expression is regulated. One is transcriptional control, in which transcription of DNA yielding mRNAs coding for a given protein or set of proteins is allowed to take place or is inhibited. The other level is translational control, in which the selection of specific mRNAs and the rate of synthesis of polypeptide chains determine which proteins and how much of a given protein

will be produced.

The basis of our understanding of molecular genetics and molecular biology comes from the detailed study of bacteria and their viruses (Hayes, 1968; Stent, 1978; Watson, 1976). These organisms provide genetic systems that can be easily maintained and observed under laboratory conditions and although, highly complex, bacteria and bacteriophage are comparatively very simple genetic elements. Much of the success of prokaryotic studies has been due to the vast number of easily obtained and characterized mutants, such as auxotrophs, deletions and conditional lethals. The advancements in prokaryotic biology bred enthusiasm to gain a comparable understanding of the molecular aspects of higher plant and animal cells. However, new difficulties are encountered in eukaryotic systems that require a change from approaches of the past to respond to questions posed in eukaryotic molecular biology. Firstly, there is a great increase in size between prokaryotes and eukaryotes. While, E. coli, some 500 fold smaller than higher plant or animal cells has a DNA content of 2.4×10^9 daltons or 3.6×10^6 base pairs whereas human cells have a DNA content of about 2×10^{12} daltons or 3×10^9 base pairs. This difference in size presents technical difficulties in analyzing the expression of genes, which may be represented only once in a very large and complex genetic structure within the nucleus of a eukaryotic cells. In addition the paucity of useful mutations in eukaryotes have required that novel physiochemical, biochemical and biological approaches be developed to gain an understanding of eukary-

otic genetic systems.

Animal viruses such as adenoviruses may serve as excellent models in which to study the nature of gene expression in eukaryotic cells. Analysis of adenovirus multiplication during productive infection suggests many similarities to host cell gene expression. The virus does not carry any known enzymes involved in gene expression into the cell and is therefore exclusively dependent upon host cell machinery for at least the initial stages of its multiplication. The double stranded viral DNA replicates in the nucleus of infected cells and transcription is carried out by cellular enzymes (Wallace and Kates, 1972; Price and Penman, 1972; Weinmann et al., 1974, 1976). Adenovirus mRNA found in the cytoplasm of infected cells possess the modifications known to be common in cellular mRNA, which are likely due to cellular enzymes. Adenovirus mRNA has a 5' methylated cap (Wold et al., 1976; Hashimoto and Green, 1976), internal m^6A residues (Sommer et al., 1976; Moss and Koczat, 1976) and a 3' terminal polyadenylated (poly A) tail (Philipson et al., 1971). The virus has the advantage that it contains a limited number of genes which simplifies the system to a technologically and conceptually approachable tool and it can be readily propagated to high titres. In addition, large quantities of purified viral DNA can be prepared and the cleavage sites of several restriction endonucleases have been established as well as portions of the genome sequenced. Adenoviruses represent defined sets of genetic information that can be used as a probe for eukaryotic gene expression and regulation by the analysis of viral transcription and translation in mammalian cells.

Adenovirus

The adenoviruses are a large group of animal DNA viruses isolated from a variety of vertebrate species (Enders et al., 1956). The adenovirus group has at least 80 members (Pereira et al., 1963), which share a common architecture. At least 31 distinct serotypes have been isolated (Beladi, 1972) from the human populations across the world. Human adenovirus infections are associated with respiratory illnesses (Hilleman and Werner, 1954), keratoconjunctivitis (Jawetz et al., 1954), and some serotypes can be found in the intestinal tract (Rosen, 1962).

The human adenovirus serotypes have been categorized into three subgroups based on their DNA homology (Lacey and Green, 1964, 1965, 1967). This classification system agrees well with groupings based on their oncogenic potential in newborn rodents (Huebner et al., 1965) and the base composition of the viral DNA molecules (Pina and Green, 1965; Green, 1970). Subgroup A viruses are highly oncogenic and have a base composition of 48-49% G+C; subgroup B are weakly oncogenic with 49-52% G+C; and subgroup C are nononcogenic with 55-60% G+C. A fourth subgroup, D based on immunological considerations contain several nononcogenic adenoviruses (MacAllister et al., 1969). The closely related types 2 (Ad 2) and 5 (Ad 5), which belong to subgroup C are by far the best characterized and most of the following information presented here relates to these two serotypes unless otherwise specified.

The virion consists of 87% protein and 13% DNA (Green and Pina, 1963) and lacks a lipid membrane envelope (reviewed by Philip-

son, 1975, 1979; Flint, 1980). The virus contains a linear double stranded DNA genome of 20-25 x 10^6 daltons (Green, 1970), which may code for some 30-40 polypeptides. The viral DNA is not circularly permuted and lacks terminal repititions or cohesive ends (Green et al., 1967a). The viral genome does contain inverted terminal repititions of about 100 nucleotides, which can give rise to single stranded pan handled circular molecules after denaturation and self-annealing (Garon et al., 1972; Wolfson and Dressler, 1972; Steenbergh et al., 1977). In virions the adenovirus genome has a circular structure with the termini joined by a pronase-sensitive linker (Robinson et al., 1973). At the 5' ends of the DNA strands a 55,000 molecular weight (MW) protein is covalently linked (Rekosh et al., 1977). The inverted terminal repeat and the 5' terminal protein are believed to be involved in viral DNA replication (Arrand and Roberts, 1979; Rekosh et al., 1977).

The adenovirus virion has an icosahedral structure of 20 triangular facets and 12 vertices with a diameter of 65-80 nm (Horne et al., 1959). The outer capsid consists of three major structural proteins, which comprise 252 capsomers of which 240 have six neighbours called hexons, which form the faces and edges of the triangular face. The remaining 12 capsomers are present at the vertices of the icosahedron and have five neighbours and are therefore referred to as pentons (Ginsberg et al., 1966). Each penton has attached an outward antennalike projection known as the fibre, which terminates in a knob like structure (Pettersson et al., 1968). After disruption of the outer capsid, the viral core consisting of a high density protein-

DNA complex is liberated (Russell et al., 1971). The virion structural proteins have been examined by SDS polyacrylamide gel electrophoresis and as many as 15 proteins have been identified (Anderson et al., 1973). The structure of the virion and the arrangement of viral polypeptides is depicted in Figure 1.

Infection of cells with adenoviruses yields different outcomes depending on the cell type and viral serotype. Infection of monkey cells with Ad 2 (Friedman, 1970) or hamster cells with adenovirus type 12 (Ad 12) (Doerfler, 1969; Mak, 1975) results in an abortive infection, which does not result in the production of virus progeny although, some limited viral functions are expressed. As well as

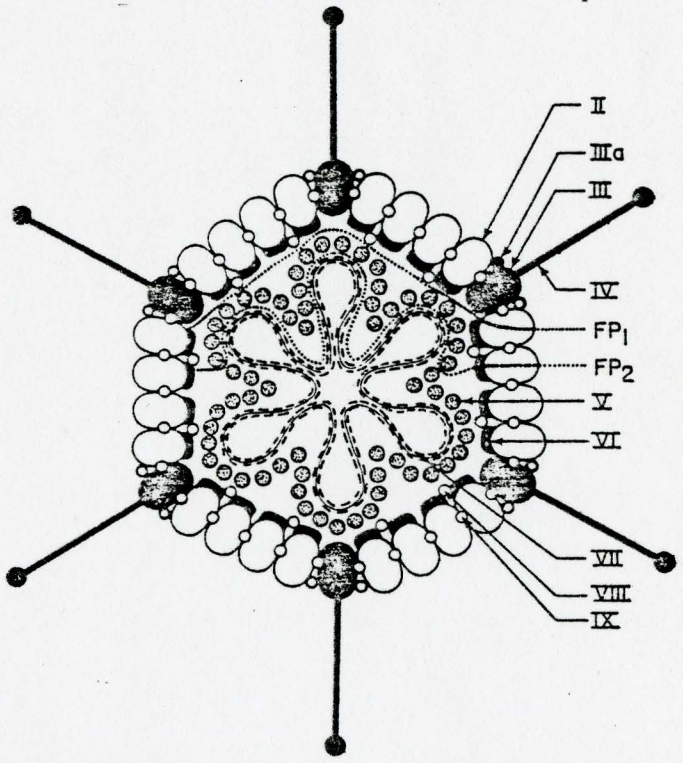


Figure 1. The proposed arrangement of adenovirus structural proteins. The model of the capsid is described by Everitt et al., 1975 and the core by Brown et al., 1975. FP1 and FP2 are fracture planes based on electron microscope data (from Flint, 1980).

the ability to induce tumours in newborn rodents with some serotypes (Trentin, 1962), rodent cells may be morphologically transformed *in vitro* by virus (Gallimore, 1974), or with sheared viral DNA (Graham *et al.*, 1974) or specific viral DNA fragments (Graham and van der Eb, 1973). The infection of permissive cells such as HeLa or KB cells results in cell death and the production of progeny virus. An outline of the events of the lytic cycle will be presented here and has been extensively reviewed by Flint and Broker (1980).

Productive Infection

The 36 hour infectious cycle of Ad 2 productive infection is conventionally divided into an early and late phase, separated by the onset of viral DNA synthesis. There are approximately 10,000 receptor sites on the plasma membrane of a KB cell to which a virion can adsorp (Lonberg-Holm and Philipson, 1969). After adsorption, the virus enters the cell by pinocytosis (Chardonnet and Dales, 1970) or by direct penetration of the membrane (Lonberg-Holm and Philipson, 1969). The virion is partially uncoated in the cytoplasm (Dales and Chardonnet, 1973) and the viral core enters the nucleus (Chardonnet and Dales, 1972) where the viral DNA is released from the core in a final uncoating stage. The uncoating phase under normal conditions is complete in about two hours (Philipson *et al.*, 1968). During productive infection adenovirus genes are expressed in an ordered fashion, which is probably achieved by the mechanisms which normally operate in mammalian cells. Transcription of adenovirus DNA is mediated by the host RNA polymerase II (Wallace and Kates, 1972; Price and Penman, 1972)

and both IA DNA strands contain coding regions (Sharp et al., 1975; Pettersson et al., 1976). Early transcription (described in detail below) is defined as viral RNA synthesis which takes place before viral DNA synthesis occurs, and is often studied in the presence of drugs such as cytosine arabinoside or fluorodeoxyuridine, which directly inhibit viral DNA synthesis (Parsons and Green, 1971; Flanagan and Ginsberg, 1962). A number of early proteins have been identified by immunological approaches (Saborio and Oberg, 1976, van der Eb et al., 1980) and in vitro translation of early viral mRNAs (Saborio and Oberg, 1976; Lewis et al., 1976; Harter and Lewis, 1978). The regions of the viral genome, which specify many of these proteins have been identified as illustrated in Figure 3. The functional roles of most of these proteins are unknown. The 72,000 MW protein is a DNA binding protein required for the initiation of DNA replication (van der Vliet and Sussenbach, 1975) and for DNA chain elongation (van der Vliet et al., 1977). Also, early proteins encoded in the left-most 11% of the viral genome are involved in transformation (Lassam et al., 1979).

Viral DNA synthesis begins about 6 to 8 hours post-infection reaching a maximum about 6 to 10 hours later (Pina and Green, 1969) and requires prior synthesis of early viral proteins (Horwitz et al., 1973). Viral DNA synthesis may be mediated by host cell DNA polymerase α or γ, which have been isolated from replicating complexes (Frenkel, 1978) or identified on the basis of drug studies (Krokan et al., 1979; van der Werf et al., 1980). Recently, viral specific enzyme activities have been implicated for at least the initiation of

viral DNA synthesis (Enomoto et al., 1981) which requires specific DNA sequences at the origin of replication and a viral terminal protein (Challberg et al., 1982). Initiation and termination of viral DNA synthesis occurs at or near both ends of the genome within an inverted terminally repeated sequence (Sussenbach and Kuijk, 1977; Winnacker, 1978) with replication believed to occur by a mechanism of strand displacement (Daniell, 1976; Rekosh et al., 1977). After the onset of the viral DNA synthesis, another set of genes is expressed. Late transcription (described in detail below) greatly changes the amounts and species of viral RNAs found in the infected cell. Late mRNA codes for the viral structural polypeptides that form the capsid and contribute to the protein-DNA core. A number of viral polypeptides including one major nonstructural protein known as 100K (Philipson, 1975, 1979; Anderson et al., 1973, 1974) have been identified to be specific to the late phase of infection. Viral polypeptides VI, VII, VIII appear in extracts of infected cells only after pulse-chase labelling, indicating that post-translational processing of larger precursor molecules occurs to generate these proteins (Anderson et al., 1973; Weber, 1976). The regions of the viral DNA encoding specific late polypeptides have been identified by selecting mRNA on restriction fragments (Lewis et al., 1975; 1977) and separated DNA strands of the viral genome (Persson et al., 1979) followed by in vitro translation. Mapping studies to determine the location of late viral proteins have also included hybrid-arrest studies (Paterson et al., 1977), in which mRNA is excluded from the population of translatable mRNA using restriction endonuclease frag-

ments of viral DNA prior to *in vitro* translation. The location of late viral proteins have also been approached through the genetic analysis of interserotypic recombinants of Ad 2 and Ad 5 (Weber and Hassell, 1979; Grodzicker *et al.*, 1977) and by a serological analysis of type-specific antigenic determinants from Ad 5 - Ad 2^{+}ND1 recombinants (Mautner *et al.*, 1975). The location of late viral polypeptides on the viral genome are illustrated in Figure 3. Structural polypeptides that are synthesized in the cytoplasm and assembled into multimeric units are transported to the nucleus where they are assembled into the adenovirion capsid structure. The process of assembly appears complex and many of the details remain obscure although, the isolation of partially assembled forms of the virion have helped elucidate this pathway. Three hexon polypeptides first assemble into trimeric hexon units (Velicer and Ginsberg, 1968) and are then transported to the nucleus possibly aided by the non-structural 100K protein (Kauffman and Ginsberg, 1976). Heterotypic marker rescue experiments of 100K gene mutants suggest that an interaction between the 100K protein and hexon polypeptides is required for the effective assembly of hexon trimers (Oosterom-Dragon and Ginsberg, 1981). These units assemble into ninemers in association with protein IX, which may act to hold the hexons together (Boulanger *et al.*, 1979) forming the capsid skeleton. Empty capsids have been isolated (Pereira and Wrigley, 1974) associated with 32,000 and 40,000 MW proteins (Evardsson *et al.*, 1976) which may act as scaffolding proteins which are not present in young or mature virions. Insertion of the viral DNA, possibly associated with core proteins V and pVII

(Evardsson et al., 1976), takes place with the left hand end of the viral genome inserted first into the empty capsid (Tibbetts, 1977). These assembly intermediates may be recovered and are associated with a virus specific protein, IVa_2, which has been referred to as a maturation protein (Persson et al., 1979). Young virions containing a full complement of viral DNA and lacking protein IVa_2 have been isolated and are physically similar to mature virions except for the presence of the precursor polypeptides for proteins VI, VII, and VIII which have not yet been processed (Ishibashi and Maizel, 1974; Evardsson et al., 1976; Weber, 1976). The final maturation step involves the cleavage of these precursor molecules by a virus associated protease to yield fully mature virions capable of infecting other cells (Bhatti and Weber, 1978).

Early Transcription

Transcription of viral RNA begins shortly after infection of the host cell and viral specific RNA accumulates slowly until the onset of viral DNA synthesis which defines the early phase of infection. Early transcription of viral DNA sequences accounts for no more than 0.1% of the total infected cell RNA (Tibbetts and Pettersson, 1974). Approximately, 25-30% of the genomic sequences are complementary to early cytoplasmic RNA (Tibbetts and Pettersson, 1974; Wold et al., 1977) of which 9-13% of the leftward transcribing strand (r-strand) is expressed (Sharp et al., 1975; Pettersson et al., 1976). There are at least five early transcription units in four widely separated regions of the viral genome. Hybridization of early mRNA to single stranded fragments of viral DNA has determined that two of these

regions are coded on the r-strand (regions E1 and E3) and two on the l-strand (regions E2 and E4) as depicted in Figure 2a (Sharp et al., 1975; Flint et al., 1976; Philipson et al., 1974; Pettersson et al., 1976). Each of the four early regions have been identified to be a unique transcription unit by nascent chain analysis (Evans et al., 1977), where labelling with ^{3}H-uridine for a very short pulse will result in a population of RNA molecules from a given transcription unit with a common 5' terminus and label accumulating at the 3' ends of the growing chains. These molecules can be fractionated by size and hybridized to viral restriction endonuclease fragments derived from the transcription unit. This will identify both the origin and the limit of transcription as the shortest nascent chains will hybridize to a DNA fragment containing the initiation site, whereas more distal fragments will detect longer RNA chains due to the presence of label only in the 3' termini of the growing chain. This analysis indicated r-strand initiation sites at 0-4 (E1) and 76-83 (E3) map units of the 100 map unit viral genome, as well as the l-strand initiation sites at 59-76 (E2) and 90-100 (E4) map units on the viral genome. Ultra violet (UV) transcription unit mapping (described below) has also demonstrated unique promotors for the same four early transcription units (Berk and Sharp, 1977a). These results also indicated that the size of the primary transcript of early region 2 was about three times larger than expected based on the size of the mRNA. This is consistent with the suggestion of Craig and Raskas (1976) that mRNA was derived from a larger precursor. These investigators detected early viral-specific polyadenylated nuclear RNA which was larger

than the early mRNA. Using very high doses of UV which result in the preferential synthesis of RNA near initiation sites, Wilson and coworkers (1979) have identified another transcription unit in tandem with E1, thereby breaking up this early region at the extreme left 11% of the genome into regions E1A and E1B. The existence of two independent transcription units within this region has also been demonstrated using the nucleoside analog, 5,6 dichloro-1-β-diribofuranosylbenzimidazole (DRB), which causes premature chain termination during RNA synthesis resulting in the accumulation of very short RNA chains containing sequences adjacent to the initiation site (Seghal et al., 1979). The precise locations for the initiation sites of early mRNAs have been determined by comparing the 5' termini of the various early mRNAs generated by RNase T1 to the DNA sequences for the appropriate regions. The initiation sites for early RNA synthesis determined in this fashion are: E1A, 1.4; E1B, 4.86; E2, 75.05; E3, 76.6; and E4, 99.1 map units (Baker and Ziff, 1980). Late during the early phase of infection, which has been noted as an intermediate time post-infection (Philipson, 1979; Nevins and Chen-Kiang, 1981), at least two additional transcription units have been described. A r-strand transcription unit initiating at 9.6 map units expresses a mRNA coding for protein IX (Spector et al., 1978; Persson et al., 1978). The other transcription unit, expressed at low concentrations (Galos et al., 1979; Flint and Sharp, 1976), is located on the l-strand from 11.3 to 15 map units and presumably codes for proteins IVa_2 (Chow and Broker, 1978). Therefore, there are at least seven separate promotors controlling unique transcript-

ion units that are expressed early during adenovirus infection. The first to be expressed is region E1A within 1 hour after infection followed by E1B, E3, E4 and then within 2 to 3 hours after infection region E2 (Nevins et al., 1979).

The cytoplasmic RNAs that result from the expression of these early regions have been examined by the elegant and powerful techniques of R-loop mapping and nuclease mapping. R loops can be formed by hybridizing RNA to complementary DNA sequences in high concentrations of formamide, where RNA - DNA hybrids are more stable than the complementary double stranded DNA (White and Hogness, 1977). The annealing of a RNA segment to a longer segment of duplex DNA under appropriate conditions results in the displacement of one DNA strand, during the formation of the RNA - DNA hybrid. These structures can be visualized in the electron microscrope as double stranded RNA - DNA hybrids

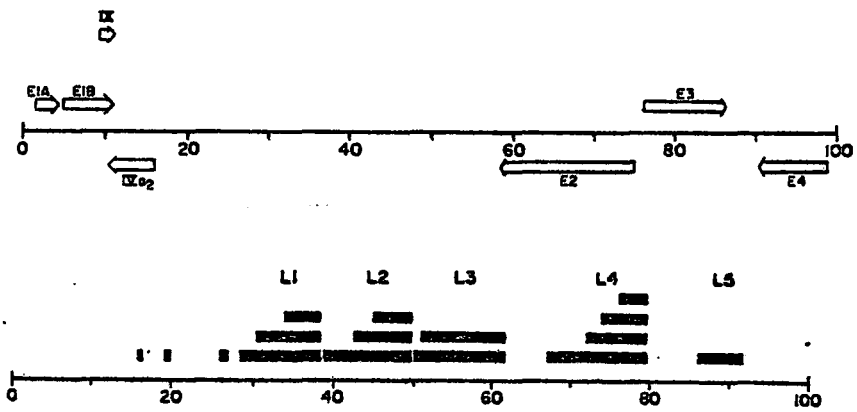


Figure 2. (A) Locations of early and intermediate (IX, IVa_2) regions of the Ad 2 genome. (B) Locations of late Ad 2 3' coterminal families of mRNA species (from Nevins and Chen-Kiang, 1981).

opposite a displaced loop of single stranded DNA. This procedure applied to the early mRNAs has allowed the determination of the precise locations of the coding regions for individual early mRNA molecules. The limits of early coding regions defined in this fashion are: E1A, 1.3-4.0; E1B, 5.0-11.1; E2, 62.4-67.9; E3, 78.6-86.1; and E4, 91.7-96.8 map units (Chow et al., 1977a; Neuwald et al., 1977).

The splicing of transcripts, which occurs during the processing of primary transcripts to generate mRNA was first described late in Ad 2 infection (Berget et al., 1977; Klessig, 1977; Chow et al., 1977b) has also been established for the biogenesis of early mRNA (Chow et al., 1979). Splicing involves the joining together of RNA sequences from two or more noncontiguous regions of the genome (reviewed by Breathnach and Chambon, 1981). This phenomenon has since been described in a large number of eukaryotic systems. Ad 2 early mRNAs are polyadenylated at their 3' termini like many cellular mRNAs (Philipson et al., 1971). The poly A tail is not encoded in the viral genome and is added post-transcriptionally (Nevins and Darnell, 1978a). This appears as a short single stranded tail when R-loops are viewed in the electron microscope. RNA - DNA hybrid molecules have also been visualized with internal loops of single stranded DNA which occur due to sequences in the mRNA that are not adjacent to each other in the viral genome but joined in the mature mRNA. Spliced cytoplasmic mRNAs have been identified for the early regions described above and the splicing sites determined measuring the lengths of the R-loops and noting the positions of single stranded loops of DNA (Kitchingham et al., 1977; Chow et al., 1979,

1980).

Spliced early mRNA molecules have also been detected through nuclease mapping in which hybrids of ^{32}P-labelled viral restriction endonuclease fragments and early cytoplasmic RNA are formed under conditions that allow only DNA-RNA annealing (Casey and Davidson, 1977) are treated with S1 endonuclease or exonuclease VII. S1 endonuclease degrades all single stranded DNA portions of the RNA-DNA hybrid and if this hybrid is formed with a spliced RNA, the tails of DNA not paired with RNA as well as the single stranded DNA loop formed due to the splice will be digested. This results in hybrids containing only DNA sequences directly complementary to and colinear with the RNA sequences with a nick at each splice point. Exonuclease VII degrades single stranded DNA from either 3' or 5' terminus, but will not degrade the single stranded DNA loop formed in RNA (containing a splice) - DNA hybrids. This results in the DNA portion of the hybrid retaining the sequences complementary to the cytoplasmic RNA as well as the sequences that are not found in the RNA due to splicing. After each reaction molecules can be analyzed by electrophoresis in denaturing alkaline agarose gels followed by autoradiography to determine the number and size of DNA segments resistant to each nuclease (reviewed by Flint and Broker, 1980). Berk and Sharp (1977b, 1978) have effectively used this method to describe spliced early cytoplasmic RNA molecules complementary to all five regions. Two classes of splices are observed in early mRNA molecules. Some splices generate a short sequence of nucleotides (50-100) linked to the 5' end of a RNA chain, while other splices join larger seg-

ments at internal positions within the RNA molecule. The spliced early mRNA species are illustrated in the detailed transcription map of Ad 2 in Figure 3.

An interesting observation that arises from such studies involves the synthesis of mRNA from region E2 coding for the DNA binding protein, 72K. The main body of the mRNA extends from coordinates 66.5 (5') to 61.6 (3') with a splice from 68.3 to 66.5 map units and a conservation of sequences from 68.5 to 68.3 map units. At early times after infection a 5' leader segment in the mRNA is encoded at 75 map units (Berk and Sharp, 1978; Chow et al., 1979). However, at intermediate and later times after infection this species declines and another species with a 5' leader at 72 map units can be detected. There is also a minor species detected with a 5' leader at 86.7 map units. Chow and coworkers (1980) have suggested these changes in splicing patterns reflects the activation of three different promotors to generate E2 mRNAs.

An A-T rich nucleotide sequence referred to as a Hogness box has been identified in DNA sequences preceding a number of eukaryotic cap sites (Konkel et al., 1978; Efstratiadis et al., 1980; Tsujimoto and Suzuki, 1979; Gannon et al., 1979; Smith et al., 1979) and upstream from the translational initiation site of D. melanogaster histone genes (Hogness in Baker and Ziff, 1980). The function of these sequences have not been established but is believed to be correlated with promotor sites for the binding of RNA polymerase near transcription initiation sites, although this sequence does not appear to be a strict requirement for the generation

of mRNA. Most Ad 2 regions show this sequence upstream from the cap site with the exception of regions E2 and IVa_2, which do not display this sequence (Baker and Ziff, 1980, 1981). It has also been found to be missing from other genes such as the late region of Simian virus 40 (Haegman and Fiers, 1978) or can be removed without affecting gene expression in vivo such as for the early region of SV40 (Benoist and Chambon, 1981). Most eukaryotic mRNAs are capped at their 5' ends with 7-methyl guanosine (Shatkin, 1976) which is added post-transcriptionally. The significance of this structural characteristic of eukaryotic mRNAs and the possible benefits of this feature are unknown, but is believed to have some role in translation (Kozak, 1978). Unique cap sites have been identified for regions E1A and E1B (Baker and Ziff, 1980), but regions E2, E3, E4 and protein IX have heterogeneous cap sites resulting from staggered 5' termini encoded within a 2-6 base region (Baker and Ziff, 1981).

Late Transcription

After the onset of viral DNA synthesis, late transcription begins and dramatically alters both the amounts and the specific seuqences of viral RNA expressed. The mechanism of the switch from the early to late phase of the infectious cycle is not well understood, but is closely tied with the onset of viral DNA synthesis (Thomas and Mathews, 1980). Inhibition of DNA synthesis using the drug cytosine arabinoside (Chow et al., 1979) or with the conditional lethal mutant ts125 at the restrictive conditions prevents the accumulation of late mRNA (Berget et al., 1976; Carter and Ginsberg, 1976). If ts125 is shifted from the permissive to the

restrictive temperature, late transcription continues even though viral DNA synthesis ceases (Carter and Ginsberg, 1976). The transition to late transcription is therefore dependent on the onset of viral DNA synthesis, but not dependent on continued viral replication. At late times after infection (15-20 hours) up to 40% of the total RNA in the infected cell is viral specific (Thomas and Green, 1969; Lucas and Ginsberg, 1971). The regions encoding late mRNA have been determined by saturation hybridization of labelled single strands of restriction endonuclease fragments of the viral genome and late mRNA. Approximately, 80% of the r-strand and 20% of the l-strand of the Ad2 genome is complementary to late viral RNA (Tibbetts et al., 1974; Sharp et al., 1975; Pettersson et al., 1976), similar results have been obtained with Ad 12 (Smiley and Mak, 1976). The vast majority of the late transcriptional products (95%) are complementary to the r-strand (Pettersson and Philipson, 1974; Zimmer et al., 1978).

Late nuclear viral RNA species have been identified corresponding to molecules large enough to represent almost a complete strand of the viral genome (Wall et al., 1972; McGuire et al., 1972; Bachenheimer, 1977). Half genome length and smaller viral RNA molecules have also been found in the nucleus of the infected cell (Parsons et al., 1971, Philipson et al., 1974). Late cytoplasmic mRNAs are significantly smaller than the majority of nuclear transcription products. These molecules contain tracts of poly A (150-200 nucleotides) at their 3' ends (Philipson et al., 1971), and a methylated 5' cap as well as internal methylated bases in the form of $^{m6}A^{m}$ and A^{m} (Moss and Koczot, 1976; McGuire et al., 1976; Sommer et al., 1976).

The relationship of the late primary transcripts and the regulation of the biogenesis of late mRNAs has generated considerable interest in recent years and is the subject of this thesis. Only a small fraction of viral nuclear RNA synthesized late during infection appears in the cytoplasm (Philipson et al., 1974). It is difficult to distinguish whether mRNA is derived from large transcripts similar to those suggested for cellular high molecular weight heterogeneous nuclear RNA (Darnell et al., 1970), or smaller transcription units possessing independent promotors.

Adenosine analogs such as toyacamycin and cordycepin, which appear to prevent processing of nuclear RNA, inhibit the accumulation of late mRNAs, but allow the synthesis of large nuclear (4-8 x 10^6 MW) viral RNA (McGuire et al., 1972; Oortmersson et al., 1975) suggesting that large nuclear transcripts represent the precursors to late mRNA species.

Analysis of nascent chains has identified the existence of nuclear viral RNA molecules much larger than polysomal viral mRNA (Bachenheimer and Darnell, 1975), although the size distribution profile of pulse labelled nuclear viral RNA also overlaps with smaller RNA molecules. This work and similar experiments using isolated nuclei (Weber et al., 1977) suggest that the majority of late transcripts are derived from a single transcription unit initiating at 11-18 map units and extending rightward to 90-100 map units generating an RNA molecule of about 26 kilobases. Nascent chain analysis performed with only very small RNA chains placed the initiation site of late transcription at 16±0.5 map units (Evans et al., 1977).

On the basis of uv transcription unit mapping of late viral nuclear RNA, Goldberg and coworkers (1977) conclude that a large RNA molecule spanning the entire region between 20 and 100 map units represents the majority of the late transcriptional products. However, these investigators do suggest the possibility of the existence of a weak secondary late promotor on the r-strand for the right hand 30% of the genome. Results obtained applying uv mapping to determine the origin of late cytoplasmic mRNAs have been interpreted to indicate that mRNA is derived from the long transcripts described above (Goldberg et al., 1978). The promotor of this transcription unit has been determined by fine structural analysis of nascent RNA sequences hybridizing to the region 11.6-18.2 map units and comparison with the DNA sequences around the promotor site place it near 16.4 map units (Ziff and Evans, 1978). An A-T rich region has been identified 31 nucleotides upstream of the 5' end of the RNA (cap

site) chain, similar to four of the early region promotors and other eukaryotic transcription units (Ziff and Evans, 1978).

Ad 2 late mRNAs have been examined by R-loop mapping defining a number of coding regions on the r-strand between 30 and 91.5 map units (Westphal et al., 1976; Chow et al., 1977a, 1980; Meyer et al., 1977). The majority of late mRNA species fall into five families that share common sequences. Different mRNA species that hybridize to the same regions of the genome have been examined by more detailed hybridization studies with very small DNA fragments. This analysis reveals the existence of mRNA species that share a common 3' terminus (McGrogan and Raskas, 1978; Nevins and Darnell, 1978b; Shaw and Ziff, 1980). The 3' common ends of late mRNA species forming five distinct families has been demonstrated by identifying common 3' oligonucleotides after T1 RNase digestion of different late mRNA species (Ziff and Fraser, 1978; Fraser and Ziff, 1978). The 3' ends of these five families map at coordinates 39, 50, 61.5, 78, and 91.5 and are designated L1-L5, respectively. Coding regions and families of late mRNAs are illustrated in Figure 2b.

The R-loops observed in the electron microscope display unpaired tails of single stranded RNA at both the 3', poly A and 5' ends (Chow et al., 1977a). The 5' terminus of late mRNAs contain an untranslated leader sequence of about 200 nucleotides joined to the body of the mRNA some distance away. The leader sequence is transcribed from at least three different sites at 16.4, 19.6, and 26.5 map units, then spliced together to form the leader sequence, which is spliced to the coding regions of the mRNAs. This tripartite

leader sequence has been found to be present on most late Ad 2 RNAs including hexon, 100K, and fibre (Berget et al., 1977, 1978; Chow et al., 1977b; Klessig, 1977; Broker et al., 1978). An additional sequence designated "i" occurrs in about 30% of 16 hour mRNAs and is derived from 22 to 23.2 map units (Chow et al., 1980). The fibre mRNA can contain additional splices of sequences from 77, 79 and/or 85 map units, between the tripartite leader and the coding region of the message (Broker et al., 1978; Chow and Broker, 1978; Dunn et al., 1978; Zain et al., 1979). Consistent with the determination of common 5' leader sequences on late mRNAs, Gelinas and Roberts (1977) have identified a single 5' undecanucleotide in ribonuclease T1 digest of total Ad 2 late mRNA extracts. The complete leader sequence for the hexon and fibre mRNAs (Akusjarvi and Pettersson, 1979; Zain et al., 1979) indicating the splice points of the leader sequences and the 5' terminus of the coding regions, as well as a lack of protein initiation codons within the leader sequence. The mechanism of the splicing process is unknown, however the most favoured model involves the looping out of RNA sequences of the primary transcript between each of the leader sequences and the coding region followed by intramolecular ligation (Klessig, 1977; Berget et al., 1978; Darnell, 1979). This would mean that each long primary transcript would generate only one mRNA species. Other models have been suggested to explain the joining of non-contiguous sequences of coding DNA found in mRNA. Intermolecular ligation of separate smaller transcripts or transcription on DNA templates with a secondary structure which brings distant sequences together or through DNA rearrangements such as

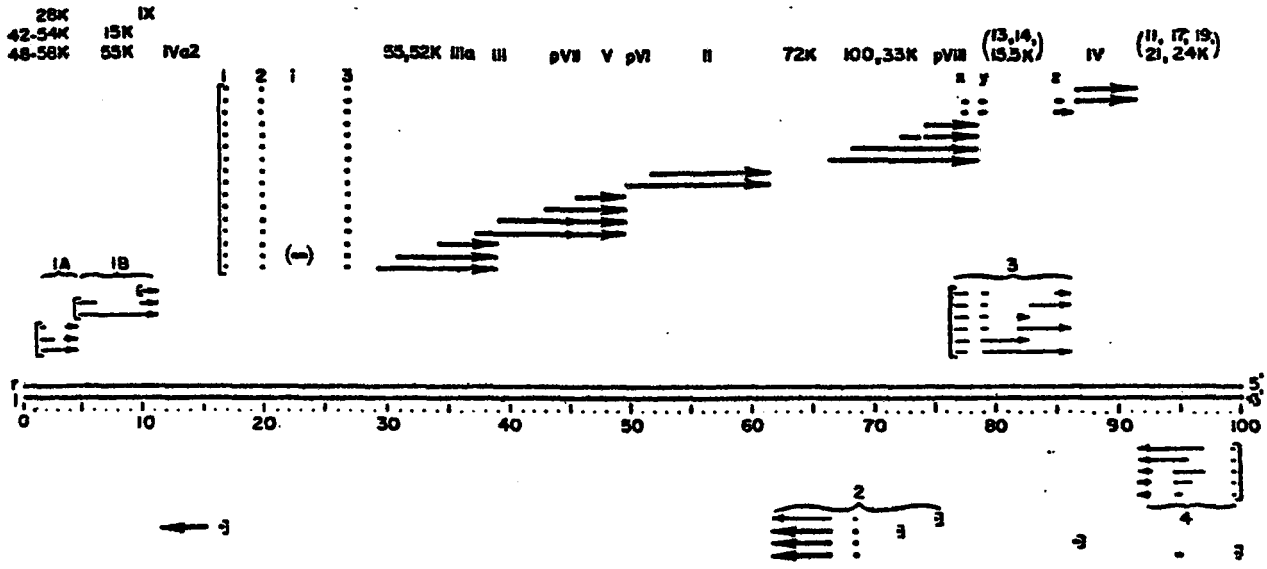


Figure 3. Map of Ad 2 cytoplasmic RNA transcripts determined by electron microscopy of RNA-DNA heteroduplexes. The Ad 2 genome is divided into 100 units representing approximately 35,500 base pairs. The r- and l-strand transcripts correspond with the direction of the arrows. The approximate locations of virus specific proteins are at the top of the map (from Chow et al., 1980).

deletions may also explain the splicing phenomenon (Flint and Broker, 1980; Darnell, 1979).

Five polyadenylation sites have been proposed for the late region on the r-strand, corresponding to the 3' end of each of the five families of late mRNAs. The polyadenylation event precedes splicing and occurs during transcription (Nevins and Darnell, 1978a) even though the polymerase continues to the termination site close to the right-most end of the genome (Fraser et al., 1979). The

termination sitesof the primary transcripts for the early regions 2 and 4 have also been located beyond the poly A addition site (Nevins et al., 1980). These observations suggest that the first critical event in the biogenesis of mRNA species is the selection of the poly A site by an appropriate endonuclease. This will determine the specific family of mRNAs, which will give rise to the production of a particular mRNA species by splicing at the 5' terminus of the primary transcript. A detailed transcription map of the late mRNA species and their locations on the Ad 2 genome is illustrated in Figure 3.

Briefly, the picture that emerges for the generation of late viral mRNAs is as follows: A primary transcript initiating at 16.4 map units transcribes the genome rightwards in equimolar amounts (Weber et al., 1977; Nevins and Darnell, 1978), and terminates near the extreme right end of the genome. The transcript is polyadenylated after selection of one of five poly A sites during transcription, capped at the 5' end and then spliced to generate individual mRNA species that are transported to the cytoplasm to be translated.

As mentioned above the possibility of another transcription unit within the major late region has been suggested and several other lines of evidence provide support for this idea. Late transcription studies involving the initiation and short pulse labelling of primary transcripts in vivo with ^{3}H-uridine followed by chain elongation in the presence of mercurated nucleotides in isolated nuclei have been reported by Weinmann and Aiello (1978). RNA molecules synthesized in this manner will preferentially accumulate label

towards the 5' ends and can be selected by affinity chromatography due to the mercurated nucleotides at the 3' ends. Hybridization to viral DNA restriction endonuclease fragments will indicate a peak at the 5' end of the RNA. Peaks of hybridization are detected at 7.5 - 17 map units and 11.3 - 18.1 map units for a rightward transcript, which is in good agreement with the location of the major late promotor at 16.4 and as well as a weak promotor within the left end 25% of the viral genome for a leftward transcript. In addition, a third 5' end is detected between coordinates 60 and 70 for a rightwards transcript. Another study examined the distribution of label in late nuclear RNA synthesized *in vivo* and *in vitro*. Late nuclear RNA was hybridized to various restriction fragments and did not yield a uniform distribution across the genome, a result which is inconsistent with the model of only one promotor and one termination site for the major rightward gene block (Zimmer *et al.*, 1978). These investigators suggest that more than one promotor-terminator regulatory element may function for late RNA synthesis. Manley and co-workers (1979) have examined the 3' ends of late nuclear RNA synthesized *in vitro* corresponding to the 5, 3' coterminal families. They were unable to detect the 3' end of a long nuclear RNA corresponding to the 5th family at 91.5 map units and have suggested the possibility of a separate promotor, which is not active *in vitro* for the synthesis of RNA for fibre. Berget and Sharp (1979) also could not detect a primary transcript with a 3' end at 91.5 *in vivo*, which may be the result of rapid processing and splicing. This event is unlikely *in vitro*, since very little splicing appears to occur and no mature fibre mRNA is synthesized.

The work presented in this thesis examines the nature of the nuclear primary transcription units, through studies of nuclear RNA, poly A containing cytoplasmic RNA, and viral polypeptides late during Ad 2 infection using the technique of uv transcription unit mapping. The results identify the existence of two transcription units which give rise to mRNA coding for the late structural proteins of Ad 2.

UV Transcription Unit Mapping

Following uv irradiation, a polar effect of uv damage on transcription of the E. coli lactose operon was observed (Starlinger and Kolsch, 1964). This effect was exploited by Sauerbier and co-workers to examine the transcriptional organization of a number of prokaryotic systems, which led to a molecular understanding of the polar effects induced by uv irradiation on transcription (Sauerbier, et al., 1970; Brautigam and Sauerbier, 1973, 1974; Hackett and Sauerbier, 1974). UV irradiation of DNA or DNA in cells produces photolesions mainly in the form of pyrimidine dimers (Hackett and Sauerbier, 1974), which results in premature chain termination of transcription without reinitiation of the RNA polymerase transcribing a damaged template past the photolesion (reviewed by Sauerbier 1975, 1976; Sauerbier and Hercules, 1978). This results in the inactivation of gene expression with DNA templates containing random photolesions due to an exponential decrease of transcription with increasing distance from the promotor. The probability that a given sequence will be transcribed is inversely proportional to the distance between that sequence and the promotor controlling its synthesis.

Figure 4 illustrates a single hypothetical transcription unit following uv irradiation and containing four genes A-D in the primary transcript. RNA polymerase binds at the promotor and RNA chain initiation occurs followed by chain elongation. During chain elongation, the transcribing RNA polymerase encounters a photolesion and the enzyme terminates the transcription process and does not resume transcription beyond the photolesion. A large population of damaged templates will give rise to a heterogeneous population of RNA chains from the transcription unit, with promotor proximal sequences being represented most often resulting in the prevalence of gene A RNA in the population. Promotor distal sequences will be found in progressively smaller amounts as is gene D RNA of Figure 4. Therefore, gene A RNA will be relatively resistant to the effects of uv compared to gene D RNA, allowing the correlation of radiosensitivity and target size to define the transcription unit.

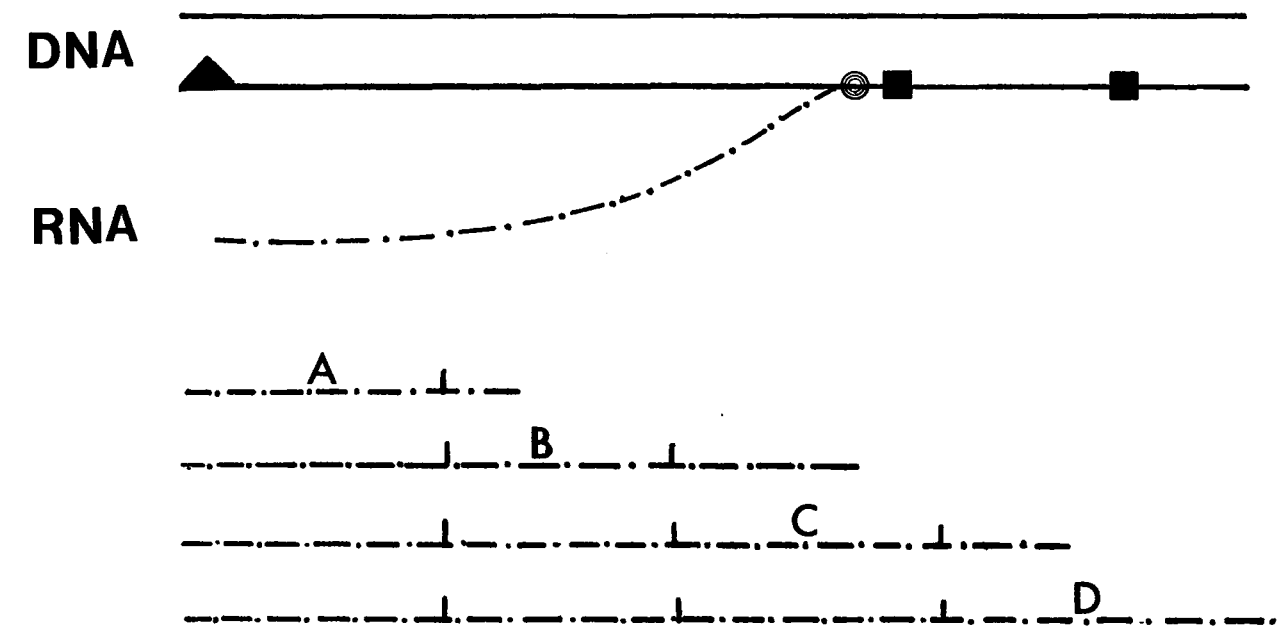
The binding of RNA polymerase to uv irradiated templates (Sauerbier et al., 1970; Ishihama and Kameyama, 1967; Chamberlain and Ring, 1973) and RNA chain initiation (Hagen et al., 1970; Sauerbier, 1973) are insensitive to uv irradiation relative to the reduction in RNA chain length. Termination and release of RNA chains and polymerase molecules at the photolesion occur with time constants similar to those for natural termination sites (Sauerbier et al., 1970).

The correlation of transcriptional inactivation and distance along the DNA (target size) assumes a random distribution of pyrimidine photoproducts. The near random distribution of pyrimidine dimers has been demonstrated within restriction endonuclease fragments of Ad 2 (Berk and Sharp, 1977a).

Figure 4

Model of uv transcription unit mapping. This model assumes a large number of DNA molecules of identical sequence with several potential transcription terminating photolesions. The first photolesion in the DNA would produce a truncated RNA molecule containing genes A and B but only a portion of gene. C would be represented in the transcription product as shown in the second RNA species illustrated in this figure. A potential photolesion (not shown) more proximal to the promotor on another DNA molecule of identical sequence would result in a shorter transcription product containing only gene A and a portion of gene B as shown in the first RNA species depicted. If only the second photolesion shown on the DNA existed then the transcription product would encode genes A, B, C and part of D. If no photolesions were induced all four genes A to D would be transcribed as shown in the last and longest RNA species.

Therefore, a large population of damaged templates with photolesions at different sites will give rise to a heterogeneous population of RNA chains from the transcription unit.



PROMOTOR

RNA POLYMERASE

PHOTOLESION

restriction endonuclease fragments of Ad 2 (Berk and Sharp, 1977a).

The determination of the transcriptional organization of bacteriophage T7, a well defined genetic system served as a critical test of the technique of uv transcription unit mapping. UV inactivation of T7 genes was assayed by protein or RNA levels with identical results (Brautigam and Sauerbier, 1973, 1974), correctly identifying the single promotor and allowing the precise positioning of fifteen T7 genes relative to their common promotor. A number of T4 genes have been positioned relative to their promotors on separate transcription units (Hercules and Sauerbier, 1973) and promotor switches for some early region T4 genes (Hercules and Sauerbier, 1974) have been determined by uv mapping. The single precursor to 16S and 23S ribosomal RNA genes of E. coli and the order of these genes within the transcription unit have been identified by this technique (Hackett and Sauerbier, 1974).

UV transcription unit mapping has also been successfully applied to several eukaryotic systems, such as in the identification of the correct direction of transcription of 45S ribosomal precursor RNA of mouse L cells (Hackett and Sauerbier, 1975); the determination of the order of the genes of the negative stranded virus, vesicular stomatitis virus (Ball and White, 1976); the identification of monocistronic transcription units for the synthesis of early vaccinia viral protein (Pelham, 1977); the identification of the single promotor for the regulation of Sendai virus transcription (Glazier et al., 1977); and the determination of the target sizes for transcription of many herpes simplex virus type 1 polypeptides (Millette, 1980).

UV transcription unit mapping offers a powerful technique for probing the transcriptional organization of many prokaryotic and eukaryotic systems.

MATERIALS AND METHODS

I. Tissue Culture

Monolayer cultures of Human KB cells and Human HeLa cells (provided by Dr. S. Mak) were maintained in modified minimal essential medium (Eagle F-11 MEM, Grand Island Biological Company (GIBCO), New York, cat. #410-1700) supplemented with 10% fetal calf serum or 10% calf serum, 1.21 mg/ml penicillin G and 100 µg/ml streptomycin (GIBCO) and sodium bicarobinate to a pH of 7.4. Confluent monolayers of KB cells were subcultured by gently scraping the cells from the plastic or glass surface with a rubber policeman. HeLa cell monolayers were subcultured by trypsinization using 1 ml of 1X trypsin-EDTA solution (GIBCO). The resulting suspensions were seeded into sterile 32 oz. glass bottles (Brockway Glass Co., Montreal) or 150 mm plastic dishes (Lux Scientific Corp., Newbury Park Co.) containing fresh medium. Cells were incubated at $37^{o}C$ in a humid atmosphere consisting of 5% CO_2 in air.

Suspension cultures of KB cells for virus propagation were maintained at a concentration of between 2.5 and 5.0 x 10^5 cells/ml in Joklik's modified MEM (spinner medium, cat. #410-1300, GIBCO) supplemented with 5% horse serum. Cells were kept in suspension by a sterile magnetic stirring bar.

II. Virus

A. Virus Strain

The wild-type strain of Adenovirus type 2 (Ad 2) obtained from Rainbow and Mak, (1970), was used throughout this study. The temperature sensitive mutant, Ad 2 tsl was obtained from J. Weber, (1976).

B. Viral Infection

1. Monolayer cultures. Monolayer cultures were used for most experiments. Cells were grown to confluency in 32 oz glass bottles or plastic 150 mm plastic dishes (1.25×10^7 cells) or in 60 mm plastic dishes (Falcon Plastics, Maryland), 3×10^6 cells). The medium was removed and the cells washed once with fresh prewarmed F-11 MEM. The larger monolayers were inoculated with 2 ml and the smaller cultures with 0.2 ml of virus suspension at a multiplicity of infection (MOI) of 50 plaque forming units (PFU) per cell in F-11 MEM + 1% fetal calf serum. Absorbtion was carried out for 60 minutes at 37°C with occasional rocking to evenly distribute the viral inocculum. Monolayers were then covered with fresh F-11 MEM + 5% calf serum.

2. Suspension cultures. Suspension cultures of KB cells were used for the preparation of Ad 2 virus. KB cells growing in suspension (about 3.5 litres) were pelleted by centrifugation (International Equipment Co. (IEC), Mass.) at 1,000 rpm at room temperature for 5 minutes and resuspended in Joklik's MEM plus 1% fetal calf serum at 1.0×10^7 cells/ml. Purified Ad 2 was added at a MOI of 10 PFU per cell and absorbed for 60 minutes at 37°C with slow and continuous agitation by a sterile magnetic stir bar. The infected cells were then diluted to 3×10^5 cells per ml in an equal mixture of the original growth medium and fresh Joklik's MEM + 5% horse serum.

Infected suspension cultures of KB cells were harvested forty-eight hours post infection by centrifugation (IEC) at 1,000 rpm for 5 minutes at 4°C. The infected cell pellet was resuspended in 30-50 ml of 20 mM Tris pH 8.1 and frozen at -60°C until purification. The

temperature sensitive mutant Ad 2 tsl was propagated in a similar manner, but absorption and the subsequent 72 hour incubation period was carried out at 33°C.

C. Virus Purification

After thawing at room temperature, the suspension described above was sonicated (Biosonic III, setting 30; Bronwill Scientific, Rochester) for two, 2 minute periods on ice. The suspension was then made 0.5% sodium deoxycholate and left at room temperature for 30 minutes with occasional gentle agitation. This viscous suspension was then adjusted to 5 mM $MgCl_2$ and treated with 7.5 µg/ml of pancreatic deoxyribonuclease (DNase, Sigman Chemical Corp., (Sigma), St. Louis, Missouri) and 2 µg/ml of ribonuclease (RNase, Sigma) at 37°C until the viscosity was reduced. The suspension was then brought to a density of 1.35 g/ml CsCl (Apache Chemical, Illinois) and subjected to two successive runs of isopycnic banding at 35,000 rpm in the ultracentrifuge (Beckman, CA; model L3-50) for 20 hours at 4°C. This CsCl is of a poor quality and must be filtered and titrated to pH 8.0 before use. The virus band was asceptically collected and the virus concentration measured by absorbance at 260 nm. One unit of absorbance at 260 nm corresponds to 3.5×10^{11} virus particles per ml (Mak, 1971). The virus was diluted to a concentration of about 5×10^{10} PFU/ml with tris-buffered-saline (136 mM NaCl, 5 mM KCl, 1.5 mM Na_2HPO_4, 12 mM glucose, 50 mM Tris pH 7.5) containing 30% glycerol and stored at -60°C. About 20 Ad2 virus particles is equivalent to 1 PFU (Green *et al.*, 1967b).

D. Plaque Assay

Viable viral titres were determined by a plaque assay. Ad 2 was plaqued on KB or HeLa cells immediately after the cells reached confluence in 60 mm plastic dishes. Ad 2 aliquots were serially diluted by ten-fold dilutions in F-11 MEM without serum and 0.2 ml of the appropriate dilutions were absorbed to the cell monolayer (in triplicate) for 1 hour at 30°C.

Two methods of plaquing were used. The sloppy agarose method consists of overlaying the plates after absorption with 10 ml of an equal volume of 1% agarose and the plaquing medium*. This mixture was kept at 45°C until the plates were ready to be overlayed. After overlaying, the plates were allowed to harden at room temperature and then incubated at 37°C for 5-6 days. To visualize the plaques, the sloppy agarose was carefully removed by inverting the plate and inserting a

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*Plaquing Medium

2 x F-11, MEM cat. #410-1100, GIBCO	40 ml	Fetal Calf serum cat. #200-6140, GIBCO	3.2 ml
BME Amino Acids (100 x) cat. #320-1051, GIBCO	2 ml	Horse Serum cat. #200-6050, GIBCO	6.0 ml
L-arginine (2.1%) Sigma	0.4 ml	Antibiotic Solution cat. #600-5295, GIBCO	1.0 ml
L-glutamine (20 mM) Sigma	1.0 ml	Fungizone (250 mg/ml) cat. #600-5295, GIBCO	0.5 ml
Yeast Extract (3%) Digco Laboratories, Michigan	1.6 ml	Sodium Bicarbonate (7.5%)	3.2 ml
Vitamin Solution (100 x) cat. #320-1040, GIBCO	1.0 ml		

spatula at the edge of the agarose. The monolayers were fixed and stained with a solution prepared by dissolving 2 g of crystal violet stain in 20 ml of methanol to which was subsequently added 144 ml of phosphate buffered saline (PBS; 0.8 g NaCl, 0.2 g KCl, 1.15 g Na_2HPO_4, 0.2 KH_2 PO_4 per litre) and 36 ml of formaldehyde. The stain was removed after 10 minutes and the cell sheets were washed with distilled water.

The neutral red method consists of overlaying the plates after absorption with 10 ml of an equal volume of 1.8% agarose and plaquing medium. After hardening at room temperature, the plates were incubated at 37°C for 6 days. After 6 days a second hard agarose overlay containing 0.3% neutral red stain (GIBCO) was added. After hardening, a further incubation period of 6-8 days at 37°C was required to visualize the plaques through the bottom of the plate on a Quebec colony counter. Clear, well defined plaques were counted and the titres calculated in PFU/ml. Ad 2 titres of 2.5 - 7.0 x 10^{10} PFU/ml were routinely obtained.

III. Radiolabelling of Cells

A. Viral DNA

Viral DNA was radiolabelled by the addition of ^{3}H-thymidine (New England Nuclear (NEN), Quebec; 20 Ci/mmole to a concentration of 10 µCi/ml to the culture medium 18 hours post-infection. The virions containing labelled DNA were then purified as described above and the DNA extracted. DNA prepared in this way had a specific activity of 4 x 10^5 cpm/µg.

B. Viral RNA

At the appropriate times post infection, the growth medium was

removed from the infected cell monolayers (150 mm, Lux) and replaced with 2 ml of prewarmed medium containing 75 μCi of ^{3}H-uridine (NEN, 37.5 Ci/mmole) to each dish and then incubated at 37°C. The dishes were periodically rocked gently to evenly distribute the labelling medium. Nuclear RNA was labelled for 7 minutes to 1 hour and cytoplasmic polyadenylated RNA was labelled for 1 hour followed by a 0.5 hour period in medium without label. The labelling periods were terminated by the addition of ice cold PBS and the cells immediately scraped from the plastic surface. The cells were collected by centrifugation (IEC) at 1,000 rpm for 5 minutes at 0°C, and the cell pellets frozen at -60°C until the RNA could be extracted.

C. Viral Protein

1. Infected cell proteins. At the appropriate times post-infection, the growth medium was removed from infected cell monolayers (60 mm, Falcon) and washed once with prewarmed PBS. The cultures were labelled in 0.5 ml of prewarmed methionine-free medium containing 30 μCi of ^{35}S-methionine (600-1200 Ci/mmole; Amersham, Oakville) and incubated for 1 hour at 37°C with periodic gentle rocking to evenly distribute the labelling medium. Medium free of the amino acid methionine was prepared according to the formulation provided by GIBCO for MEM (cat. #410-1700) except for the addition of methionine. The labelling period was terminated by the addition of ice cold PBS and the cells were immediately collected by scraping with a rubber policeman followed by centrifugation (IEC) at 1,000 rpm for 5 minutes at 0°C. The infected cell pellet was stored at -60°C until it was prepared for electrophoresis.

2. Virion structural proteins. Virions assembled with radioactively labelled structural proteins were prepared in spinner KB cultures. At 16 and 28 hours post-infection cells were concentrated to 1 x 10^7 cells/ml in prewarmed methionine-free medium containing 15 μCi/ml of ^{35}S-methionine and incubated for 1 hour at 37°C with constant agitation. After each labelling period the cells were diluted to 3 x 10^5 cells/ml in equal volumes of conditioned and fresh Joklik's medium. Incubation of the spinner culture was continued at 37°C until 48 hours post-infection, and then the virions were purified as described above. The virus band was dialyzed for 4 hours against 10 mM Tris pH 7.4 and concentrated with aquacide (Calbiochem., CA.) before being dissolved with an equal volume of 2 x electrophoresis sample buffer.

IV. Ultra-Violet Irradiation

At an appropriate time after infection, the medium was removed and the infected cells were washed once with prewarmed PBS. The infected cell monolayers were then exposed to ultra-violet (uv) irradiation of 254 nm. Dosimetry of the uv germicidal lamp (General Electric G8T) was carried out using a J-225 short wave uv meter (Ultra-Violet Products, Inc., San Gabriel, Ca.). The incident dose rate employed under these conditions was 1-5 joules/m^2sec. with doses ranging from 0 to 60 joules/m^2.

V. Nucleic Acid Extraction and Cellular Fractionation

A. Viral DNA Extraction

Purified virus was dialyzed for four hours at 4°C against 10 mM Tris, 5 mM EDTA, pH 8.0. The virus suspension was adjusted to 0.25%

SDS and mixed gently resulting in a clear, viscous suspension to which 1 mg/ml of pronase B (pre-digested for 3 hours at 37°C, Calbiochem.) was added. The mixture was incubated for 30 minutes at 37°C then extracted two to three times with an equal volume of redistilled phenol saturated with 50 mM Tris pH 8.0. Each extraction was for 5 minutes with gentle inversion of the extraction vessel; vigorous shaking was avoided. Phases were separated by centrifugation (IEC, 3,500 rpm, 5 min, 4°C). The final aqueous phase was made 0.15 M NaCl and the DNA was precipitated by the addition of two volumes of cold 95% ethanol. After overnight precipitation at -20°C, the DNA was pelleted (Sorval, 8,000 rpm, 20 min, 0°C). The pellet was washed once with cold 70% ethanol and then air dried. Finally, the pellet was dissolved in 10 mM Tris pH 7.4 and the DNA concentration was estimated by spectrophotometry at a wavelength of 260 nm, assuming that an abosrbance of 1 is given by a 50 µg/ml solution of DNA. Under these conditions, 12.8 µg of viral DNA could be routinely isolated per absorbance unit of purified virus suspension.

B. Preparation of Nuclear and Cytoplasmic Fractions

All manipulations were carried out at 0°C. Cells were collected by centrifugation (IEC, 1,000 rpm, 5 min) and washed with isotonic buffer (0.15 M NaCl, 10 mM EDTA, 10 mM Tris, pH 7.8). The pellets were resuspended to 2 x 10^7 cells/ml in isotonic buffer and an equal volume of isotonic buffer containing 1.6% NP40 (Bethesda Research Laboratories (BRL), Maryland) was added. When cells were being fractionated specifically for cytoplasmic isolation, 1% naphthalene-1, 5-sulfonic acid was added to the isotonic buffer prior to cell

lysis. This mixture was kept on ice for 10 minutes with periodic gentle shaking, and cell lysis was monitored by phase contrast microscopy. Nuclei were collected by centrifugation (IEC, 750 rpm, 5 min) and the supernatant was withdrawn and used as the cytoplasmic fraction. The nuclear pellet was withdrawn and used as the cytoplasmic fraction. The nuclear pellet was washed twice with isotonic buffer, and finally resuspended in 10 mM Tris, 10 mM EDTA, pH 7.3. Occasionally, a brief period (5-10 sec) of sonication was required to resuspend the nuclear pellet.

Monitoring of cell lysis by phase contrast microscopy indicated a cytoplasmic fraction free of nuclei and unlysed cells; and a nuclear fraction containing no obvious unlysed cells, although some cellular debris was detected.

Cells that have been labelled with ^{3}H-thymidine for 30 minutes were fractionated in this fashion and greater than 99% of the acid insoluble radioactivity was recovered in the nuclear fraction.

C. RNA Extraction

1. Nuclear RNA. RNA was extracted from the separated nuclear fraction by a modification of the method of Warner et al. (1966).

Only sterile extraction vessels and reagents were used. The nuclear suspension was made 50 mM NaHAc, 10 mM EDTA, pH 5.1 and SDS was added to 1%. After 30 minutes with periodic gentle shaking at room temperature, the lysate was extracted at 65^{o}C with an equal volume of distilled phenol-chloroform-isoamyl alcohol (75:24:1) saturated with the same buffer described above but lacking SDS and containing 1.0 mg/ml of 8-hydroxyquiniline. After each 5 minute extraction the

mixture was cooled in a methanol-ice bath and the phases separated by centrifugation (IEC, 3,000 rpm, 5 min, 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 NaHAc and the nucleic acids were precipitated overnight at -20°C by the addition of two volumes of cold 95% ethanol. The nucleic acids were pelleted (Sorval, 8,000 rpm, 30 min, 0°C) and washed with cold 70% ethanol until no phenol odour could be detected; usually one to two washings were sufficient. After air drying the pellet was taken up in 10 mM Tris pH 7.4, and adjusted to 10 mM $MgCl_2$ followed by the addition of 100 µg/ml of pancreatic deoxyribonuclease (DNase; Worthington, New Jersey or Boerhinger Mannheim, Germany; electrophoretically pure). The solution was digested for 1 hour at 37°C. After digestion, SDS was added to 0.5% and the mixture re-extracted as above, once at 65°C and then repeated at room temperature. The solution was made 0.15 M NaHAc and the RNA precipitated with two volumes of cold 95% ethanol overnight at -20°C. The RNA was pelleted as above and taken up in 5 mM Tris pH 7.3. In the following studies it was necessary that RNA extraction be highly reproducible. Therefore, extraction volumes were maximized, while still remaining convenient to handle and the extractions performed carefully.

2. Cytoplasmic RNA. Only sterile extraction vessels and reagents were used. The cytoplasmic extract was made 0.5% SDS and 100 µg/ml of poly vinyl sulphate was added. This mixture was extracted with an equal volume of distilled phenol-chloroform-isoamyl alcohol (50:49:1) containing 1.5 mg/ml of 8-hydroxyquiniline, saturated with 50 mM Tris pH 7.6, 1 mM EDTA. After 5 minutes of extraction with gentle inversion

of the tube at room temperature, the phases were separated by centrifugation (IEC, 3,500 rpm, 5 min, 20°C). The aqueous phase was removed and re-extracted twice as above, followed by one extraction with chloroform-isoamyl alcohol (49:1). Finally, the aqueous phase was made 0.15 M NaHAc and the RNA precipitated with two volumes of cold ethanol overnight at -20°C. The cytoplasmic RNA was pelleted by centrifugation (Sorval, 8,000 rpm, 30 min, 0°C), air dried and resuspended in 5 ml of sterile water. RNA concentrations were estimated by abosrbance at 260 nm, assuming that an absorbance of 1 corresponds to 42 µg/ml of RNA.

D. Polyadenylated RNA Isolation

A polyurydilate (poly U) sepharose column was prepared to isolate the polyadenylated RNA (poly A RNA) from the extracted cytoplasmic RNA (Taylor and Tse, 1976). The column matrix was prepared by swelling 0.4 g of poly U sepharose-4B beads (Pharmacia, Fine Chemicals, Uppsala, Sweden), in 20 ml of 1 M NaCl for one hour at room temperature. The beads were gently poured into a 10 cc disposable syringe containing a glass fibre filter and fitted with an 18 G needle, which pierces a sterile silicon stopper to regulate the column's flow. The column is provided with a 50 cc syringe fitted with a 18 G needle as a reservoir above the column and piercing a rubber plunger fitting the top of the column. The column was washed with 30 ml of high salt buffer I (HSB I, 0.7 M NaCl, 50 mM Tris pH 7.5, 10 mM EDTA pH 7.5, and 25% formamide). The formamide used in buffers applied to the column was purified by ion exchange with mixed bed resin (Bio-Rad, Ca.) in 5% w/v, swirled for 1-2 hours and then filtered through a glass fibre filter. To 5.0 ml of extracted cytoplasmic RNA in sterile water (maximum 100 absorbance

units of RNA), 5.0 ml of 2 x HSB I was added and the mixture slowly loaded on the column, while collecting the void volume. The void volume was reloaded and allowed to flow through the column. The column was then washed with 25 ml of HSB I followed by a 25 ml wash with HSB II (0.5 M NaCl, 50 mM Tris pH 7.5, 10 mM EDTA pH 7.5, 50% formamide). Polyadenylated RNA was isolated by applying 10 ml of elution buffer (10 mM Tris pH 7.6, 10 mM EDTA pH 7.5, 0.2% SDS, 90% formamide) to the column and collecting 0.5 ml fractions through a 22 G needle. A 5 µl aliquot of each fraction was assayed by liquid scintillation counting to identify the labelled polyadenylated RNA. The peak fractions were pooled and the polyadenylated RNA was precipitated with an equal volume of cold 0.8 M ammonium acetate in 95% ethanol overnight at -20°C. The poly A RNA was pelleted by ultracentrifugation (Beckman, L3-50, 35,000 rpm, 0°C, 2 hrs) in polyalymer tubes. The pellet was washed with cold 0.4 ammonium acetate in 50% ethanol and ultracentrifugation repeated. The poly A pellet was air dried and resuspended in 1 ml of 5 mM Tris pH 7.3.

E. Control DNA and Carrier RNA

E. coli DNA (Sigma) used as a control to assess the level of non-specific binding of RNA during nitrocellulose filter hybridization; and yeast RNA (British Drug House, (BDH), England), used as a carrier during hybridizations were purified. Each nucleic acid was dissolved in 10 mM Tris pH 7.4 and made up to 0.5% SDS before being repeatedly extracted with phenol-chloroform-isoamyl alcohol (75:24:1) saturated with 50 mM Tris pH 7.4. The final aqueous phase was adjusted to 0.15M

NaCl and the nucleic acids precipitated with two volumes of cold 95% ethanol. After precipitation at -20°C, overnight the nucleic acids were pelleted by centrifugation and the pellets air dried. The pellets were dissolved in 10 mM Tris pH 7.4 and their concentrations estimated by spectrophotometry at 260 nm.

VI. Manipulations with DNA

A. Cleavage of DNA with Restriction Endonucleases

Viral or plasmid DNA was digested with EcoRI (Bethesda Research Laboratories), HindIII, BamHI or BglII (Boehringer Mannheim) in buffers recommended by the suppliers. DNA was usually incubated with 0.25 units of restriction endonuclease per microgram of DNA at 37°C for 2-4 hours. Digests were analyzed by electrophoresis on vertical 1% agarose gels.

B. Recovery of Viral Restriction Fragments from Agarose Gels

Preparative amounts of viral DNA (400 µg) were digested with EcoRI or BamHI and the resulting DNA fragments electrophoresed through a horizontal agarose slab gel. After electrophoresis the separated DNA fragments were recovered by inserting strips of filter paper (3MM, Whatman, England) backed by dialysis membrane into slits cut in the gel in front of the DNA bands and continuing electrophoresis until the DNA is collected in the paper. Collection of the fragments can be monitored by examination under ultra-violet light. The filter and dialysis membrane were then removed onto a piece of parafilm and separated from one another. After blotting up any DNA that may be on the dialysis membrane, the filter paper was placed in a 1.5 ml Eppendorf tube, which had been pierced at the bottom with a hot 26 G needle and was inserted

into the top of a polypropylene tube (12 x 75 mm, Falcon Plastics). The tubes were spun for 2 minutes at 5,000 rpm in a bench top centrifuge so that the DNA eluting from the paper was collected in the bottom tube. The strip of filter paper was further soaked with 0.2 ml of TNE-SDS buffer (10 mM Tris pH 7.6, 10 mM NaCl, 1 mM EDTA, 0.2% SDS), incubated for 30 minutes at room temperature and again centrifuged. This step may be repeated two to three times to ensure that all of the DNA has been eluted from the paper. Finally, the DNA solution was adjusted to 3% NAHAc, and precipitated with two volumes of cold 95% ethanol, overnight at -20°C or held at -60°C for 1-2 hours. After precipitation, the DNA was collected by ultracentrifugation (Beckman, L3-50) at 35,000 rpm for 45 minutes. The DNA pellet was washed with 95% ethanol, dried at room temperature, and dissolved in 10 mM Tris pH 7.6, 1 mM EDTA.

Recovery well above 70% is routinely obtained with this technique and the DNA recovered was biologically active and can be recleaved (Girvitz, et al., 1980).

C. Strand Separation of Plasmid DNA

PBR 322 containing HindIII inserts of Ad 2 restriction fragments was digested with restriction endonucleases for the shortest possible time to achieve complete digestion but minimize the nicking properties of the enzymes used. In order to resolve the complementary strands of DNA after digestion, the solution was made 20 mM EDTA and 0.2 M NaOH. After 10 minutes at room temperature, the alkali-denatured DNA was subjected to vertical electrophoresis through 1.4% agarose gels in 30 mM Tris, 36 mM NaH_2PO_4, 1 mM EDTA, pH 7.8. Gels were usually run for 12

hours at 40 volts with continuous circulation of the electrophoresis buffer. DNA was visualized under short-wave uv illumination after staining the gel with ethidium bromide (EtBr, 0.5 µg/ml).

VII. Molecular Cloning

The preparation of large amounts of pure viral DNA fragments was achieved by molecular cloning. All cloning experiments were performed under level B containment as specified by the Medical Research Council of Canada (1979) guidelines.

1. DNA ligation. The E. coli plasmid pBR322 (obtained from R. McKinnon, McMaster University) was used as the amplification vector (Bolivar et al., 1977). pBR322 carries the drug resistance genes for ampicillin and tetracycline with a single HindIII restriction site in the tetracycline resistance gene (Figure 5). For the ligation of viral DNA fragments into pBR322, both plasmid and viral DNA to be inserted were digested with HindIII and then the enzyme was heat inactivated at 56°C for 15 minutes. Both DNA solutions were incubated together in 10 mM $MgCl_2$, 20 mM dithiothreitol, 50 mM Tris pH 7.7, 1 mM ATP, 50 µg/ml BSA and 0.1 unit of T4 DNA ligase (New England Biolabs, Ma.) per µg DNA at 4°C for 18-24 hours. The reaction was terminated by heating at 56°C for 15 minutes.

2. Transformation and screening for recombinant clones. Overnight cultures of E. coli LE 392 were diluted 1:100 into Luria broth (5 g yeast extract, 5 g NaCl, 10 g tryptone, 1 g glucose, per litre) and incubated about 2 hours (OD_{660} of 0.5). Approximately, 100 ml of cells were collected by centrifugation (IEC, 3000 rpm, 4°C, 10 min)

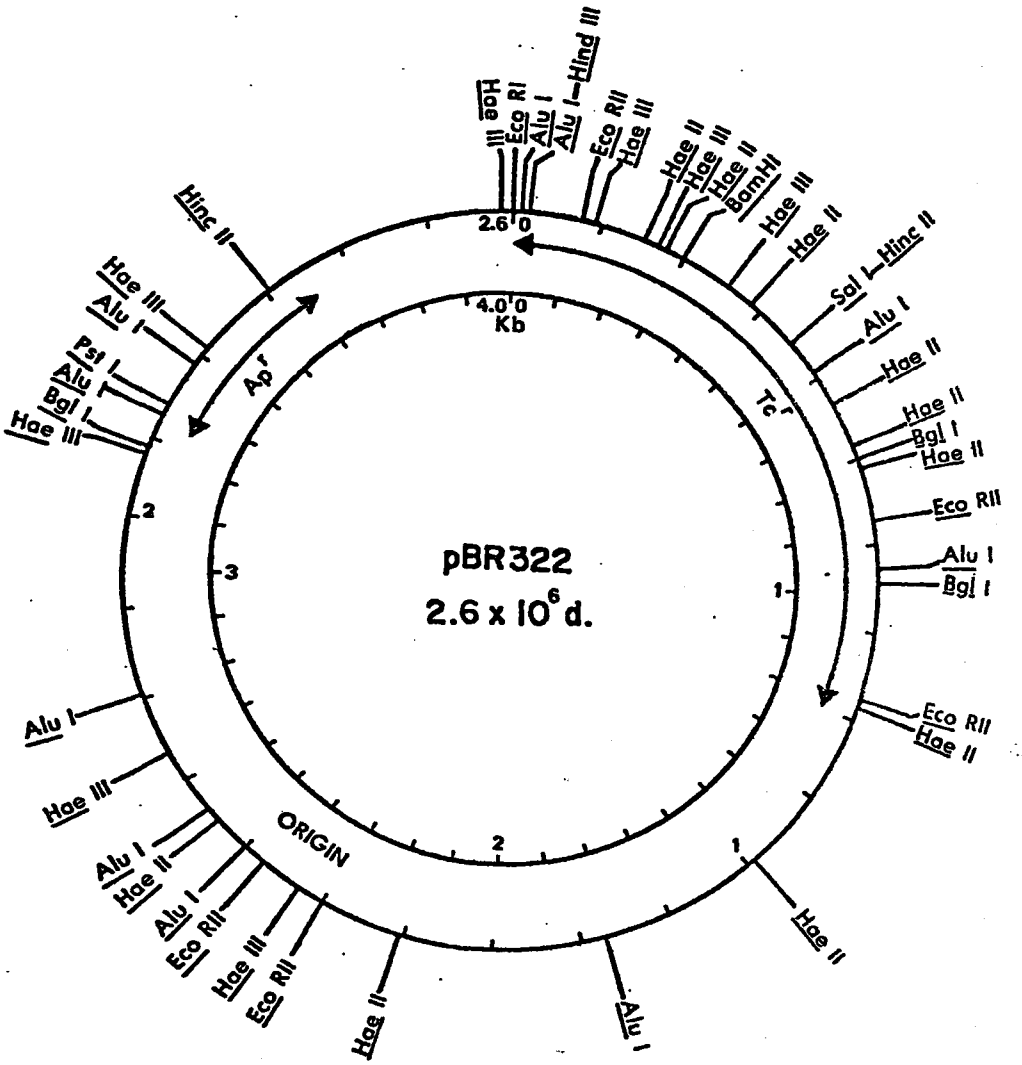


Figure 5: The circular restriction map of pBR322. The map is divided into units of 1 x 10^5 daltons (outer circle and 0.1 kilobases (inner circle). The size and position of the ampicillin (Ap^r) and tetracycline (Tc^r) resistant genes are shown. The unique HindIII and BamHI restriction cleavage sites are found within the tetracycline resistant gene (Bolivar et al., 1977).

and resuspended in 25 ml of 5 mM Tris pH 7.6, 7.5 mM $CaCl_2$, and incubated at 4°C for 20 minutes. The cells were again collected by centrifugation and resuspended in 2 ml of Tris-$CaCl_2$ plus 440 ng of ligated DNA. After 1 hour on ice with periodic agitation, the cells were heat shocked at 42°C for 2 minutes and added immediately to 8 ml of warm Luria broth. The bacteria were then serially diluted in pre-warmed Luria broth, and 0.2 ml aliquots from each dilution were plated onto Luria agar (1.8% noble agar in Luria broth) containing 20 µg/ml of ampicillin and incubated at 37°C overnight. Ampicillin resistant colonies were picked and transferred to duplicate gridded plates of Luria agar plus 20 µg/ml of ampicillin or Luria agar plus 10 µg/ml of tetracycline and incubated at 37°C overnight. The ampicillin resistant-tetracycline sensitive colonies were picked and grown in 10 ml of Luria broth plus 20 µg/ml of ampicillin with continuous shaking at 37°C.

3. Screening of plasmid DNA. The 10 ml cultures described above were incubated until the cultures reached an absorbance at 660 nm of approximately 0.6. A 1 ml aliquot was added to 1 ml of 40% glycerol in numbered vials and stored at -20°C for later use. Chloramphenical (Boerhringer Mannheim) was added to the remaining cells to a concentration of 50 µg/ml and the culture incubated for a further 12-18 hours at 37°C with continuous shaking. Cells were collected by centrifugation and plasmid DNA prepared using the method described by Birnboim (1980). Cell pellets were resuspended in 1 ml of Luria broth, transferred to 1.5 ml Eppendorf tubes (Brinkmann Instruments, N.Y.) and

centrifuged (Eppendorf, Model 5412) at room temperature for 15 seconds. The supernatant was removed by aspiration and the cell pellet resuspended in 100 µl of 25 mM Tris pH 8.0, 10 mM EDTA, 50 mM glucose and 2 mg/ml lysozyme (Sigma). This mixture was incubated at $0^{o}C$ for 30 minutes followed by the addition of 200 µl of alkaline SDS (0.2 N NaOH, 1% SDS) and gently mixed by inversion of the tube. After 5 minutes at $0^{o}C$, 150 µl of 3 M NaHAc pH 4.8 was added and the tubes mixed by inversion, and incubated for a further 60 minutes at $0^{o}C$. The samples were then centrifuged for 5 minutes and the supernatants transferred to a second Eppendorf tube followed by the addition of two volumes of cold 95% ethanol and the nucleic acids precipitated overnight at $-20^{o}C$. The nucleic acids were collected by centrifugation, the supernatant removed by aspiration and the pellet dissolved in 50 mM Tris pH 8.0, 0.1 M NaHAc. This was reprecipitated in two volumes of cold 95% ethanol and the final pellet dissolved in 50 µl of 10 mM Tris pH 7.4, 1 mM EDTA. Aliquots of this extract were digested with HindIII and screened on analytical 1% agarose gels to identify specific recombinant DNA plasmids.

4. Preparative recombinant plasmid DNA purification. For preparative recombinant plasmid DNA purifications approximately 1 litre of bacteria carrying the plasmid of interest was grown in Luria broth supplemented with 20 µg/ml of ampicillin to about OD_{660} of 0.6. Chloramphenical was added to a concentration of 50 µg/ml and incubation was continued for 12-18 hours at $37^{o}C$ with continuous shaking. To isolate the plasmid DNA, the Birnboim method (1980) described above, was scaled up 50-fold. The final nucleic acid pellet obtained

after ethanol precipitation was dissolved in 10 mM Tris pH 7.5, 1 mM EDTA, 1.54 g/ml CsCl (BDH), 0.2 mg/ml EtBr, and the DNA banded to equilibrium at 35,000 rpm for 44 hours at 10°C. Banded plasmid DNA was recovered from the gradient using a 1 ml disposable syringe with a 25 G needle, and then banded a second time as above. The plasmid DNA recovered from the second gradient was extracted with three changes of CsCl saturated isoamyl alcohol to remove the EtBr, and the DNA was extensively dialyzed against 10 mM Tris pH 7.5, 1 mM EDTA. The plasmid DNA solution was then made 0.15 M NaHAc and precipitated by the addition of two volumes of cold 95% ethanol. After overnight precipitation at -20°C, the plasmid DNA was pelleted (Sorval, 8,000 rpm, 20 min, 0°C) and the pellet air dried and dissolved in 2 ml of 10 mM Tris pH 7.4. Plasmid DNA concentration was estimated from the absorbance at 260 nm of the solution, assuming that an absorbance of 1 is given by a 50 μg/ml solution of DNA. This estimation suffers from the possible contamination of the plasmid DNA with bacterial RNA. Therefore, a second method of comparing the EtBr fluorescence of a known standard and plasmid DNA on an agarose gel was also employed.

VIII. Nucleic Acid Hybridization

A. Filter Hybridization

The method of Gillespie and Spiegelman (1965) was used. Viral DNA, viral DNA restriction fragments, recombinant DNA plasmids or control DNA (usually *E. coli*) in 0.1 x SSC (1 x SSC (standard saline citrate); 0.15 M NaCl, 0.015 M sodium citrate) was boiled for 10 minutes and then directly put on ice. The DNA solution was diluted to the appropriate concentration with cold 2 x SSC and 5 ml was filter-

ed under suction onto nitrocellulose filters (0.45 micron pore size; Sartorius, Germany) previously equilibrated with 2 x SSC. Each filter received 0.5 to 5 μg of DNA. The filters were washed extensively with 2 x SSC, dried at 37°C for 2 hours and then baked at 80°C for 4 hours to immobilize the DNA.

Nuclear RNA labelled with ^{3}H-uridine was partially hydrolyzed in 0.2 N NaOH at 0°C for 10 minutes and then neutralized with 0.5 M hepes (GIBCO). This solution was made 2 x SSC, 0.1% SDS, 0.75 mg/ml yeast RNA and 1 ml was incubated with each filter at 65°C for 24 hours in a tightly stoppered vial. Hybridization reactions were carried out in duplicate or triplicate in all experiments. After hybridization the filters were extensively washed with 2 x SSC under suction, treated with 20 μg/ml of pancreatic ribonuclease at room temperature for 1 hour, and then washed again with 2 x SSC. Finally, the filters were placed in glass scintillation vials, air dried and dissolved in 1 ml of ethyl acetate for 30 minutes at 37°C (Kobayashi and Harris). The radioactive RNA that hybridized to each filter was assayed by liquid scintillation counting using 10 ml of aquasol-2 (NEN, cat. #NEF-952) scintillation cocktail.

B. Blot Hybridization

1. Transfer of DNA from agarose gels to nitrocellulose sheets. DNA was transferred from gels to nitrocellulose sheets using a modification of the "Southern blot" technique (Southern, 1975). Briefly, after electrophoresis, agarose gels were soaked in two changes of 0.25 M HCl for 12 minutes each to partially depurinate the DNA, followed by two changes of 0.5 M NaOH plus 1 M NaCl for 15 minutes each to denature

and fragment the DNA. This was followed by a neutralization step in 0.5 M Tris pH 7.5 plus 3 M NaCl with two changes for 30 minute periods. DNA was then transferred from the gel to a nitrocellulose sheet by blotting for 2-4 hours. The sheet was then gently rinsed in 6 x SSC and baked for 4 hours at 80°C.

2. Hybridization of ^{3}H-RNA to nitrocellulose transfers. Nitrocellulose sheets with bound DNA were preincubated at 65°C in sealed plastic bags with 2 x SSC, 0.1% SDS and 0.75 mg/ml yeast RNA for 2 hours. The preincubation mixture was replaced with 2 x SSC, 0.1% SDS, 0.5 mg/ml yeast RNA and 3-6 x 10^{6} cpm of ^{3}H-RNA that has been partially hydrolyzed by incubation for 10 minutes at 0°C in 0.2 N NaOH and then neutralized with hepes (Goldberg et al., 1977). Hybridizations were carried out for 30 hours at 65°C. After hybridization, the sheets were washed extensively with 2 x SSC and then treated with 20 µg/ml of pancreatic ribonuclease in 2 x SSC for 1 hour at room temperature followed by repeated washings with 2 x SSC. After air drying the sheets were sprayed with an intensifier (En-^{3}Hance; NEN) and exposed to Kodak XAR-5 film pre-sensitized by a short exposure to a flash of white light (Laskey and Mills, 1975). The fluorograms were exposed at -60°C for 10-60 days and developed in a Kodak automatic X-Ray processor.

IX. Electrophoresis

A. Agarose Gels

1. Horizontal Electrophoresis. A horizontal electrophoresis apparatus was constructed similar to the Eltech design (Eltech, Burlington)

and was used for the preparative isolation of Ad 2 restriction fragments. Ad 2 DNA (400 µg) digested with EcoRI was electrophoresed through 1% agarose (Miles Laboratories, South Africa) in electrophoresis buffer (40 mM Tris, 5 mM NaHAc, 1 mM EDTA, pH 7.8) and BamI digested Ad 2 DNA was electrophoresed through 0.85% agarose in electrophoresis buffer. Approximately, 500 ml of hot agarose was poured onto a glass plate within the plexiglass chamber of the apparatus and a 15 x 0.8 x 3 mm well was formed near the cathode end of the gel. After hardening, the well former and the end blocks were removed and electrophoresis buffer was poured into the end chambers. The restriction enzyme digest was diluted to 2 ml with electrophoresis buffer and 0.35 ml of sample buffer (40 mM Tris, 20 mM EDTA, pH 7.8 + 15% ficoll) was added before being placed in the well. Electrophoresis was carried out at 1-2 volts per cm for 24-48 hours at room temperature with continuous circulation of the electrophoresis buffer. After about 1-2 hours of running the sample well was filled with electrophoresis buffer, the entire gel covered with saran wrap and a lid put over the apparatus to prevent dessication. The migration of DNA bands could be monitored under uv by the addition of 0.5 µg/ml of EtBr to the agarose and the electrophoresis buffer.

2. Analytical electrophoresis. Vertical 1% agarose slab gels were used for the analysis of restriction digests of viral and plasmid DNA. The gels were formed between two glass plates separated by 3 mm thick spacers. After the hot agarose was poured between the plates a 13 tooth comb was set in the top of the gel to form the running channels. After hardening, the comb was removed and the glass plates

were tightly mounted on a vertical electrophoresis apparatus. To about 20 µl of DNA solution, 5 µl of sample buffer was added and the samples were loaded into the wells. The gels were run at 30 volts for 12-14 hours in electrophoresis buffer at room temperature. After electrophoresis the gel was stained with 0.5 µg/ml of EtBr and the DNA bands were visualized under the uv light. The buffers and stain are identical to those described above.

B. SDS-Polyacrylamide Gel Electrophoresis

SDS-polyacrylamide gel electrophoresis (SDS-PAGE) was performed using the discontinuous buffer system described by Laemmli (1970) for the separation of proteins by molecular weight. Gels were formed as slabs 0.15 mm thick between glass plates. A stacking gel of 5% acrylamide and 0.16% bisacrylamide containing 12 sample slots was cast above the running gel of 15% acrylamide and 0.86% bisacrylamide.

Samples were prepared for electrophoresis by disruption of 10 µl of sample with an equal volume of 2 x SDS sample buffer (1 x SDS sample buffer, 62.5 mM Tris pH 6.8, 2% SDS, 10% glycerol, 0.001% bromophenol blue, 0.1 M dithiothreitol) as described by Anderson and Gesteland (1972). Prepared samples were heated in a water bath at 100°C for 2 minutes and then 10 µl of sample were loaded in each slot. Electrophoresis was carried out at 80 to 120 volts for 12-14 hours with the electrophoresis buffer of Laemmli (1970). After electrophoresis, the gels were stained with 0.05% Coomassie brilliant blue (in 25% isopropanol, 6.5% acetic acid), destained in 30% methanol and dried under vacuum. Autoradiograms were exposed for 24 to 48 hours on Kodak RPR medical X-ray film.

X. Quantitation of Viral Polypeptide Bands

To quantify the radioactivity present in a viral polypeptide after SDS-PAGE, the autoradiogram was used as a guide for the excision of the appropriate radioactive polypeptide bands from the dehydrated gel. The dry gel fragments were dissolved in 0.2 ml of 30% H_2O_2 at 37°C for 24 hours and then mixed with 10 ml of aquasol (NEN, Cat. #NEF-934) and assayed by liquid scintillation counting.

Another method made use of the Joyce Lobel MKIII CS double beam recording microdensitometer. The relative amount of radioactive protein in each band was determined from the area under each peak after scanning the autoradiogram. A calibration curve for Kodak RPR film was constructed through an increasing gradient of exposure to the film and measurement of the corresponding film densitites. All film densities to be used as experimental measurements, fell on the linear part of the characteristic curve of the film.

RESULTS

I. Isolation and Purification of Ad 2 DNA Fragments

Ad 2 transcription unit mapping experiments assayed by DNA-RNA hybridization require a source of purified viral DNA fragments of known origin on the physical map of the viral genome. Bacterial restriction endonucleases cleave DNA at specific nucleotide sequences. A large number of bacterial restriction enonucleases capable of generating Ad 2 DNA fragments have been isolated and physical maps of the Ad 2 genome identifying the specific restriction endonuclease cleavage sites have been determined for several of these enzymes (Tooze, 1980). The restriction maps for the endonucleases, Eco RI, Bam HI, Hind III, and Bgl II are shown in Figure 6. Ad 2 DNA fragments generated by restriction endonucleases were isolated and purified by preparative electrophoresis on horizontal agarose gels or by molecular cloning.

A. Preparative Horizontal Gel Electrophoresis

I. Efficiency of isolation of Ad 2 DNA fragments. Ad 2 DNA was digested with Eco RI and then loaded into a single well of a preparative horizontal 0.85% agarose gel and subjected to electrophoresis. The Ad 2 genome contains five Eco RI cleavage sites, which generates six fragments, denoted A-F by increasing electrophoresis mobility (Fig. 6). The six Ad 2 Eco RI fragments can be clearly separated by electrophoresis and visualized under UV light after staining with ethidium bromide as shown in Fig. 7.

Each of the Ad 2 DNA restriction fragments may be recovered

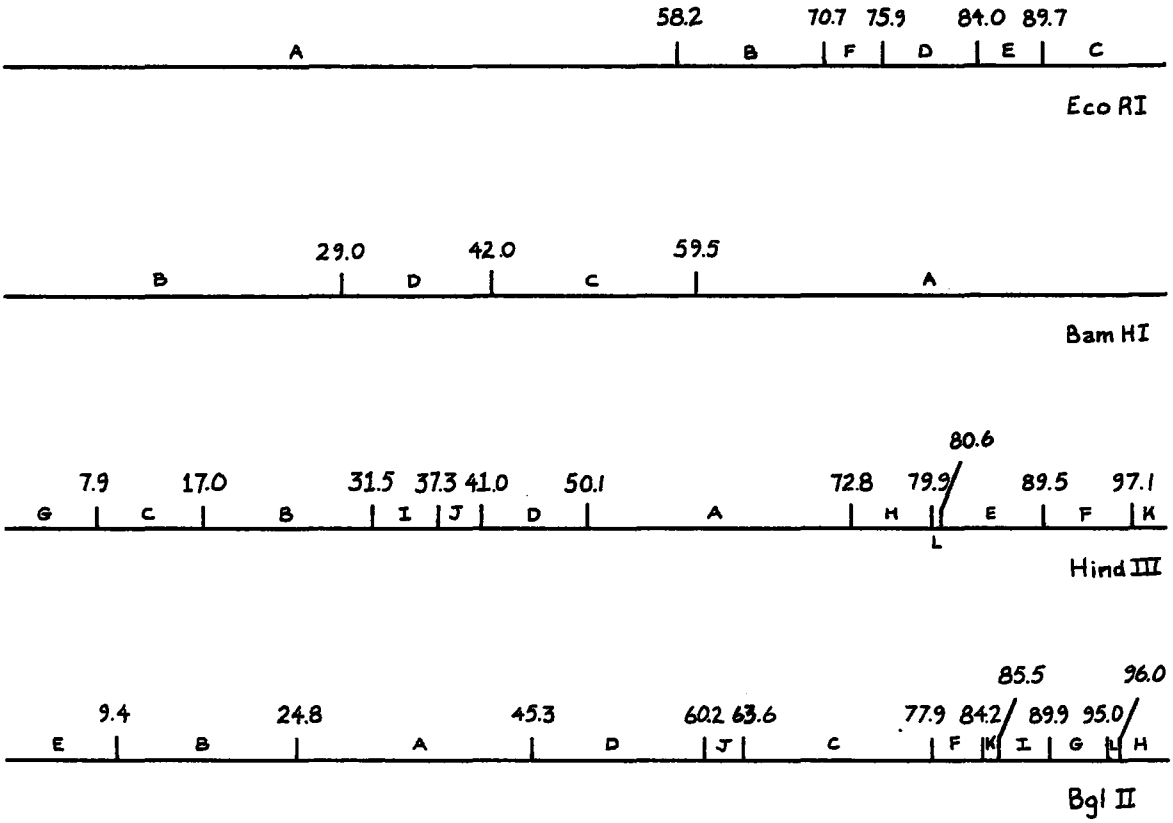
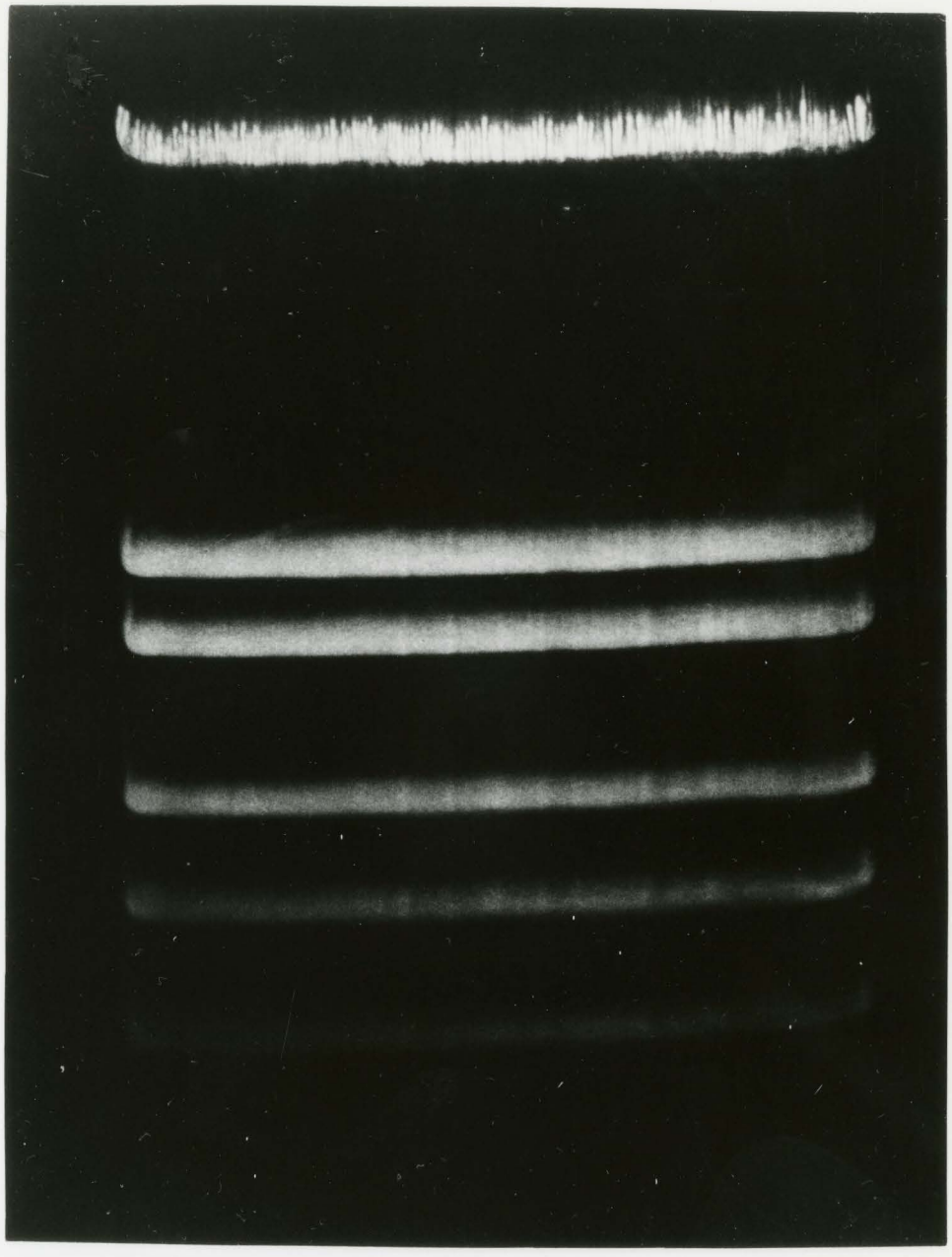


Figure 6: Restriction endonuclease cleavage maps of Ad 2 DNA

The physical map of the Ad 2 genome is divided into 100 map units with each unit representing 1% of the genome or approximately 350 nucleotide pairs. The vertical lines represent specific enzyme cleavage sites and their coordinates in map units are indicated. Each fragment is named alphabetically on the basis of size (Tooze, 1980).

Figure 7

Preparative horizontal gel electrophoresis. Ad 2 DNA (400 µg) cleaved with 0.25 units of Eco RI per µg of DNA for 4 hours at 37°C and subjected to electrophoresis through 0.85% agarose at 30 volts for 36 hours. The six Ad 2 Eco RI fragments are labelled A to F by increasing electrophoretic mobility. The gel is stained with ethidium bromide before being visualized under UV light.


A
B
C
D
E
F

from the agarose gel by electrophoresis onto strips of filter paper backed by dialysis membrane and then eluted as described in Materials and Methods. The amount of each individual fragment recovered was determined by either liquid scintillation counting of ^{3}H-labelled DNA or by spectrophotometry of unlabelled DNA. The percentage recovery was calculated relative to the expected amount of each fragment based on the DNA input and fragment size. As shown in Table 1,yields above 70% and often 80% were routinely obtained. It was found that recovery increased if two cycles of collection onto filter strips were used for bands containing more than 200 μg of DNA.

2. Purity of Ad 2 DNA restriction fragments. The purity of the isolated fragments were assessed by electrophoresis on vertical 1% agarose gels. Fig. 8 shows the typical Eco RI cleavage pattern of Ad 2 DNA as well as the migration of each of the fragments A to F isolated from the preparative horizontal agarose gel. No cross-contamination between fragments is apparent from this isolation. A trace amount of Ad 2 Eco RI C fragment is occasionally observed with isolated B fragment due to a small amount of streaking during the preparative gel electrophoresis and is not a property of the collection method. The Ad 2 Eco RI B fragment isolated in this manner may be subjected to a second cycle of preparative gel electrophoresis and collection to attain a purity similar to that of the other isolated fragments. The efficiency of recovery for the second cycle of electrophoresis and collection is comparable to that illustrated in Table 1.

TABLE 1

Recovery of Ad 2 DNA fragments generated by cleavage with Eco RI and isolated from preparative agarose gels

Expt. #	DNA input (µg)	Ad 2 DNA fragments (% recovery)					
		A	B	C	D	E	F
1	150	77	75	76	82	80	67
2	200	67	82	91	84	82	89
3	300	81	88	78	88	70	90
4	400	79	83	78	98	88	95
5	500	47	71	82	71	80	85

The amount of each fragment recovered was determined by liquid scintillation counting for expt. #1 (input 150 µg of ^{3}H-DNA at 4.4×10^{5} cpm/µg) and by spectrophotometry for expts. #2-5 in which unlabelled DNA was used. The % recovery for each fragment was calculated from the expected amounts based on the fragment size.

3. Cleavage of the Ad 2 Eco RI A fragment. DNA recovered using the filter paper method could be recleaved with other restriction enzymes. Complete digestion could be achieved by increasing the enzyme concentration to 1 unit per μg of DNA with the addition of 1 mg/ml of bovine serum albumin to the reaction mixture. The Ad 2 Eco RI A fragments represents the left hand 58.2% of the viral genome and contains two of the three Bam HI cleavage sites present on the genome (Ad 2 Bam HI cleavage sites; 29.0, 42.0, and 59.5 map units; Fig. 8, lane 5). The Ad 2 Eco RI A fragment was cleaved with Bam HI to generate 3 fragments; B, C, and D (Fig. 8, lane 4). These fragments were then isolated on a preparative 0.65% agarose gel, collected and checked for purity on the analytical agarose gel shown in Fig. 8 (lane 1 to 3). The results indicate that complete digestion of the Eco RI A fragment was achieved with Bam HI and no apparent cross contamination of the DNA fragments was present after isolation and purification.

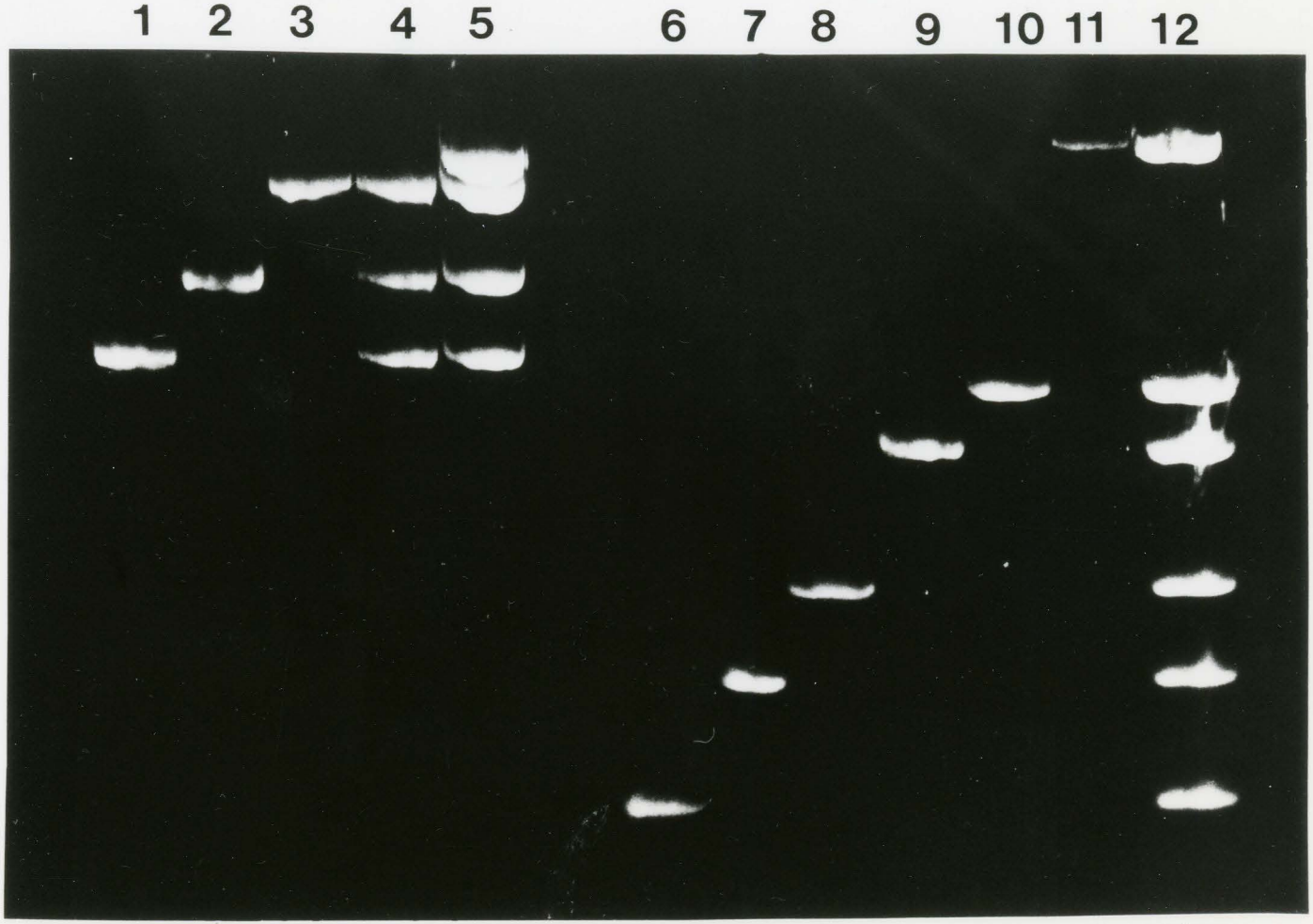
The isolation and recovery of DNA from preparative agarose gels provides a reliable source of good quality viral DNA fragments, but has several disadvantages. Firstly, smaller DNA fragments are available in limited total amounts per electrophoretic run. Also the method can be time consuming and requires many runs for a continuous source of experimental DNA. Finally, absolute purity for all fragments in a single run cannot be guaranteed and is dependent upon differences in relative electrophoretic mobility.

B. Preparation of Ad 2 fragments by molecular cloning.

1. Screening of recombinant DNA plamids. Pure viral DNA fragments were also prepared by molecular cloning. Ad 2 DNA digested to complet-

Figure 8

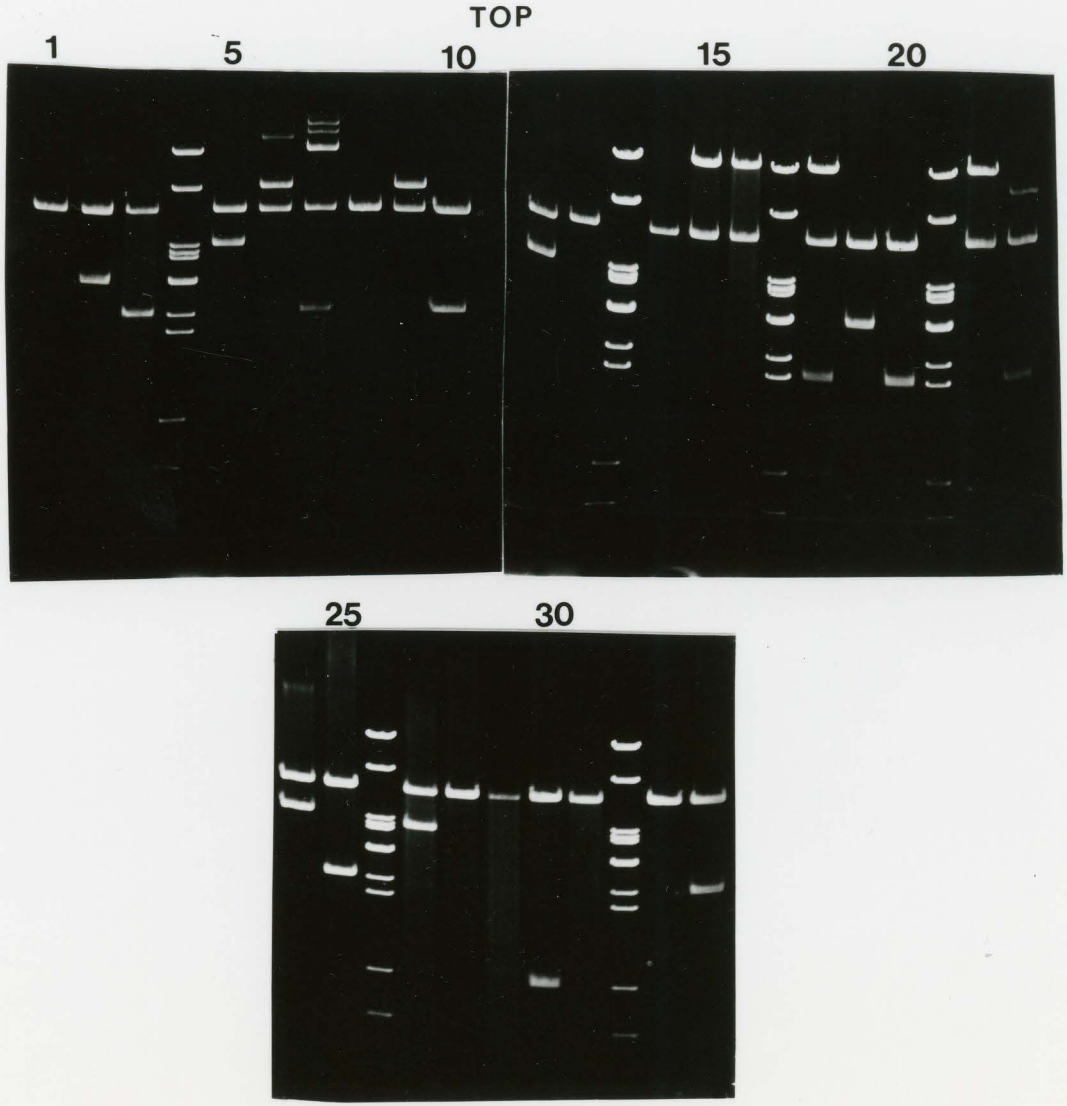
Isolation of Ad 2 DNA restriction fragments. The purity of isolated DNA restriction fragments was investigated on 1% analytical agarose gels. Fragments were subjected to electrophoresis at 40 volts for 12 hours. Lanes: (1-3) isolated Bam HI fragments D, C, and B, respectively, generated by recleavage with Bam HI from the isolated Ad 2 Eco RI A fragment; (4) Eco RI A fragment digested with Bam HI; (5) Bam HI digested Ad 2 DNA; (6-11) isolated Eco RI fragments F to A, respectively; (12) Eco RI digested Ad 2 DNA. The gel was stained with ethidium bromide before being visualized under UV light.


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

ion with Hind III was ligated with T4 DNA ligase to plasmid pBR322 DNA cleaved at the unique Hind III cleavage site within the tetracycline resistance gene. E. coli LE 392 were transformed with 400 ng of ligated DNA and ampicillin resistant, tetracycline sensitive colonies were selected with an efficiency of 21% as described in Materials and Methods. Individual colonies were grown in Luria broth containing ampicillin and then amplified with chloramphicol. The recombinant plasmid DNA was isolated from the selected colonies (Birnboim, 1980) followed by digestion with Hind III and then screened on analytical 1% agarose gels to identify specific recombinant plasmids. Approximately, 120 colonies were screened of which 28 are compared to marker Hind III digested DNA shown in Fig. 9. Of the 10 possible internal Ad 2 Hind III DNA fragments, 8 (A, B, C, D, F, H, I and J; Figs. 6 and 9) were identified, spanning a wide variety of regions across the viral genome. The very small fragment L (79.9 - 80.6 map units) may have run off the bottom of the analytical gels and was not identified, but may be responsible for some of the ampicillin resistant, tetracycline sensitive colonies that appear to contain pBR322 DNA without a viral DNA insert. In addition, the E fragment (80.6 - 89.5 map units) was not identified during the screening of recombinant DNA plasmids. Three Ad 2 Hind III-pBR322 recombinant DNA plasmids, B, F and H spanning regions 17.0 - 31.5, 89.5 - 97.1, and 72.8 - 79.9 map units respectively were chosen for large scale plasmid preparation. In addition, clone 1010 of pBR322, which carries the Ad 2 Bam HI C DNA insert spanning the region 42.0 - 59.5 map units (obtained from Dr. F.L. Graham, McMaster University) was utilized in the following experiments. Figure 10 shows each of the

Figure 9

Screening of Ad 2 Hind III-pBR322 recombinant DNA plasmids. Ampicillin resistant, tetracycline sensitive colonies of E. coli LE 392 transformed with pBR322 containing Hind III Ad 2 DNA fragments were grown and the recombinant plasmid DNA was extracted from each culture. The extracted DNA was digested with Hind III and screened on analytical 1% agarose gels. Lanes 4, 13, 19, 26, and 32 contain marker Ad 2 DNA digested with Hind III. The bands are denoted A to K with decreasing size. The F/G band migrates as a single wide, intense band. All other lanes show the results of the Hind III digest of pBR322 alone (lanes 1, 8, 12, 14, 28, 29, 31, and 33) or with Ad 2 fragments. Fragments: (A (lanes 15, 16, 22); B (lanes 6, 9); C (lanes 5, 11, 24); D (lane 27); F/G (lanes 2, 19); H (lanes 3, 7, 10, 25, 34); I (lanes 20, 23); J (lane 30). Electrophoresis was carried out at 40 volts for 12 hours through 1 % agarose and the gel was stained with ethidium bromide before being visualized under UV light.


TOP
1
5
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25
30

Ad 2 Hind III-pBR322 DNA plasmids digested with Hind III and compared to marker Ad 2 DNA digested with Hind III. The Ad 2 Bam HI C-pBR322 plasmid DNA digested with Bam HI and compared to marker Ad 2 DNA digested with Bam HI is also shown in Fig. 10.

2. Definitive identification of Ad 2 Hind III F. Hind III digestion of Ad 2 DNA yields 11 identifiable fragments. The viral DNA fragments C, D, and E are close in molecular weight and not always well resolved on agarose gels as in Fig. 10 due to overloading of DNA. Frequently, these fragments may be seen as three separate well resolved bands as in Fig. 9. The DNA fragments F and G also represent similar sized fractions of the viral genome and always appear as a single wider intense band on agarose gels. The G fragment represents the left terminal 7.9% of the genome and would not be expected to be ligated to Hind III digested pBR322 due to the absence of a Hind III site at the left hand terminus. However, it was necessary to prove that the viral DNA insert of the recombinant DNA plasmid, which comigrated with the Ad 2 F/G band was indeed the Hind III F fragment. The Hind III F fragment contains three Bgl II cleavage sites and no such sites occur within the Hind III G fragment. A double digest of the plasmid with Hind III and Bgl II (Fig. 11, lane 3) results in the disappearance of the band, which comigrates with the F/G band. Four additional bands of faster electrophoretic mobility would be expected from the Bgl II digestion of Hind III F (Fig. 6) representing about 5.1%, 1.1%, 1.0% and 0.4% of the total genome. Two additional bands of expected electrophoretic mobility, (one representing the 5.1% band and the other a combined band

Figure 10

Ad 2-pBR322 recombinant DNA plasmids. Selected colonies carrying recombinant DNA plasmids were prepared in 1.0 litre cultures and the plasmid DNA extracted. Aliquots of the extracted plasmid DNA were digested with Hind III or Bam HI and analyzed on a 1% agarose gel. Lane 1, marker Ad 2 DNA digested with Hind III showing bands A to K with bands C/D/E as a single wide band and F/G as a single wide band. Lane 2, Ad 2 Hind III B; lane 3, Ad 2 Hind III F/G; lane 4, Ad 2 Hind III H; lane 5, marker Ad 2 DNA digested with Bam HI generating 4 bands denoted A to D with increasing electrophoretic mobility; lane 6, Ad 2 Bam HI C. The common band in lanes 2, 3, 4, and 6 is pBR322. Electrophoresis was carried out at 40 volts for 12 hours through 1% agarose and the gel was stained with ethidium bromide before being visualized under UV light.

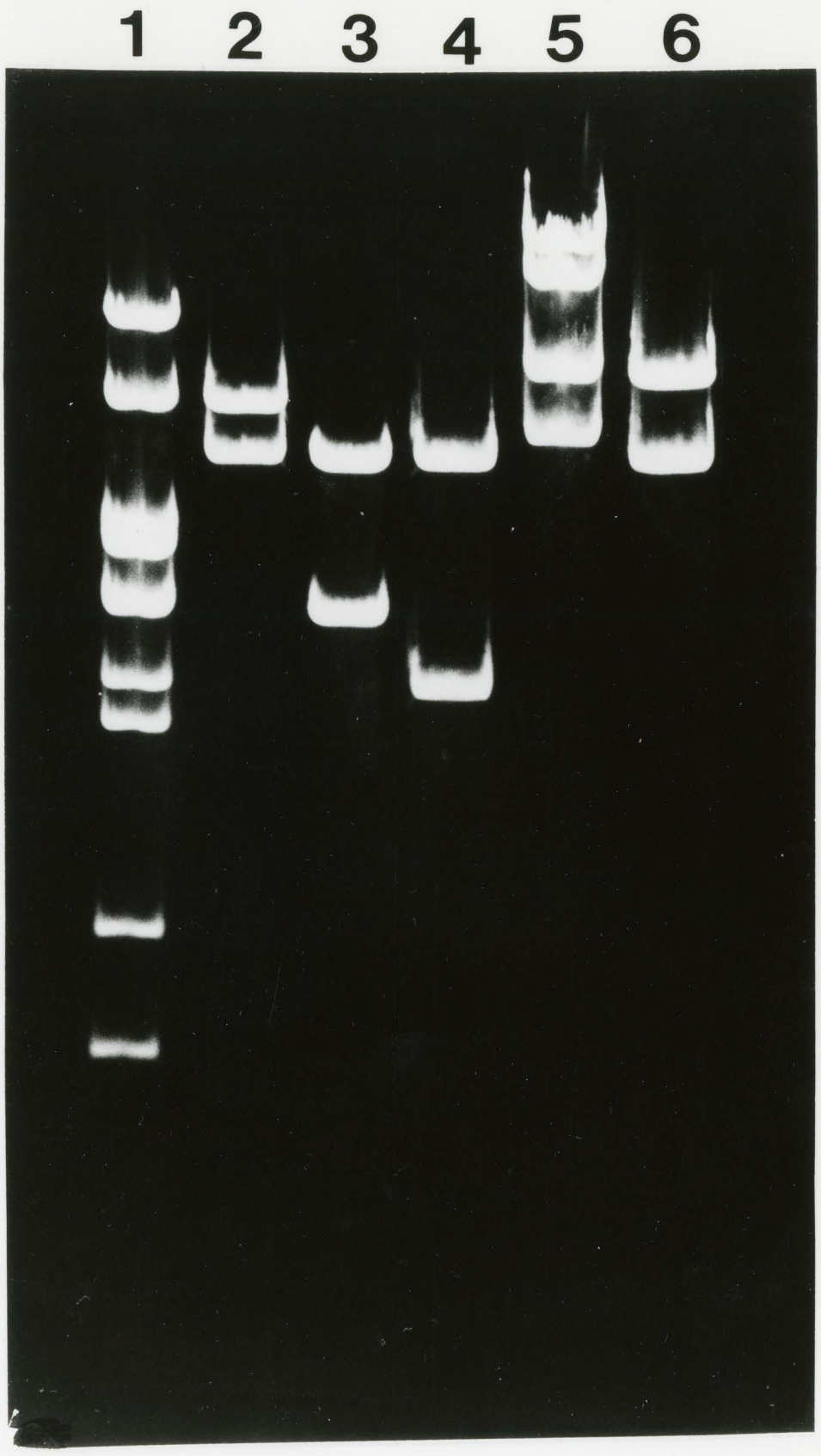
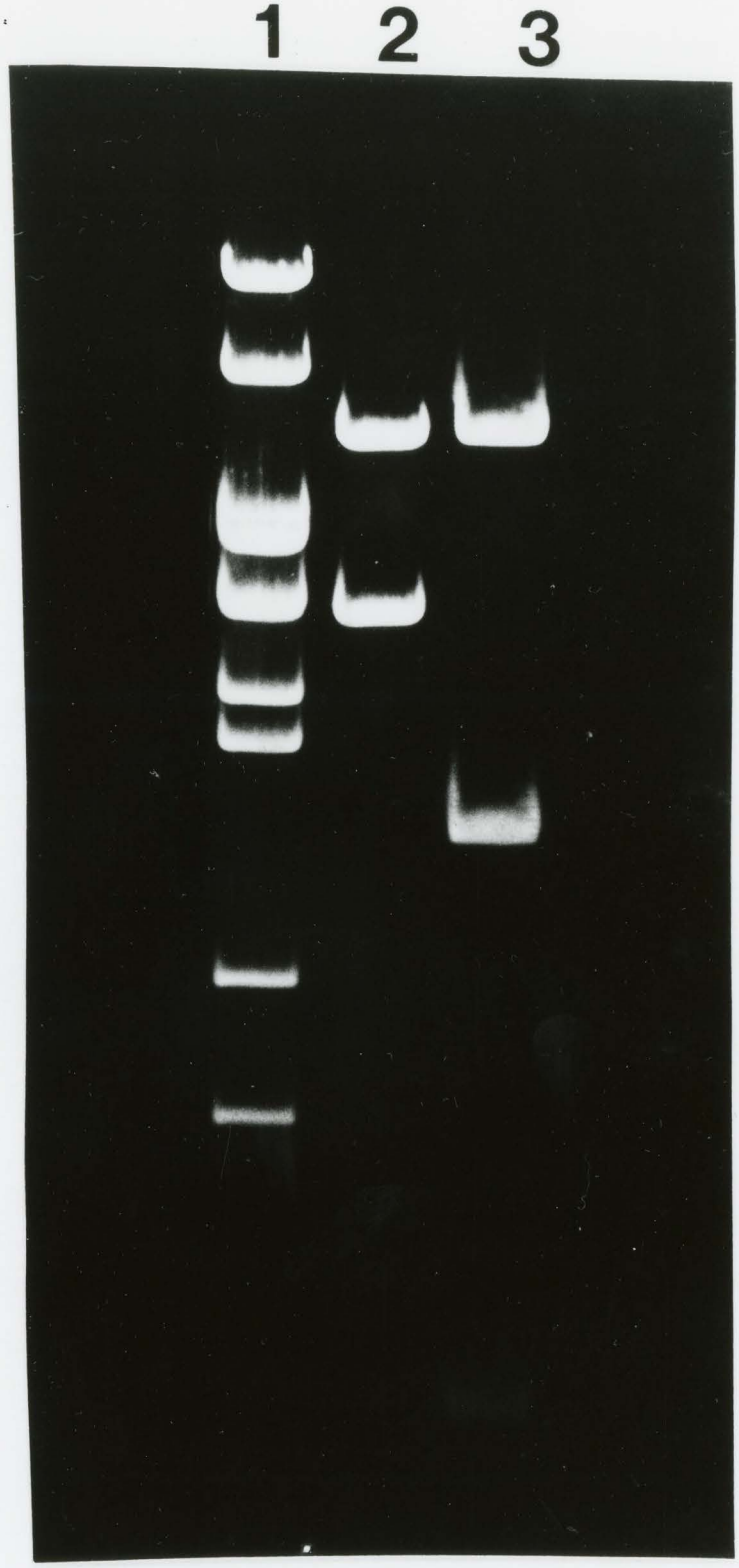

1
2
3
4
5
6

Figure 11

Identification of Ad 2 Hind III F - pBR322. Electrophoresis through 1% agarose was carried out at 40 volts for 12 hours and the gel was stained with ethidium bromide before being visualized under UV light. Lane 1, Hind III digested Ad 2 DNA; lane 2, Hind III F/G - pBR322 DNA digested with Hind III; lane 3, Hind III F/G-pBR322 DNA digested with Hind III and Bgl II, (a faint band is close to the bottom of the lane).


1
2
3

representing the 1.1% plus 1.0% bands) were observed (Fig. 11, lane 3), while the smallest band would be expected to run off the bottom of the gel. Therefore, the identity of the viral insert of the recombinant DNA plasmid, which comigrates with the Ad 2 Hind III F/G band is Ad 2 Hind III F.

II. Saturation Filter Hybridization

1. Saturation filter hybridization of Ad 2 DNA and infected cell RNA. Ad 2 transcription was assayed by filter hybridization of Ad 2 DNA and radioactively labelled RNA extracted from Ad 2 infected cells. In order to quantitate the relative levels of transcription in subsequent experiments it was essential that hybridizations were performed under conditions of DNA excess. To determine the relative amounts of nucleic acids required for the transcriptional analysis experiments, saturation hybridizations were carried out with full length Ad 2 DNA or several Ad 2 DNA fragments. Nitrocellulose membrane filters were prepared with increasing amounts of heat denatured DNA immobilized on the filters. Approximately 1 x 10^8 Ad 2 infected KB cells were labelled with 37.5 µCi/ml of ^{3}H-uridine at 15 hours post-infection for 1 hour at 37°C. The infected cell nuclei were isolated and the RNA extracted. Hybridization of viral DNA immobilized on the filters with either a 16 µl or 32 µl aliquot of the extracted Ad 2 infected cell nuclear RNA was carried out at 65°C for 24 hours in a 1 ml reaction of 2.0 x SSC, 0.1% SDS and 0.75 mg of yeast RNA. After hybridization was complete, the filters were washed extensively and treated with 20 µg/ml of ribonuclease for 1 hour at room temperature before liquid scintillation count-

ing. Figure 12 shows the saturation hybridization of Ad 2 DNA and 2.5×10^5 or 1.25×10^5 acid precipitable counts per minute (cpm) of Ad 2 infected cell nuclear ^{3}H-RNA. The saturation hybridization reactions plateau at 15 and 9 µg of viral DNA respectively, indicating the minimum concentration of viral DNA required to meet the conditions of DNA excess for the amounts of nuclear DNA used. Similar hybridization reactions with several different preparations of Ad 2 infected cell nuclear RNA indicates that between 19 and 24% of the ^{3}H-uridine incorporated into nuclear RNA during the labelling period is viral specific.

Assuming an even distribution of transcription along the length of the viral genome, a similar number of µg equivalent of viral restriction fragment DNA (where 1 µg equivalent is the fraction of 1 µg of Ad 2 DNA represented by the size of the restriction fragment) should be required to attain similar saturation by hybridization results with the same amounts of nuclear ^{3}H-RNA as in Fig. 12. The hybridization characteristics of the Ad 2 Bam HI D (Fig. 13A) and Eco RI E (Fig. 13B) DNA fragments, which represent diverse regions of the viral genome were determined. Approximately, 2.5×10^5 acid precipitable cpm of nuclear ^{3}H-RNA was hybridized to increasing amounts of each fragment. Figure 13 shows that the plateau for the hybridization of the Ad 2 infected cell nuclear RNA occurs at 14 µg equivalent of Bam HI D DNA fragment, similar to that found for the full length genome DNA. The Eco RI E fragment reached the hybridization plateau at approximately 5 µg equivalent using the same amount of nuclear RNA, suggesting that transcription may not occur evenly along the length of the viral genome. Alternatively, processing of viral nuclear RNA during the 1 hour

Figure 12

Saturation filter hybridization of Ad 2 DNA and ^{3}H-uridine labelled Ad 2 infected cell nuclear RNA. Hybridizations were carried out in 1 ml reactions in tightly stoppered vials for 24 hours at 65°C. Filters were loaded with increasing amounts of Ad 2 DNA and hybridized to a 16 µl aliquot of 1.25 x 10^{5} cpm (open circles) or 32 µl aliquot of 2.5 x 10^{5} cpm (closed circles) of Ad 2 infected cell nuclear ^{3}H-RNA. Each point is the result of assaying duplicate filters and the background of hybridization supplied by control filters with varying amounts of E. coli DNA has been subtracted.

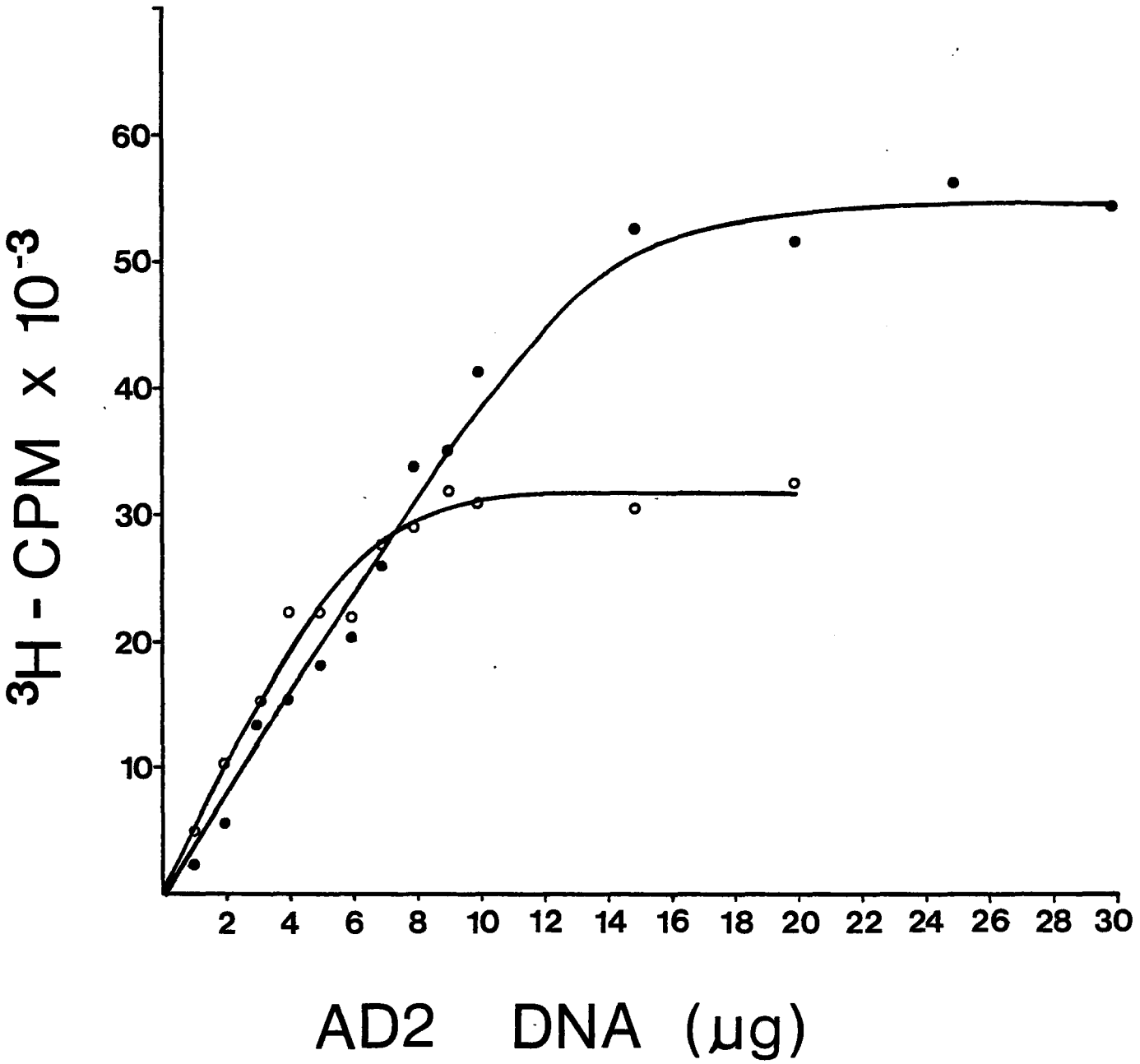
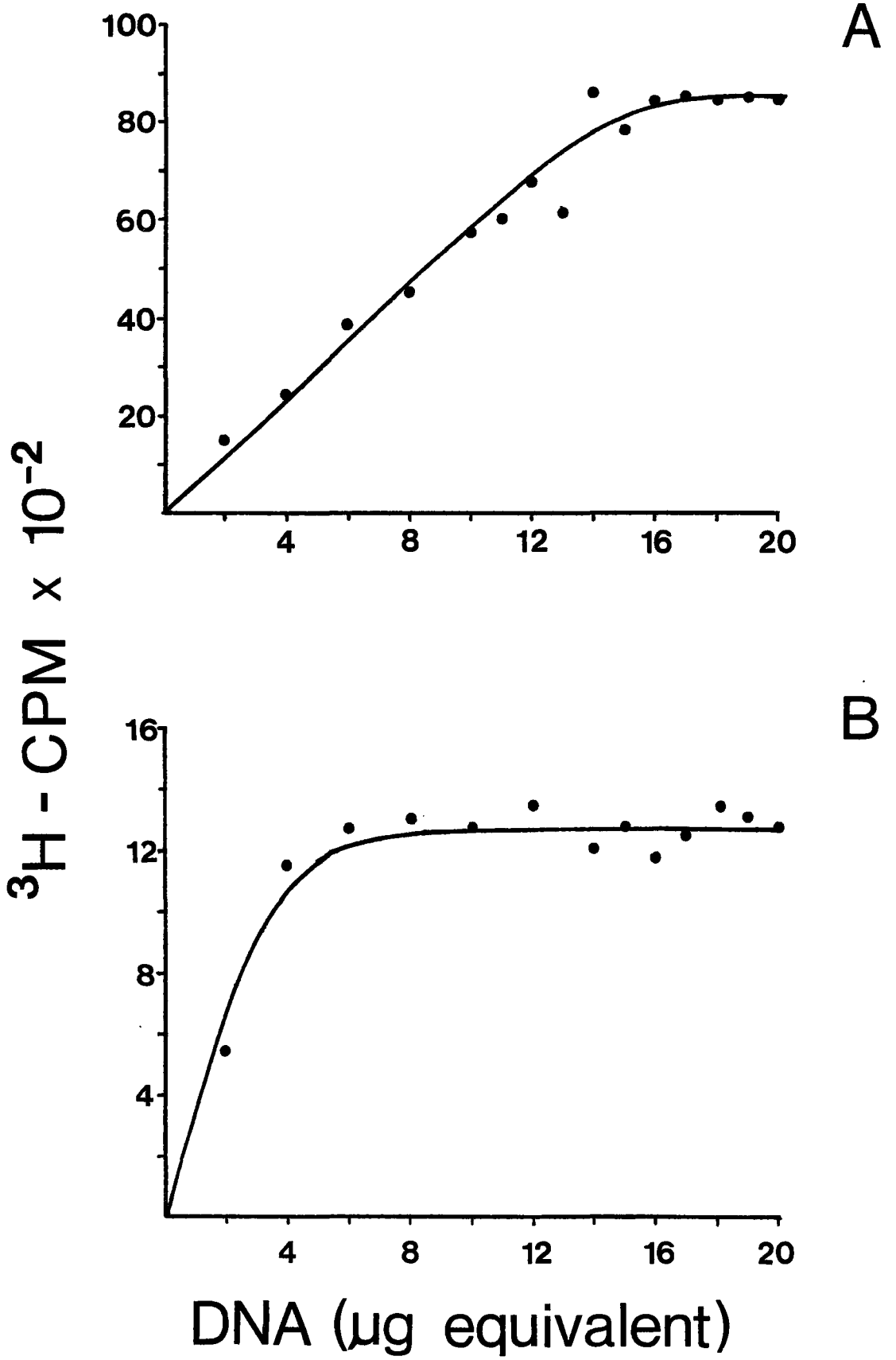

^{3}H - CPM x 10^{-3}
60
50
40
30
20
10
2
4
6
8
10
12
14
16
18
20
22
24
26
28
30
AD2 DNA (µg)

Figure 13

Saturation filter hybridization of Ad 2 Bam HI D and Eco RI E DNA with ^{3}H-uridine labelled Ad 2 infected cell nuclear RNA. Hybridizations were carried out in 1 ml reactions in tightly stoppered vials for 24 hours at 65°C. Filters were loaded with increasing amounts of Ad 2 Bam HI D DNA (Fig. 13A) or Ad 2 Eco RI E DNA (Fig. 13B) and hybridized with 2.5 x 10^{5} cpm of Ad 2 infected cell ^{3}H labelled nuclear RNA. Each point is the result of assaying duplicate filters and the background supplied by control filters with varying amounts of E. coli DNA being subtracted.


A
B
100
80
60
40
20
16
12
8
4
4
8
12
16
20
^3H - CPM x 10^{-2}
DNA (µg equivalent)

labelling period may affect the distribution of transcriptional differentially along the length of the genome.

In subsequent experiments, 20 μg equivalent of viral DNA restriction fragments were chosen to satisfy the condition of DNA excess. Test filters with greater concentrations of restriction fragment were included with each preparation of RNA to insure that conditions of DNA excess were present.

III. Specificity of Filter Hybridization

1. Ad 2 DNA and KB cell RNA. A spinner culture of 1.0×10^8 KB cells was labelled with 20 μCi/ml of ^{3}H-uridine for 2 hours during the logarithmic growth phase of the cell culture and the whole cell RNA was extracted. Ad 2 DNA was hybridized with nuclear ^{3}H-RNA from Ad 2 infected KB cells and compared with the extent of hybridization of Ad 2 DNA and KB cell ^{3}H-RNA under the hybridization conditions described above. Table 2 shows that only a very small proportion of the ^{3}H-RNA extracted from KB cells hybridized to Ad 2 DNA while a significant proportion of the ^{3}H-RNA extracted from Ad 2 infected KB cell nuclei late during infection hybridized to Ad 2 DNA indicating a high degree of specificity of the filter hybridization assay.

2. Control DNA and Ad 2 infected cell nuclear RNA. ^{3}H-RNA extracted from Ad 2 infected KB cell nuclei late during infection was hybridized to DNA from a variety of sources to test the specificity of the filter hybridization assay. Table 3 shows that extensive hybridization occurs with Ad 2 DNA immobilized on nitrocellulose filters. Ad 2 nuclear ^{3}H-RNA binding to filters containing salmon sperm, rat, *E. coli* or pBR322 DNA was very poor and only slightly above the hybridization reaction

TABLE 2

Specificity of Hybridization

RNA Source	CPM of Input RNA	Hybridized CPM	% Hybridization
Ad 2 infected KB cell nuclei	2.5×10^5	58,740	23.5
KB Cell	2.5×10^5	206	0.08
Ad 2 infected KB cell nuclei	1.25×10^5	27,988	22.4
KB Cell	1.25×10^5	268	0.21

^3H RNA from KB cells or Ad 2 infected KB cell nuclei was hybridized to 20 µg of Ad 2 DNA immobilized in nitrocellulose filters.

TABLE 3

Specificity of Hybridization

DNA Source	Amount of DNA per Filter (μg)	Hybridized CPM
Ad 2	20	58,740
Salmon sperm	10	202
	20	235
Rat	10	105
	20	117
E. coli	10	173
	20	126
pBR322	20	109
-	0	103

Each hybridization reaction contained 2.5×10^5 cpm of ^{3}H-RNA extracted from Ad 2 infection KB cells nuclei at 16 hours post-infection. Rat DNA was obtained from Dr. M. Reuben, McMaster University and Salmon sperm DNA was obtained from Dr. F.L. Graham, McMaster University.

with filters containing no DNA. This indicates that the binding of RNA to filters carrying DNA sequences is a highly specific event and should provide a reliable assay system to examine Ad 2 transcription.

In subsequent experiments, purified E. coli DNA was used as a control to assess the background level of ^{3}H-RNA binding to filters carrying Ad 2 DNA or DNA fragments.

IV. Ultraviolet Transcription Unit Mapping by DNA-RNA Hybridization

A. Late Nuclear RNA

1. Effect of uv irradiation on Ad 2 transcription. Equal numbers of Ad 2 infected KB cells were exposed to uv light with increasing doses from 10 to 60 joules/m^2 at 15 hours post-infection. After a further 0.5 hour incubation period at 37°C to allow equilibriation of the system, these cultures were labelled with 37.5 μCi/ml of ^{3}H-uridine for 15 minutes. The labelling period was terminated by the addition of ice cold PBS and the cells were immediately harvested from the plastic dishes and collected by centrifugation. An equal number of infected cells was not exposed to uv irradiation but subjected to the identical procedures as the uv irradiated cells. All manipulations with each set of cultures were carried out simultaneously and under identical conditions.

The nuclei and cytoplasm were fractionated from each culture and the RNA was extracted from the isolated nuclear fraction as described in Materials and Methods. Equal aliquots of total nuclear ^{3}H-RNA from each culture were precipitated in 5% trichloroacetic acid at 0°C for 20 minutes and then filtered onto nitrocellulose filters. The filters were dissolved in ethyl acetate and the ^{3}H-RNA assayed by

liquid scintillation counting. The total amount of acid precipitable ^{3}H-RNA obtained for uv irradiated cultures was then expressed as a fraction of that obtained for non-irradiated cultures. The "surviving fraction" defined in this way was then plotted as a function of uv dose. Figure 14 indicates that uv irradiation inhibits transcription with increasing doses. The multi-component nature of the survival curve is thought to reflect the presence of transcription units of different sizes but of similar abundance, which would be expected in assaying total nuclear RNA.

The uv sensitivity of viral specific transcription was determined by hybridization of the total nuclear ^{3}H-RNA and Ad 2 DNA. Equal aliquots of the total nuclear ^{3}H-RNA from each culture were hybridized to 20 μg of Ad 2 DNA on nitrocellulose filters for 24 hours at 65°C in 1 ml reactions of 2 x SSC, 0.1% SDS and 0.75 mg/ml of BSA. After extensive washing with 2 x SSC and treatment with 20 μg/ml of ribonuclease for 1 hour at room temperature, the hybridized nuclear ^{3}H-RNA was determined by liquid scintillation counting. Transcription of viral specific nuclear RNA labelled late during infection is also inhibited by uv irradiation with increasing dose (Fig. 15). The fact that the curve shows less components than Fig. 14 suggests less variation in transcription unit size for the viral RNA as compared to the total cellular RNA. The effect of uv irradiation on transcription has been utilized in a number of studies (Sauerbier and Hercules, 1978) to investigate the transcriptional organization for the expression of both prokaryotic and eukaryotic genes. Such studies require the ability to examine the transcription at various locations on the genome.

Figure 14

Effect of uv irradiation on the total nuclear transcription of Ad 2 infected cells. Nuclear ^{3}H-RNA from Ad 2 infected cells exposed to uv light with increasing doses from 10 to 60 joules/m^2 at 15 hours post-infection was acid precipitated onto nitrocellulose filters and assayed by liquid scintillation counting. The surviving fraction of total nuclear ^{3}H-RNA at each dose was determined by comparing the acid precipitable ^{3}H-RNA cpm of the uv irradiated cultures to control non-irradiated cultures. Each point represents the average of duplicate filters from each culture which were within 10% of each other.

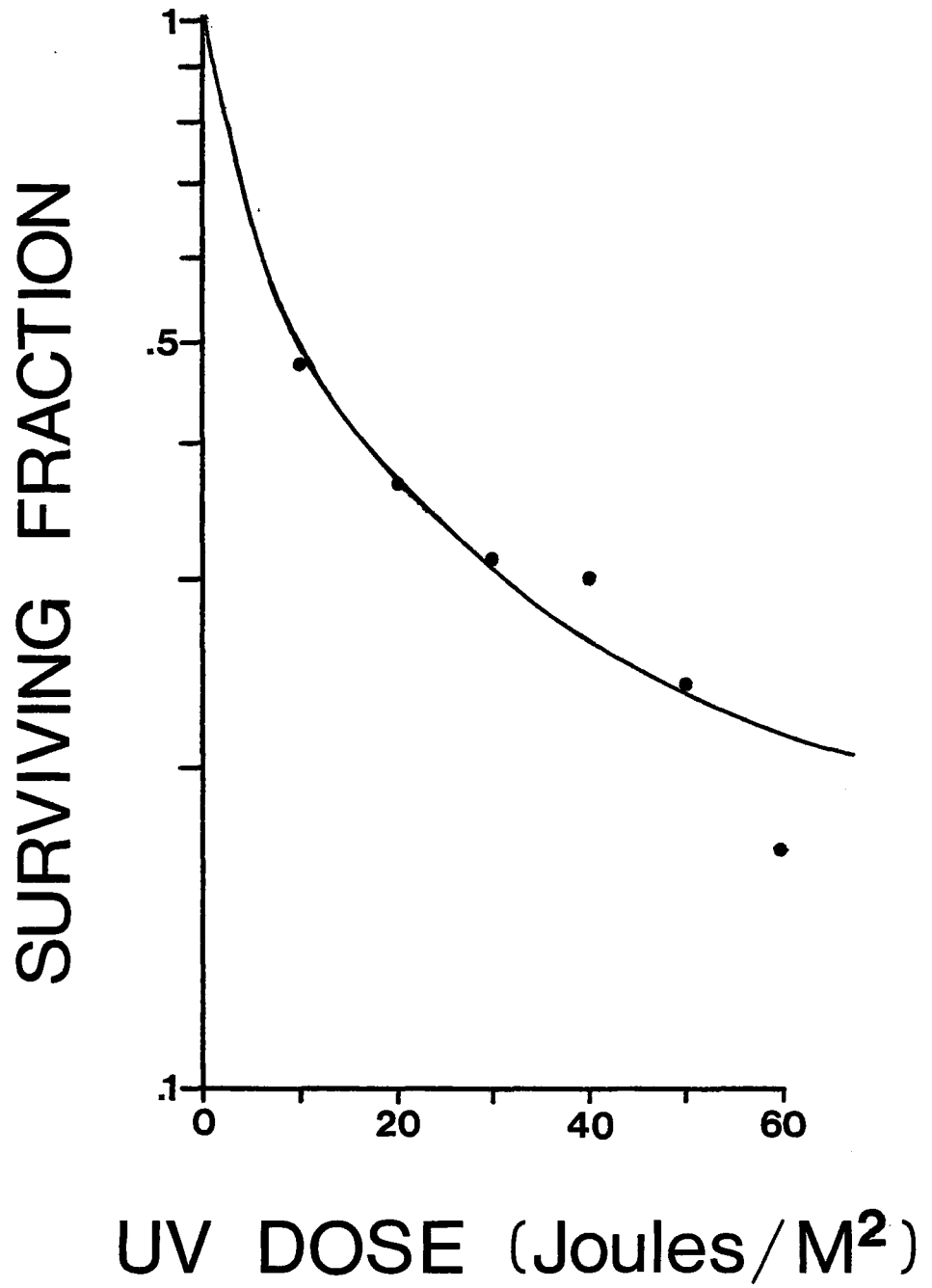
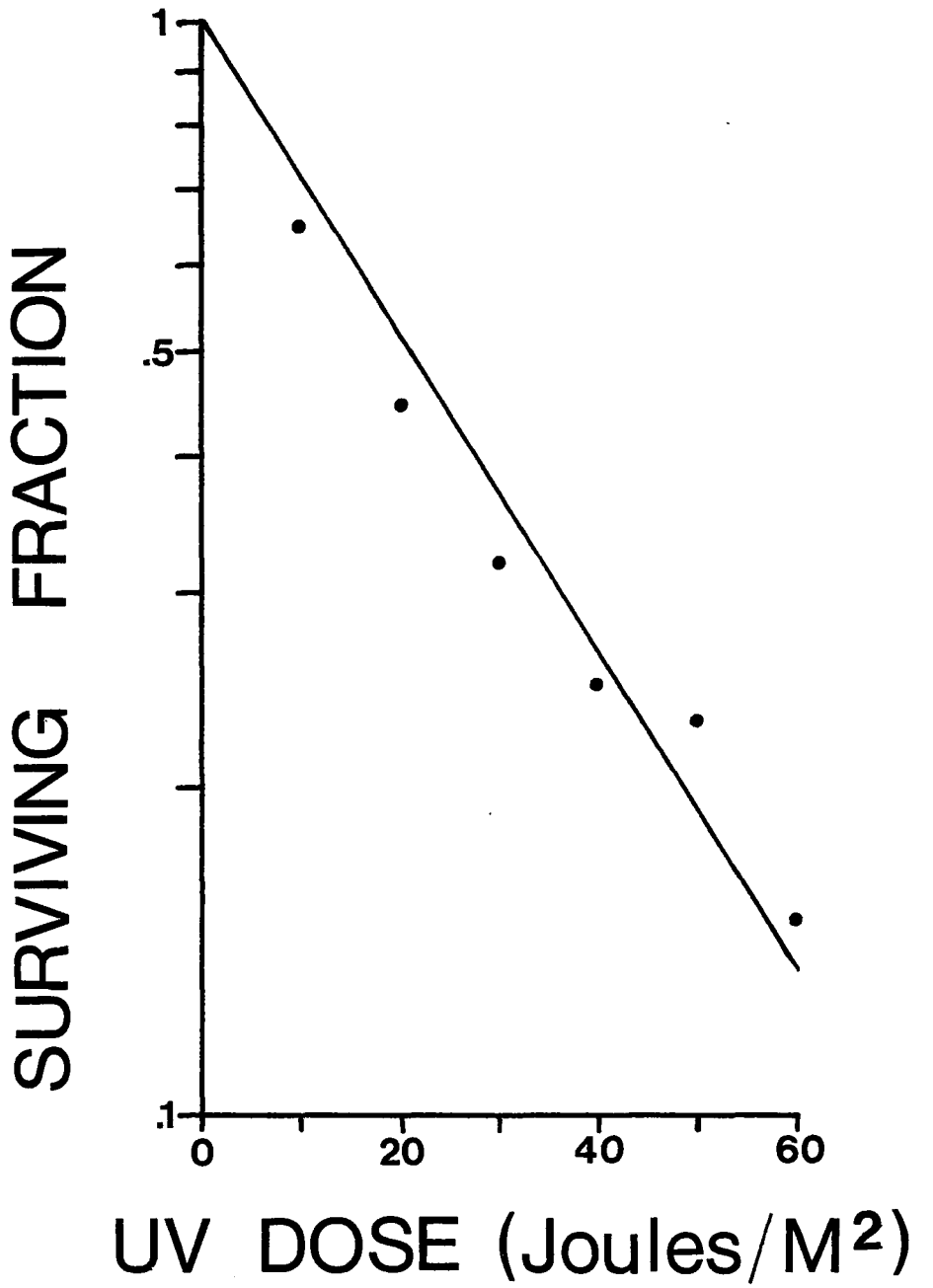

SURVIVING FRACTION
1
.5
.1
0
20
40
60
UV DOSE (Joules/M²)

Figure 15

Effect of uv irradiation on viral specific transcription. Nuclear ^{3}H-RNA from Ad 2 infected cells exposed to uv light with increasing doses from 10 to 60 joules/m^2 at 15 hours post-infection was hybridized to 20 μg of Ad 2 DNA on nitrocellulose filters for 24 hours at 65°C and then assayed by liquid scintillation counting. The surviving fraction of viral specific nuclear ^{3}H-RNA at each dose was determined by comparing the hybridized cpm of ^{3}H-RNA from the uv irradiated cultures and control unirradiated cultures. Each point represents the average of duplicate filters from each culture, which were within 10% of each other.


SURVIVING FRACTION
1
.5
.1
0
20
40
60
UV DOSE (Joules/M2)

2. UV sensitivity of viral specific transcription at various sites along the Ad 2 genome. As described earlier, the uv sensitivity of transcription can be correlated to the relative target size of a transcription unit(s) as synthesis of RNA from promotor proximal regions is inhibited at a lower rate than promotor distal regions following uv irradiation. The transcriptional organization of Ad 2 late in infection was investigated by comparing the hybridized ^{3}H-RNA from uv irradiated and non-irradiated cultures to DNA restriction fragments representing different regions of the viral genome.

Equal numbers of Ad 2 infected cells were uv irradiated with a dose of 30 joules/m^{2} at 15 hours post-infection or left unirradiated and incubated at 37^{o}C for a further 30 minutes before being labelled with ^{3}H-uridine for 15 minutes. Ad 2 nuclear RNA from non-irradiated and uv irradiated cells was extracted and equal aliquots hybridized to 20 µg equivalent of seven different Ad 2 DNA restriction fragments. The surviving fraction of virus specific RNA, which hybridized to each region was plotted against the genome position of the right hand terminus of each DNA fragment. Figure 16 shows typical results. A gradient of increasing uv sensitivity beginning towards the left hand end of the genome and continuing towards the right with the greatest uv sensitivity of viral transcription within the Bam HI C fragment (42.0 - 59.5 map units) was observed. Towards the right of this region the uv sensitivity of transcription changes abruptly and shows a marked decrease in the suppression of transcription caused by uv irradiation. Table 4 gives the surviving fraction for each of the genome regions examined in three independent experiments.

The Eco RI F fragment (70.7 - 75.9 map units) was found to be the most resistant to the effects of uv irradiation with the remainder of the genome (to 100 map units) displaying an increase in the uv sensitivity of transcription. The slight decrease in the uv sensitivity of transcription for the extreme right hand end of the genome represented by the Eco RI C fragment shown in Fig. 16 was a consistent observation in this type of experiment.

Late during Ad 2 infection, the vast majority (95%) of transcription occurs from the rightward transcribing strand (r-strand) (Pettersson and Philipson, 1974; Zimmer et al., 1978). If transcription begins toward the left hand end of the genome and continues within a single transcription unit to the extreme right hand end, the uv sensitivity of transcription would be expected to increase exponentially with increasing target size. This would be expected to yield a single continuous curve on a plot of the logarithm of the surviving fraction against a linear representation of the genome position of the various DNA fragments. Figure 16 shows two gradients of increasing uv sensitivity of transcription suggesting that two transcription units are responsible for the production of late viral nuclear RNA.

3. UV dose response of viral specific transcription at specific sites along the Ad 2 genome. A more detailed examination of nuclear transcription during late Ad 2 infection at specific sites along the viral genome was undertaken. Two regions within each of the two gradients of uv sensitivity of trans-

Figure 16

Ultraviolet sensitivity of viral specific transcription at various sites along the Ad 2 genome. Cultures of Ad 2 infected cells were exposed to uv irradiation at 30 joules/m^2 at 15 hours post-infection or left unirradiated before being labelled with ^{3}H-uridine for 15 minutes. The nuclear ^{3}H-RNA was extracted and equal aliquots hybridized to 20 µg equivalent of Ad 2 DNA restriction fragments on nitrocellulose filters for 24 hours at 65°C. The filters were washed extensively with 2.0 x SSC and treated with 20 µg/ml of ribonuclease for 1 hour at room temperature before being dissolved in ethyl acetate and assayed by liquid scintillation counting. The surviving fraction of viral specific transcription for each region examined was determined by comparing the hybridized cpm of the ^{3}H-RNA from the uv irradiated cultures, and unirradiated cultures. Each point represents the average of triplicate samples.

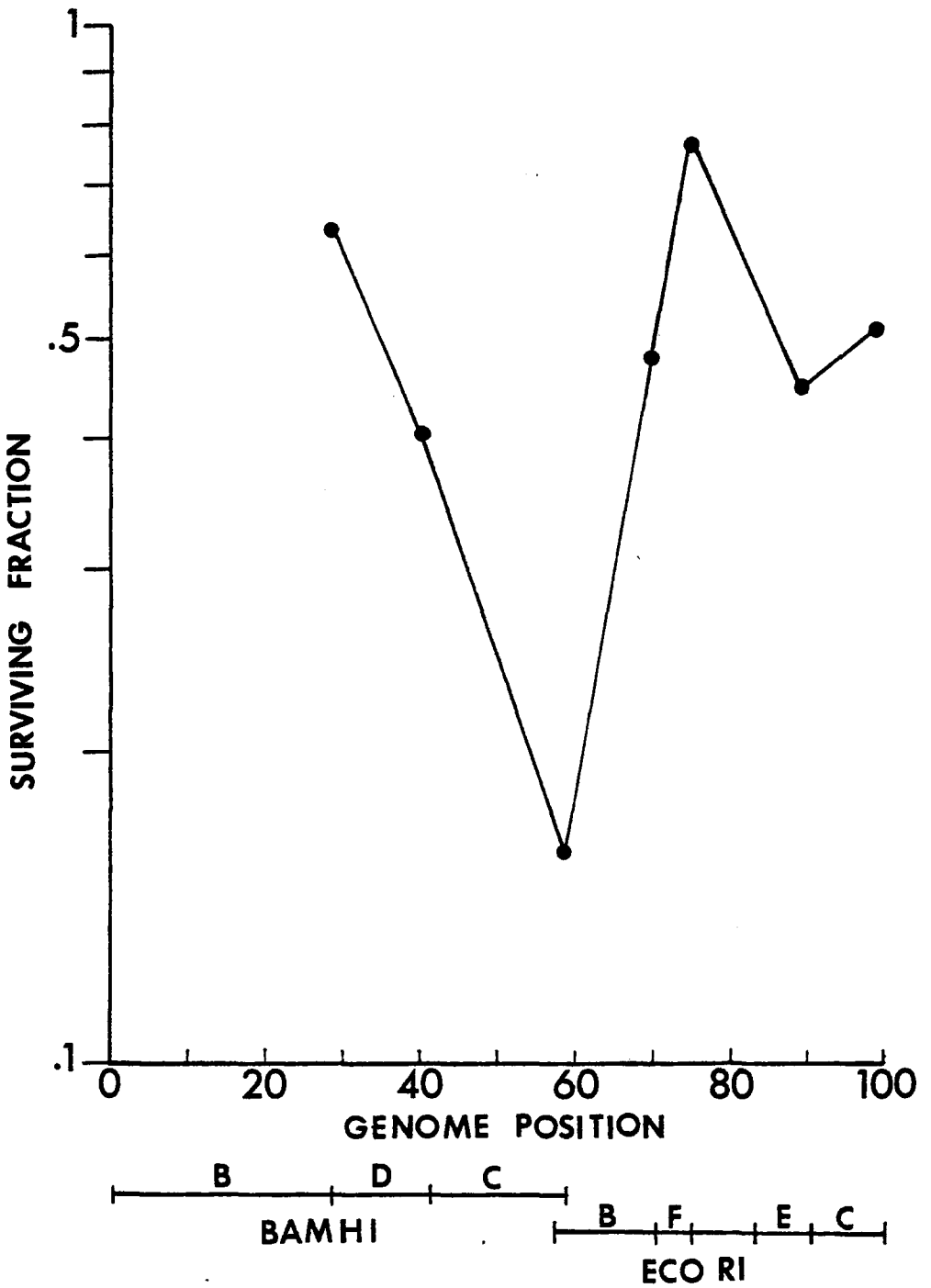

1
.5
.1
SURVIVING FRACTION
0
20
40
60
80
100
GENOME POSITION
B
D
C
BAMHI
B
F
E
C
ECO RI

TABLE 4

UV sensitivity of viral specific transcription at various sites along the Ad 2 genome

Fragment	Surviving Fraction		
	Expt. 1	Expt. 2	Expt. 3
Bam HI B	0.63	0.56	0.65
D	0.40	0.36	0.47
C	0.17	0.24	0.25
Eco RI B	0.48	0.47	0.39
F	0.76	0.78	0.82
E	0.44	0.42	0.60
C	0.51	0.46	0.64

Viral specific nuclear ^{3}H-RNA from three independent similar experiments comparing the surviving fraction from Ad 2 infected cells uv irradiated at 15 hours post-infection or left unirradiated before being labelled with ^{3}H-uridine for 15 minutes at 15.5 hours post-infection.

cription described above were chosen for a more detailed transcriptional analysis.

Recombinant DNA plasmids of Ad 2 DNA restriction fragments cloned into the vector pBR322 provided a convenient source of pure Ad 2 DNA fragments. The fragments chosen for the following study were Hind III B, Bam HI C, Hind III H, and Hind III F, representing regions 17.0 - 31.5, 42.0 - 59.5, 72.8 - 79.9, and 89.5 - 97.1 map units respectively.

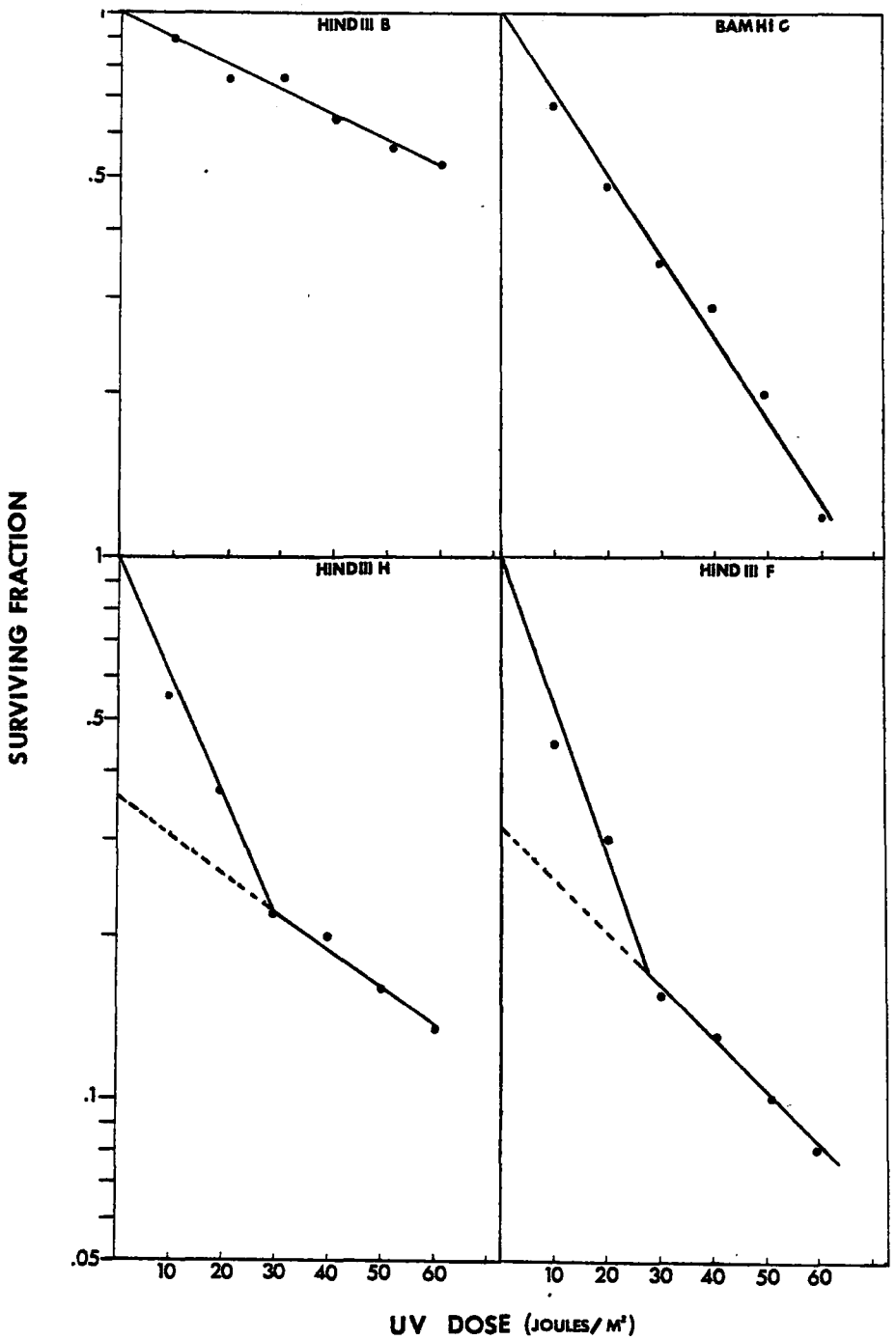
Seven cultures of 2.5×10^7 KB cells were infected with Ad 2 and incubated at 37°C. At 23.5 hours post-infection, the medium was removed from each monolayer and samples from six of the cultures were exposed to doses of uv irradiation from 10 to 60 joules/m^2. Fresh, prewarmed medium was added after irradiation and the infected cells were incubated for a further 30 minutes at 37°C before being labelled with 37.5 µCi/ml of ^{3}H-uridine for 15 minutes at 37°C. Maniputaltions on all cultures were under identical conditions.

Equal aliquots of nuclear ^{3}H-RNA was hybridized to 20 µg equivalent of viral DNA fragments of the recombinant DNA plasmids on nitrocellulose filters. The surviving fraction was determined at each uv dose for the various regions of the genome by comparing the hybridized cpm of ^{3}H-RNA from uv irradiated and control non-irradiated infected cultures. Typical results for the uv dose response of late transcription for the four regions of the genome examined are illustrated in Fig. 17. Results for three similar experiments are shown in Table 5. Transcription toward the left hand portion of the genome represented by the Hind III B fragment spanning the region 17.0 to 31.5 map units (Fig. 17) shows a single component exponential decrease

of transcriptional survival with increasing uv dose. However, this region is relatively resistant to the effects of uv irradiation by comparison to other regions of the genome suggesting a promotor proximal location. Transcription probed within the region of the genome represented by Bam HI C (42.0 - 59.5 map units) has a similar single component exponential response to the effects of increasing uv irradiation, but shows a far greater uv sensitivity of transcription than that displayed within the Hind III B fragment region. This suggests that the relative target size of transcription has increased from left to right along the genome, consistent with the idea of a single transcription unit originating within or before the Hind III B fragment region and continuing into the Bam HI C fragment region. Further to the right along the genome, the uv transcriptional analysis of the region represented by Hind III H (72.8 - 79.9 map units) and Hind III F (89.5 - 97.1 map units) fragments (Fig. 17) shows a different manner of uv inactivation of transcription. The survival curves for both of these regions consist of two components suggesting that more than one population of viral specific nuclear RNA were transcribed from these regions of the viral genome. Both Hind III H and F fragment regions show survival curves with a highly uv sensitive component and also a second relatively less uv sensitive component. This suggests that within these regions towards the right hand side of the genome, late viral specific nuclear RNA is derived from two separate transcription units; one very long transcription unit and the other significantly shorter.

Figure 17

Ultraviolet dose response of viral specific transcription at specific sites along the Ad 2 genome. Cultures of Ad 2 infected cells were exposed to increasing doses of uv irradiation from 10 to 60 joules/m^2 at 23.5 post-infection or left unirradiated, before being labelled with ^{3}H-uridine for 15 minutes. The nuclear ^{3}H-RNA was extracted and equal aliquots hybridized to 20 µg equivalent viral restriction fragment DNA carried by recombinant DNA plasmids on nitrocellulose filters for 24 hours at 65^{o}C. The filters were washed extensively with 2.0 x SSC, and treated with 20 µg/ml of ribonuclease for one hour at room temperature before being dissolved in ethyl acetate and assayed by liquid scintillation counting. The surviving fraction of viral specific transcription at each dose for the four regions examined was determined by comparing the hybridized cpm of the ^{3}H-RNA from uv irradiated cultures and control non-irradiated cultures. Each point represents the average of triplicate samples.


HIND III B
BAM HI C
HINDIII H
HIND III F
SURVIVING FRACTION
1
.5
.1
.05
10
20
30
40
50
60
UV DOSE (JOULES/M²)

It was possible that processing of nuclear RNA for the biogenesis of cytoplasmic messenger RNA during the 15 minute labelling period may have had some influence on the data generated in this experiment. To test this possibility, a similar experiment was performed with uv irradiation of infected cells at 23.5 hours post-infection and minimizing the labelling period to 7 minutes with 60 μCi/ml of ^{3}H-uridine at 24 hours post-infection. Minimizing the possible effects of nuclear RNA processing by a shortened labelling period yields very similar results to the experiment described above (Table 5, expt. 2).

This type of uv dose response experiment was also repeated with uv irradiation of Ad 2 infected cells at 15 hours post-infection followed by a 15 minute labelling period with ^{3}H-uridine at 15.5 hours post-infection. Again, similar results were obtained (Table 5, expt. 3), although the proportion of the second components for the Hind III H and Hind III F fragment regions were found to be somewhat higher than at 24 hours post-infection (Table 6).

The slopes (K) of the various survival curves for the four regions of the viral genome examined were obtained by linear regression analysis (Table 5) and are correlates of the relative target size of the transcription products assayed (ie. an increase in slope indicates an increase in the relative target size of the transcription unit). The standard error of the slopes involved in this type of experiment.

Extrapolation to a uv dose of 0 joules/m^{2} (see Fig. 17) for the second component of the uv survival curve found for the Hind III H and

TABLE 5

K ($\times 10^{-2} \pm$ standard error $\times 10^{-2}$) Values of the Survival Curves from UV Dose Response Experiments

Experiment #	Hind III B	Bam HI C	Hind III H	Hind III F
			1st Component	
1	1.06±0.07	3.40±0.17	4.93±0.28	5.88±0.82
2	1.22±0.06	4.30±0.25	4.75±0.79	6.93±1.14
3	1.44±0.16	3.80±0.28	6.54±1.65	7.12±0.30
			2nd Component	
1			0.95±0.17	2.24±0.12
2			1.20±0.09	2.10±0.27
3			1.41±0.44	2.42±0.40

K, the slope of each of the survival curves and the standard error of the slope was determined for each region by linear regression analysis from the data of the uv dose response of late Ad 2 transcription experiments. Experiment 1, infected cells were uv irradiated at 23.5 hours post-infection followed by a 15 minute labelling period with ^{3}H-uridine at 24 hours post-infection. Experiment 2, infected cells were uv irradiated at 23.5 hours post-infection followed by a 7 minute labelling period with ^{3}H-uridine at 24 hours post-infection. Experiment 3, infected cells were uv irradiated at 15 hours post-infection followed by a 15 minute labelling period with ^{3}H-uridine at 15.5 hours post-infection.

and Hind II F fragment regions suggests that at least one third of nuclear transcription within these regions of the viral genome is dereived from the second shorter (less uv sensitive) transcription unit at 24 hours post-infection (Table 6).

In Figure 18, the uv inactivational cross section, the K value for each survival curve from the experiments described above was plotted against the genome position of the right hand terminus of each DNA restriction fragment. This correlates the relative target size of the transcription units to the uv sensitivity of transcription at the various regions along the viral genome late during Ad 2 infection. Extrapolation of the uv inactivation cross section to the abscissa should indicate the location of the site of initiation of RNA synthesis. Determination of the intercept by linear regression analysis of the data presented in Fig. 18 suggests that the longer transcription unit originates at approximately 17 map units and continues towards the right hand end of the genome. A second transcription unit originates at approximately 63 map units and also extends towards the extreme right hand end of the genome. These results suggest that two separate transcriptional events are responsible for the synthesis of viral nuclear RNA late during Ad 2 infection.

4. Reproducibility of the extraction of nuclear ^{3}H-RNA. The uv transcription unit mapping experiments performed to investigate the transcriptional organization of Ad 2 are critically dependent on the ability to extract nuclear RNA reproducibly from separate Ad 2 infected cell cultures. The experimental procedures involved in the generation of different nuclear RNA preparations is a lengthy and multistaged

TABLE 6

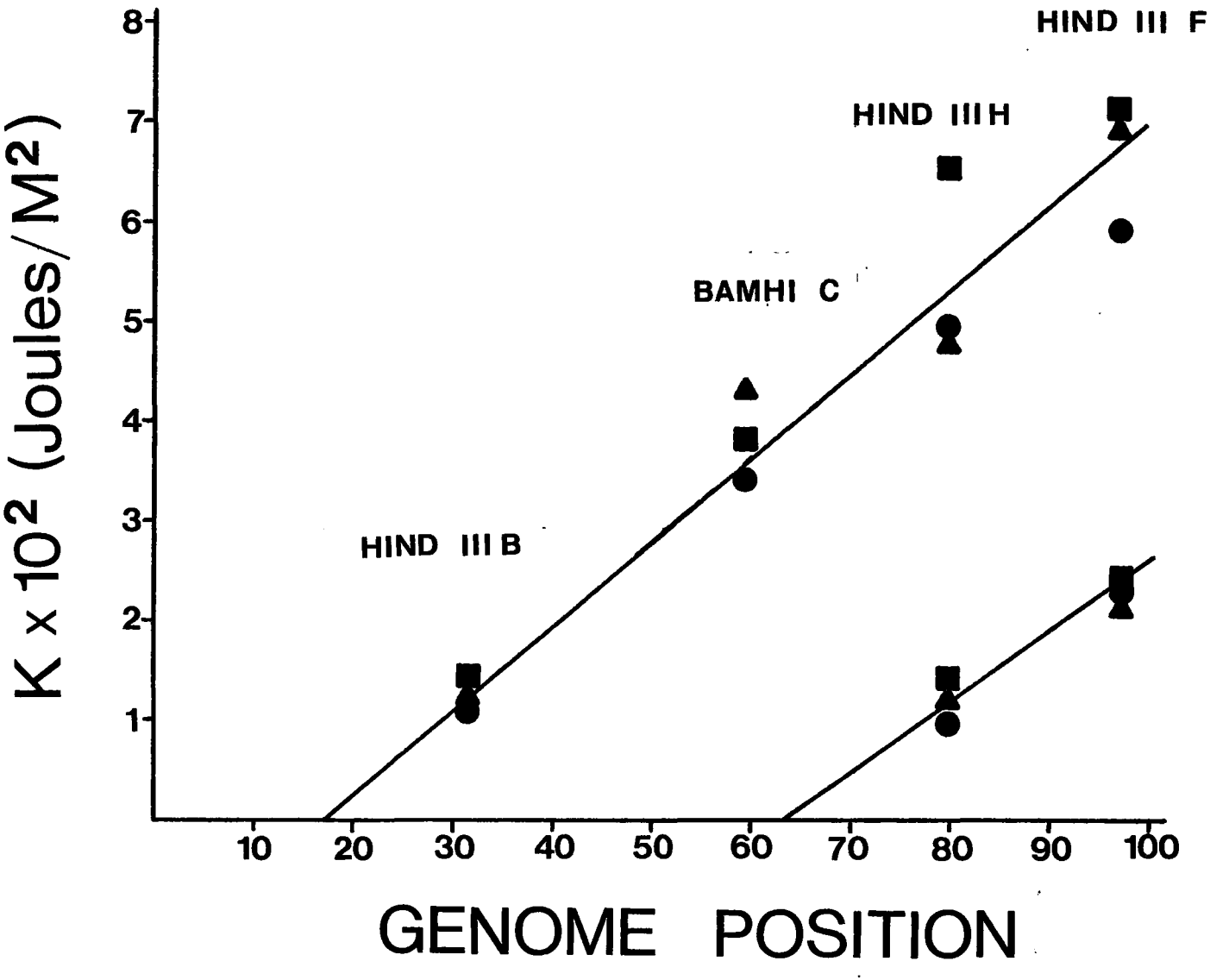
Proportion of Ad 2 Late Transcription from the Second Component of Regions Hind III H and Hind III F

Experiment #	Hind III H	Hind III F
1	0.37	0.32
2	0.32	0.38
3	0.55	0.59

The intercept for the second component of the survival curves from the uv dose response of late Ad 2 transcription experiments was determined by extrapolation of the curve to a dose of 0 joules/m^2. Experiment 1, infected cells were uv irradiated at 23.5 hours post-infection followed by a 15 minute labelling with ^{3}H-uridine. Experiment 2, infected cells were uv irradiated at 23.5 hours post-infection followed by a 7 minute labelling period with ^{3}H-uridine. Experiment 3, infected cells were uv irradiated at 15 hours post-infection followed by a 15 minute labelling period with ^{3}H-uridine.

Figure 18

UV inactivational cross-section. UV sensitivities of the production of late Ad 2 transcripts plotted against the genome position of the right hand terminus of the DNA sequences represented by Ad 2 DNA restriction fragments Hind III B, Bam HI C, Hind III H and Hind III F. The K values are the slopes of the inactivation curves for the four different regions of the genome investigated and were determined by linear regression analysis from the data of the uv dose response of late Ad 2 transcription experiments. The genome position intercepts were determined by linear regression analysis. The closed circle, triangle and box symbols are K values of the three experiments described in Table 4.


HIND III F
HIND III H
BAMHI C
HIND III B
K x 10² (Joules/M²)
8
7
6
5
4
3
2
1
10
20
30
40
50
60
70
80
90
100
GENOME POSITION

protocol, which provides the potential for experimental error at a variety of steps.

To test the reproducibility of nuclear RNA extraction, three separate, identical cultures of 2.5×10^7 Ad 2 infected KB cells and a fourth culture of 7.5×10^7 Ad 2 infected KB cells was labelled with ^{3}H-uridine at 15.5 hours post-infection for 15 minutes. The nuclear ^{3}H-RNA was extracted and hybridized to the Ad 2 RNA fragment Bam HI C.

Duplicate 5 µl aliquots of nuclear ^{3}H-RNA from each culture was acid precipitated onto nitrocellulose filters and assayed by liquid scintillation counting. Table 7 shows that the acid precipitable cpm are very similar for the separately extracted cultures of 2.5×10^7 infected cells and was approximately one third of the acid precipitable cpm of the culture containing 7.5×10^7 infected cells. Hybridization data for duplicate samples of nuclear ^{3}H-RNA from each culture to 20 µg equivalent of Ad 2 Bam HI C fragment DNA immobilized on nitrocellulose filters is also presented in Table 7. The results indicate that the extraction of nuclear ^{3}H-RNA from separate cultures is sufficiently, quantitatively reproducible and independent of cell numbers between $2.5 - 7.5 \times 10^7$ cells to perform uv transcription unit mapping experiments accurately when care and patience is exhibited during the technical procedures.

5. Distribution of ^{3}H-labelled late nuclear RNA sequences. Nuclear RNA labelled with ^{3}H-uridine at 15.5 hours post-infection for 15 minutes was extracted from Ad 2 infected KB cells and hybridized to Ad 2 DNA restriction fragments, Bam HI B, D, and C and Eco RI B, F, E and C. The ^{3}H cpm, which hybridized to each fragment was divided by the size of the respective fragment and plotted against the genome position

TABLE 7

Reproducibility of the Extraction of Nuclear ^{3}H-RNA

Culture	# of Ad 2 Infected Cells	TCA cpm	Hybridized cpm
A	2.5×10^{7}	18,644	29,660
		18,475	27,157
B	2.5×10^{7}	17,850	26,645
		18,122	25,094
C	2.5×10^{7}	18,660	27,246
		18,731	26,717
D	7.5×10^{7}	55,994	ND
		56,626	ND

Nuclear ^{3}H-RNA from 3 identical Ad 2 infected cultures (A-C) and one culture containing 3 times the number of infected cells (D) was extracted under identical conditions and equal aliquots from each preparation was assayed in duplicate for trichloroacetic acid (TCA) precipitable cpm. Duplicate samples of each nuclear ^{3}H-RNA preparation was also assayed by hybridization to 20 µg equivalents of Ad 2 DNA fragment Bam HI C.

ND = not done

of the region represented by each of the DNA fragments (Fig. 19A).

A separate nuclear ^{3}H-RNA preparation labelled at 24 hours post-infection for 7 minutes was hybridized to recombinant DNA plasmids carrying the Ad 2 restriction fragments Hind III B, Bam HI C, Hind III H and Hind III F. The ^{3}H cpm per unit genome length was plotted against the genome position of each DNA fragment (Fig. 19B).

The distribution of the incorporation ^{3}H-uridine into viral specific RNA sequences in both the experiments described above shows a marked asymetry between the left hand 60% of the genome and the remaining right hand 40% of the genome. Nuclear transcription of the left hand 60% of the genome occurs approximately 3.5 fold more frequently than the right hand 40% in Fig. 19A and approximately 5 fold more frequently in Fig. 19B.

This type of experiment has been repeated with HeLa cells, KB cells in spinner culture, and also utilizing shortened labelling periods (4 minutes) at 15 and 24 hours post-infection. The results consistently show a greater (3.5-6 fold) level of transcription for the left hand 60% of the Ad 2 genome. This suggests that many of the late transcripts that originate near the left hand end of the genome terminate within the Eco RI B fragment (58.2 - 70.7 map units), which is consistent with the idea that more than one promotor-terminator regulatory element functions for the synthesis of late nuclear RNA during Ad 2 infection.

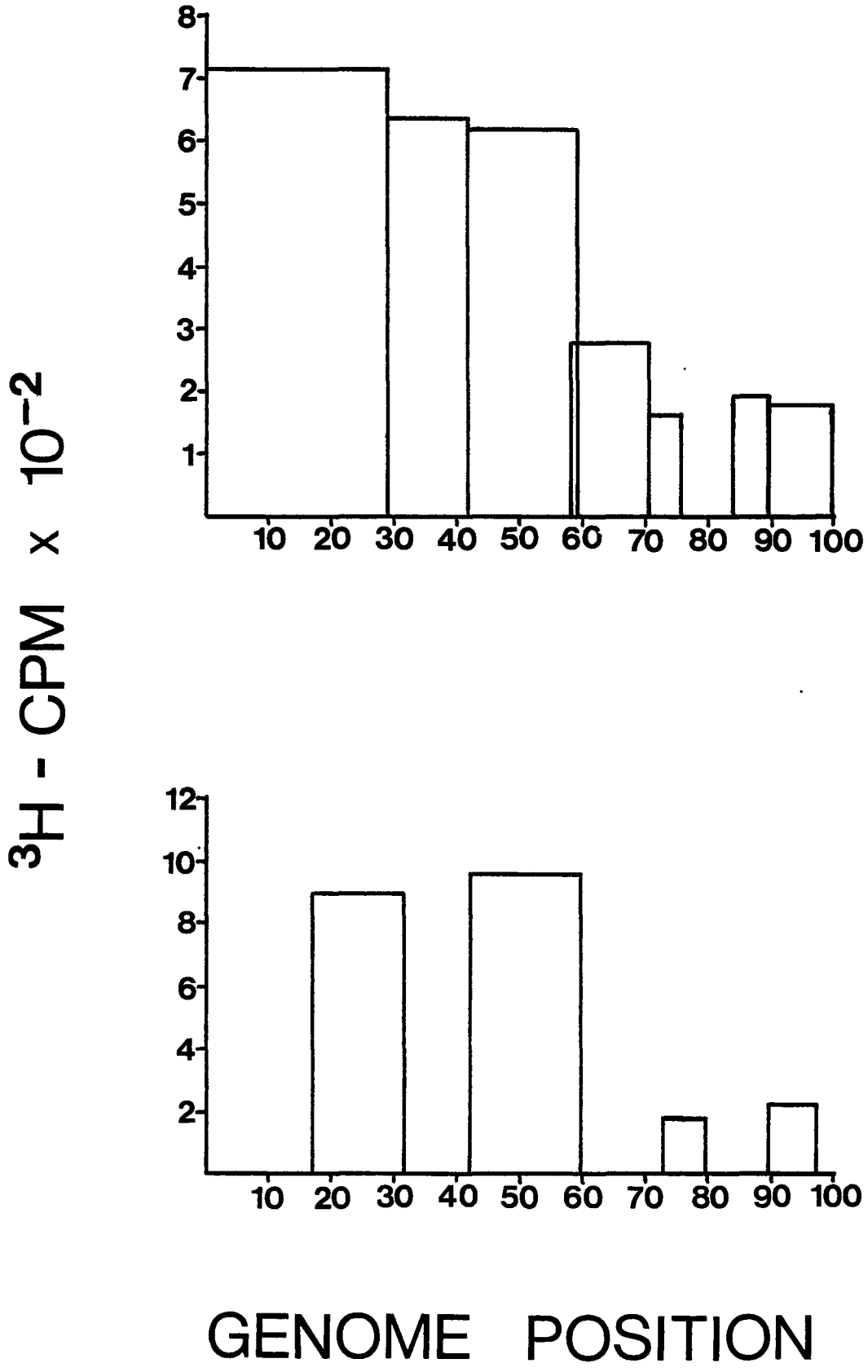
6. Hybridization of late nuclear Ad 2 ^{3}H-RNA to separated strands of Ad 2 DNA fragments. At late times during Ad 2 infection, after the onset of viral DNA synthesis, the vast majority (more than 95%) of newly synthesized viral RNA is derived from the rightward transcribing

Figure 19

Distribution of late nuclear ^{3}H-RNA sequences. Hybridization of nuclear ^{3}H-RNA to 20 µg equivalent of Ad 2 DNA restriction fragments on nitrocellulose filters was carried out for 24 hours at 65°C. The filters were washed extensively with 2.0 x SSC and treated with 20 µg/ml of ribonuclease before being dissolved in ethyl acetate and assayed by liquid scintillation counting.

A. Infected cells were labelled with 37.5 µCi/ml of ^{3}H-uridine at 15.5 hours post-infection for 15 minutes. The nuclear ^{3}H-RNA was extracted and hybridized to Ad 2 DNA fragments Bam HI B, D, and C and Eco RI, B, F, E, and C.

B. Infected cells were labelled with 60 µCi/ml of ^{3}H-uridine at 24 hours post-infection for 7 minutes. The nuclear ^{3}H-RNA was extracted and hybridized to recombinant DNA plasmids carrying Ad 2 DNA fragments Hind III B, Bam HI C, Hind III H and Hind III F.


^{3}H - CPM x 10^{-2}
8
7
6
5
4
3
2
1
10 20 30 40 50 60 70 80 90 100
12
10
8
6
4
2
10 20 30 40 50 60 70 80 90 100
GENOME POSITION

strand (r-strand) (Petterssen and Philipson, 1974; Zimmer et al., 1978). Late nuclear transcription assayed by DNA-RNA hybridization using Ad 2 DNA fragments containing both DNA strands in the experiments described above assumes that the transcripts measured in this manner are derived only from the r-strand. Significant levels of transcription occurring from the leftward transcribing (l-strand) would critically affect the interpretation of uv transcription unit mapping experiments. The two component nature of the survival curves for the transcription of DNA sequences represented by the Hind III H and Hind III F DNA fragments (Fig. 17), suggesting that more than one transcription unit is responsible for late transcription in these regions could be explained by the presence of significant levels of l-strand transcription in addition to the expected r-strand transcripts.

The origin (r/l strand) of newly synthesized late Ad 2 RNA was investigated by hybridization of ^{3}H-RNA to separated strands of Ad 2 DNA restriction fragments Hind III H and Hind III F by the Southern blot method (Southern, 1975). Approximately, 4 μg of the recombinant DNA plasmids, Ad 2 Hind III H - pBR322 and Ad 2 Hind III F - pBR322 was digested with Hind III and denatured in 0.2N NaOH for 10 minutes at room temperature before being subjected to electrophoresis through 1.4% agarose gels. The separated strands of Ad 2 DNA fragments Hind III H and Hind III F are denoted as fast or slow strands on the basis of electrophoretic mobility and are well resolved as seen at the bottom of the agarose gel in Fig. 20 (lanes 2 and 7, respectively). The band immediately above the separated fast and slow strands of the Ad 2 restriction fragments are the separated strands of pBR322, which

are not well resolved in these gels. The two bands of slower electrophoretic mobility are the respective Ad 2 restriction fragment and pBR322, which probably reannealed after being loaded on the gel. The two recombinant DNA plasmids were linearaized by digestion with Bam HI, alkali denatured and run on the agarose gels. The separated strands of the total linearized plasmids were also well resolved as seen in Fig. 20 (lanes 4 and 5). Ad 2 ^{3}H-DNA was digested with Hind III (Fig. 20 lanes 1 and 8) as was Ad 2 Hind III H-pBR322 (Fig. 20, lane 3) and Ad 2 Hind III F-pBR322 (Fig. 20, lane 6) and run on the gels to serve as markers.

Each of the agarose gels displayed in Fig. 20 was blotted onto nitrocellulose filters. The blot of one gel (Fig. 20A) was hybridized with nuclear ^{3}H-RNA extracted from Ad 2 infected cells labelled with ^{3}H-uridine for 15 minutes at 24 hours post-infection. The blot of an identical agarose gel (Fig. 20B) was hybridized with nuclear ^{3}H-RNA extracted from Ad 2 infected cells that were uv irradiated at a dose of 30 joules/m^{2} at 23.5 hours post-infection and labelled with ^{3}H-uridine for 15 minutes at 24 hours post-infection. The hybridized blots were exposed to x-ray film for 30 days at -60°C to obtain the autoradiograms shown in Fig. 21.

Nuclear ^{3}H-RNA from both unirradiated and uv irradiated Ad 2 infected cells hybridized to the fast strands of the separated strands of Ad 2 DNA fragments Hind III H and Hind III F (Fig. 21A and B, lanes 2 and 7). Hybridization of nuclear ^{3}H-RNA from uv irradiated Ad 2 infected cells and the fast strand of Hind III F produced a faint band on the reproduction of the autoradiogram (Fig. 21, lane 7), which is

Figure 20

R-strand specific transcription of late nuclear RNA. I. Strand separation of Ad 2 DNA restriction fragments. Two identical 1.4% agarose gels were electrophoresed for 16 hours at 40 volts. The gels were stained with ethidium bromide and visualized under uv light before being prepared for Southern blotting. Lanes: (1 and 8) Hind III digested Ad 2 marker ^{3}H-DNA; (2) denatured Hind III digested Ad 2 Hind III H-pBR322; (3) Hind III digested Ad 2 Hind III H-pBR322; (5) denatured Bam HI digested Ad 2 Hind III F-pBR322; (6) Hind III digested Ad 2 Hind III F-pBR322; (7) denatured Hind III digested Ad 2 Hind III F-pBR322.

A. Agarose gel containing separated strands of Ad 2 Hind III H and Hind III F DNA fragments, which was blotted by the Southern technique for hybridization with nuclear Ad 2 ^{3}H-RNA labelled for 15 minutes with ^{3}H-uridine at 24 hours post-infection.

B. Agarose gel containing separated strands of Ad 2 Hind III H and Hind III F DNA fragments, which was blotted by the Southern technique for hybridization with nuclear ^{3}H-RNA extracted from Ad 2 infected cells uv irradiated at a dose of 30 joules/m^{2} at 23.5 hours post-infection and labelled with ^{3}H-uridine for 15 minutes at 24 hours post-infection.

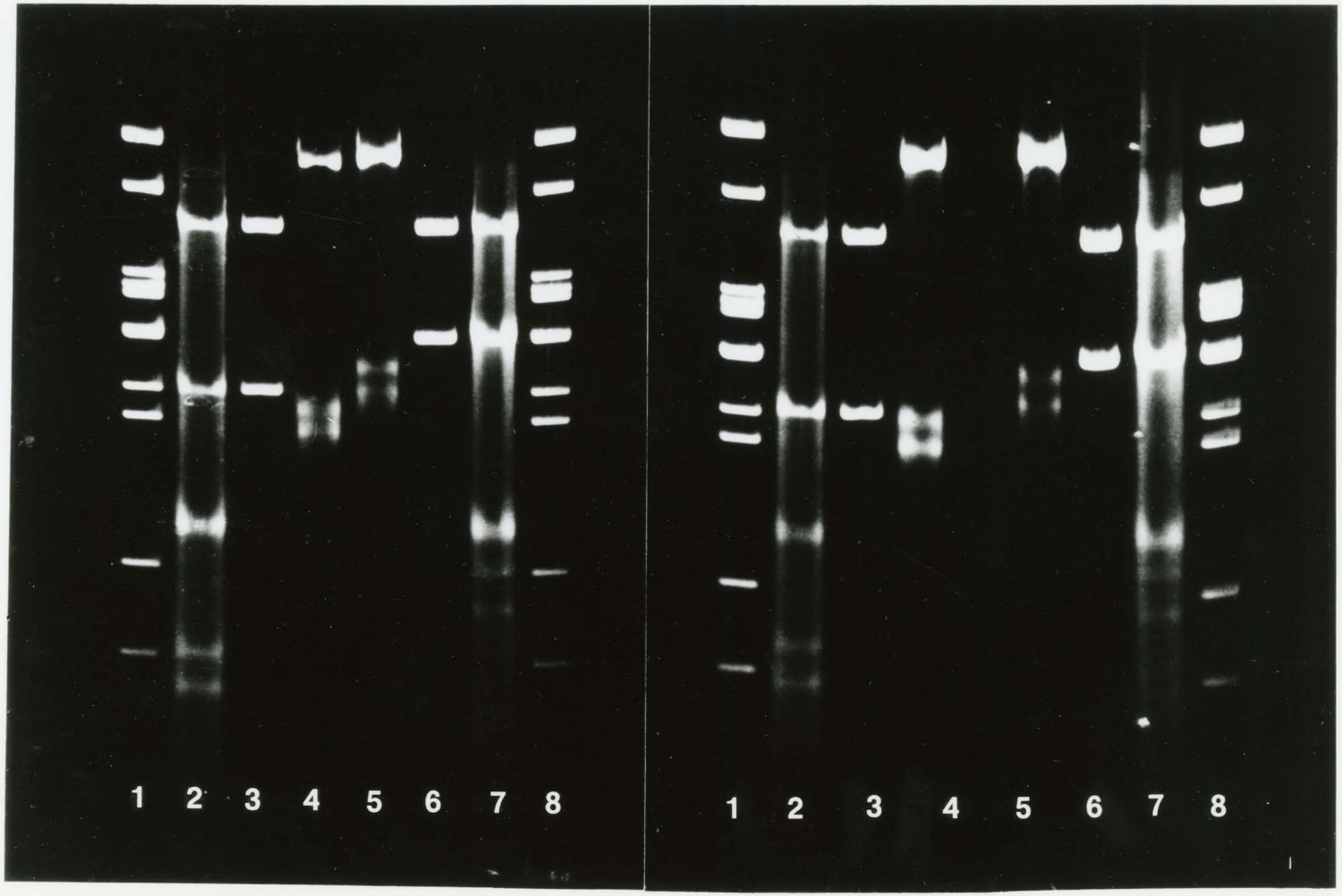

A. NO UV
B. 30 J/M2
1 2 3 4 5 6 7 8
1 2 3 4 5 6 7 8

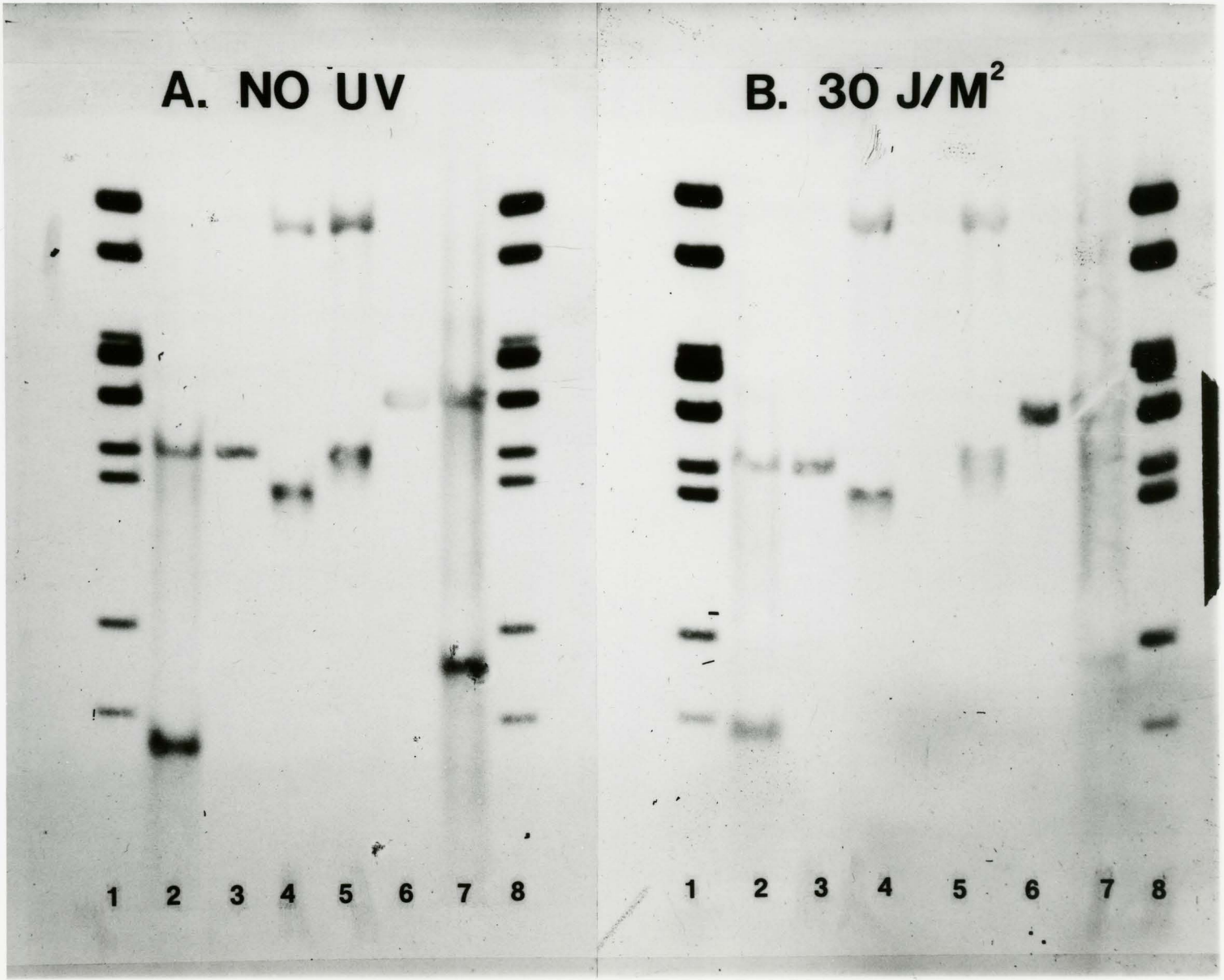
Figure 21

R-strand specific transcription of late nuclear RNA. II. Hybridization of late nuclear Ad 2 ^{3}H-RNA to Southern blots.

Hybridization of Southern blots were carried out in a 10 ml reaction within a sealed plastic bag for 30 hours at 65°C. After hybridization, the blots were washed extensively with 2.0 x SSC, treated with 20 µg/ml of ribonuclease for 1 hour at room temperature, air dried and finally sprayed with ^{3}H-enhancer before being exposed to x-ray film for 30 days at -60°C. Lanes: (1 and 8) Hind III digested Ad 2 marker ^{3}H-DNA; (2) denatured Hind III digested Ad 2 Hind III H-pBR322; (3) Hind III digested Ad 2 Hind III H-pBR322; (4) denatured Bam HI digested Ad 2 Hind III H-pBR322; (5) denatured Bam HI digested Ad 2 Hind III F-pBR322; (6) Hind III digested Ad 2 Hind III F-pBR322; (7) denatured Hind III digested Ad 2 Hind III F-pBR322.

A. Southern blot hybridized with nuclear ^{3}H-RNA extracted from Ad 2 infected cells labelled with ^{3}H-uridine for 15 minutes at 24 hours post-infection.

B. Southern blot hybridized with nuclear ^{3}H-RNA extracted from Ad 2 infected cells uv irradiated at a dose of 30 joules/m^{2} at 23.5 hours post-infection before being labelled with ^{3}H-uridine for 15 minutes at 24 hours post-infection.


A. NO UV
B. 30 J/M^2
1 2 3 4 5 6 7 8
1 2 3 4 5 6 7 8

quite clear on the original autoradiogram. Hybridization was also detected with the fast strands of the separated strands of the linearized plasmids, Hind III H-pBR322 and Hind III F-pBR322 DNA (Fig. 21, lanes 4 and 5). Hybridization of Ad 2 infected nuclear ^{3}H-RNA was not detected with any of the slow strands of the separated strands from Ad 2 DNA restriction fragments or total linearized plasmid.

This experiment was repeated using nuclear ^{3}H-RNA extracted from Ad 2 infected cells labelled with ^{3}H-uridine for 15 minutes at 15.5 hours post-infection. The agarose gels used for the blots are shown in Fig. 22 and the results of hybridization are shown in Fig. 23. Again, nuclear ^{3}H-RNA from uv irradiated (Fig. 23B) or non-irradiated Ad 2 infected cells hybridized to the fast strands of the separated strands of Ad 2 DNA restriction fragments Hind III H and Hind III F. No hybridization could be detected with the slow strands of the restriction fragments.

Flint and coworkers (1975, 1976) have identified the fast and slow strands of the separated DNA strands of viral restriction fragments by hybridization of ^{32}P-labelled DNA of fast and slow bands with excess unlabelled r and l-strands of Ad 2 DNA. The fast strand of restriction fragments derived from the right hand 60% of the Ad 2 genome was found to correspond to the r-strand of Ad 2 DNA. The experiments described above indicate that late Ad 2 transcripts detected by the Southern blot technique are transcribed from r-strand sequences of the regions examined and no transcripts derived from the l-strand could be detected using nuclear ^{3}H-RNA from non-irradiated uv irradiated Ad 2 infected cells.

Figure 22

R-strand specific transcription of late nuclear RNA.

I. Strand separation of Ad 2 DNA restriction fragments.

Approximately, 4 μg of Ad 2 Hind III H-pBR322 and Hind III F-pBR322 DNA was electrophoresed on a 1.4% agarose gel at 65 volts for 9 hours. The gel was stained with ethidium bromide and visualized under uv light before being prepared for Southern blotting. Lanes: (1) denatured Hind III digested Ad 2 Hind III H-pBR322 DNA; (2) Hind III digested Ad 2 marker ^{3}H-DNA; (3) denatured Hind III digested Ad 2 Hind III F-pBR322 DNA.

A. Agarose gel, which was blotted onto a nitrocellulose filter for hybridization of nuclear ^{3}H-RNA extracted from Ad 2 infected cells labelled with ^{3}H-uridine for 15 minutes at 15.5 hours post-infection.

B. Agarose gel, which was blotted onto a nitrocellulose filter for hybridization of nuclear ^{3}H-RNA extracted from Ad 2 infected cells uv irradiated at a dose of 30 joules/m^{2} at 15 hours post-infection before being labelled with ^{3}H-uridine for 15 minutes at 15.5 hours post-infection.

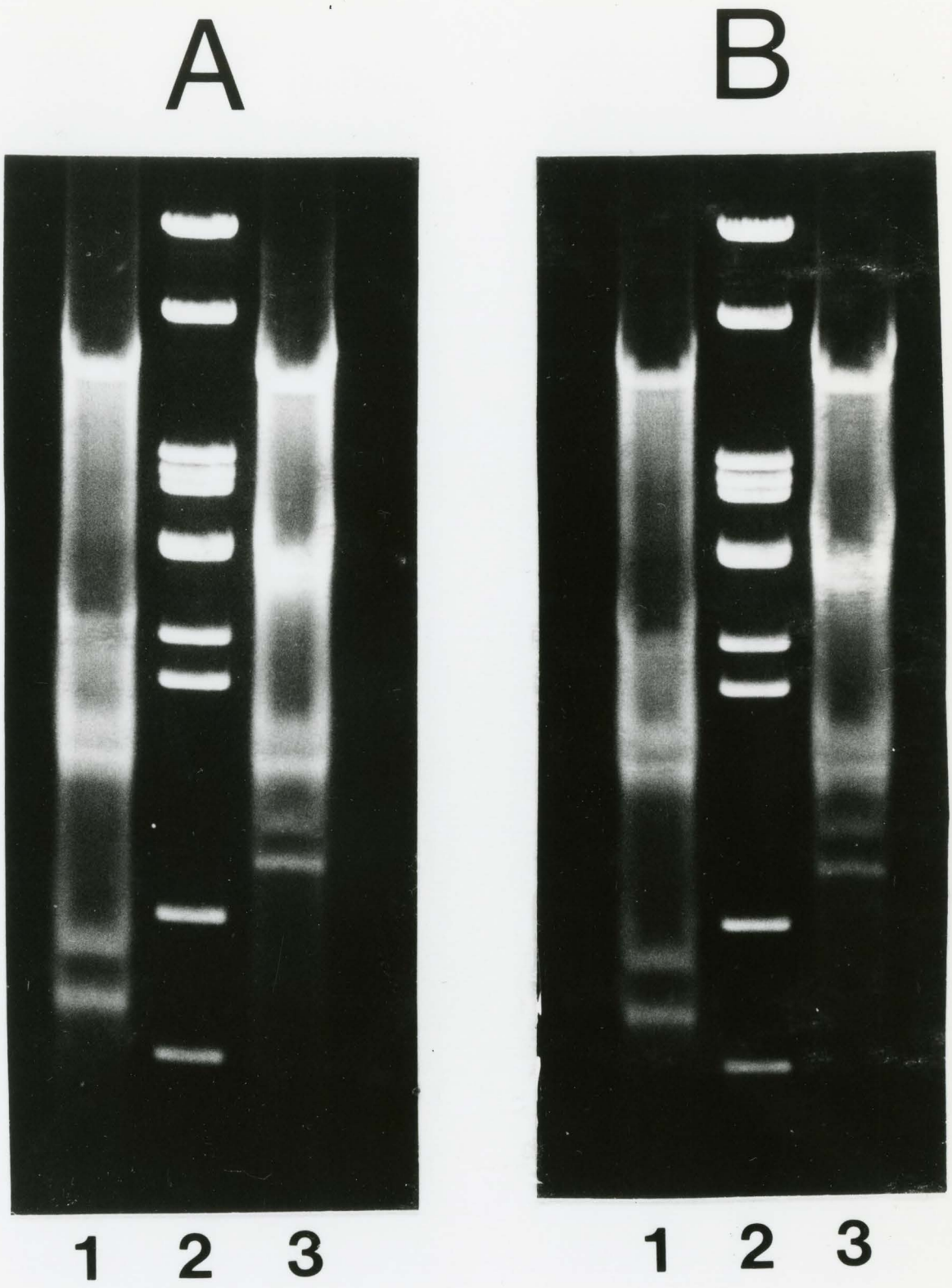

A
B
1 2 3
1 2 3

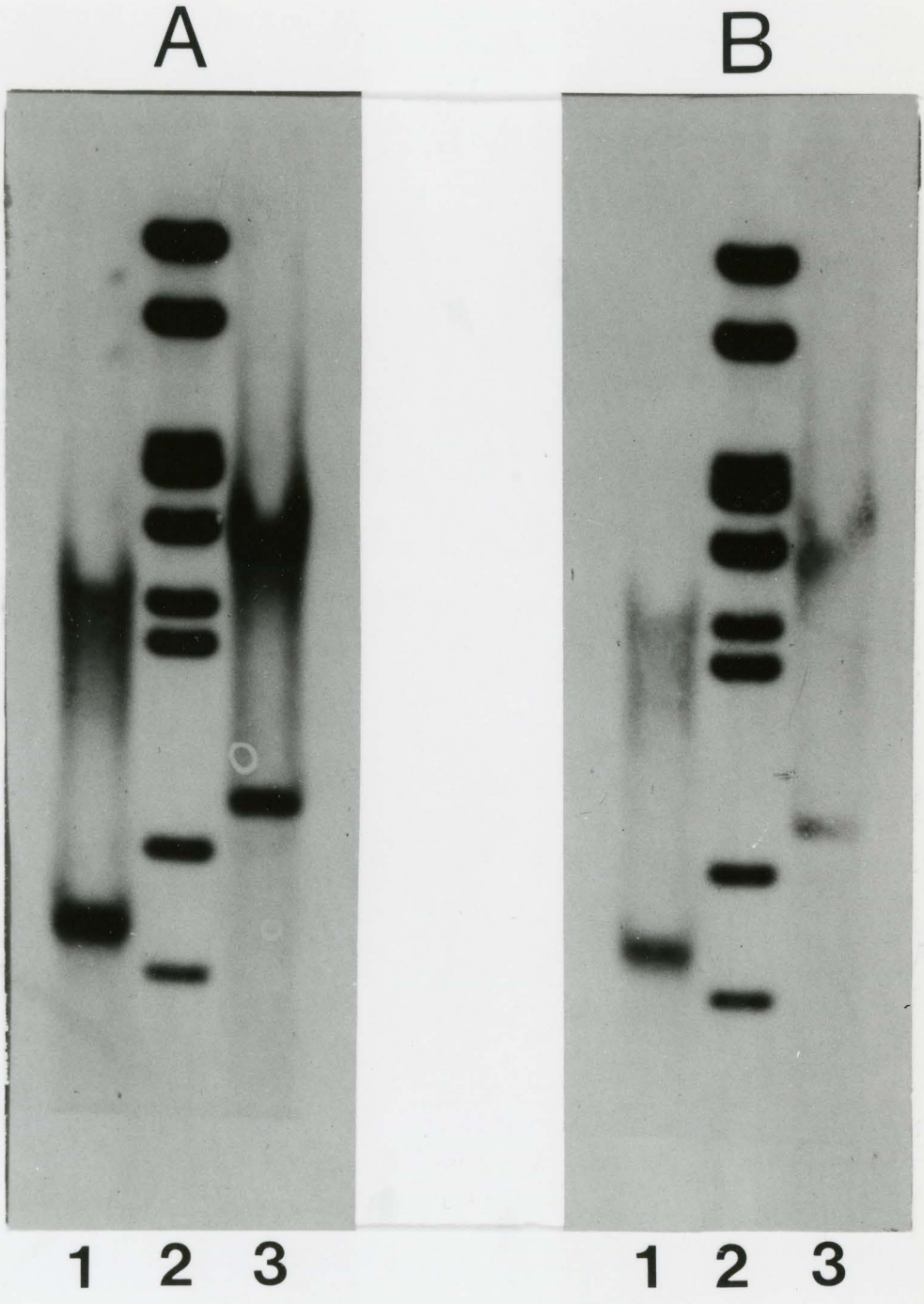
Figure 23

R-strand specific transcription of late nuclear RNA. II. Hybridization of late nuclear Ad 2 ^{3}H-RNA to Southern blots.

Hybridization to southern blots was carried out in a 7 ml reaction within a sealed plastic bag for 30 hours at 65°C. After hybridization, the blots were washed extensively with 2.0 x SSC, treated with 20 µg/ml of ribonuclease for 1 hour at room temperature, air dried and finally sprayed with ^{3}H-enhancer before being exposed to x-ray film for 30 days at -60°C. Lanes: (1) denatured Hind III digested Ad 2 Hind III H-pBR322 DNA; (2) Hind III digested Ad 2 marker ^{3}H-DNA; (3) denatured Hind III digested Ad 2 Hind III F-pBR322 DNA.

A. Southern blot hybridized with nuclear ^{3}H-RNA extracted from Ad 2 infected cells labelled with ^{3}H-uridine for 15 minutes at 15.5 hours post-infection.

B. Southern blot hybridized with nuclear ^{3}H-RNA extracted from Ad 2 infected cells uv irradiated at a dose of 30 joules/m^{2} at 15 hours post-infection before being labelled with ^{3}H-uridine for 15 minutes at 15.5 hours post-infection.


A
B
1 2 3
1 2 3

B. Late cytoplasmic polyadenylated RNA.

1. UV sensitivity of viral specific cytoplasmic polyadenylated (polyA) RNA at various sites along the Ad 2 genome. The effects of uv irradiation of viral RNA synthesis on the production of viral cytoplasmic poly A RNA was examined by DNA-RNA hybridization.

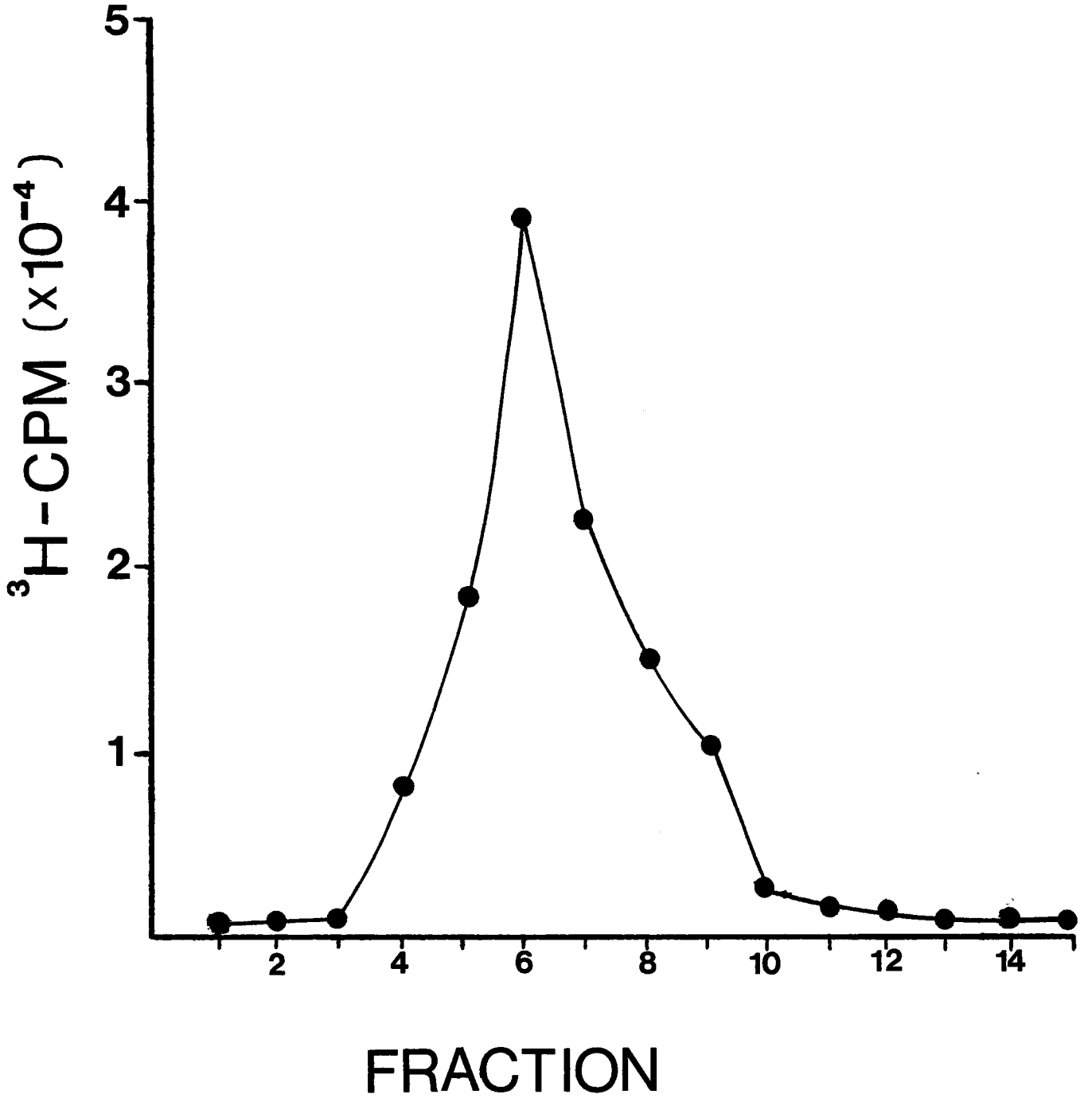
Identical cultures of KB cells grown in monolayer were infected with Ad 2 and uv irradiated at a dose of 30 joules/m^2 at 15 hours post-infection or left unirradiated. After a further incubation period of 30 minutes at 37°C, the infected cells were labelled with ^{3}H-uridine for 1 hour. The cytoplasm and the nuclei on nonirradiated or uv irradiated infected cells was fractionated and the cytoplasmic RNA was extracted. The isolated cytoplasmic RNA was loaded onto a column containing poly U sepharose 4B beads as described in Materials and Methods and the poly A RNA was eluted with a high formamide buffer into 0.5 ml fractions. The peak fractions (Fig. 24) were pooled and used as a source of purified cytoplasmic poly A for hybridization to Ad 2 DNA restriction fragments.

Equal aliquots of ^{3}H-poly A RNA from non-irradiated and uv irradiated Ad 2 infected cells were hybridized to 20 µg equivalent of Ad 2 DNA restriction fragments Bam HI B, D, C and Eco RI B, F, E, and C. The surviving fraction of poly A RNA production was determined by comparing the hybridized cpm of ^{3}H-poly A RNA from uv irradiated and non-irradiated cultures at each of the regions of the genome examined.

The surviving fraction was plotted against the genome position of the right hand terminus of each DNA fragment (Fig. 25). A gradient of increasing uv sensitivity beginning in the Bam HI B fragment and

Figure 24

Isolation of cytoplasmic polyadenylated RNA. Ad 2 infected cells were labelled with 50 μCi/ml of ^{3}H-uridine for 1 hour at 15 hours post-infection and the cytoplasmic RNA was extracted. The cytoplasmic RNA was loaded onto a column of poly U sepharose 4B in a high salt, low formamide buffer. The poly A ^{3}H-RNA was eluted from the column with a low salt, high formamide buffer and collected in 0.5 ml fractions. A 5 μl aliquot of each fraction was assayed by liquid scintillation counting. The peak fractions (4-9) were pooled, ethanol precipitated, collected by centrifugation and dissolved in 5 mM Tris pH7.4.


5
4
3
2
1
^{3}H-CPM (x10^{-4})
2
4
6
8
10
12
14
FRACTION

continuing towards the right of the genome with the greatest uv sensitivity of poly A RNA production within the Bam HI C fragment was observed. Towards the right of this region the uv sensitivity decreases and an increase in the surviving fraction is observed for the Eco RI B and F regions followed by a second increase in the uv sensitivity, which is similar in pattern to the results described in Fig. 16 for newly synthesized nuclear viral specific RNA. The extreme right hand end of the genome represented by the Eco RI C fragment consistently yielded the observation of a dramatic elevation in the hybridized cpm of ^{3}H-poly A RNA from uv irradiated cultures as compared to the non-irradiated culture. This experiment was repeated several times and although considerable variation was observed in the absolute surviving fraction of ^{3}H-poly A RNA production at each region, a similar pattern to that described above consistently emerged from these experiments. Table 8 gives the surviving fraction for each of the genome regions examined in three independent experiments. The uv sensitivities observed at the right hand 40% of the genome is inconsistent with the idea of a single transcription unit for the production of late poly A RNA.

^{3}H-poly A RNA examined at the DNA sequences represented by the Eco RI F fragment was very resistant to the effects of uv irradiation and often appeared to be stimulated as in Fig. 25. The significance of this result is unclear.

The uv stimulation of viral specific ^{3}H-poly A RNA production found in the cytoplasm of Ad 2 infected cells at the extreme right hand end of the genome represented the Eco RI C region was an unexpect-

Figure 25

Ultraviolet sensitivity of viral specific poly A RNA at various sites along the Ad 2 genome. Cultures of Ad 2 infected cells were exposed to uv irradiation at 15 hours post-infection or left unirradiated before being labelled for 1 hour with ^{3}H-uridine. The cytoplasmic RNA was extracted and the poly A RNA isolated. Equal aliquots of ^{3}H-poly A RNA were hybridized to 20 µg equivalent of Ad 2 DNA restriction fragments on nitrocellulose filters for 24 hours at 65^{o}C. The filters were washed extensively with 2.0 x SSC, treated with 20 µg/ml of ribonuclease, dissolved in ethyl acetate and assayed by liquid scintillation counting. The surviving fraction of viral specific ^{3}H-poly A RNA was determined by comparing the hybridized cpm from uv irradiated and non-irradiated cultures. Each point represents the average of triplicate samples.

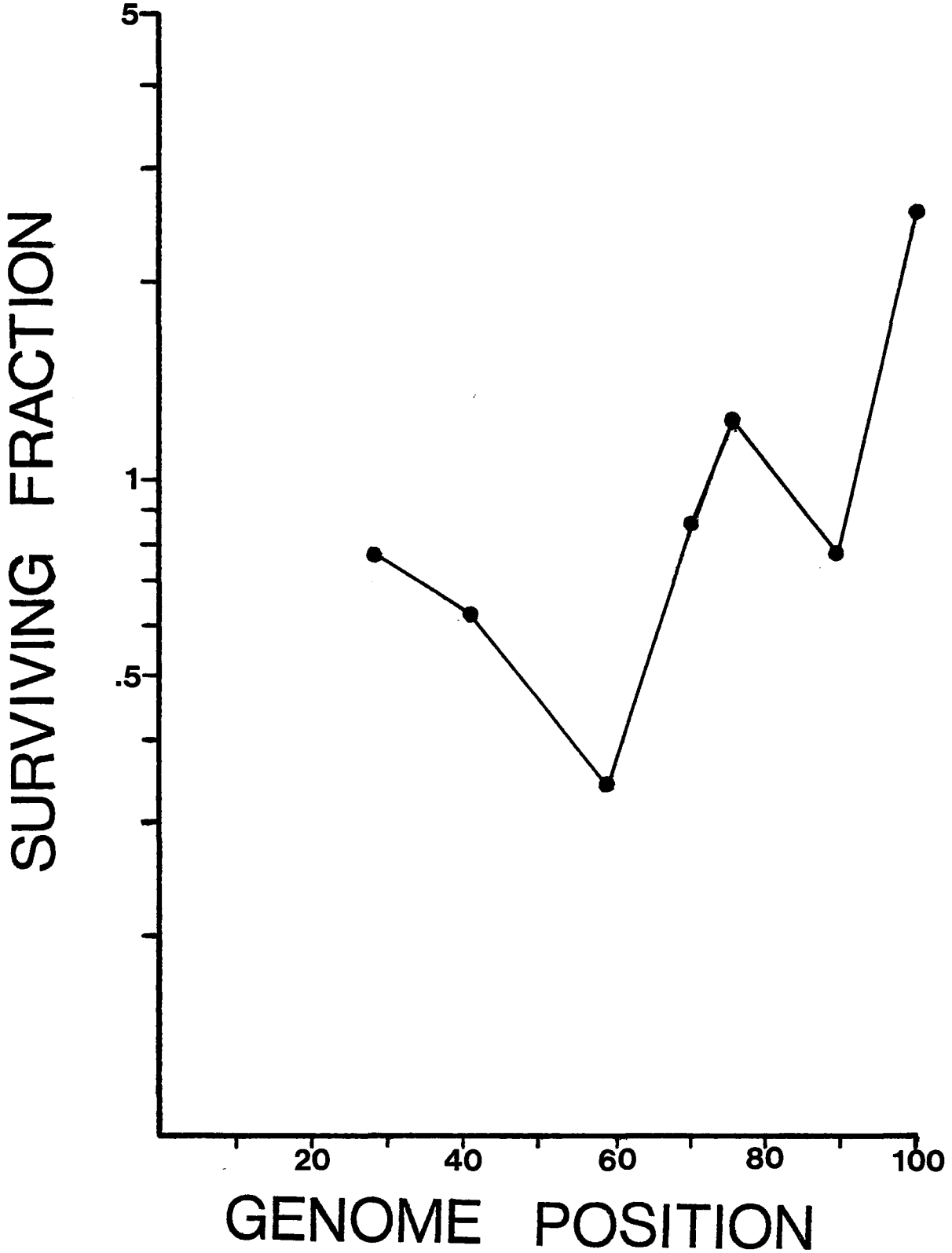

SURVIVING FRACTION
5
1
.5
20
40
60
80
100
GENOME POSITION

Table 8

UV sensitivity of viral specific poly A RNA production at various sites along the Ad 2 genome

Fragment	Surviving Fraction Expt. 1	Expt. 2	Expt. 3
Bam HI B	0.76	0.71	0.95
D	0.62	0.55	0.66
C	0.35	0.46	0.39
Eco RI B	0.84	0.65	1.05
F	1.30	0.92	1.24
E	0.79	0.73	0.88
C	2.55	1.22	2.00

Viral specific cytoplasmic ^{3}H-poly A RNA from three independent similar experiments comparing the surviving fraction from Ad 2 infected cells uv irradiated at 15 hours post-infection or left unirradiated before being labelled with ^{3}H-uridine for 1 hour at 15.5 hours post-infection.

ed and puzzling observation. It was thought possible that the uv irradiation selectively stimulated the transport of mRNA coded by this region for the expression of the fibre protein from the nucleus to the cytoplasm. Alternatively, the small decrease in the uv sensitivity of nuclear transcription observed for this region in Fig. 16 may represent a small amount of 1-strand specific transcription of early region four that was not detected in the Southern blots in Figs. 21 and 23, but may be amplified in the poly A RNA fraction. This latter possibility was tested by hybridization of ^{3}H-poly A RNA to separated strands Ad 2 restriction fragments on Southern blots.

2. Strand specificity of poly A RNA. Approximately 4 µg of the recombinant DNA plasmids, Ad 2 Hind III H-pBR322, which overlaps the Ad 2 Eco RI F fragment and Hind III F-pBR322, which includes the majority of the Ad 2 Eco RI C fragment region was digested with Hind III and denatured in 0.2N NaOH for 10 minutes at room temperature. The alkali denatured DNA was subjected to electrophoresis on a 1.4% agarose gel. The separated fast and slow strands of Hind III H and Hind III F DNA were resolved towards the bottom of the agarose gel, as described previously, and are shown in Fig. 26 (lanes 1 and 3). Each of the two identical agarose gels shown in Fig. 26 was blotted on nitrocellulose filter sheets and hybridized with ^{3}H-poly A RNA isolated from the cytoplasm of Ad 2 infected cells uv irradiated at 15 hours post-infection of left unirradiated and labelled with ^{3}H-uridine for 1 hour at 15.5 hours post-infection. The hybridized blots were exposed to x-ray film and the autoradiograms obtained are shown in Fig. 27.

Figure 26

Strand specificity of Poly A RNA. I. Strand separation of Ad 2 DNA restriction fragments. Approximately 4 μg of Ad 2 Hind III H-pBR322 and Hind III F-pBR322 DNA was electrophoresed on a 1.4% agarose gel at 40 volts for 16 hours. The gel was stained with ethidium bromide and visualized under uv light before being prepared for Southern blotting. Lanes: (1) denatured Hind III digested Ad 2 Hind III H-pBR322 DNA; (2) Hind III digested Ad 2 marker ^{3}H-DNA; (3) denatured Hind III digested Ad 2 Hind III F-pBR322 DNA.
A. Agarose gel, which was blotted onto a nitrocellulose filter for hybridization of ^{3}H-poly A RNA isolated from Ad 2 infected cells labelled with ^{3}H-uridine for 1 hour at 15.5 hours post-infection.
B. Agarose gel, which was blotted onto a nitrocellulose filter for hybridization of ^{3}H-poly A RNA isolated from Ad 2 infected cells uv irradiated at a dose of 30 joules/m^{2} at 15 hours post-infection before being labelled with ^{3}H-uridine for 1 hour at 15.5 hours post-infection.

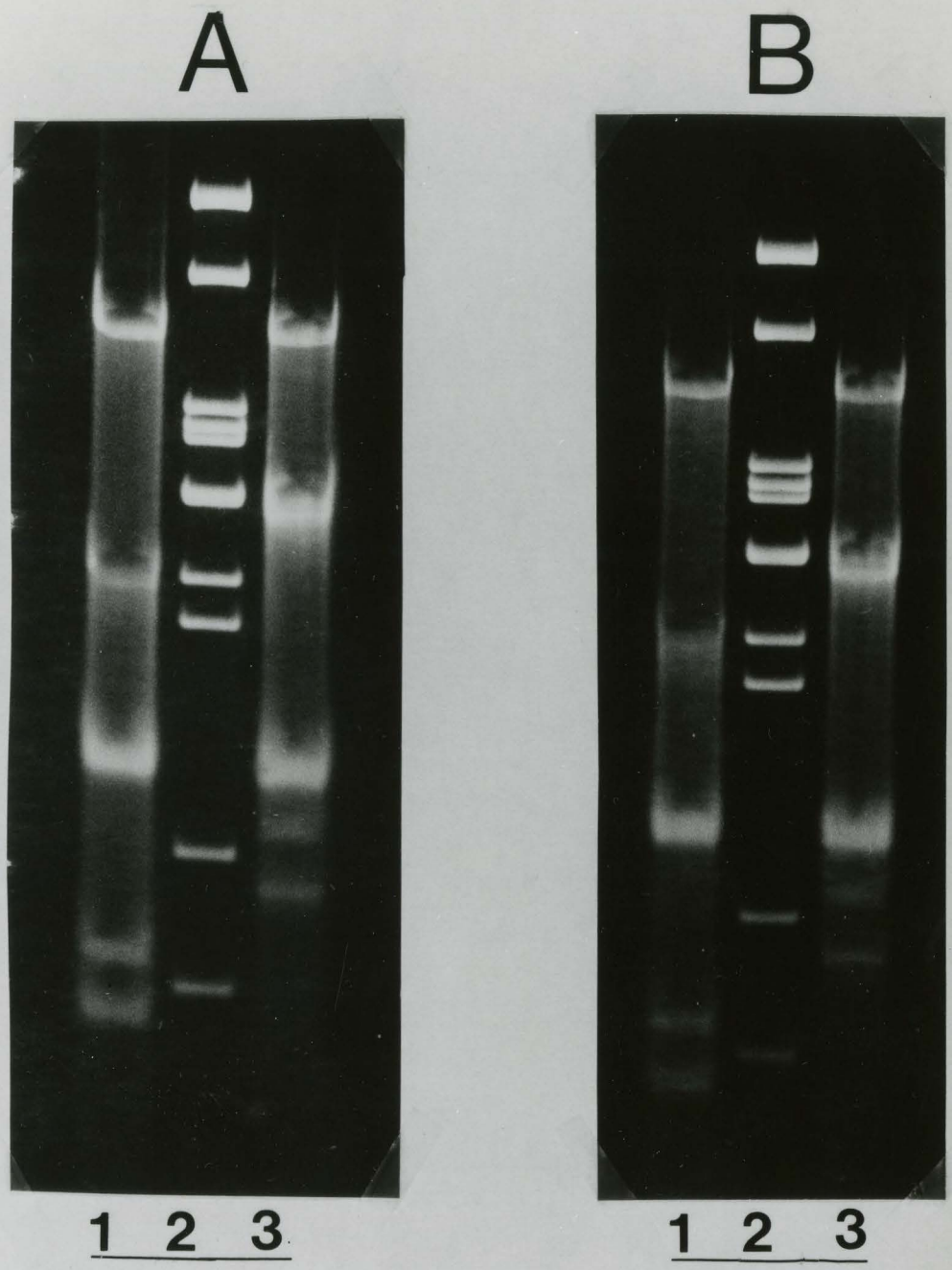
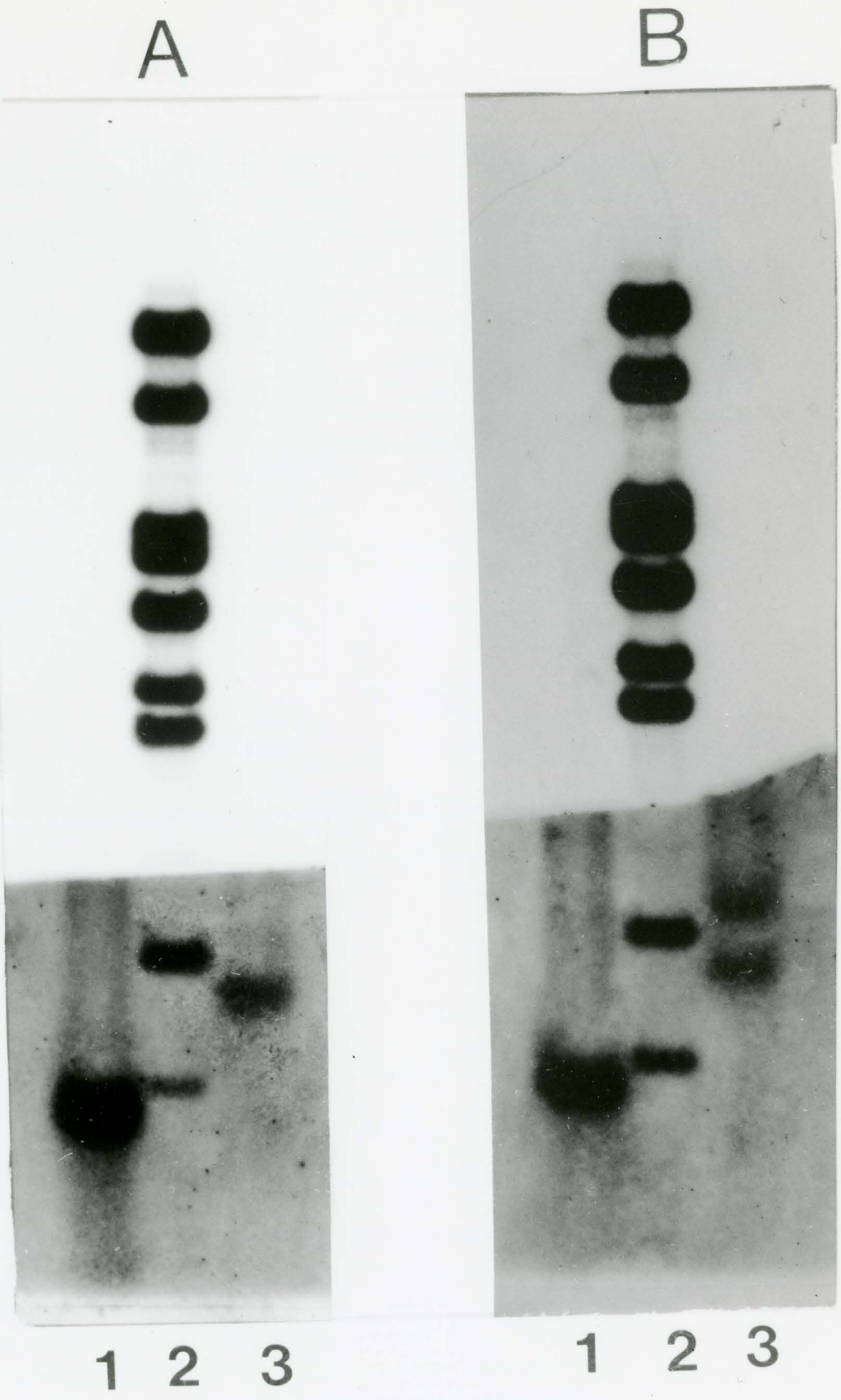

A
B
1 2 3
1 2 3

Figure 27

Strand specificity of Poly A RNA. II. Hybridization of late Ad 2 ^{3}H-poly A RNA. The blots were cut prior to hybridization such that only the bottom of the blot containing both of the separated strands of the Ad 2 restriction fragments would participate in the hybridization reaction. Hybridization to Southern blots were carried out in a 5 ml reaction within a sealed plastic bag for 30 hours at 65^{o}C. After hybridization, the blots were washed extensively with 2.0 x SSC, treated with 20 µg/ml of ribonuclease for 1 hour at room temperature, air dried and finally sprayed with ^{3}H-enhancer before being exposed to x-ray film for 60 days at -60^{o}C. Lanes: (1) denatured Hind III digested Ad 2 Hind III H-pBR322 DNA; (2) Hind III digested Ad 2 marker ^{3}H-DNA; (3) denatured Hind III digested Ad 2 Hind III F-pBR322 DNA.

A. Southern blot hybridized with cytoplasmic ^{3}H-poly A RNA isolated from Ad 2 infected cells labelled with ^{3}H-uridine for 1 hour at 15.5 hours post-infection.

B. Southern blot hybridized with cytoplasmic ^{3}H-poly A RNA isolated from Ad 2 infected cells uv irradiated at a dose of 30 joules/m^{2} at 15 hours post-infection before being labelled with ^{3}H-uridine for 1 hour at 15.5 hours post-infection.


A
B
1 2 3
1 2 3

^{3}H-poly A RNA from both the non-irradiated and uv irradiated Ad 2 infected cells hybridized to the fast strand of the separated strands of Ad 2 Hind III H DNA (Fig. 27A and B, lane 1). No hybridization to the slow strand was detected indicating that poly A RNA produced from this region of the viral genome is derived only from the r-strand at 15.5 hours post-infection as determined by the Southern blotting technique. ^{3}H-poly A RNA from unirradiated Ad 2 infected cells hybridized to the fast strand of the separated strands of Ad 2 Hind III F DNA and no hybridization to the slow strand was detected (Fig. 27A, lane 3) similar to the results observed with nuclear ^{3}H-RNA (Fig. 21A, lane 7). However, ^{3}H-poly A RNA from the uv irradiated Ad 2 infected cells clearly hybridized to both the fast and slow strands of the separated strands of Ad 2 Hind III F DNA. This suggests that uv irradiation of Ad 2 infected cells may stimulate some transcription of early region four coded for by the l-strand at the extreme right-hand end of the viral genome. The transport of poly A RNA, coded for by the l-strand of this region, may have been stimulated by uv irradiation allowing the detection of l-strand specific poly A RNA on Southern blot (Fig. 27) and probably accounts for the results observed in Fig. 25 for the Eco RI C fragment hybridizations with ^{3}H-poly A RNA. In addition, the finding of consistently higher values for the surviving fraction at each of the various regions examined with poly A RNA (Table 8) as compared to nuclear RNA (Table 4) may be a result of a transport effect by uv irradiation.

V. UV Transcription Unit Mapping by Gene Product Inactivation.

A. Late Ad 2 Proteins

1. Time course of synthesis of late Ad 2 proteins. The reduction of late Ad 2 gene products by uv irradiation was examined to investigate the transcriptional organization of late Ad 2 gene expression. As described previously, the uv sensitivity of the expression of a given gene produced is related to the relative target size of the transcription unit from which that gene is derived. Comparing the uv sensitivities of a number of late viral proteins from different regions of the genome should reflect the relative target size of the transcription unit(s) expressed late during Ad 2 infection.

The induction of late Ad 2 protein synthesis is dependent on the initiation of viral DNA synthesis (Thomas and Mathews, 1980), which occurs at approximately 5 to 6 hours post-infection (Green et al., 1970). Consequently, in order to detect the effect of uv irradiation on the synthesis of late viral proteins it was necessary to uv irradiate the infected cells after the initiation of viral DNA synthesis, but before large amounts of late viral specific polypeptides are produced. It was also of importance to determine the time of ^{35}S-methionine labelling which would permit the detection of sufficient synthesis of all the late Ad 2 polypeptides to be examined following uv irradiation of the infected cells.

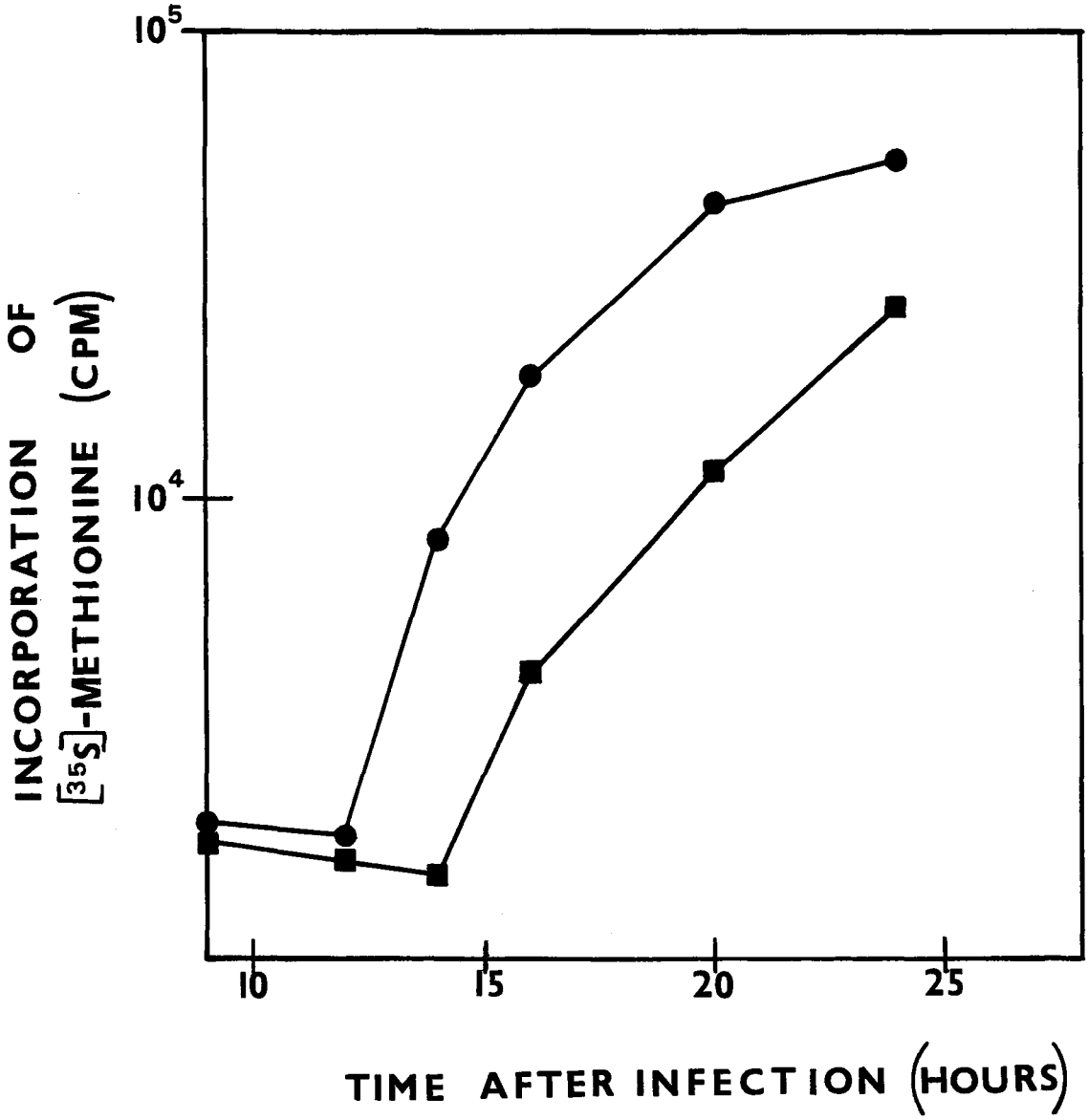
KB cells were infected at 50 PFU/cell with Ad 2 and subsequently uv irradiated at a dose of 20 joules/m^2 at 8 hours post-infection. Similar non-irradiated infected cultures were kept as controls. The infected cells were then pulse labelled for 1 hour with 35 μCi of ^{35}S-

methionine at various times after uv irradiation. An aliquot of each sample was subjected to SDS-polyacrylamide gel electrophoresis. The gel was stained, destained, dried under vacuum and finally exposed to x-ray film to obtain an autoradiogram of the labelled infected cell proteins. A variety of easily detectable late Ad 2 polypeptides similar to those reported previously (Anderson et al., 1973, 1974; Lewis et al., 1975, 1977; Weber, 1976) were observed. Individual bands were excised from the dried gel employing the autoradiogram as a guide and their radioactivity was assayed by liquid scintillation counting. Typical results for the time course of polypeptide synthesis are shown in Fig. 28 for hexon, the gene II product. It can be seen that for the non-irradiated culture that significant synthesis of Ad 2 hexon polypeptide was detectable by 14 hours post-infection and continued until at least 24 hours post-infection, at which point the experiment was terminated. It can also be seen that uv irradiation of the infected cells resulted in a considerable reduction in the synthesis of the hexon protein compared to non-irradiated infected cells. Since uv irradiation occurred prior to the appearance of detectable amounts of late protein synthesis, the reduction in protein synthesis observed is thought to result from damage to viral DNA rather than from damage to pre-existing viral specific mRNA. The uv inactivation of viral polypeptide synthesis is, therefore, thought to result from the induction of transcription terminating lesions in the viral DNA. The fact that this uv radiation induced depression of polypeptide synthesis was reduced at later times after irradiation suggests that at least some of these lesions can be repaired.

Figure 28

Time course for the detection of the hexon protein. Ad 2 infected KB cells from a non-irradiated culture and from cultures uv irradiated at 8 hours post-infection with 20 joules/m^2 were pulse labelled for 1 hour with ^{35}S-methionine at different times after infection. These samples were processed and subjected to SDS-polyacrylamide gel electrophoresis and autoradiography. The hexon polypeptide bands were excised from the dried gel, dissolved in 1 ml of 30% H_2O_2 and assayed for radioactivity by liquid scintillation counting.

Closed circles, 0 dose; closed boxes, 20 joules/m^2.

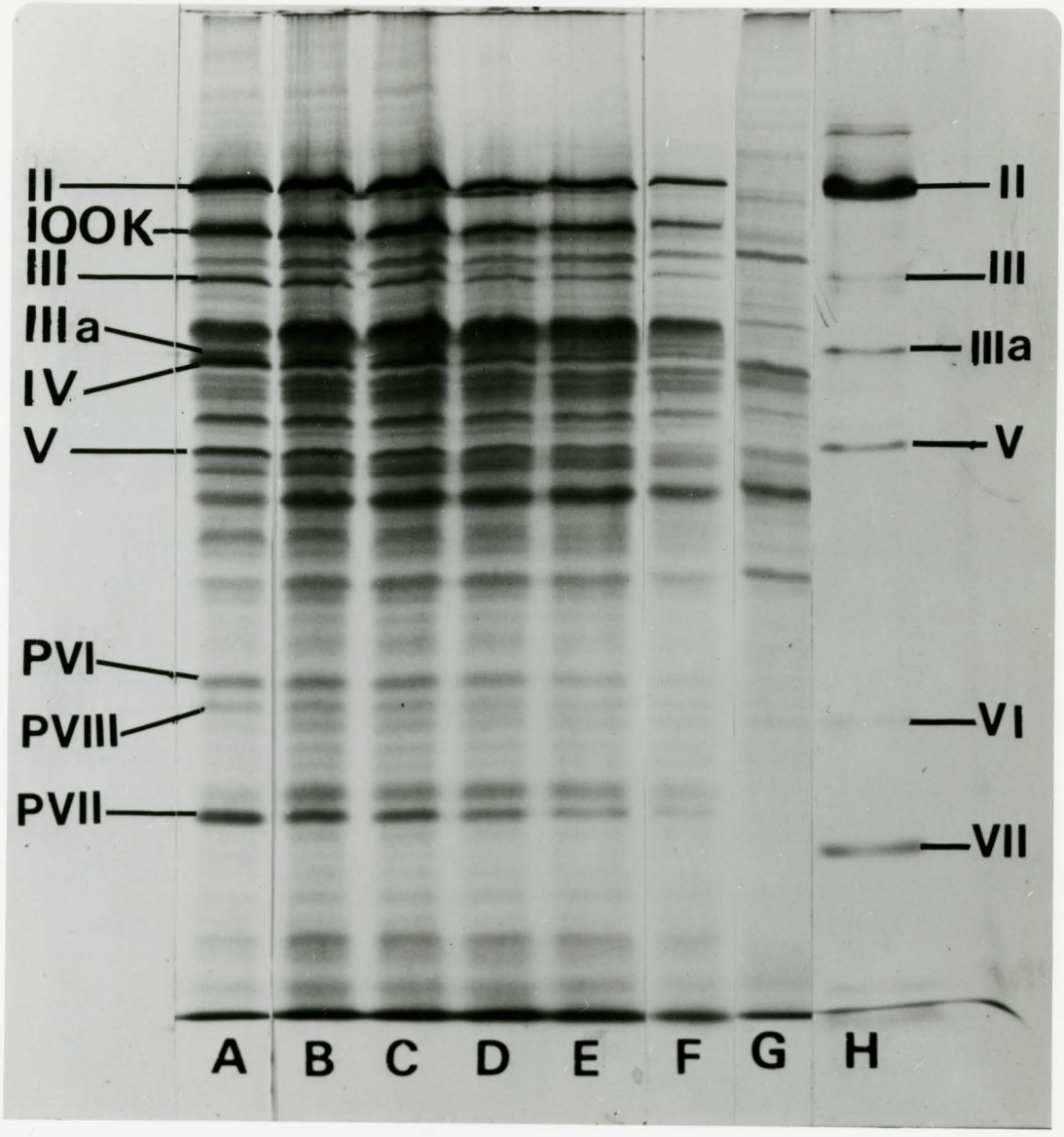

10^5
10^4
INCORPORATION OF [35S]-METHIONINE (CPM)
10
15
20
25
TIME AFTER INFECTION (HOURS)

2. Differential uv inactivation of late Ad 2 polypeptide synthesis. In order to determine the relative uv inactivation for the synthesis of the different late Ad 2 polypeptides, Ad 2 infected KB cells were uv irradiated with a series of increasing doses at 10 hours post-infection and were pulse labelled with ^{35}S-methionine for 1 hour at 16 hours post-infection. An equal volume of each sample extract, obtained from an equal number of infected cells, was analyzed by SDS-polyacrylamide gel electrophoresis and autoradiography. A typical autoradiogram is shown in Fig. 29. It can be seen that increasing uv doses to the infected cells result in a differential reduction in polypeptide synthesis for the different late Ad 2 proteins. Employing the autoradiogram as a guide, protein bands were excised from the dried gel and dissolved in 30% H_2O_2 before their radioactivity was assayed by liquid scintillation counting. From the radioactivity obtained in each polypeptide band, inactivation curves for the synthesis of the various late Ad 2 proteins were determined. The average of the results from two polyacrylamide gels of the same experiment are shown in Figs. 30 and 31.

A comparison of the uv inactivation curves for the genes IIIa and IV demonstrates that the uv sensitivity is not merely a function of gene size, since proteins IIIa and IV are of similar molecular weight (Anderson et al., 1973), but yield very different inactivation profiles. Furthermore, an increase in the amount detected for one of the proteins, pVI, the precursor to protein VI was observed following uv irradiation, as compared to a decrease for all other late viral proteins examined. This may result from a uv radiation effect on the

Figure 29

SDS-polyacrylamide gel autroadiogram of Ad 2 infected cell cell proteins. SDS-polyacrylamide gel autoradiograms of labelled extracts of Ad 2 infected cells after various doses of uv irradiation at 10 hours post-infection. The infected cells were labelled for 1 hour with ^{35}S-methionine at 16 hours post-infection. (A) 0, (B) 5, (C) 10, (D) 15, (E) 20, and (F) 25 joules/m^2; (G) mock-infected KB cells; (H) purified ^{35}S-methionine labelled Ad 2. Electrophoresis was carried out on SDS-15% acrylamide and 0.86% bisacrylamide for 12 hours at 120 volts. After electrophoresis, the gel was stained with coomassie brilliant blue, destained, and dried under vacuum before being exposed to x-ray film for 48 hours.


II
IOOK
III
IIIa
IV
V
PVI
PVIII
PVII
II
III
IIIa
V
VI
VII
A
B
C
D
E
F
G
H

processing of pVI occurring during the 1 hour labelling period. Both pVI and pVII are processed following their synthesis (Anderson et al., 1973), and as a consequence the amount of labelled protein detected in the corresponding polypeptide bands may not truly represent the amount of these proteins actually synthesized during the labelling period.

Lewis and coworkers (1975, 1977) have translated in vitro RNA from Ad 2 infected cells selected by hybridization to different regions of the Ad 2 genome to locate many of the genes for late Ad 2 proteins. Genes coding for polypeptides IIIa, III (penton), V (minor core), pVI, pVII (major core precursor) and II (hexon) are located within the left hand 60% of the viral genome, whereas genes 100K and IV (fibre) have been assigned to the right 40% of the viral genome. The location of these genes on the Ad 2 genome is consistent with several other studies analyzing interserotypic recombinants (Mautner et al., 1975; Grodzicker et al., 1977; Weber and Hassell, 1979), or by R-loop mapping of RNA sequences derived from these genes (Chow et al., 1977). The proposed positions for these genes can be found in Fig. 3. By means of hybridization techniques and cell free translation these genes representing the majority of late Ad 2 proteins have been assigned to the r-strand of the viral DNA (Oberg, Mak, and Philipson, personal communication).

Transcription from a single promotor on the r-strand would result in a significantly greater inactivation of protein synthesis for genes 100K and IV as compared to gene II, since genes 100K and IV are both distal to gene II. However, it can be seen from Figs. 30 and 31 that the inactivation of polypeptide synthesis for genes 100K and IV is in fact far less than that for gene II, suggesting the existence of

Figure 30

UV inactivation of late Ad 2 proteins. Relative rates of synthesis of late Ad 2 proteins from infected cells, as a function of uv dose for the proteins known to be coded for by the first 60% of the Ad 2 genome. The points of the graph were obtained from evaluating autoradiograms (Fig. 29) by comparing the radioactivity incorporated into protein bands during a 1 hour pulse label with ^{35}S-methionine at 16 hours post-infection from Ad 2 infected cells uv irradiated at 10 hours post infection or left nonirradiated. Each point to the average of results from two separate SDS-polyacrylamide gels of the same experiment. Closed squares, gene pVI; open circles, gene IIIa; open triangles, gene V; closed circles, gene III; open squares, gene pVII; closed triangles, gene II.

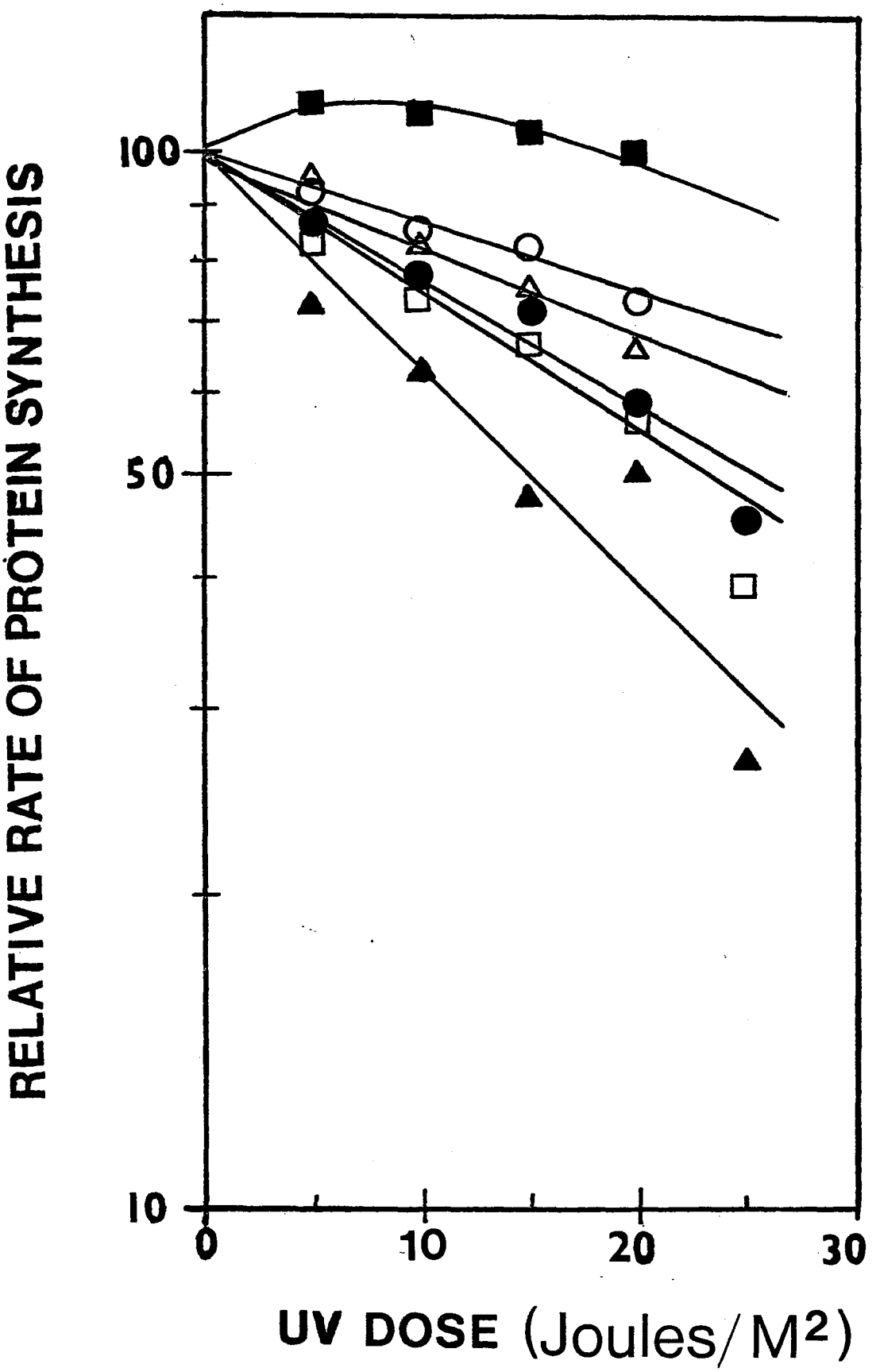
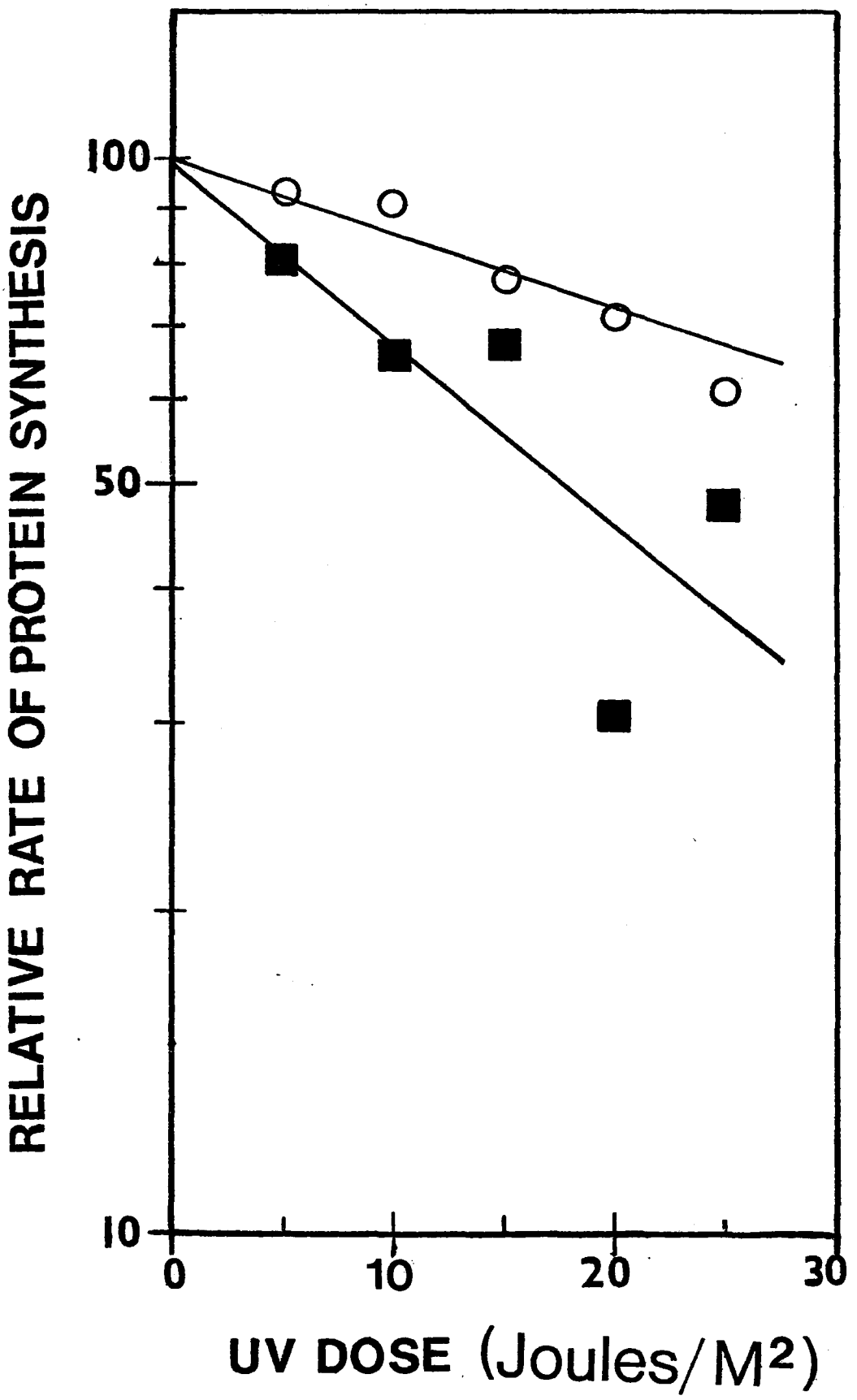

RELATIVE RATE OF PROTEIN SYNTHESIS
100
50
10
0
10
20
30
UV DOSE (Joules/M²)

Figure 31

UV inactivation of late Ad 2 proteins. Relative rates of synthesis of late Ad 2 proteins from infected cells, as a function of uv dose for the proteins known to be coded for by the right 40% of the Ad 2 genome. The points of the graph were obtained from evaluating autoradiograms (Fig. 29) by comparing the radioactivity incorporated into protein bands during a 1 hour pulse label with ^{35}S-methionine at 16 hours post-infection from Ad 2 infected cells uv irradiated at 10 hours post-infection or left unirradiated. Each point is the average of results from two separate SDS-polyacrylamide gels of the same experiment. Open circles, gene 100K; closed squares, gene IV.


RELATIVE RATE OF PROTEIN SYNTHESIS
100
50
10
0
10
20
30
UV DOSE (Joules/M2)

a second transcriptional unit encoding genes 100K and IV. The inactivation curves shown in Figs. 30 and 31 indicate that the first transcription unit has the gene order IIIa, V, III, and II, whereas the second unit consists of gene 100K followed by gene IV. Weber and Hassell (1979) also determined this exact gene order as well as their approximate locations on the viral genome by the extensive study of interserotypic recombinants between ts mutants of Ad 2 and Ad 5.

It is seen from Figs. 30 and 31 that, with the exception of gene pVI, increasing uv doses resulted in an exponential decrease for each gene product synthesized. The exponential slope of each uv inactivation curve reflects the uv sensitivity of the genes coding for the various late Ad 2 proteins. K, the slope of each inactivation curve, is assumed to be proportioned to the length along the DNA from the promotor to the 5'-terminus of each gene as has been found in other systems (Hercules and Sauerbier, 1973; Ball and White, 1976). Data from duplicate gels of the same experiment were fitted by linear regression analysis to obtain the K values shown in Table 9. The standard error of the slopes shown give an indication of the experimental error involved in this type of experiment.

A similar analysis to that for the data of viral specific nuclear RNA (Fig. 18) has been applied here in Fig. 32, the inactivation cross section, K for each of the different late proteins is plotted against a proposed position on each transcription unit. The proposed positions of the late genes considered have been assigned on the basis of the gene order determined here, the molecular weights of Ad 2 late gene products (Anderson et al., 1973), the assignment of late

TABLE 9

Ultraviolet Sensitivity of Late Ad 2 Gene Expression

Gene	K (x 10^{-2}) (m^2/joules)[a]	Relative radiosensitivity[b] Expt. 1	Expt. 2[c]
IIIa	1.58±0.18	0.34±0.07	0.22±0.03
V	2.15±0.50	0.47±0.15	0.34±0.04
III	2.83±0.18	0.61±0.11	0.55±0.05
pVII	3.18±0.39	0.69±0.16	ND[d]
II	4.62±0.51	1.00±0.22	1.00±0.09
100K	1.87±0.38	0.41±0.12	0.40±0.05
IV	3.92±0.80	0.85±0.36	0.78±0.07

a. Data points from duplicate gels of Expt. 1 were fitted by linear regression analysis to obtain the standard errors shown.

b. Relative radiosensitivity as compared to gene II.

c. Cells were uv irradiated at 9 hours post-infection and pulse labelled at 15 hours post-infection for 0.5 hours. The relative radiosensitivity of each protein was determined from the autoradiograms by microdensitometry.

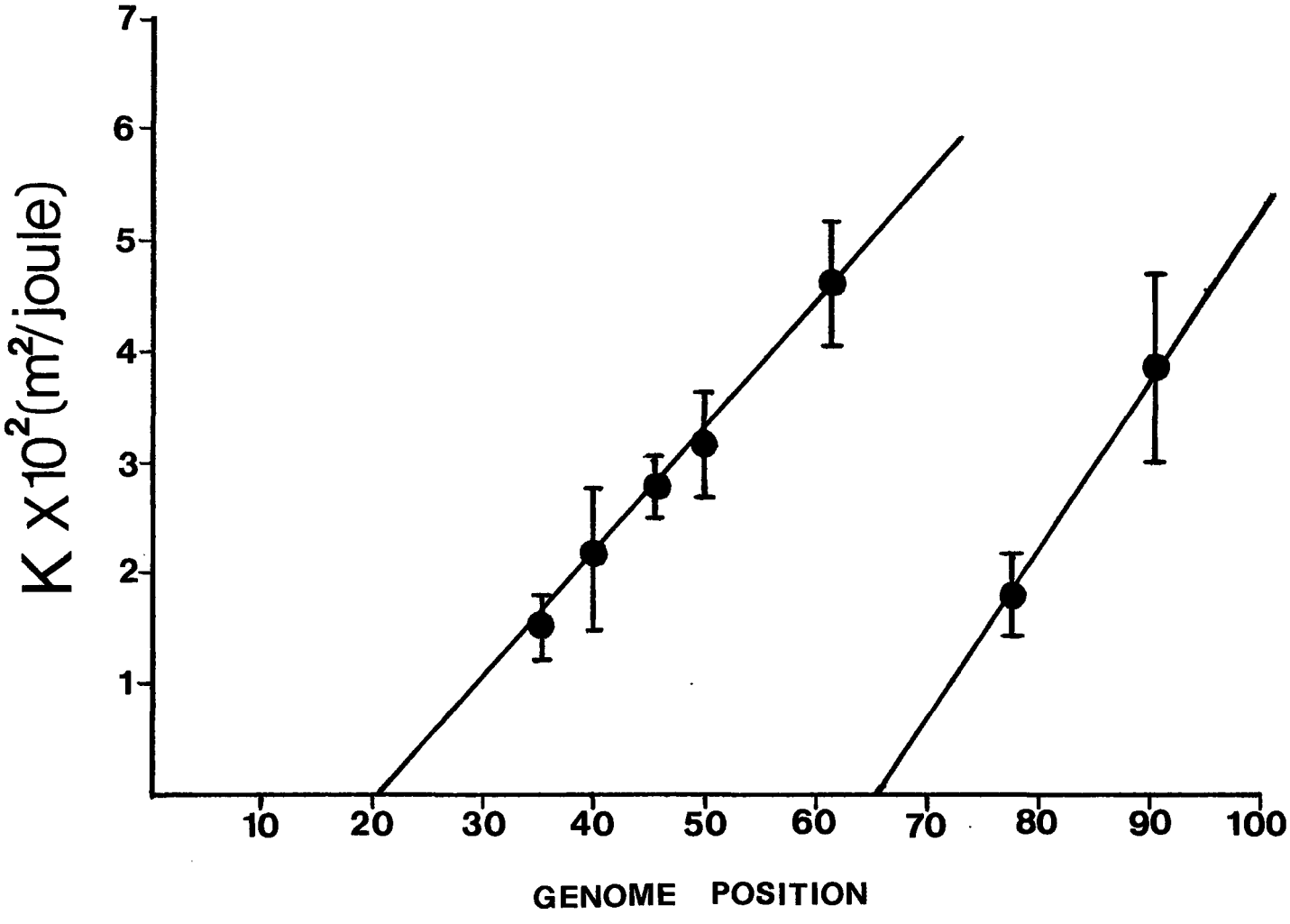
d. Not done.

polypeptides to restriction enzyme fragments (Lewis et al., 1975, 1977), the determination of the location of late genes by examining intersera-typic recombinants (Mautner et al., 1975; Grodzicker et al., 1977; Weber and Hassell, 1979), and by R-loop mapping of RNA sequences derived from these genes (Chow et al., 1977a). The plot of the K value for each gene within a single transcription unit versus its physical distance along the Ad 2 DNA yields a linear curve as expected. The intercept on the abscissa approximates the physical location on the Ad 2 genome for the initiation of each transcript. These results reflect the findings of the uv studies with viral specific nuclear RNA and indicate that late Ad 2 transcription occurs from two transcription units with the expression of genes 100K and IV from a transcription unit originating at approximately 60-65 map units on the Ad 2 genome.

This experiment has been repeated several times with slight variations in the time of uv irradiation as well as in the time of pulse labelling. In one experiment Ad 2 infected KB cells were uv irradiated at 9 hours post-infection and pulse labelled for 0.5 hours at 15 hours post-infection. The relative radiosensitivity of each protein was determined from the autoradiograms by microdensitometry and are shown in Table 9. In all experiments the radiosensitivity of genes 100K and IV was found to be less than that for gene II, indicating the existence of two transcription units on the r-strand for late Ad 2 gene expression.

Figure 32

UV inactivation cross section. UV sensitivities of the production of late Ad 2 proteins plotted against the proposed genome position of the gene coding for each polypeptide. The K values are the slopes of the inactivation curves for each gene product, and were determined by linear regression analysis of data from the inactivation curves of late Ad 2 protein synthesis from Figs. 30 and 31.


K X 10^2(m^2/joule)
7
6
5
4
3
2
1
10
20
30
40
50
60
70
80
90
100
GENOME POSITION

B. Ad 2 Precursor Polypeptides

1. The Ad 2 - ts1 mutation. In order to determine the relative uv sensitivity of the Ad 2 precursor polypeptides by uv transcription unit mapping it would be necessary to totally inhibit the specific proteolytic cleavage of these molecules. The primary gene products could then be analyzed in the same manner as the other Ad 2 late proteins described above.

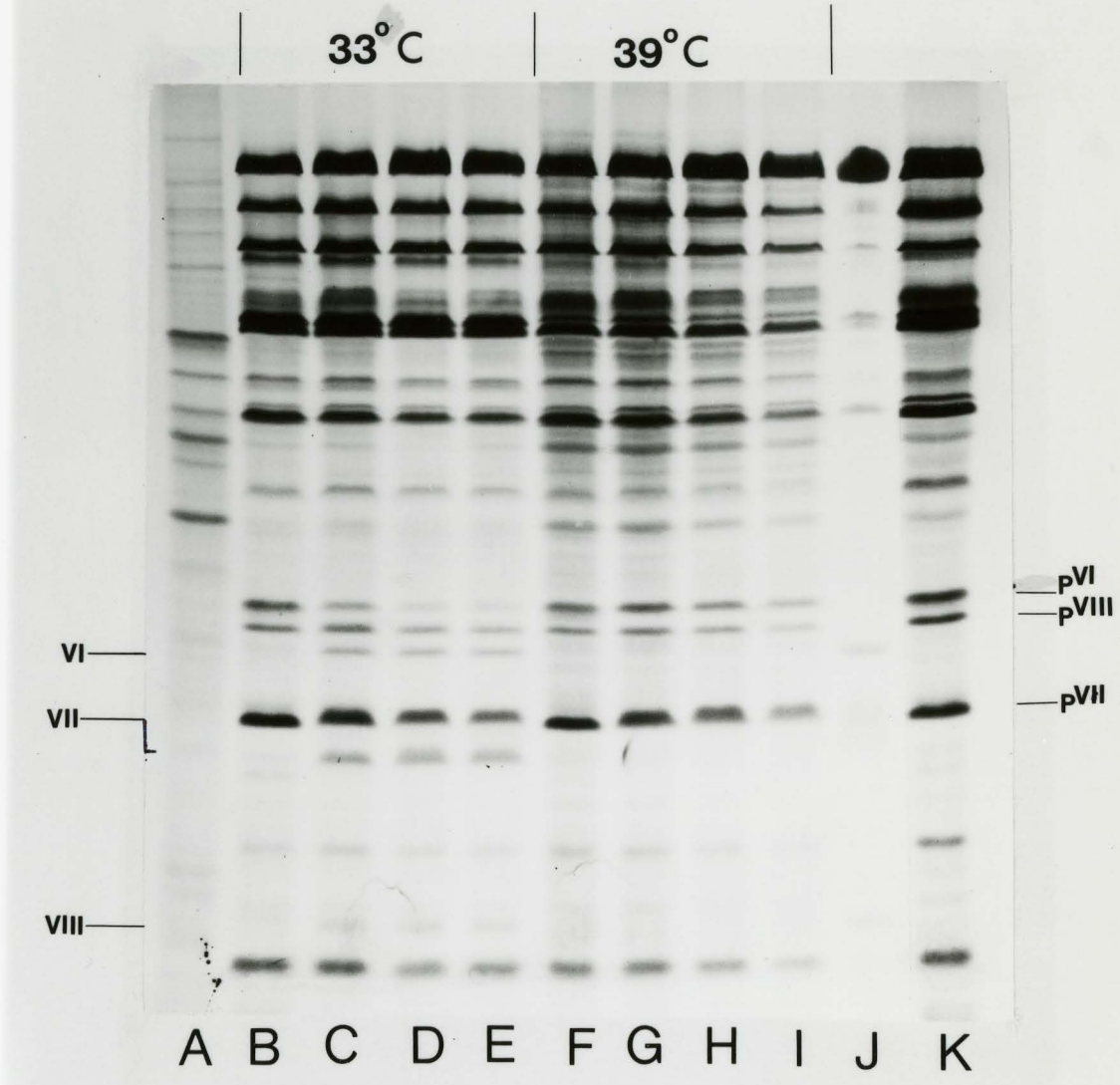
Fortunately, a characterized conditional lethal mutant was available to perform such a study. The temperature sensitive mutant ts1 of Ad 2 has been isolated and characterized by Weber (1976; Amin et al., 1977). This mutation, ts1, blocks the translational processing of the virus specified precursor polypeptides at the restrictive temperature (39°C). At the restrictive temperature, ts1 produces a normal yield of non-infectious physical particles, which contain a full complement of Ad 2 primary gene product structural proteins including pVI, pVII, and pVIII, but lack protein VI, VII, and VIII. Examination of ts1 infected cell extracts analyzed by SDS-polyacrylamide gel electrophoresis and autoradiography demonstrates that all of the expected viral induced polypeptides are synthesized during a 1 hour pulse at late times after infection, except for the cleavage products of the precursor polypeptides (Weber, 1976). It is therefore believed that the ts1 lesion affects only the function of processing of viral precursor polypeptides.

The Ad 2 mutant, ts1 (obtained from Dr. J. Weber, Sherbrooke, Quebec) was prepared at the permissive temperature (33°C) by serial passage in KB cells and purified as described in Materials and Methods.

A titre of 2.4 x 10^{10} PFU/ml was attained by titration on HeLa cells at 33°C. After serially passaging ts1, it was necessary to test that the ts1 lesion remained intact. Therefore, a pulse-chase experiment was performed at both the restrictive and permissive temperatures. Monolayers of KB cells were infected with ts1 at an MOI of 50 PFU/cell and incubated at either the restrictive temperature, 39°C or the permissive temperature, 33°C. Infected cultures incubated at 39°C were labelled at 12 hours post-infection for 1 hour with 40 µCi/ml of ^{35}S-methionine. Similarly, cultures incubated at 33°C were pulse labelled at 22 hours post-infection for 1.5 hours. After the pulse label, infected cultures at both temperatures were chased with 100X cold methionine for 0, 4, 16, and 24 hours and then harvested at the end of each chase. The labelled infected cell extracts were then analyzed by SDS-polyacrylamide gel electrophoresis and autoradiography. Figure 33 clearly shows that viral induced polypeptides, pVI, pVII, and pVIII are not processed during the pulse label or subsequent chases to proteins VI, VII and VIII, respectively, when cells were infected with ts1 and incubated at the restrictive temperature. Contrarily, a progressive shift in radioactivity occurs during the chases from proteins pVI, pVII, and pVIII to positions that comigrate with virion compoenents, VI, VII, and VIII, respectively, when cells were infected with ts1 and incubated at the permissive temperature. Therefore, the viral stock of ts1 is defective in the processing function at the restriction temperature.

Figure 33

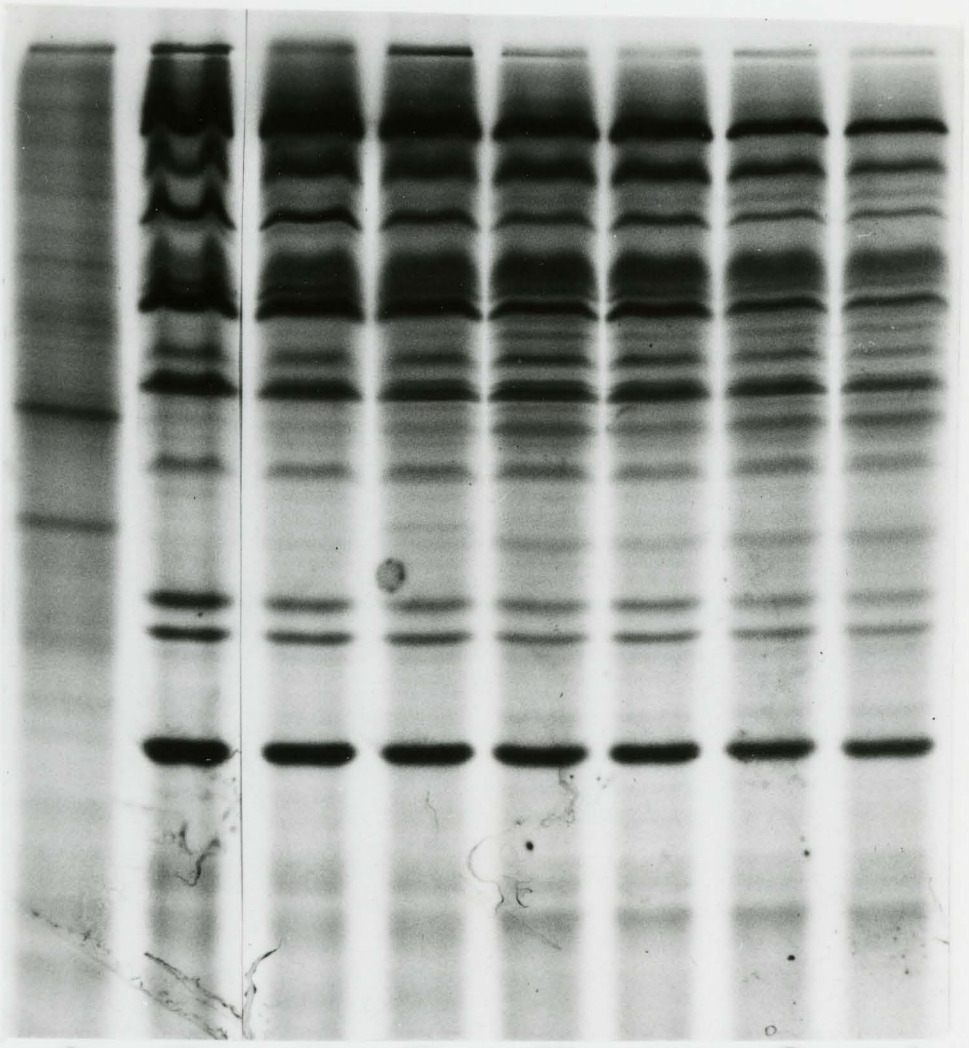
Pulse-chase of ts1 at 33° and 39°. SDS-polyacrylamide gel autoradiogram of a pulse-chase experiment with ts1 at the permissive and non-permissive temperatures. Infected cultures of KB cells incubated at 33°C and 39°C were pulse labelled for 1.5 hours at 22 hours post-infection or 1 hour at 12 hours post-infection, respectively. After the pulse label, infected cultures were chased with 100X cold methionine for varying periods and then harvested. (A) mock-infected KB cells; at 33°C: (B) pulse labelled; (C) 4 hour chase; (D) 16 hour chase; (E) 24 hour chase; at 39°C: (F) pulse labelled; (G) 4 hour chase; (H) 16 hour chase; (I) 24 hour chase; (J) purified Ad 2 virion components; (K) Ad 2 infected whole cell extracts labelled at 16 hours after infection for 0.5 hours at 37°C. Protein VIII is rather faint on the original autoradiogram and difficult to observe in the reproduction of the autoradiogram. After electrophoresis, the gel was stained with coomassie brillian blue, destained, and dried under vacuum before being exposed to x-ray film for 72 hours.


33°C
39°C
VI
VII
VIII
pVI
pVIII
pVII
A B C D E F G H I J K

2. UV inactivation of Ad 2 precursor polypeptides, pVI, pVII, and pVIII. In order to determine the relative uv inactivation for the synthesis of Ad 2 precursor polypeptides, pVI, pVII, and pVIII, ts1 infected KB cells were uv irradiated at 6.0 hours post-infection and labelled with ^{35}S-methionine at 12 hours post-infection. An equal volume of each sample extract, obtained from an equal number of infected cells, was analyzed by SDS-polyacrylamide gel electrophoresis (Fig. 34). The intensities of the protein bands of interest were determined by microdensitometry and the area under each peak was measured by planimetry. Inactivation curves for the synthesis of the individual ts1 proteins were determined by the difference in peak area between proteins from non-irradiated and uv irradiated ts1 infected cells and are shown in Fig. 35. The genes coding for proteins pVI and pVII have been located within the left hand 60% of the Ad 2 genome and the gene coding for gene pVIII in the right hand 30% of the genome before gene IV and after gene 100K (Lewis et al., 1975, 1977; Chow et al., 1977). The relatively small protein, pVI is translated from a 27S mRNA, which is significantly larger than required to code for pVI, while the much larger hexon (gene II) protein is coded by a 24S mRNA (Anderson et al., 1974). In cell free translation experiments of mRNA selected on DNA restriction fragments, Lewis et al. (1975, 1977) found pVI and hexon closely linked to the same region of the genome. Recently, Akusjarvi and Persson (1981) have elegantly established that the coding region for protein pVI is coded for very close to the 3' side of the much larger hexon gene on the viral genome. The 27S mRNA identified for pVI containsboth the

Figure 34

SDS-polyacrylamide gel autoradiogram of ts1 infected cell proteins. SDS-polyacrylamide gel autoradiograms of labelled extracts of ts1 infected cells after various doses of uv irradiation at 6 hours post-infection. The infected cells were labelled for 1 hour with ^{35}S-methionine at 12 hours post-infection. (A) mock-infected KB cells; (B) 0; (C) 7.5; (D) 10; (E) 12.5; (F) 15.0; (G) 17.5; (H) 20.0 joules/m^2. After electrophoresis, the gel was stained with coomassie brilliant blue, destained and dried under vacuum before being exposed to x-ray film for 72 hours.


A B C D E F G H

pVI and hexon sequences with separate initiator codons, although only the first smaller gene (pVI) is expressed from this mRNA species. The 24S mRNA species is thought to represent sequences coding only for the hexon protein.

The uv inactivation curves for the synthesis of proteins, pVI and pVII in Fig. 35A are grouped with the other inactivation curves for the synthesis of proteins known to be encoded in the left hand 60% of the viral genome. The inactivation curve for the synthesis of protein pVIII in Fig. 35B is grouped with the inactivation curves for the synthesis of proteins encoded towards the right hand end of the viral genome. The relative radiosensitivity of each protein determined by comparing the slopes of the uv inactivation curves (Fig. 35) are illustrated in Table 10. Unlike the uv inactivation experiments with Ad 2, an exponential inactivation curve for the precursor protein, pVI was observed with the Ad 2 mutant tsl. This is thought to result from the inhibition of processing of this protein during the labelling period. The results indicate that both of the relatively small precursor proteins, pVI and pVII have very similar inactivation profiles, suggesting they are located near each other on the transcription unit. The synthesis of hexon appears to be somewhat more sensitive to the effects of uv irradiation suggesting hexon is derived from sequences further along the transcription unit. This may reflect the difference in size between the pVI and hexon genes even though they are closely linked on the viral genome. The finding of the uv sensitivity for protein pVIII between 100K and IV is consistent with the gene order determined through other studies (Lewis et al., 1977; Chow et al., 1977). As in the previous experiments with Ad 2, synthesis of both 100K and IV proteins show less uv sensitivity than hexon (gene II)

Figure 35

UV inactivation of late ts1 (Ad 2) proteins. Relative rates of synthesis of late ts1 proteins from infected cells, as a function of UV dose. (A) Proteins known to be coded for within the left hand 60% of the Ad 2 genome. (B) Proteins known to be coded for within the right hand 40% of the Ad 2 genome.

The relative radiosensitivity was determined by microdensitometry of autoradiograms (Fig. 34) over the linear portion of the characteristic curve of the x-ray film, where density α log (relative exposure). Thus for each protein band, A, the area under the peak of the scan is given by $A = K_1 \log_e$ (relative exposure) or relative exposure $= e^{A/K_1}$ where A = area under the peak of densitometer scan, K_1 is a constant. Assuming the amount of radioactive protein, P in each band is proportional to the relative exposure given to the film R. Then $R \alpha P$, $P = K_2 R$ and $P = K_2 e^{A/K_1}$. Thus $\frac{P_o}{P} = \frac{e^{A_o/K_1}}{e^{A/K_1}} = e^{\frac{(A_o - A)}{K_1}}$, where P_o = relative rate of protein synthesis at 0 UV dose and P = relative rate of protein synthesis at UV dose D. Thus, the K value for each protein is given, $K = \ln\frac{P_o}{P} = \frac{A_o - A}{K_1}$ or K value $\alpha (A_o - A)$.

Figure 35A

Closed circles, gene III; closed triangles, gene pVIII; closed boxes, gene pVI; open circles, gene II.

Figure 35B

Closed circles, gene 100K; closed boxes, gene pVIII; closed triangles, gene pVI.

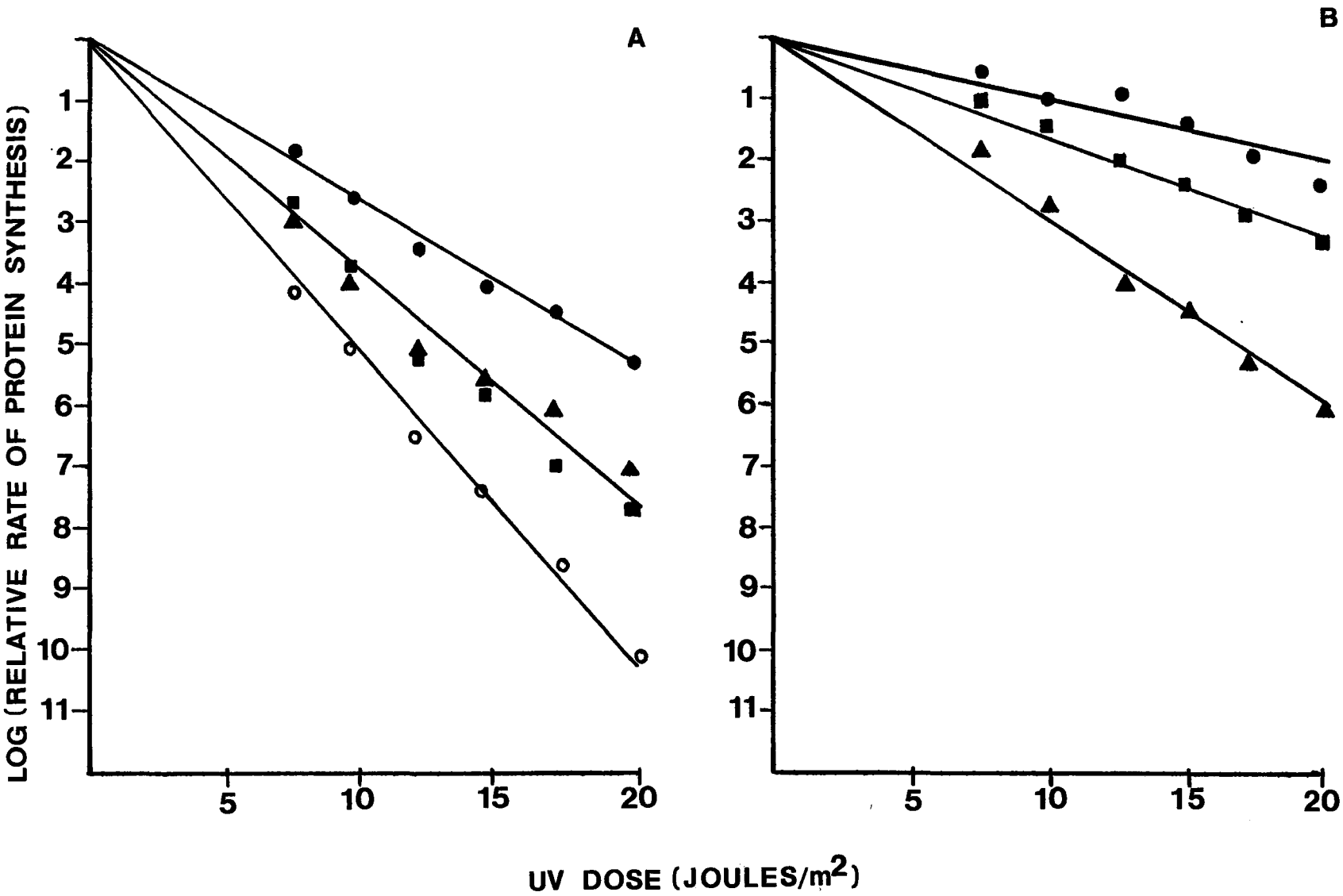

A
B
LOG (RELATIVE RATE OF PROTEIN SYNTHESIS)
1
2
3
4
5
6
7
8
9
10
11
5
10
15
20
UV DOSE (JOULES/m2)

Table 10

Ultraviolet Sensitivity of ts1 (Ad 2) Gene Expression

Gene 4	Relative Radiosensitivity Expt. 1	Expt. 2
100K	0.26±.04	ND[a]
pVIII	0.36±.02	0.35±.04
III	0.53±.04	ND
IV	0.64±.05	ND
pVII	0.74±.07	0.70±.07
pVI	0.81±.06	0.77±.07
II	1.00±.08	1.00±.06

The slopes of uv inactivation curves (expt. 1, Fig. 35) were determined by linear regression analysis and compared to the slope of the most uv sensitive protein (II) to obtain the relative radiosensitivity for each protein.

[a]ND - not done

suggesting they are not expressed from a single continuous transcription unit originating towards the left hand end of the viral genome.

Discussion

The many aspects of the molecular biology of eukaryotic gene expression have become a major focus of scientific research in recent years. A central issue in this area is the regulation and nature of primary transcription in the nucleus of eukaryotic cells which produce the cytoplasmic mRNAs that through translation ultimately allow the expression of polypeptides encoded in the DNA.

The study presented here has examined late adenovirus type 2 gene expression in KB cells by uv transcription unit mapping experiments. Results of such experiments suggest that late during Ad 2 infection relatively long polycistronic RNA molecules, originating from only two transcription units, are responsible for the expression of cytoplasmic mRNAs coding for many viral polypeptides.

I. The origin of mRNA

Unlike prokaryotic gene expression, where individual blocks of related genes (operons) are transcribed into polycistronic mRNA sequences containing multiple translational initiation and termination codons (Kiho and Rich, 1965), eukaryotes produce monocistronic mRNAs (Petersen and McLaughlin, 1973) coding for individual proteins involved in common pathways from diverse regions of the genome (Ruddle and Creagan, 1975). The regulatory mechanisms involved in the expression of eukaryotic mRNAs has been the subject of intensive study in recent years.

High molecular weight heterogeneous nuclear RNA (hnRNA) up to 45,000 nucleotides in length (Greenberg and Perry, 1971) is the most abundant RNA synthesized during short pulse labelling with ^{3}H-uridine in mammalian cells (Penman, 1966). Only about 20% of the label appearing in the unstable hnRNA appears in the cytoplasm (Philipon et al., 1974). The finding of some hnRNA molecules with capped 5' termini and 3' polyadenylated tails has led to the suggestion that hnRNA is the direct precursor of mRNA (Darnell et al., 1970). However, the size distribution of the short pulse labelled RNA overlaps with that of cytoplasmic mRNA. This makes it difficult to determine whether the rapid synthesis and turnover of hnRNA and the production of cytoplasmic mRNA truely represent a precursor-product relationship or are entirely separate events.

The synthesis of nuclear RNA and its relationship to mRNA production has been investigated using adenovirus provides a model system for the study of eukaryotic gene expression (Philipson, 1977). The adenovirus DNA is transcribed in the nuclei of infected cells by cellular enzymes (Wallace and Kates, 1972; Price and Penman, 1972) into RNA which is larger than polysomal viral mRNA (McGuire et al., 1976; Craig and Raskas, 1976). The viral specific RNA accumulates in the nucleus of the infected cells much faster than RNA sequences appear in the cytoplasm and only about 20% of the viral RNA labelled during a short pulse ever appears in the cytoplasm (Philipson et al., 1974). Some of the nuclear viral specific RNA molecules are capped at the 5' termini (McGuire et al., 1976) and contain 3' polyadenylated tails (Philipson et al., 1974).

Like other eukaryotic mRNAs, adenovirus polysomal mRNA species are monocistronic (Persson et al., 1977) and contain capped 5' termini and 3' polyadenylated tails (Philipson et al., 1971).

The nature of the primary nuclear transcripts has been investigated late during Ad 2 infection by nascent chain analysis both in vivo (Bachenheimer and Darnell, 1975) and in isolated nuclei (Weber et al., 1977; Vennstrom and Philipson, 1977). Ad 2 infected cells or isolated nuclei labelled with a very short pulse (1-3 min) of ^{3}H-uridine results in a population of RNA molecules from a given transcription unit with a common 5' terminus and label accumulating at the 3' ends of the elongating chains. These molecules were separated on sucrose gradients and subsequently hybridized to viral DNA restriction fragments along the length of the genome. This will identify the approximate origin and limit of transcription as the shortest nascent chains will hybridize to a DNA fragment containing the initiation site, whereas initiation distal fragments will detect longer RNA chains due to the accumulation of label only in the 3' termini of the growing chain. These studies determined that the short nascent RNA molecule originates in the left 30% of the Ad 2 genome (Vennstrom and Philipson, 1977) within the region of 11-18 map units (Weber et al., 1977). These studies also indicate that the nascent RNA chains increase in size as the right hand end of the genome is approached. This is believed to demonstrate that a single primary transcript within the high molecular weight hnRNA spans almost the entire length of the genome from the origin of RNA synthesis to the right hand end of the genome. These long transcripts have been

proposed as the precursors to viral mRNA (Bachenheimer and Darnell, 1975; Weber et al., 1977). However, the size distribution profiles overlap with smaller, polysomal sized RNA molecules, which does not clearly allow a precursor-product relationship to be established between the long nuclear transcript and mRNA production.

The transcriptional organization for the expression of late Ad 2 genes examined by uv transcription unit mapping is the focus of this thesis. The technique was pioneered by Sauerbier and his colleagues (Sauerbier, 1975, 1976; Sauerbier et al., 1970; Brautigam and Sauerbier, 1973, 1974; Hackett and Suerbier, 1974; Hercules and Sauerbier, 1973, 1974) for the investigation of the transcriptional organization of a number of prokaryotic systems and has also been found to be a valuable tool in examining eukaryotic transcription (reviewed by Sauerbier and Hercules, 1978). Following uv irradiation of DNA capable of undergoing transcription, photolesions (primarily thymidine dimers) causing the premature termination of transcription are induced in the DNA. The transcription of DNA sequences are differentially sensitive to uv irradiation dependent on the position of the sequence relative to the site of initiation of transcription. Consequently, for each transcription unit, uv irradiation would result in a preferential inhibition of transcription for promotor-distal portions of the transcript compared to promotor-proximal positions of the transcript. Therefore, the probability that any given DNA sequence will be expressed becomes inversely proportional to the distance along the DNA between the promotor for trans-

cription and the DNA sequence.

II. UV transcription unit mapping of late Ad 2 gene expression assayed by nucleic acid hybridization.

The relative target size of late Ad 2 transcription unit(s) was examined by comparing the uv sensitivities of transcription at different regions of the viral genome. The effects of uv irradiation on Ad 2 transcription was investigated at the level of viral specific nuclear RNA synthesis and also as reflected in the production of late cytoplasmic polyadenylated RNA.

The uv sensitivity of Ad 2 specific nuclear RNA synthesis was assayed by the hybridization of nuclear RNA labelled with ^{3}H-uridine at 15.5 hours post-infection for 15 minutes following uv irradiation to Ad 2 DNA restriction fragments representing diverse regions of the viral genome. Consistent with the idea of long nuclear transcripts, an increase in uv sensitivity of transcription was observed beginning within the left hand 30% of the genome and continuing to approximately 60 map units (Fig. 16) suggesting a continually increasing target size for RNA synthesis within a single transcription unit to this position on the viral genome. However, to the right of this region, the increasing uv sensitivity of viral specific nuclear transcription expected for a full genome length RNA chain was not observed. Instead, all the regions examined in the right hand 40% of the genome showed a decrease in uv sensitivity relative to the Bam HI C fragment (42.0-59.5 map units) with transcription of the Eco RI F fragment (70.7-75.9 map units) being most resistant to the effects of uv

irradiation (Fig. 16). This suggests that another transcriptional initiation has occurred between 59.5 and 75.9 map units on the Ad 2 genome. Transcription of the Eco RI B fragment (58.2 - 70.7 map units) displays a uv sensitivity intermediate between Bam HI C and Eco RI F in this type of experiment (Table 4) and may represent the transcription of a portion of one transcription unit initiated at a significant distance away near the left hand end of the genome and as well as transcription at the beginning of another transcription unit. Therefore, the uv sensitivity of transcription observed in the region of the genome represented by the Eco RI B fragment may be the result of a uv sensitive portion of one transcription unit and a relatively uv resistant portion of another transcription unit. If this was indeed the case for the results observed with the Eco RI B fragment then in order for the transcription of the Eco RI F fragment to be even less uv sensitive, it would mean that many of the transcripts originating near the left hand end of the genome would have to terminate within the Eco RI B fragment region. Alternatively, the majority of transcripts assayed at the Eco RI F fragment could originate from a shorter and therefore, less uv sensitive transcription unit, presumably by a high frequency of initiation at the more uv resistant transcription unit. To the right of the Eco RI F fragment, nuclear transcription again shows an increase in uv sensitivity as expected from the continuation of transcription towards the end of the genome. The extreme right hand end of the genome shows a slight decrease in uv sensitivity (Table 4), which will be discussed below.

Goldberg and coworkers (1977) performed very similar uv mapp-

ing experiments with late nuclear Ad 2 RNA and also observed an exponential increase in the uv sensitivity of transcription beginning near the left hand end of the viral genome and continuing toward the right of the genome. These investigators also observed a decrease in the uv sensitivity of viral transcription at the same site (70.7-75.9 map units) as the data discussed above. However, this alteration in the expected continuous exponential decay of transcription was of a lesser magnitude than that observed in the experiments described above (Table 4). These authors conclude that the majority of late Ad 2 transcription that occurs in the nucleus of infected cells begins near the left end of the genome and continues within a single transcription unit to the right hand end of the genome producing a very long primary transcript. Goldberg et al. (1977) also suggested the existence of a weaker second promotor for the expression of another transcription unit for the synthesis of late nuclear RNA for the right hand 30% of the Ad 2 genome. It is interesting to note that Goldberg et al. (1977) also observed a slight decrease in the uv sensitivity of transcription of the Eco RI C fragment (89.7-100 map units) similar to that observed in Figure 16. The reasons for the differences in the data of Goldberg et al. (1977) relative to Figure 16 and Table 4 described above is unclear, although a similar pattern of uv effects on late transcription could be observed in both investigations for this type of experiment.

A more detailed analysis of the response of Ad 2 nuclear transcription to uv irradiation was examined at four separate regions of the genome using recombinant DNA plasmids with viral DNA restrict-

ion fragment inserts; HindIII B (17.0-31.5 map units), Bam HI C (42.0-59.5 map units), HindIII H (72.8-79.9 map units) and HindIII F (89.5-97.1 map units). An exponential decrease of transcriptional survival was observed with increasing uv dose for both the HindIII B and Bam HI C region of the genome (Fig. 17). Transcription of Ad 2 DNA sequences represented within the Bam HI C fragment was more sensitive to the effects of uv irradiation than transcription represented within the HindIII B fragment region as determined by the difference in the slopes of the transcriptional inactivation curves for both of these regions (Table 5). This indicates that transcription of DNA sequences initiated within or before the HindIII B fragment region continues into the sequences represented by the Bam HI C fragment producing a relatively long RNA chain spanning a large portion of the genome (17.0-59.5 map units). Further to the right of this region along the viral genome within fragments HindIII H and HindIII F, the pattern of uv inactivation of transcription shows two components (Fig. 17) suggesting that more than one population of late viral specific nuclear RNA was transcribed from these regions of the viral genome. Late Ad 2 nuclear transcription within these regions consists of both a highly uv sensitive component and also a second relatively less uv sensitive component (Table 5). These findings indicate that the synthesis of a nuclear transcript originating within or before the HindIII B fragment region continues towards the right hand end of the genome producing very long RNA molecules. In addition, a second transcription unit produces a shorter nuclear RNA species expressing DNA sequences located within the right hand 30% of the viral genome. Approximately, one third to a half of the DNA transcribed within this

region is derived from this additional transcription unit (Table 6). The finding of a very long transcript as well as a shorter one within this region of the genome suggests that a mechanism exists for the read through of the second initiation site of RNA synthesis within or before the HindIII H fragment region.

An apparently inconsistent result was observed comparing the data of experiments represented in Fig. 16 and Fig. 17. In the single dose experiment performed at 30 joules/m^2 examining the uv sensitivity of transcription at different regions of the viral genome shown in Fig. 16 the maximal uv sensitivity of transcription was observed within the BamHI C fragment (42.0-59.5 map units). Transcription further along the genome within the EcoRI F fragment (70.7-75.9 map units) was observed to be significantly less uv sensitive. However, data extrapolated from Fig. 17 at a dose of 30 joules/m^2 does not show the same maximal uv sensitivity of transcription within the BamHI C fragment. Transcription within the HindIII H fragment (72.8-79.8 map units), which overlaps sequences within the EcoRI F fragment, was observed to have a greater uv sensitivity at a dose of 30 joules/m^2 in Fig. 17.

The explanation for this difference is not clear. However, the difference in the DNA sequences used for each experiment may be significant. The EcoRI F fragment purified from agarose gels and the HindIII H fragment obtained from a recombinant plasmid share common sequences but each fragment also encodes unique sequences. If the relatively small EcoRI F fragment contains a site for the initiation of transcription within the unique sequence as well as a lack of potential transcription terminating lesions, it may show significant resistance to the effect of uv irradiation compared to the HindIII H fragment.

In addition there was a significant difference in the time of uv irradiation during the late phase of infection between these experiments. The single dose experiment (Fig. 16) was performed with the uv dose given at 15 hours post infection as compared to 23.5 hours post infection in the multi-dose experiment (Fig. 17). When the latter experiment was repeated with uv irradiation at 15 hours post infection (Tables 5 and 6, experiment #3), transcription at the HindIII H fragment appeared to be less uv sensitive than at later times of uv irradiation. This suggests that the proportion of RNA synthesis may be variable at different times during the late phase of Ad 2 infection.

Weinmann and Aiello (1978) have investigated the locations for the initiation of RNA synthesis on the Ad 2 genome during the late phase of Ad 2 infection. This study examined the initiation and short pulse labelling of primary transcripts with ^{3}H-uridine *in vivo* followed by RNA chain elongation in isolated nuclei with mercurated nucleotides. RNA molecules synthesized in this manner will preferentially accumulate label at their 5' ends and can be selected by affinity chromatography due to the mercurated nucleotides at the 3' ends. Hybridization of the newly synthesized RNA molecules to viral DNA restriction fragments or separated strands of viral DNA restriction fragments have determined a r-strand specific initiation site for RNA synthesis between 7.5-18.1 map units, which is in good agreement with the location of the major late promotor at 16.4 map units (Evans *et al.*, 1977; Ziff and Evans, 1978). As well a second r-strand specific initiation site at 60-70 map units was also identified consistent with the results from uv mapping experiments discussed above. Weinmann and Aiello (1978) have also suggested the possibility that both long transcripts, including a readthrough product of this second promotor and independent initiations in the 60-70 map unit region of the genome occur simultaneously.

A plot of the uv sensitivities of viral specific nuclear transcription from Table 5 against the genome position of each of the regions examined provides a view of the relative target size for transcription (Fig. 18) at each region of the genome. As described above transcripts derived from both a very long transcription unit spanning the majority of the genome and a second relatively short transcription unit expressing DNA sequences from the right hand 30-40% of the genome was observed. Extrapolation of this data (Fig. 18) suggests that the longer transcript originates at approximately 17 map units on the viral genome, which is consistent with the initiation site for late RNA synthesis described by other studies, (Evans et al., 1972; Ziff and Evans, 1978; Weinmann and Aiello, 1978). The shorter transcription unit was found to originate at approximately 63 map units on the viral genome, which is in good agreement with the r-strand initiation site observed at 60-70 map units by Weinmann and Aiello (1978).

Although several studies have found that late Ad 2 transcripts are derived almost exclusively from the r-strand (Petterson and Philipson, 1974; Zimmer et al., 1978; Winmann and Aiello, 1978) it was necessary to examine the strand specificity of late Ad 2 RNA used in the uv transcription unit mapping experiments. If significant levels of l-strand transcripts were also being assayed by hybridization to the DNA restriction fragments, the uv sensitivities determined for the various regions of the genome would represent a combination of transcription units of different sizes and could thus account for the two component nature of the inactivation curves observed within the HindIII H and HindIII F fragment regions. This was examined by hybridization of late nuclear ^{3}H-RNA to separated strands of Ad 2 DNA restriction

fragments on Southern blots. Figure 20-24 shows that hybridization was detected only with one DNA strand (fast strand), presumably the r-strand (Flint et al., 1975, 1976) and no hybridization was detected with the other DNA strand of HindIII H and HindIII F DNA fragments using late nuclear ^{3}H-RNA from non-irradiated or uv irradiated Ad 2 infected cells. This indicates that certainly the vast majority of nuclear RNA assayed in the experiments described above is derived only from the r-strand of Ad 2 DNA within the regions examined and, therefore, provides a reliable assay for the primary transcripts synthesized during a 15 minuts or shorter pulse label at late times during Ad 2 infection. Late cytoplasmic ^{3}H-poly A RNA from non-irradiated infected cells also hybridized only to the fast strands of the separated strands of HindIII H and HindIII F DNA fragments. Hybridization of late cytoplasmic ^{3}H-poly A RNA from uv irradiated infected cells was also detected only for the fast strand of the separated strands of the HindIII H DNA fragment. However, both the fast and the slow strands of the HindIII F DNA fragment hybridized to this same ^{3}H-poly A RNA preparation (Figs. 26 and 27). This suggests that in uv irradiated cells at least a small amount of the HindIII F region l-strand specific RNA was synthesized in the nucleus during the 1 hour pulse label, although this was not detected using newly synthesized nuclear RNA on Southern blots (Figs. 20-24). This probably accounts for the slight consistent decrease in the uv sensitivity of nuclear transcription observed at the extreme right hand end of the genome represented by the Eco RI C fragment (Table 4). In addition, the detection of l-strand specific RNA sequences only from

the HindIII F fragment region using ^{3}H-poly A RNA from uv irradiated infected cells suggests that only a very small amount of RNA was synthesized as the primary transcript, but selectively transported to the cytoplasm as poly A RNA.

The uv sensitivity of cytoplasmic poly A RNA synthesis was also examined at various sites along the genome and much the same pattern of uv inactivation was observed as that found for nuclear virus specific RNA (Fig. 25). Again, an increasing gradient of uv sensitivity beginning near the left hand end of the genome and continuing toward the right of the genome with the most uv sensitive region between 42.0-59.5 map units (Bam HI C fragment) was observed. This is believed to indicate that the poly A RNAs are derived from a primary transcript of a single transcription unit spanning most of the left hand 60% of the genome. To the right of the Bam HI C fragment region the uv sensitivity of poly A RNA production decreases as was also observed for nuclear RNA (Fig. 16), which is inconsistent with the synthesis of a single late primary transcript of increasing target size extending to the right hand end of the genome. This supports the contention presented earlier that a second transcription unit is also operative for the expression of viral DNA sequences within the right hand 30-40% of the genome. The decreased uv sensitivity of poly A RNA observed at the Eco RI C fragment (89.7-100 map units, Fig. 25) is believed to be an amplification of a similar phenomenon observed with nuclear RNA (Fig. 16) and is thought to be the result of early region IV expression from 1-strand specific transcription as described above (Results section IV B: 2).

Goldberg and coworkers (1978) have compared the uv sensitivity of the production of cytoplasmic RNA to that of nuclear RNA at various sites along the Ad 2 genome to investigate the nature of the primary transcript giving rise to poly A RNA. If cytoplasmic poly A RNAs were derived from individual monocistronic transcription units, and not from the long transcripts described above, then it would be expected that the production of poly A RNA would be much less uv sensitive than production of long nuclear transcripts. These investigators determined that the uv sensitivity of poly A RNA production increased continuously from near the left hand end of the genome to the right hand end of the genome as did nuclear RNA and in fact was even more uv sensitive for the left hand 80% of the genome. Goldberg and coworkers (1978) concluded that poly A RNA production is derived from a long nuclear transcript synthesized from a single transcription unit initiating near the left hand end of the genome and continuing to the right hand end of the genome. The reasons for the differences in the data of Goldberg and coworkers (1978) and this study (Fig. 25) using similar systems is not clear and may represent some intrinsic difference in the experiments performed, such as the differences in uv doses employed. A comparison of the uv sensitivity of poly A RNA production and nuclear transcription was also examined through uv dose response experiments in the study by Goldberg and coworkers (1978). These experiments show that the uv sensitivity of poly A RNA production was greater than or at least equal to the sensitivity of nuclear transcription at all regions examined. Indeed the production of poly A RNA appeared more uv sensitive than nuclear transcription, although the slopes of

the poly A RNA inactivation curves were approximately the same at each region of the genome examined and only the uv sensitivity of nuclear transcription changed with genome position, which appears to be inconsistent with other experiments of the same study (Goldberg et al., 1978) described above. The uv sensitivity of poly A RNA production assayed on viral DNA restriction fragments would be expected to be as or slightly less uv sensitive if poly A RNA were derived from the long nuclear transcript dependent upon the extent of the poly A RNA coded for within the region represented by the restriction fragment. Therefore, the significance of the finding of poly A RNA production being more uv sensitive than nuclear transcription at most regions of the genome examined (Goldberg et al., 1978) is unclear. The present study has found that the uv sensitivity of poly A RNA production was somewhat less than that observed for nuclear transcription at all regions of the genome examined (Tables 4 and 8). A closer examination of the data from the uv dose response experiments of Goldberg and coworkers (1978) reveal that the uv inactivation curves for the right hand portion of the genome (70.7-89.7 maps) also consist of two components similar to those described in Fig. 17. Looked at in this light, these inactivation curves are consistent with the hypothesis that nuclear RNA synthesis occurs from more than one transcription unit for this region of the genome at late times during Ad 2 infection.

If a primary transcript was initiated at 16.4 map units on the Ad 2 genome and was transcribed rightwards from a single transcription unit, which terminates near the right hand end of the genome as suggested in several studies (Bachenheimer and Darnell, 1975; Weber et al.,

1977; Goldberg et al., 1978; Nevins and Darnell, 1978; Fraser et al., 1979), this would predict that an equimolar distribution of newly synthesized RNA would be found across the entire length of the genome from 16.4 map units. This prediction was tested by the examination of the incorporation of ^{3}H-uridine into nuclear RNA during a short pulse label of late Ad 2 infected cells at various regions across the genome. The hybridization of pulse labelled late nuclear RNA to Ad 2 DNA restriction fragments is shown in Fig. 19. A definite asymmetry was observed in the cpm detected per unit length of each restriction fragment between the left hand 60% and the remaining right hand 40% of the genome. In a number of experiments performed under various conditions, it was consistently observed that the viral DNA restriction fragments from the right hand 40% of the genome hybridized between 3.5-6 fold less labelled nuclear RNA. This is also consistent with observations from saturation hybridizations (Fig. 13), where much more Bam HI D fragment (29.0-42.0 map units) DNA was required than Eco RI E fragment (84.0-89.7 map units) DNA to completely hybridize an equal amount of nuclear ^{3}H-RNA. Figure 19 suggests that many of the transcripts initiated near the left hand end of the genome terminate before reaching the right hand end of the genome, probably within the Eco RI B fragment region (58.2-70.7 map units).

For an asymmetry of approximately 4 fold about 80% of the total late nuclear transcription would be derived from within the left hand 60% of the genome and about 20% of total late nuclear transcription from the remaining 40% of the genome. Table 6 suggests that between one third to a half of the transcription between 72.8-97.1

map units originate from a second, less uv sensitive transcription unit. Therefore, only about 10-13% of the transcripts originating near the left hand end of the genome would continue through to the right hand end and about 66-70% terminate probably within the Eco RI B fragment region of the genome.

Zimmer and coworkers (1978) examined the distribution of RNA labelled with a short pulse of ^{3}H-UTP in isolated nuclei. If there were only one terminator and one promotor for the major rightward gene block, the amount of RNA labeled during in vitro elongation and termination should increase as the right end of the genome is approached. From the results of the study, these investigators also suggested that more than one promotor-terminator regulatory unit functions for the expression of late Ad 2 r-strand specific transcription.

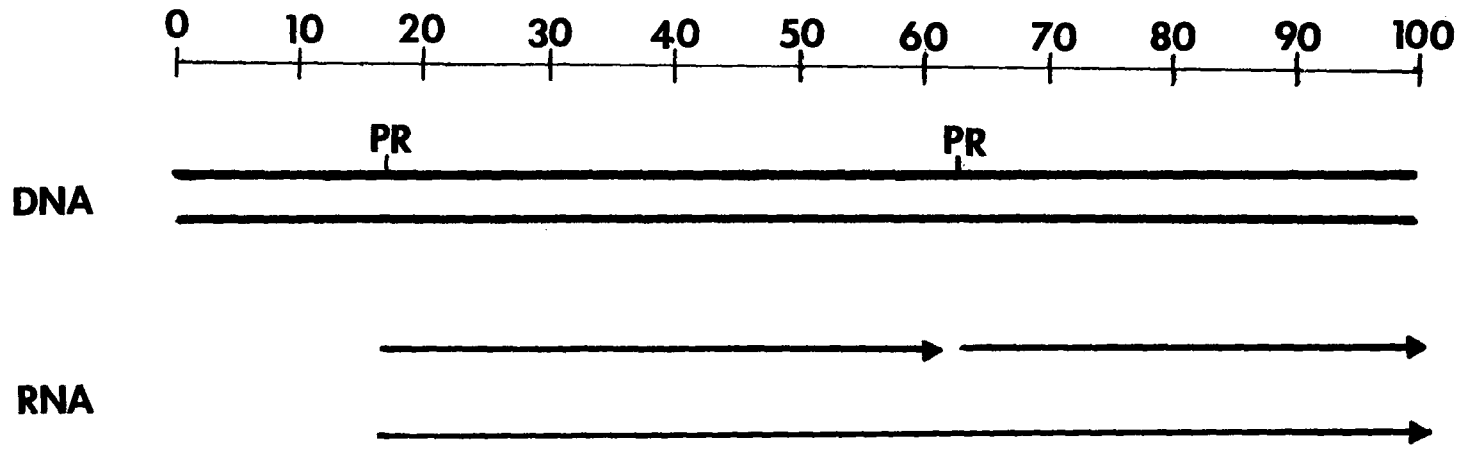
III. Model of late Ad 2 r-strand transcription

Based on the results of the experiments discussed above a possible model for late Ad 2 transcription is presented in Fig. 36. The model consists of two separate sites for the initiation of RNA synthesis known as promotors (PR) on the r-strand of Ad 2 DNA at approximately 17 and 63 map units. The majority of transcripts originate from the promotor at approximately 17 map units and terminate between 58.2-70.7 map units, possibly before the promotor at approximately 63 map units. Another transcription unit originates at approximately 17 map units and continues towards the right hand end of the genome. In addition, a third transcript originates at approximately 63 map units and also continues towards the right hand end of the genome.

The currently accepted model found in the literature for the transcription of Ad 2 late genes originates primarily from Darnell's laboratory and the work of his colleagues described throughout this thesis. The model described by Darnell (1979) is at variance with the model described above and the major features of his model are summarized here. The proposed model suggests that transcription of late Ad 2 genes initiate at a promotor located at approximately 17 map units on the viral genome and continues towards the extreme right hand end of the genome terminating at a specific site. This results in a single very long nuclear transcript representing all of the late Ad 2 genes. The primary nuclear transcript then undergoes a complex set of RNA processing step, which includes splicing, 5' capping and 3' polyadenylation. This process produces five families of cytoplasmic mRNA that can be translated into late Ad 2 proteins (Nevins and Darnell, 1978). The experiments supporting Darnell's model have been presented earlier in this work including a discussion of the differences and similarities in the data of this study with those of Darnell and coworkers. The differences in these two models of late transcription appears to result primarily from a difference in interpretation of the data from the uv transcription unit mapping experiments reported here and elswhere (Darnell, 1979).

Figure 36

Model of late Ad 2 r-strand transcription. The three possible transcription units for the expression of late Ad 2 RNA (see text for details).



AD 2 LATE TRANSCRIPTION

It is interesting to note at this point that the major late promotor at 16.4 map units (Evan et al., 1977; Ziff and Evans, 1978) has also been found to be expressed at early times after infection (Kitchingham and Westphal, 1980), but at a rate much less than that found at late times (Nevins and Wilson, 1981). During early infection, transcription of RNA from this promotor was found to terminate between 60 and 70 map units on the Ad 2 genome and not continue towards the right hand end of the genome (Nevins and Wilson, 1981). This indicates that some mechanism must exist to prevent readthrough and allow termination of this transcription unit, within this region of the genome and may also be operative during late infection.

An A-T rich nucleotide sequence has been identified preceding the cap site for a number of eukaryotic transcripts including most but not all of the transcripts for adenovirus gene expression (Baker and Ziff, 1980, 1981). These A-T rich regions, which vary in their precise sequence have been associated with eukaryotic promotor sites. The initiation site for RNA synthesis of some eukaryotic transcripts appear to be lacking in such a sequence (Haegman and Fiers, 1978), while the removal of this sequence when present for some initiation sites does not prevent expression of the transcript (Benoist and Chambon, 1981). Therefore, the functional significance of this particular sequence and the essential requirements that specify promotor regions are not yet clear. However, the region of the Ad 5 genome, between 61.7 and 71.4 map units has been sequenced (Kruijer et al., 1981) and Ad 2 is known to share a very close sequence homology (Flint, 1980). An examination of this sequence reveals

an A-T rich sequence, which occurs at approximately 63.9 map units with no other such sequences close to this site and followed by a long open reading frame from about 64.8-73.0 map units containing sequences coding for 100K protein. The finding of such a sequence does not necessarily identify it as a promotor site and a possible role for this sequence has not been implicated in the initiation of RNA synthesis within this region. However, its location is consistent with an initiation site for the transcription unit described in Fig. 36.

The existence of gene splicing, where RNA sequences from two or more non-contiguous regions of a genome are joined together to form a monocistronic mRNA has now been identified in a large number of eukaryotic systems (reviewed by Breathnach and Chambon, 1981). Splicing of adenovirus RNAs has provided much of the earliest work in this area and spliced mRNAs have been identified both early and late after infection (Berget et al., 1977; Klessig, 1977; Chow et al., 1977a,b, 1979, 1980; Chow and Broker, 1978). The 5' terminus of late mRNAs containsan untranslated leader sequence of about 200 nucleotides joined to the body of the mRNA some distance away. Portions of the leader sequence are transcribed from at least three different sites at 16.4, 19.6, and 25.6 map units, then spliced together to form the leader sequence, which is then spliced to the coding regions of the mRNAs. This leader sequence has been found to be present on most late Ad 2 RNAs including sequences coding for 100K and fibre coded in the right hand 40% of the Ad 2 genome (Berget et al., 1977, 1978; Chow et al., 1977b; Klessig, 1977; Broker et al., 1978). The fibre mRNA can contain additional splices from 77, 79, or 85 map units (Broker et al., 1978; Chow and

Broker, 1978; Dunn et al., 1978; Zain et al., 1979).

The functional significance of splicing, its precise mechanism and a demonstration of the universiality of such a mechanism await further study, but are clearly essential to an understanding of eukaryotic gene expression.

The mechanism by which splicing occurs may be envisaged in several ways. Firstly, several individual primary transcripts could be cleaved and fragments ligated (intermolecular ligation). This seems very unlikely as one might expect a more random pattern of spliced mRNAs than is observed and would add a very high degree of unnecessary complexity to an already complex phenomenon. Spliced sequences could also arise by genetic rearrangements or deletions followed by transcription. Alternatively, a change in the secondary structure of transcribing DNA molecules might allow the realignment of DNA sequences before they are transcribed by RNA polymerase. The most favoured model for the mechanism of splicing involves the transcription of a long primary transcript followed by the looping out of RNA sequences (introns) which are not to be included in the mature mRNA and intramolecular ligation of those sequences (exons) forming the mature mRNA.

It is difficult to imagine how the spliced 5' tripartite leader sequence could be joined to RNA sequences that might arise from a separate transcription unit originating at about 63 map units on the Ad 2 genome described earlier by this latter splicing mechanism. It is possible that mRNAs derived from this transcription unit may not contain the 5' tripartite leader sequence. Alternatively, this transcription unit could be expressed without invoking the existence of a

separate promotor. If a region of intramolecular sequence complementarity exists between the end of the tripartide region and at about 63 map units on the r-strand, an alteration in secondary structure may occur causing the looping out of the DNA strand between these regions followed by transcription from the promotor at 16.4 map units and subsequent splicing. The mRNAs that would result from such a process would then contain the 5' tripartide leader sequence and identical cap sites found on late mRNA molecules (Gelinas and Roberts, 1977). Whatever, the precise mechanism may be, mRNAs which translate late viral specific polypeptides are derived from this transcription unit as discussed below.

IV. UV sensitivity of late Ad 2 protein synthesis

A number of studies have examined the transcriptional organization of both prokaryotes and eukaryotes by assaying specific polypeptides in uv transcription unit mapping experiments (reviewed by Sauerbier and Hercules, 1978). The synthesis of specific polypeptides after uv irradiation was found to be differentially inactivated and reflected the relative target size of the gene coding for a given polypeptide within the transcription unit.

Late Ad 2 mRNAs code for the viral structural polypeptides that form the capsid and contribute to the protein-DNA core. Several late specific viral polypeptides have been identified in lytically infected cells (Anderson et al., 1973) and many of these polypeptides have also been identified by cell-free translation of polysomal RNA from the lytically infected cells (Anderson et al., 1974; Oberg et al., 1975). The approximate locations of the coding regions for the late

viral specific proteins have been determined through the selection of mRNAs by hybridization to viral DNA restriction fragments and the corresponding viral polypeptide translation products synthesized in a cell-free system (Lewis et al., 1975, 1977), or by R-loop mapping of late cytoplasmic RNAs (Chow et al., 1977a), or by the genetic analysis of interserotypic recombinants of adenovirus mutants (Mautner et al., 1975; Grodzicker et al., 1977; Weber and Hassell, 1979).

The transcriptional organization of late Ad 2 gene expression was approached through uv transcription unit mapping experiments by examining the uv sensitivities of the synthesis of late Ad 2 proteins for which the approximate gene locations are known. Ad 2 infected cells were uv irradiated with increasing doses after the onset of DNA synthesis and before late viral proteins could be detected, followed by ^{35}S-methionine labelling when late proteins were being synthesized. Differential uv sensitivities were observed for the uv inactivation of late viral proteins known to be coded for by approximately the left hand 60% of the genome (Fig. 30). The relative radiosensitivities (Table 9) derived from the uv inactivation profiles such as those displayed in Fig. 30 suggest that the gene order for this region is IIIa, V, III, pVII, and II, which is consistent with other studies described above. The uv sensitivities for the synthesis of late viral proteins known to be coded for by approximately the right hand 40% of the genome (Fig. 31, Table 9) did not increase relative to gene II, the most uv sensitive gene located to the left of this region. The gene order suggested by the relative uv sensitivities for this region is 100K followed by IV, (Table 9), which is also consistent with the findings of other

studies described above. Often a shoulder was observed for the uv inactivation curve for the synthesis of the 100K protein. The significance of this is unclear. Transcription from a single promotor initiating a single transcription unit on the r-strand would result in a significantly greater inactivation of protein synthesis for genes 100K and IV relative to gene II, since genes 100K and IV are both distal to gene II. The decrease in the uv sensitivity of protein synthesis for genes 100K and IV (Fig. 31) suggest that another, less uv sensitive r-strand transcription unit encodes genes 100K and IV. This is consistent with the findings of the uv transcription unit mapping experiments described earlier, which identified a second r-strand transcription unit within this region. The majority, if not all of the transcription of these genes appears to be derived from this transcription unit as judged from the single component uv inactivation of protein synthesis for these genes and the correlation of the uv sensitivity and gene position on the Ad 2 genome (Fig. 32).

The uv sensitivity of the synthesis of precursor polypeptides, pVI, pVII, and pVIII was examined in a similar manner utilizing the ts1 mutant of Ad 2, which fails to allow the processing of the precursor proteins to their respective products found in the virion (Weber, 1976). The synthesis of pVI and pVII were found to have approximately the same UV inactivation kinetics, suggesting that genes pVI and pVII are closely linked on the genome (Fig. 35A). The proximity of the nucleotide sequences for genes pVI and II (Akusjarvi and Persson, 1981) would then suggest the gene order to be pVII, pVI and II. The location of gene pVIII on the Ad 2 genome has been suggested to occur

between genes 100K and IV (Lewis et al., 1977), which is consistent with the relative uv inactivation profiles observed for the synthesis of proteins 100K, pVIII and IV (Fig. 35B). Again, these proteins coded for by approximately the right hand 40% of the genome were less sensitive to the effects of uv irradiation than protein II coded for to the left of this region suggesting the expression of another transcription unit encoding genes 100K, pVIII, and IV.

Late cytoplasmic mRNAs complementary to the r-strand of Ad 2 DNA have been found to occur within five families, L1-L5 of mRNA species that share a common 3' terminus (McGrogan and Raskas, 1978; Nevins and Darnell, 1978; Shaw and Ziff, 1980) as shown in Figs. 2 and 3. The genes of interest represented in each family are L1-IIIa; L2-V, III, pVII; L3-pVI, II; L4-100K, pVIII; L5-IV. If transcription is required past the coding region of each gene to the 3' coterminal end within each family, the expected uv sensitivity of protein synthesis should be similar for all the genes of a given family. The finding that proteins, encoded by genes supposedly within a single family of mRNAs with common 3' termini were differentially uv sensitive (Tables 9 and 10) suggests that prematurely terminated transcripts may give rise to a translatable RNA molecule containing one coding region upstream of a uv induced photolesion, but not containing downstream sequences.

The rate of uv inactivation of gene expression was roughly similar, whether nuclear RNA synthesis or protein synthesis was assayed during late Ad 2 infection (Figs. 18 and 32). It has been suggested that the majority of uv-induced transcription terminating lesions on

the DNA are pyrimidine dimers (Sauerbier, 1975). Assuming this and using the data of Rainbow and Mak (1973) for the rate of thymidine dimer induction in Ad 2 DNA i.e., 7.5×10^{-2} dimers/single strand/joule/m^2, it can be calculated that one lethal hit corresponds to about 0.7 thymine dimers when assaying proteins and about 0.85 thymine dimers when assaying nuclear RNA. This difference may represent repair of the thymine dimers in the 6 hours between the UV irradiation and the pulse label when assaying proteins compared to the 0.5 hour interval employed when assaying nuclear RNA.

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