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RESTRICTION OF HUMAN ADENOVIRUS TYPE 2 REPLICATION IN CHINESE HAMSTER CELLS

BY

RACHEL LEVI RADNA

A dissertation submitted to the Graduate Faculty in Biochemistry in partial fulfillment of the requirements for the degree of Doctor of Philosophy, The City University of New York.

1985

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4/23/85
Date

Chairman of Examining Committee

4/29/85
Date

Horst Schulz
Executive Officer

Supervisory Committee

ABSTRACT

Restriction of adenovirus type 2
replication in Chinese hamster cells

by

Rachel Levi Radna

Advisor: Professor Harvey L. Ozer

A study of human adenovirus type 2 (Ad2)infection in Chinese hamster cells (CHO, V79) was undertaken. It was found that Ad2 infected Chinese hamster cells, however, multiplicities of infection greater than 1 pfu/cell needed. The peak of viral DNA synthesis was delayed. No progeny virus was made and the viral late genes were expressed differentially with the structural proteins synthesized at a level lower than the 100K protein. Hexon is moderately reduced and fiber is not detectible. This overall pattern is similar to that obtained in adenovirus infection of monkey cells (CV-1); however, the reduction is more pronounced in CHO. The low level of fiber in CHO could

explain the failure to observe progeny virus.

The role of SV40 T antigen as a helper to adenovirus infection of Chinese hamster cells was examined. A cell line was developed in CHO which constitutively synthesized T antigen from an integrated SV40 genome. Infection of such Chinese hamster cells and conventional CHO cells with an adenovirus-SV40 hybrid (AdND1) did not result in progeny virus or increased fiber synthesis.

Chinese hamster X human cell hybrids were infected with adenovirus; some hybrids were found to correct the defect in Ad2 replication. Those hybrids which had many human chromosomes produced progeny virus. The reduced hybrids did not, however, some reduced hybrids overcame the block to fiber protein synthesis. It was not possible to assign the responsible function to a unique human chromosome.

ACKNOWLEDGEMENTS

It is with deep gratitude that I express my thanks to Dr. Harvey L. Ozer for guiding me through this thesis research, and patiently overseeing my growth as a scientist. His ability to imaginatively define and enthusiastically undertake research problems is an inspiration.

Many thanks also to Drs. Uta Francke and Lawrence Feldman for making cell lines and viruses available for this study.

I thank my fellow graduate students and post doctoral fellows for sharing in the experience.

This thesis is dedicated to my parents, Dr. Bezalel and Mrs. Rebeka Levi. The support and understanding of my entire family during this endeavor made its completion possible.

I would like to give special thanks to my husband Richard and my son Rudy, and my father-in-law, Dr. Rudolf Radna, for the encouragement, support and understanding given to me throughout the period of these studies.

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CHAPTER 1: INTRODUCTION

Adenoviruses and human adenovirus, in particular, have been the subject of intensive study. This introduction will not review the entire subject of adenovirology, but rather present those facts most relevant to this present study.

In 1953 Rowe placed adenoids that were surgically removed from children into tissue culture; he noticed that the cultures showed degeneration after a time. He was then able to serially transmit this degenerative effect to established lines of human cells (e.g. HeLa) by filtered culture fluid. The agent responsible was called adenoid degenerating agent. In the next few years, many viruses were isolated belonging to this same general group. Viruses with similar properties were isolated from other animal species including monkeys, dogs, mice and cattle. In 1956 the name "adenoviruses" was given to the group (Tooze, 1980).

There are at least 80 members of the adenovirus group, categorized into six subgroups according to the natural host species. All share some common general

properties. The best studied of the adenoviruses are the human strains. At least 37 distinct serotypes have been recognized (Wigand et al., 1982). Adenoviruses can be classified into five groups: A, B, C, D and E. These are subgrouped on basis of oncogenicity, DNA:DNA homology, and clinical presentation.

It was first shown by Horne et al. (1959) that the infectious particle is 65-80nm in diameter and is composed of an inner core and an outer icosahedral shell (20 equilateral triangular faces). The virus is non-enveloped and is comprised essentially of DNA and protein. The outer capsid is composed of 252 capsomers, 240 of which have six neighbors and are called hexons, while 12 are at the vertices, have five neighbors and are called pentons. Each penton unit is formed of a base ("penton base") anchored in the body of the capsid, from which projects an antenna-like structure, the fiber, which terminates in a knob. Figure 1 shows a diagramatic representation of a typical adenovirus.

The adenovirus genome is a linear molecule of double stranded DNA with a molecular weight of 23 X 10^6 daltons for serotypes 1,2 and 5 (Green, 1967). The genome codes for 20-30 proteins at least 15 of which are incorporated into the viral particle. Table 1 describes some of the viral proteins.

If the double-stranded DNA is melted and the single

Figure 1: Model of the arrangement of structural proteins and viral DNA in the virion. The positions of the structural polypeptides are indicated by Roman Numerals (Tooze, 1980).

Figure 1

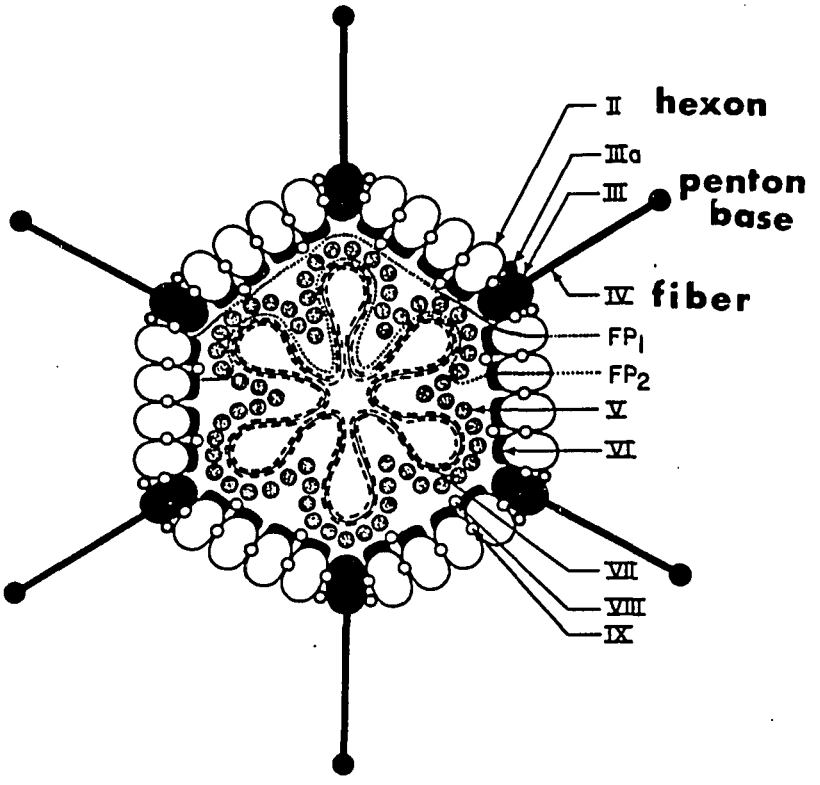


Table 1

Structural polypeptides of adenovirus 2[a]

Protein	Molecular weight	No./virion	Polypeptide	Polypeptide molecular weight
Hexon	313,000–380,000	240	II	90,000–120,000 (3/hexon)
Penton	600,000–515,000	12	III	85,000 (5–6/penton)
Fiber	183,000–207,000	12	IV	60,000–65,000 (3/fiber)
Major core protein	18,000	~1000	VII	18,500
Minor core protein	48,000	~200	V	48,500
Hexon-associated I (all)	50,000	~450	VI	24,000
Hexon-associated II	15,000	n.k.	VIII	13,000
Hexon-associated III (peripentonal)	n.k.[b]	n.k.	IIIa	66,000
Hexon-associated I (groups of 9)	n.k.	n.k.	IX	12,500

a from Tooze (1980)
b n.k., not known

strands of the DNA are allowed to reanneal, circles of DNA are formed, indicating that there are inverted repeat structures at the two ends of the viral DNA (Garon et al., 1972;Wolfson and Dressler, 1971). Adenoviral DNA has a viral-encoded protein covalently linked at both of its 5' termini (Robinson et al., 1973).

The interaction of human adenoviruses with different species of mammalian cells leads to several different responses. When adenovirus infects the cells of its permissive host, such as HeLa cells in culture, a productive course of infection is followed. Adenovirus enters the cell after it becomes attached to specific receptors found on the surface of the cell. This is achieved by the process of pinocytosis (Chardonnet and Dales, 1970a;1970b), or by penetration of the membrane (Lonberg-Holm and Philipson, 1969). Once the virus is inside the cell, uncoating begins by the loss of the penton capsomers. The pentonless particle is then transported to the nucleus where it becomes associated with nuclear pores. Here the remaining capsomers are removed; the DNA-protein core alone enters the nucleus (Chardonnet and Dales, 1972).

The infectious cycle is divided into early and late phases delineated by the onset of viral DNA replication. Philipson et al. (1975) as well as Sharp and Gallimore (1975) mapped Ad2 specific early mRNA to four discrete regions in the viral genome, two in each

strand (designated l and r): regions E1A, E1B, and E3 from the r strand and regions E2A, E2B, and E4 from the l strand. The E1A region encodes function(s) which are essential for the expression of other early region genes (Berk et al.,1979). Figure 2 shows the viral genome and summarizes the major viral transcription patterns.

Five major species of Ad2 early mRNA can be detected. Chow et al. (1977) showed that many of these RNA species are spliced; that is, they contain covalently linked sequences transcribed from noncontiguous regions in the Ad2 genome. It follows that a large number of virus-specific polypeptides are synthesized in the early phase of infection. One of these early proteins produced in large amounts is the 72K protein, encoded in region E2B. It was found to have a functional role in that it binds to single stranded DNA; hence it is also referred to as DNA binding protein (DBP). It plays a role in DNA replication, probably both in initiation (van der Vliet and Sussenbach, 1975) and elongation (van der Vliet et al., 1977). It also may have a role late in infection, since 5 different mutant viruses with an alteration in the DNA binding protein were able to overcome the block to late gene expression in monkey cells (Klessig, 1979) as discussed later. It also is thought to have a role in viral assembly (Nicolas et

Figure 2: Map of the Ad2 genome.
Early RNAs are depicted by thin arrows and late RNAs by thick arrows. The proteins are designated by numbers. Roman numerals designate the capsid proteins. Polypeptides synthesized as larger precursor proteins have the prefix p (i.e.,pVI). Taken from Genetic Maps (1984) Stephen O'Brien Ed.

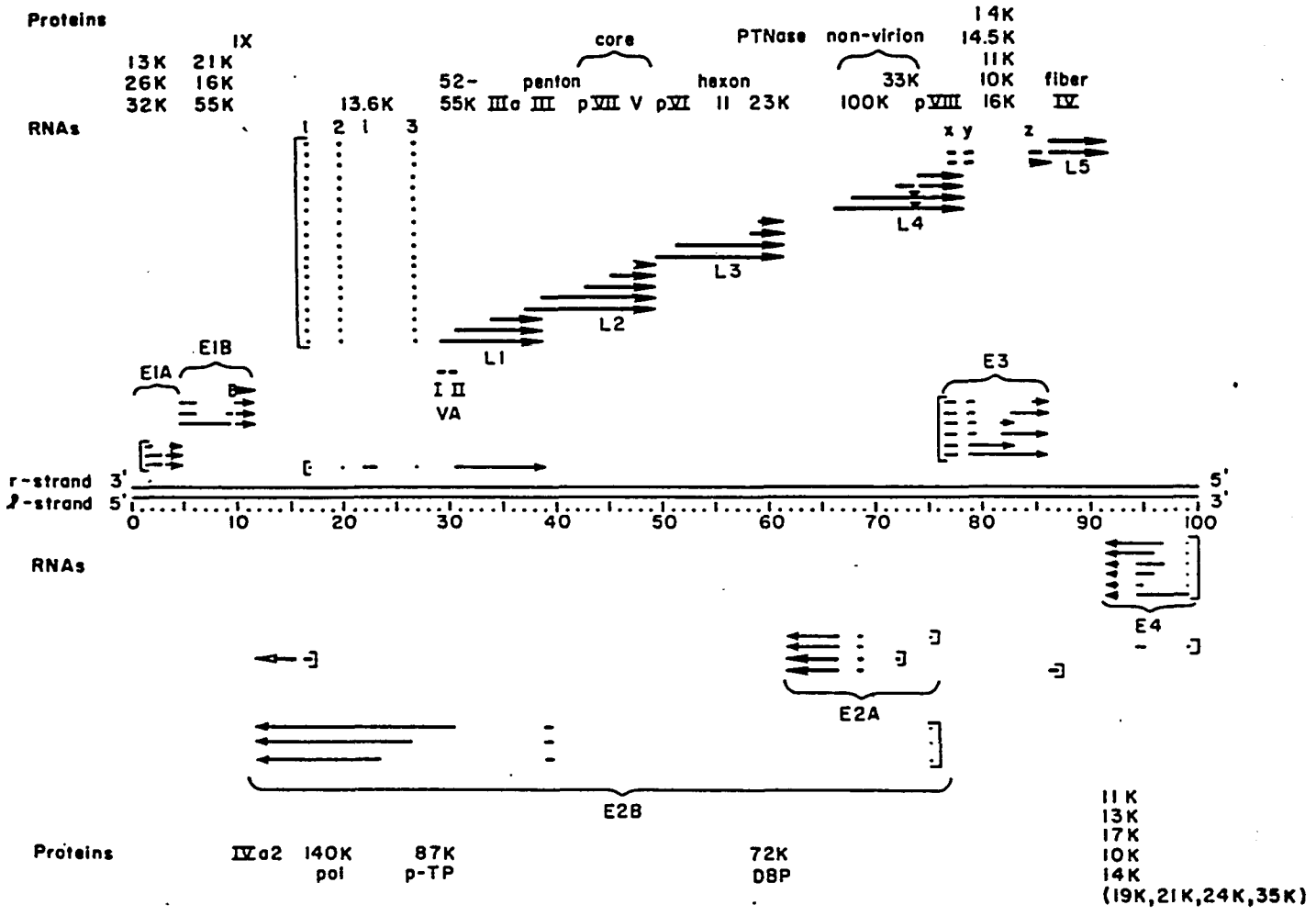

Proteins
IX
13K 21K
26K 16K
32K 55K
13.6K
52-55K IIIa III
penton
core
pVII V pVI II 23K
hexon
PTNase
non-virion
33K
100K pVIII
14K
14.5K
11K
10K
16K
fiber
IV
RNAs
1 2 1 3
x y
z
L5
L4
L3
L2
L1
E1B
E1A
I II
VA
E3
r-strand 3'
l-strand 5'
5'
3'
0 10 20 30 40 50 60 70 80 90 100
RNAs
E4
E2A
E2B
Proteins
IVa2 140K 87K
pol p-TP
72K
DBP
11K
13K
17K
10K
14K
(19K,21K,24K,35K)

Figure 2

al., 1983). The initiation of viral DNA synthesis requires early gene products. However, once progeny DNA molecules appear, DNA synthesis becomes independent of protein synthesis.

Replication of adenovirus DNA is semiconservative (Bellet and Younghusband, 1972). The model for the mechanism of adenoviral DNA replication was originally proposed by Daniell (1976). Synthesis is initiated at the left end or the right end, and copying of one of the two parental strands proceeds to completion, fully displacing the other strand. This synthesis proceeds in the 5'—>3' direction (Type I synthesis). The self-complementary ends of the displaced DNA strand hybridize to form a circular intermediate. This intermediate contains a double-stranded region which is used as an initiation site for replication of this structure, resulting in a second double-stranded viral genome (Type II synthesis). It was first postulated by Rekosh et al. (1977) and subsequently demonstrated in vitro (Challberg and Kelly, 1982) that the terminal protein (as an 80,000 dalton precursor protein) functions directly as a primer by covalently binding deoxycytidine as the 5' terminal nucleotide of the new DNA chain.

By 15 to 20 hrs after the initiation of infection, and after the onset of DNA replication, 20-40% of the

total RNA in the cell is viral in origin, and viral mRNA has almost completely replaced cellular mRNA on the polyribosomes. During this late phase of infection there appears a nuclear RNA species that is considerably larger than mRNA (see Figure 2). Adenovirus 2 late mRNA species carry 5' capped structures that resemble cellular mRNA. In addition, the majority of late mRNA have a 5'-terminal sequence, 150-200 nucleotide in length, transcribed from three sites in the Ad2 DNA located in the r strand (tripartite leader) distant from the body of the message. The major late promotor site was determined to be between 16.3 and 16.5 map units. Examination of the DNA sequence in this region revealed a 10 nucleotide sequence identical to the capped sequence of the late mRNAs. The sequences surrounding this promotor site have interesting properties; the immediate preceding sequence is purine rich, the promotor sequence itself can be arranged in a secondary structure which includes a single stranded loop containing the start site, and 31 nucleotides upstream from the 5'end of the RNA is a sequence TATAAAA, which is similar to sequences observed at a equivalent position in a number of eukaryotic transcription units. A model of transcription initiated at a site near 16.4 map units gives rise to very large RNA molecules which include the 3 segments

of mRNA to be spliced into late mRNAs. A mechanism by which intervening sequences are spliced followed by intramolecular ligation is proposed to generate the mRNA molecules found in the cytoplasm (Klessig 1977; Berget et al., 1978). It follows that the mRNA for many late proteins including hexon, 100K and fiber are processed from a large precursor RNA initiated from a single promotor. This point is discussed in more detail later.

After the late messages are transcribed and translated, the structural proteins are transported from the cytoplasm to the nucleus, where assembly of the virion takes place. The first steps in assembly can be defined as the formation of the major structural units of the capsid; the hexon, fiber and penton base, from monomeric polypeptide chains during the late phase of viral infection. It was reported by Oostrom-Dragon and Ginsberg (1981) that the 100K protein provides an essential function in the assembly of nascent hexon polypeptide chains into mature hexons. The 100K protein is a viral nonstructural protein which is synthesized in large amounts during late infection. It is thought that the 100K protein functions in the capacity of a scaffolding protein to assist in transport and assembly of hexon. There are two distinguishable classes of hexon trimers which can be separated on the basis of their charge (Boulanger,

1975). It is postulated by Oostrom-Dragon and Ginsberg that to obtain these two classes of hexons, the 100K protein may hold the hexon in a particular conformation to allow specific folding of the chains. These trimers take part in higher orders of assembly to form the groups of nine hexons associated with the faces of the adenovirus icosahedron. The fiber polypeptide is glycosylated (Dorsett and Ginsberg, 1975) and associated into a trimeric structure with a sedimentation coefficient of 6S. A 10.6S penton which consists of fiber and penton base (Carstens and Weber, 1977) has also been isolated. The penton interacts with the 12S hexons to yield intermediate particles which are probably precursors of infectious virions. Several types of particles are produced late in infection in addition to intact infectious virions. One type consists of capsids without DNA and are called empty particles or top components (Ishibashi and Maizel, 1974). Another type called incomplete particles contains viral DNA sequences which are shorter than full-length virion DNA (Burlingham et al., 1974). Both types of particles have been suggested to be intermediates in the assembly of adenovirus, based on studies with viral temperature sensitive (ts) mutants. In the case of H5ts142, an Ad5 mutant ts in fiber, empty capsids accumulated at the non-permissive temperature. When the temperature was lowered to

the permissive temperature, particles were detected into which viral DNA appeared to be entering. It was also determined that the left end of the viral DNA appears to enter the empty capsid first during assembly (Chee-Sheung and Ginsberg, 1982). The requirement for concurrent and continued viral DNA synthesis for the assembly of normal virus particles, and that these processes may be physically coupled has been reported by Weber et al. (1985). Between 4,000 - 10,000 progeny viral particles are produced per permissive cell (Green and Daesch, 1961).

When human adenovirus infects cells other than those of its natural host, a semi-permissive or abortive course of infection ensues. The particular course of infection depends on both the nature of the infecting virus and the type of host cell. Group C (1,2,5,6) adenoviruses can replicate nearly as well in hamster cells as in human cells (Takahashi, 1972). Infection of rat embryo cells with these viruses shows a moderate decrease in progeny produced (1,000-2,000 pfu/cell). Different tissues within the rat embryo differ in their ability to support viral growth.

Younghusband et al. (1979) reported that when C57 black mouse cells were infected by Ad5, early viral antigens (e.g. DNA binding protein) are made in most cells, but fewer cells make late antigens and the yield of infectious virus is much lower (< 0.2 infectious

units per cell) than that in semi-permissive rat cells (Gallimore, 1974). Silverstein and Strohl (1985) reported that in Ad2 infection of mouse B3T3 cells, both early and late virus-specific antigens were synthesized. The yield of progeny virus varied from 0.1-10 infectious units per cell, depending on experimental conditions. In contrast, hamster cells are completely nonpermissive to adenovirus type 12. Adenovirus 12 enters hamster cells; early mRNA and proteins are made; but replication of viral DNA does not take place (Doerfler, 1968).

In some cases, infections as above result in a permanent alteration of the growth properties of the cell, similar to oncogenesis, and are associated with persistent expression of some viral functions. This is known as transformation. When DNA from eight different Ad2 transformed rat cells was analyzed (Sambrook et al., 1975; Sharp et al., 1974; Gallimore et al., 1974) as to the nature of the viral sequences they contained, it was found that viral sequences were integrated into the cellular genome. None of the transformants contained a complete adenovirus genome; the only viral DNA sequences which were common to all these cell lines contained the left-hand 14% of Ad2 DNA, also referred to as the E1A region. Transfection with purified DNA from this region alone can result in growth transformation (Houweling et al.,

1980). Similar results have been obtained with other adenoviruses and other cell systems. The frequency of transformation varies over a wide range but is typically low. The low frequency and integration of subgenomic fragments is likely related to the fact that most Ad-cell interactions are associated with cell death due to viral gene expression. Consequently stable associations logically occur in the absence of an intact viral genome. This model is supported by observations with other cytopathic DNA tumor viruses, as the Papovaviruses (Small et al., 1982) and exceptional Ad2 or Ad5 cell interactions in which all, or virtually all, of the Ad genome persists. Examples of the latter include transformation of CREF cells which are non-permissive for virus replication (Dorsch-Hasler et al., 1980), transformation by a viral ts mutant defective in virus DNA synthesis (H5ts125, Mayer and Ginsberg, 1977) and transformation with a deletion mutant which is defective in viral genome expression in a host-dependent manner (Van Doren et al., 1984). Integration of viral sequences may also occur in acute infection but there is no evidence that such is an essential feature of viral replication (Fanning et al.,1978) The viral DNA intermediate in integration has not been defined; proposals include circular forms which have been noted as minor species or due to the terminal repeats (Graham, 1984) in addition to the linear

genome.

The best studied semipermissive infection involves Ad2 or Ad5 infection of monkey cells. There is a decrease by a factor of about 1,000 in the amount of progeny virus produced when adenovirus infects primary African green monkey cells (AGMK) as compared with human cells. The adsorption and entry appear to be normal (Feldman et al., 1966). It was reported by Friedman et al. (1970) that both the onset and the rate of viral DNA synthesis are comparable. Early proteins are synthesized; however, the synthesis of some late adenovirus proteins is reduced. This defect is corrected by coinfection with SV40 (see later for details). This latter phenomenon is known as enhancement.

There is an ongoing controversy regarding the failure of monkey cells to synthesize some late viral proteins. The protein synthesizing machinery of monkey cells were examined, as it was suggested that viral mRNA could not be efficiently translated by the monkey cell polyribosomes. It was demonstrated by Eron et al. (1975) that viral messages extracted from adenovirus infected monkey cells were translated <u>in vitro</u> as efficiently as viral messages extracted from enhanced infection. On the other hand, it was observed by Nakajima et al. (1973) that polyribosomes from monkey cells could not translate viral mRNA efficiently in

vitro. They observed the ability of monkey cell polyribosomes from enhanced infections to translate viral messages more efficiently, postulating that coinfection with SV40 induced the synthesis of new initiation factor(s) and, therefore, translation takes place.

On the other side of the controversy, it was suggested that failure to synthesize late viral protein rested at the level of mRNA production. Klessig and Anderson (1975) found the steady-state levels of cytoplasmic late mRNAs encoding late viral proteins were reduced by a factor of 2-10, resulting in a comparable reduction in synthesis of most late viral proteins. They found a drastic reduction in the synthesis of fiber polypeptide, however; it was reduced by a factor of greater than 100 even though the steady-state level of mRNA encoding this protein was reduced by a factor of only 5-10 (Klessig and Chow, 1980; Anderson and Klessig, 1983). Multiple studies demonstrated that there is a less severe decrease of other late viral proteins in the unenhanced infection. Klessig and Anderson (1975) also reported that some polypeptides such as 100K and IVa2 were synthesized at a normal amount. Futhermore, they reported that a similar pattern of synthesis took place when late messages from this unenhanced infection were translated in vitro. The discrepancy between the amount of fiber

mRNA transcribed and that which ends up translated into fiber protein was addressed in Klessig and Chow's 1980 study. They reported that the fiber mRNA had a normal, capped tripartite leader at its 5'terminus, but that a large proportion of these molecules contained long sequences between the tripartite leader and the main body which were not spliced out of the initial transcript during maturation of the fiber mRNA. The presence of these RNA species suggests that splicing of the fiber message in the monkey cell line CV-1 cells may be somewhat anomalous. More recently, Anderson and Klessig (1984) looked at the 5'ends of fiber messages from several different productive and semi-permissive infections. They report a direct correlation between synthesis of the fiber polypeptide in vivo and the presence of the "x" and/or "y" ancillary leaders on messages encoding the fiber polypeptide. They speculate that since only the fiber message can contain the x or y leader sequence and the fiber protein is the only late protein to be glycosylated, these leader sequences play a role in the synthesis of this glycoprotein. This post-transcriptional model was supported by cell fusion studies (Zorn and Anderson, 1981). They demonstrated that Ad2 fiber protein is expressed upon infection of 50-100 cell colonies of recently fused monkey and human cells by use of immunofluorescence techniques whereas infected monkey cells alone were negative. Hybrid

cells were verified by the presence of visual markers (latex beads) from each cell. Since fiber production could be rescued in Ad2 infected monkey cells fused to uninfected human cells, it was postulated that human cells contain a "dominant" factor that acts in trans and overcomes the block to fiber synthesis in monkey cells. Moreover, reconstructed cells from infected human karyoplasts (nucleus-containing bodies) and monkey cytoplasts expressed fiber, whereas cells contructed from infected monkey karyoplasts and human cytoplasts did not. Hence, these results seem more consistent with the hypothesis that the block involves a nuclear event which interferes with the formation of functional mRNA. It is also suggested from the reconstruction experiments that the translational machinery of monkey cells is competent to translate functional fiber mRNA synthesized in human cells. Finally, it should be noted that differences in results obtained by the different groups may be a reflection of the varied responses of the different monkey cell lines to infection by Ad2. For example the VERO line of monkey cells is even permissive for this virus (Eron, 1975).

It was first reported by Rabson (1964) that coinfection with simian virus 40 (SV40) increased the replication of adenovirus in monkey cells to a level comparable to that found in human cells. This

responsible factor must be an early function since SV40 does not replicate its DNA during coinfection (Nakajima and Oda, 1975). This interpretation is supported by the fact that some SV40 group A mutants (early gene encoding SV40 T antigen) are not good helpers for adenovirus growth in monkey cells. The exact nature of the helper function is not known, but it is encoded by the region of the SV40 genome between 18 and 22 map units, since infection with Ad2-SV40 hybrid virus (e.g., Ad2ND1) that contains a segment encoding only the carboxyl terminus of the SV40 large T antigen is permissive (Lewis et al., 1969).

Klessig (1977) reported the isolation of several host range mutants of adenoviruses 2 (Ad2hr400-403) and 5 (Ad5hr404) which multiply efficiently in monkey cells without the aid of helper virus. This mutation was mapped to the gene encoding the 72K DBP (Klessig and Grodzicker 1979), more specifically the result of a point mutation in the amino terminal part of the adenovirus DNA binding protein (Anderson, 1983; Kruijer, 1981). As noted previously, the 72K protein is required for viral DNA replication (Ginsberg, 1975). Since viral DNA replication in unenhanced monkey cells proceeds normally, the mutation must effect a second functional domain of the protein which is needed for late gene expression.

Somatic cell fusion permits cells of different

types and of different species to be fused together. The resultant cell contains the genetic information of both parental cell types (Ozer and Jha, 1977). Cell fusion occurs spontaneously in culture at a low rate. With the aid of Sendai virus, the efficiency of fusion can be increased markedly. Since that observation a number of fusion techniques have been reported, the most useful of which is polyethylene glycol (PEG). The resulting binucleate or multinucleate cells formed by fusion of parental cells of different species have been termed heterokaryons. These cells are viable and capable of limited division. Those nuclei which enter mitosis together are usually reconstituted as a single unit. Cells in which a single nucleus contains chromosomes of both parental species are called synkayons or somatic cell hybrids.

Chromosomes may be lost from hybrid cells at a fairly steady rate following fusion. When the chromosomes of one parental genome are lost preferentially over many generations, the resulting daughter cells have a chromosome complement that consists of a resident set of chromosomes from one parent with the addition of a few chromosomes from the other. It is possible to identify the additional chromosomes and to try to correlate their presence with the expression of genes of this parent. This preferential chromosome loss provides a tool for

mapping chromosomes. The human genome is quite amenable to this technique as human chromosomes are characteristically lost from human-rodent cell hybrids. Cell hybrids can be probed for the study of host cell functions required for virus infection. In a non-permissive or semi-permissive system (i.e. restrictive system), the block to viral replication could be found anywhere along the route of normal viral growth, i.e. adsorption, expression of early genes, DNA synthesis, late gene expression and virion assembly. The block could be due to the fact that a host cell product essential to virus infection is absent in the restrictive host. Alternatively, the viral proteins may interact inefficiently or ineffectively with the relevant cell protein. Replication of viral particles may occur when cell fusion takes place between permissive and restrictive cells. Restoration of viral replication may also occur when cells of different semipermissive cell lines are fused to form hybrids if these cell lines complement each other, i.e. defective in different processes.

It has been shown by Miller et al. (1974), Lemons et al. (1977) and Brown et al. (1979) that permissivity often is dominant when such experiments are performed. When a situation arises in which the permissive host is also the one whose chromosomes are preferentially lost, it becomes possible to identify the

chromosome(s) whose gene products are required for viral replication. It was demonstrated by Miller et al. (1974) that the presence of human chromosome 19 in mouse X human hybrid cells was responsible for the susceptibility to polio virus. This particular chromosome (19), together with chromosome 6 was implicated in the productive infection of Baboon M7 Type C Virus in Chinese hamster X human cell hybrids as reported by Brown et al. (1979). However, they were unable to exclude a function coded for by human chromosome 3. In both cases, the relevant human gene encoded a surface membrane receptor. Francke and Francke (1981) were able to demonstrate the requirement for the long arm of human chromosome 11 in the replication of Herpes simplex virus type 1 in hybrids between restrictive chinese hamster and permissive human fibroblast. The gene product was not defined biochemically but it functioned at a step subsequent to viral DNA synthesis. Garver et al. (1980) studied SV40 infection of hybrids involving semi-permissive Chinese hamster cells and permissive monkey cells. Permissivity was dominant and correlated with monkey chromosome 12 (related to human chromosome 11) but all hybrids contained large numbers of primate chromosomes. The site of restriction analysed was after viral uncoating since all studies were performed with infection by viral DNA, bypassing (other) potential blocks in adsorption etc. A few genes

responsible for susceptibility to virus were mapped in mouse cells using somatic cell hybrids (Oie et al., 1978). No studies have been reported on adenovirus infection of clonally isolated synkaryons, although two laboratories have investigated adenovirus infection shortly after cell fusion. Weber and Stich (1969) studied the replication of Ad2 and Ad12 in heterokaryons produced by fusion of permissive (human HEp2) cells and hamster (BHK21) cells non-permissive for Ad12 replication and restrictive for Ad2. They found by immunofluorescence that infection of the heterokaryons led to the formation of intranuclear inclusion bodies and viral antigens. They later reported (Weber and Stich 1970) that when they fused Ad12 infected BHK21 cells with HEp2, they failed to see viral inclusion bodies or V-antigen. It led them to conclude that the presence of the HEp2 cytoplasm or nucleus at the time of infection influences the expression of the viral genome. As noted earlier, Zorn and Anderson (1981) observed that reconstituted cells involving monkey and human cells were permissive for Ad2.

The fact that adenovirus uses the host cell machinery to replicate its DNA makes it a good model for the study of host cell factors involved in DNA replication. It was with the aid of mutant cells that DNA replication in prokaryotic cells was deciphered.

The need for eukaryotic mutant cell lines to study mechanisms of DNA replication is obvious. There has been a dearth of eukayotic DNA synthesis mutant cells and their isolation has been difficult. However, the established Chinese hamster cell lines CHO and V79 are particularly amenable to recovery of mutants.

Since one of the major interests of our laboratory is the host cell factors involved in DNA replication, considerable effort has been devoted to the isolation of temperature sensitive DNA replication mutants in these cell lines. At the time that this study was begun, there was no report of a DNA virus which replicated in them. This study commenced originally as the replication of Mouse adenovirus FL strain in CHO, with the rationale that the CHO cells were more likely to replicate the mouse adenovirus than the human strain, mouse being closer to hamster phylogenetically. It was then reported by Horwitz and Longiaru (1981) that adenovirus replicated its DNA in CHO, though they failed to observe synthesis of late proteins. It was at this time that the switch to the study of adenovirus type 2 in CHO was made. The reason for the switch was that the human viruses had been studied extensively, and there were many reagents that would be available for the study of the Ad2-CHO system.

It was therefore, the focus of this thesis to characterize the properties of Ad2 infection of

semipermissive CHO and V79 cells. Analysis of viral replication in mutant and wild type cells may be restricted in some manner. A study of this restriction should provide insight into cell functions needed by the virus in different aspects of the virus-cell interaction. Since these viruses are rather well characterized in terms of their genome structure and molecular biology, they provide probes or models for different aspects of cellular macromolecular biosynthsis in so far as they utilize host functions. The use of somatic cell hybrids was exploited to assign which human chromosome(s) are responsible for the susceptibility to adenovirus type 2.

CHAPTER 2: MATERIALS AND METHODS

2.1 CELL LINES AND TISSUE CULTURE PROCEDURES

2.1.1 Mammalian cell lines and culture conditions

Cell lines used in these studies and the sources from which they were obtained are listed in Tables 2, 3 and 4. Cells were grown in Dulbecco's modified Eagle's medium (DME, M.A. Bioproducts) supplemented with 10% Fetal Bovine Serum (FBS, M.A. bioproducts), penicillin "G" phosphate (Gibco, .05gm/l) and streptomycin sulfate (Gibco, .05gm/l) in a 10% CO_2 atmosphere at 37°C. The human X Chinese hamster cell hybrids were expanded for the initial experiment and stored at an early passage. All repeat experiments were performed with freshly plated cells two passages from frozen storage. Dialysed FBS was required for experiments involving expression of Dihydrofolate Reductase (DHFR) and was prepared in the following manner: dialysis tubing (Spectrapor, S/P) was soaked and boiled for 10 min in 2% $NaHCO_3$,0.001 M ethylenediaminetetracetate (EDTA). The tubing was then washed and boiled in distilled H_2O. Serum was dialyzed against Hanks'

Table 2

Designation of Chinese hamster cell lines

Cell line designation	Source	Laboratory	Comment
CHO-K1	Ovary derived fibroblast	Chasin	wild-type parent (proline auxotroph)
DUK	CHO-K1	Sharp	DHFR deficient (isolated by Chasin)
T2	CHO-K1	Siminovitch	resistant to multiple drugs
TNT	T2	Ozer	selected for spontaneous resistance to thioguanine (HPRT deficient)
AK412	CHO-K1		HPRT deficient used as parent for cell hybrids
V79, Don	Lung derived fibroblast		Wild Type (WT)
E36-34	V79	Francke	HPRT deficient, used as WT and for cell hybrids
A23	Don	Francke	Thymidine Kinase deficient, used as parent for cell hybrids
CHE	Embryo fibroblast	Francke	not established cell line

Table 3

Designation of Primate Cell lines

Cell line designation	Source	Laboratory	Comment
HeLa	human cervical ca	Feldman	Wild Type
HEK	human embryo kidney	Feldman	not established
HS74	human fetal bone marrow derived fibroblasts	Smith	not established
CV1	African Green Monkey Kidney	Berg	wild type permissive for SV40 semi-permissive for Ad2

Table 4

Designation of
CH X Human Cell Hybrids obtained from U. Francke
(Francke and Francke, 1981)

V79 X human fibroblasts

Thesis Designation	Literature Designation	Passage History
X-7A	X-7A	
UF1	XV-G1	p8AF10
UF5	XV-18B-6a-K2	p9AF8
UF6	XV-F18AG	p5AF8
UF7	XV-18B-7a-N4	p8AF8
UF8	XV-18A-10q-C1	p5AF10
UF18	XVII-10A-12A	p2AF2,8

V79 X human leukocytes

UF3	XII-5E	p3AF2
UF10	XII-4A	p5AF6
UF16	XII-4A-HAT	p5AF6
UF17	XII-2D aza	p5AF12
UF2	XIII-5B	p3AF4
UF4	XIII-3A	p16AF6
UF9	XIII-4D-1c aza	p1AF5,7,2
UF14	XIII-7A	p4AF3,5
UF19	XIII-7A aza	p2AF1,14

Table 4 cont.

Thesis Designation	Literature Designation	Passage History
CHO X human fibroblast		
UF11	XXIV-A2b	p5
UF12	XXIV-Aga	p4
UF13	XXIV-AIIab	p10
Don X human		
UF15	XXI-51B	p5AF3

a Roman Numeral indicates cell fusion experiment.
b p8AF10 means initially frozen at passage 8 (p8), thawed and passaged for an addition 10 passages (AF10).

Balanced Salt Solution (Gibco) for 2 days at 4°C with 2 changes of dialysis fluid. The dialyzed serum was collected and divided into 10 ml aliqots in sterile glass tubes and stored at -20°C. Immediately before use, the tube was thawed, mixed with DME, and sterilized by filtration (0.45 u, Nalgene) to prepare the complete dialyzed serum medium. This medium was supplemented with proline and used to select for the DHFR+ phenotype.

Cells were passaged by brief treatment with a trypsin-EDTA mixture (0.5 gm/liter trypsin and 0.2 gm/liter EDTA, M.A.Bioproducts); cells were concentrated by centrifugation at 500 X g for 5 min. Cell counts were obtained with a Royco Tissue Cell Counter after dilution in phosphate buffered saline (PBS) (0.15M NaCl, 3mM NaH_2PO_4, 7mM Na HPO_4, pH 7.4). Cell lines were stored in Nunc Cryotubes frozen in liquid N or in a -70°C freezer in complete medium containing 10% dimethyl sulfoxide (DMSO).

2.1.2 Transfection of mammalian cells by the DNA-$CaPO_4$ co-precipitation technique (Wigler et al., 1978)

Twenty hours prior to transfection, cells were seeded at 5 X 10^5 per 100mm Petri dish in complete medium. Four hours prior to tranfection,

cells were refed with DME supplemented with 5% fetal bovine serum. DNA precipitates were prepared as follows: a double strength DNA-$CaCl_2$ solution was prepared containing DNA at 40 ug/ml in 10mM tris, 1mM EDTA pH 7.6 (TE). 2.5M $CaCl_2 \cdot 2H_2O$ (Malinckrodt) was added dropwise with constant agitation to a final concentration of 250mM. This DNA-$CaCl_2$ solution was then added dropwise, to a polypropylene tube (Falcon) containing an equal volume of double-strength HEPES buffered saline (2X HBS) (280 mM NaCl, 50mM HEPES, 1.5mM $Na_2HPO_4 \cdot 12\ H_2O$, pH 7.05), while bubbling vigorously by mouth through a 1 ml plugged plastic pipette. The flocculent DNA-calcium phosphate precipitate was then resuspended and added dropwise to recipient culture Petri dishes (1 ml per 100mm Petri dish). Cultures were incubated for 4 hours, the medium was then aspirated and cultures were refed with complete medium. After 3 days the cells were trypsinized and seeded into Petri dishes containing DHFR selective medium. (section 2.1.1)

2.2 VIRUS PREPARATION AND PROCEDURES

2.2.1 Virus Stocks

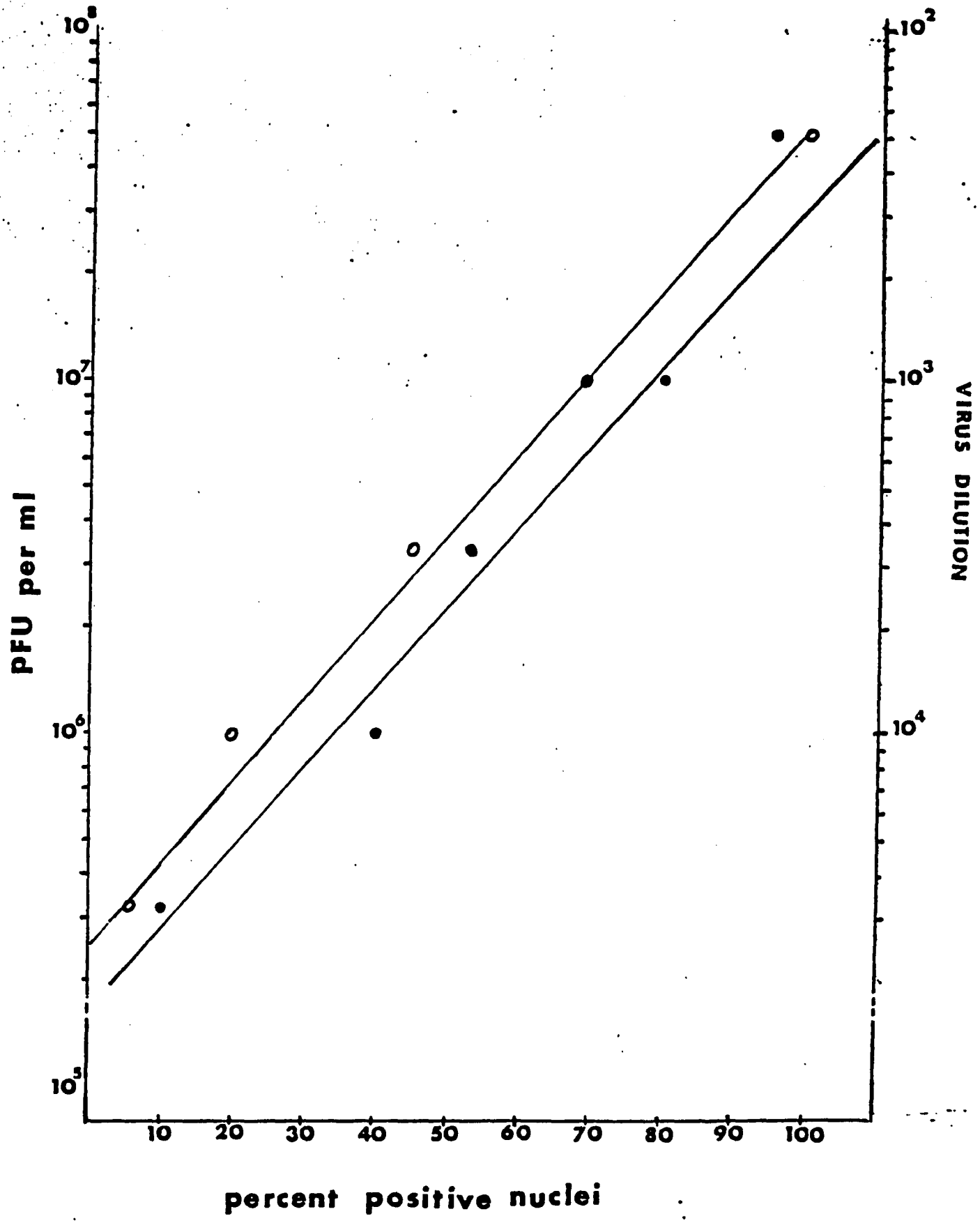
Adenovirus type 2 (Ad2) was grown in HeLa suspension culture and purified on $CsCl_2$ gradients The virus was prepared by Dr. Lawrence Feldman and two different virus pools were kindly provided for these studies. In some cases, purified virus (pool 2) was dialyzed against 10mM Tris-1mM EDTA, pH 7.9, for 1 day with 2 changes of buffer. Glycerol was added to a final concentration of 20% after dialysis. Aliquots, were stored at $-70^{o}C$. When virus was thawed for use, a limited amount was diluted in DME containing 1% FBS for infection and the remainder refrozen. Repeated freezing and thawing was avoided.

I titered these virus pools using an immunofluorescence assay (IF) and, in the case of pool 2, a plaque assay, as well. This was done as follows: HeLa coverslip cultures were infected with varying dilutions of stock virus. These cultures were fixed at 24 hr pi and processed for immunofluorescence as described elsewhere in the Materials and Methods. The percentage of nuclei positive for 72K protein (a viral-encoded protein expressed early in infection) was determined. The plaque assay was performed as described in the following section. Virus pool 2 was

found to contain 1.0 X 10^{10} plaque forming units (pfu)/ml. Therefore, it was possible to relate the percent of cells positive for 72K to virus concentration in pfu/ml. Figure 3 is the graphic representation of these data. Dilution of virus preparation and pfu/ml were plotted against percent 72K positive nuclei. This standard titration curve served to determine the concentration of other virus pools. From the graph, it was calculated that Ad2 pool 1 had a concentration of 1.7 X 10^{10} pfu/ml. Identical results were obtained whether IF was performed with infected HeLa (as shown) or CV-1 (data not shown).

Non defective Ad2ND1 was provided by Dr. Terry Grodzicker of Cold Spring Harbor Laboratory. Host range mutants (Ad2hr400 and Ad2hr401) were purified on CsCl gradients and provided by Dr. Daniel Klessig, University of Utah, Medical School. In the case of Ad2ND1, virus, in the form of a crude cell lysate, was either used directly (Pool 1, 1.4 X 10^{8} pfu/ml) or after preparation of a virus stock in this laboratory. Pool 1 virus was diluted 1:20 in DME containing 1% FBS and used to infect confluent monolayers of CV-1 cells (2 ml per 75 cm flask). After adsorption for 2 hours, virus was removed and the culture refed with 10 ml of complete medium. Cells were harvested after 3-4 days at complete cytopathic effects (cpe) by repeated freezing and thawing the cells in the original medium.

Figure 3: Ad2 Standard Titration Curve HeLa coverslip cultures were infected with Ad2 virus pool 1 and pool 2 at varying dilutions. The coverslips were fixed at 24 hr pi and processed for immunoflourescence as descibed elsewhere in these Materials and Methods. PFU were determined on HEK cells as described elsewhere in these Materials and Methods. Pool 1 (o) and Pool 2 (●).


PFU per ml
10^8
10^7
10^6
10^5
VIRUS DILUTION
10^2
10^3
10^4
10
20
30
40
50
60
70
80
90
100
percent positive nuclei

Cell debris was removed by centrifugation at 2000 X g for 10 minutes. The supernatant was designated as the Ad2ND1 virus, pool 2, and was divided into aliquots for storage at at -20°C. Its titer was 5 X 10^7 infectious units per ml as determined by IF (See Table 20 in Chapter 3.3).

2.2.2. Virus growth curves

Infection for assessment of virus production was performed by infecting cell monolayers in 25 cm flasks (Falcon, T25) with virus at a multiplicity of infection (MOI) of 0.5-1.0 pfu/cell. Two tenths of a milliliter of virus suspension was added to cells for adsorption as above. The monolayers were then washed carefully 3 times with 5 ml DME to remove unadsorbed virus, which would interfere with measurement of virus produced. Five milliliters of complete medium was added and the cultures incubated at 37°C. Samples were harvested immediately following adsorption 0 time pi value, within the first day pi for an eclipse value, and 3 days and 5 days pi for measurement of virus production. At the set times, the monolayers were frozen at -20°C.

2.2.3 Virus Titration by Plaque Assay

In the case of the viral growth curve, the infected cells were frozen at the appropriate times pi. The cultures were subsequently frozen and thawed three times to effect cell shearing and the release of virus particles into the medium. Duplicate samples were pooled and centrifuged at 1,500 rpm for 15 minutes in a counter top IEC centrifuge. For these and other virus preparations, serial 10 fold dilutions were made in DME with 1% fetal bovine serum, and 0.2 ml of the virus sample was plated (in duplicate) on confluent early passage human embryo kidney cells (HEK). The Petri dishes were incubated at 37°C for 2 hours with rocking every 15 minutes. Following adsorption, the virus was removed and the monolayer was overlayed with a 0.65% Noble agar (Difco) solution in DME containing 5% fetal bovine serum and 0.05 M $MgCl_2$. The overlay was allowed to solidify at room temperature (15-20 minutes) before returning the Petri dishes to the incubator at 37°C. Ten days later, the Petri dishes were stained with neutral red (3.3 mg/ml neutral red (sodium salt), Gibco) either by adding 4ml of a 1:25 dilution of stock neutral red in DME and incubating the Petri dishes for 2-4 hours before removing excess dye, or by making the same dilution in agar and adding a solid overlay to the Petri dishes to be read the

following day. Plaques were counted with the aid of a light box.

2.3 ASSAYS FOR VIRAL PROTEINS

2.3.1 Antibodies and immune reagents

Antibody source and origin are summarized in Table 5. Monoclonal antibodies to Ad2 proteins were prepared as ascites fluid and received lyophilized. Upon reconstitution, they were diluted in PBS with 1% Bovine Serum Albumin (BSA, Calbiochem), filtered and stored at $4^{o}C$ when used for IF. They were used undiluted for immunoprecipitation. Staph A (Pansorbin, Calbiochem-Behring) is a 10% suspension of fixed staphylococcus aureus cells of the Cowan I strain in PBS. A typical lot binds at least 2.0 mg human IgG per 100 mg cells. Preliminary control experiments were performed to determine the proper condition for the antibody and Staph A immunoprecipitation. The amounts used (2 ul antibody, 100 ul pansorbin) were demonstrated to be at saturation for 25 ul of infected HeLa lysate and the total extract from Chinese hamster cells or their cell hybrids as determined by comparison of extracts before and after a single immunoprecipitation and/or by repeated immunoprecipitations. One exception involved the 100K protein in which the monoclonal antibody gave incomplete precipitation as noted in Figure 19. In most studies the rabbit antiserum, which did not

TABLE 5

Description of antibody source and Specificity

Antibody	Source	Specificity	Laboratory
R∝100K	polyclonal (serum)	Ad2 100K protein	H. Ginsberg
M∝100K	monoclonal (ascites)	Ad2 100K protein	C. Cepko/ P. Sharp
M∝Hexon	monoclonal (ascites)	Ad2 Hexon trimers and ninemers only	C. Cepko/ P. Sharp
R∝Fiber	polyclonal (serum)	Ad2 Fiber free and assembled	C. Anderson
M∝Fiber	monoclonal (ascites)	Ad2 Fiber free and assembled	C. Cepko/ P. Sharp
R∝72K	polyclonal (serum)	Ad2 72K (DBP) protein	C.Anderson
R∝Ad5	multivalent (serum)	Ad2 or Ad5 virus particles	M.A. Bioproducts
M∝T (pAb 101) (pAb 108)	monoclonal (purified)	SV40 T antigen (carboxyl terminal region) (amino terminal region)	E. Gurney

a R = rabbit
b M = mouse

show this problem, was utilized. Reconstruction experiments in which I mixed varying amounts of infected and uninfected lysates verified that I could detect small amount of viral proteins against a background of cellular proteins.

Fluoroisothiocyanate conjugated rabbit anti mouse IgG and goat anti rabbit IgG were purchased from Miles-Yeda and used at recommended dilutions of 1:32 and 1:20 in PBS respectively.

Rhodamine-Bovine serum albumin was used as a counter stain (NIH Reagent) at final concentration of 1:40.

2.3.2 Immunofluorescent Staining of Monolayer Cells

Cells were seeded on four rectangular coverslips (11mm by 22mm No 2, Cat no. 6663-Q10, Thomas Scientific) in a 60mm Petri dish. The next day, the subconfluent coverslips were transferred to an empty Petri dish and 50 ul of the virus suspension of appropriate concentration was applied directly to the coverslip. The Petri dish containing the infected coverslip was then incubated in a humidified incubator at 37^{o}C for 2 hours. At the end of the infection, 5ml of complete medium was added. At different times post infection the coverslips were fixed by the method of Solomon et al.

(1979). Medium was removed and the coverslips were washed twice with PBS at room temperature. The coverslips were then incubated at room temperature overnight in a freshly prepared 3.7% formaldehyde solution. The formaldehyde solution was then removed and the coverslips were stored in PBS at 4°C. To permeabilize the cells, the coverslips were incubated for 2 minutes at -20°C in a cold 50% PBS: 50% acetone solution, followed by a 5 minute incubation in cold 100% acetone at -20°C and again 2 minutes in the 50% mixture. To stain the cells, coverslips were transferred to a dry 150 mm Petri dish which served as a holder. The appropriate primary antibody was applied to the coverslips and they were incubated for 45 minute in a humidified chamber at 37°C. Subsequently the coverslips were washed 3 times in PBS before the appropriate fluorescein conjugated antiglobulin was applied to the coverslips and again they were incubated for 45 min. The coverslips were then washed three times in PBS and once in distilled H_2O before they were mounted, cell side down, onto a slide using 50% glycerol as mounting media. The slides were viewed with a Leitz Microscope Ortholux Model equipped for fluorescence microscopy with filters for fluorescein and rhodamine. Positive and negative cells were counted per high power field (63X) under oil immersion and the percent positive cells was determined. Several fields and at least 200 cells were counted for each determination.

2.3.3 Virus Infection and Radiolabelling of Proteins

Cells were infected with Ad2 at at a MOI of greater than 50 pfu/cell for hamster cells and a MOI of 1-10 pfu/cell for human and CV1 cells in the following manner. The appropriate dilution of virus was made in 1% fetal bovine serum, and 0.2 ml of the virus suspension was added to cells in duplicate 60 mm Petri dishes. The virus was allowed to adsorb for two hours at 37^{0}C with rocking every 15 minutes. The virus was then removed, and cells were refed with complete medium. For pulse label, at the determined time post infection, the cells were starved for methionine by washing the monolayer with warmed PBS, and incubating with methionine-free DME (M.A.Bioproducts) for one hour prior to the addition of radioactive label. The cells were labeled with methionine free medium containing 100uci/ml ^{35}S Methionine (New England Nuclear, 1143.4 ci/mMole) for two hours (2 ml per 100mm Petri dish, 1ml per 60mm Petri dish). For the"long" label, at the determined time pi, the medium was replaced with a methionine free labelling solution containing 10% FBS, 10% DME and 10uci/ml ^{35}S methionine (5ml per 60mm Petri dish).

2.3.4 Extraction of Radiolabeled Protein and TCA Precipitation

At the end of the labelling period (pulse or long labels), the cells were scraped into the medium with a rubber policemen and transferred to a 15 ml centrifuge tube. Duplicates were pooled. The cells were sedimented by centrifugation in a counter top IEC centrifuge for 5 minutes at setting #4 (500 X g) at room temperature. The cell pellets were washed with PBS and frozen at -70°C. All subsequent procedures were performed at 4°C. Each pellet (the equivalent of two 60 mm Petri dishes) was suspended with 1ml of sonication buffer (0.14 M NaCl, 0.01 M Hepes, pH 8.5, 0.01 M EDTA and freshly added Phenylmethylsulfonyl Fluoride (PMSF) at 250 ug/ml). Samples were sonicated at 0.1 milliamps for 3 minutes in a Sonic Oscillator (Model DF101, Raytheon). The sonicates were then centrifuged at 1,500 rpm for 5 minutes and the supernatant was recentrifuged in a microfuge at 10,000 rpm for 1 minute. The resulting supernatant was referred to as the whole lysate.

For trichloroacetic acid (TCA) precipitation, duplicate 5ul aliquots of the lysates were added to glass culture tubes containing 1 ml PBS. One milliliter of a 20% TCA solution was added to each tube to effect a final concentration of 10% TCA. The tube was

then incubated on ice for 1 hour. The precipitates were collected on GFA filters under suction. The tubes and filters were washed with 5% TCA and cold 95% ethanol. The filters were dried under a heat lamp (Infra-Radiator, Fisher) and placed into scintillation vials. To each vial 1 ml NCS (tissue solubilizer, Amersham) was added. Vials were capped and put into a 50°C oven for 10 minutes. The vials were cooled before 10 ml of liquiflour (160 ml liquiflour concentrate in 3.784 liters toluene) was added and radioactivity determined by liquid scintillation spectroscopy (Beckman LS-250).

2.3.5 Immunoprecipitation

Immunoprecipitates of viral proteins to be analyzed by polyacrlamide gel electrophoresis (PAGE) were prepared in two ways. Equal volumes of whole lysates or varied volumes containing equal TCA precipitable radioactivity were precleared by incubation with Staph A for 1 to 2 hrs on ice. Samples were centrifuged in a microfuge and the pellets containing staph A and any non-specifically bound material were discarded. The antibody was added to the supernatants and allowed to incubate overnight at 4°C. 100 ul of staph A was then added to the samples and re-incubated on ice for 1 hr. The pellets were

collected by 1 min centrifugation in a microfuge and washed vigorously 3-5 times in pellet washing buffer (1M NaCl, 0.002M EDTA, 1% NP40 and 0.001M Tris PH 7.5). After the last wash, 20ul of 3X sample buffer (30% glycerol, 1.5% B-mercaptoethanol, 0.95 SDS, 0.18M Tris, pH 6.8, and a few grains of Bromophenol Blue) and 30 ul of doubly distilled H_2O was added to each sample. The samples were resuspended by mixing with a vortex and heated at 90^0C for 2 minutes to dissociate the immunoprecipitate. The samples were centrifuged in a micofuge and were loaded on a gel. Typically one-half of the samples were used per lane.

2.3.6 Polyacrylamide gel electrophoresis (PAGE) (Laemmli,1970)

For the preparation of a vertical 12.5% polyacrylamide gel, 20 ml of a separating gel solution was made up in the following manner. A 30% acrylamide (Biorad) and 0.8% bis (Biorad) stock solution was diluted appropriately in lower tris buffer (0.4% SDS, .15 M Tris-HCl, pH 8.8) plus 60 ul 10% ammonium persulfate (Biorad). The solution was put into a 125 ml side arm flask and then degassed. Nine microliters of Temed (Biorad) was added and mixed thoroughly. The solution was then poured into a space which was formed

when 2 glass plates were clipped together with spacers (1mm in width) on 3 sides and sealed with hot 1.7% agar (Nobel, Difco). A 0.1% SDS solution was layered on top of the gel to provide a smooth interface. After the gel was polymerized (about 30 min), a 10 ml stacking gel solution was prepared. The bis-acrylamide stock solution was diluted to a final concentration of 3% acrylamide in upper tris buffer (0.5 M Tris- HCl, pH 6.8, 0.4% SDS) plus 60 ul 10% ammonium persulfate and 20 ul Temed. A 12-well (1mm width) teflon comb was placed into the stacking gel after it had been poured on top of the separating gel. It was then allowed to polymerize undisturbed. After polymerization has taken place (about 30 min), the bottom spacer was removed and the glass plates were clipped onto a vertical gel apparatus (Aquaboque, L.I.) and the space between the glass plate and the wall of the apparatus was sealed with hot agar. Gel running buffer (0.025 M Tris, 0.19 M Glycine, 0.1% SDS) was added into the upper chamber and leakage into the lower chamber was checked. The lower chamber was then filled and a syringe with a bent needle was used to remove air bubbles at the bottom of the gel. The comb was then removed and the gel was subjected to 150 V for 10 min before the samples were loaded. Twenty five microliter samples were loaded per well and electrophoresis was performed for 4-5 hours at 150 V.

2.3.7 Fixation and preparation of gel for autoradiography

Following electrophoresis, the gel was removed and placed into a glass Petri dish with gel fixer solution (10% TCA, 10% Acetic Acid, 30% Methanol) for 2 hours or overnight. The fixer solution was then removed and replaced by Enhance (Autoradiography Enhancer, NEN) for 1 hour with gentle agitation. After removal of the Enhance, the gel was rehydrated for at least 1 hour in H_2O. A piece of Whatman 3mm paper was cut to appropriate size and placed on a gel dryer apparatus (Hoefer). The gel was carefully placed on top of the filter without distortion, covered with saran wrap, and dried with heat under vacuum using a dry ice-acetone trap. The dried gel was placed in an X-ray film holder cassette with Kodak X-Omat AR-2 film. The cassette was wrapped in aluminum foil and placed at $-70^{\circ}C$ for the duration of exposure. The film was exposed for varying lengths of time depending on the experiment. However all the companion gels presented in this thesis were exposed for the same length of time. The X-ray film was developed manually by conventional photographic processing methods, i.e. 3-5 minutes in developer (Pix-developer, Picker), 1 min in stop solution (Indicator Stop Concentrate,

Kodak) and 5 minutes in fixer solution (Pix-fixer, Picker). Densitometry on autoradiograms was performed on a Scanner Model Joyce Automatic Recording Microdensitometer (Model MK 111c Joyce, Loebl, and Co).

2.3.8 Immunoblotting Procedure (Towbin et al., 1979)

After the completion of electrophoresis, the gel was removed and placed into electroblot buffer (192mM glycine, 20% methanol, 24mM Tris, pH 8.3). The gel, in contact with a nitrocellulose filter (BA 85, Schleicher and Schuell), was placed into the Electoblot Apparatus (Hoeffer). After transfer was complete (16 hours at 0.1 amps), the marker lanes were removed and stained 2 min with amido black (0.1g amido black, 45% methanol, 10% acetic acid) and destained for 15 min in 10% acetic acid, and 45% methanol. This was done to verify complete transfer of proteins to the nitrocellulose filter and for molecular weight determination.

For the blotting reaction, the filters were placed in a heat-sealable plastic bag ("seal-a-meal", Dazey) and incubated 1 hour in blocking buffer containing 3% gelatin in Tris buffered saline (TBS) (50mM NaCl, 20mM Tris, pH 7.5). At the end of the incubation period, the bag was opened at a corner and the blocking buffer

was squeezed out and replaced with a primary antibody solution (i.e. antibody to the viral protein) in 1% gelatin. The bag was resealed and incubated on a shaker for 1 hour at room temperature. The filters were then washed in TBS four times, placed into new bags and incubated with goat anti mouse immunoglobulin conjugated with horse radish peroxidase (Bio Rad) for 1 hour on a shaker at room temperature. The filter was then washed four times and developed in a solution of 0.05% 4 chlor-1-napthol, 0.01% H_2O_2 in TBS. When the filter was fully developed (approximately 15 minutes), it was rinsed with H_2O and photographed. It may be stored in the dark at 4°C (in H_2O) for several months.

2.4 PROCEDURES FOR DNA ANALYSIS

2.4.1. Determination of Viral DNA Synthesis (Horwitz et al.,1981)

Cultures were infected with Ad2 as previously described. At appropriate times pi, the cells were pulse labelled with ^{3}H-thymidine (TdR) (S.A 79 Ci/mM) at 5.0 uci/ml for one hr at 37^{o}C followed by a 30 min chase in 2 X 10^{-5} M TdR. Incorporation was terminated by scraping the cells into the medium and pelleting them at the 500 X g. The pellet was washed with PBS and resuspended in 0.15 M NaCl for immediate analysis on freshly prepared alkaline sucrose gradients.

Gradients were preformed in cellulose nitrate tubes (0.5 by 4 in.) for centrifugation in the SW 27.1 rotor of a Beckman ultracentrifuge. A cushion of 0.5 ml of $CsCl_2$ in water (ρ=1.8) was placed in the bottom of the tube followed by a linear 15 ml gradient (5 to 20% sucrose) prepared in 1.0 M NaCl, 0.19 N NaOH, and 0.01 M EDTA. 0.5 ml of the NaOH-NaCl-EDTA solution (without sucrose) containing 0.5% sodium deoxycholate was placed over the preformed gradient. Cells in 0.5 ml of 0.15 M NaCl were carefully layered onto the gradients. Centrifugation was performed at 4^{o}C for 16 hours at 24,000 rpm. Gradients were fractionated with

a polystaltic pump through a hollow tube placed 1.5 cm above the bottom of the tube. Fractions were collected drop-wise (approximately 0.5 ml per fraction). Under these conditions, replicated cell DNA sedimented onto the $CsCl_2$ cushion and virus DNA appeared in mid gradient.

2.4.2 Recombinant Plasmid DNA

Plasmid DNA's were obtained from other laboratories and used without further genetic manipulation. Purified pAdD26SV(A)#1 was provided by R.Kaufman and used directly. pMKSV40(Bgl I-R)(6-1) was provided by Y. Gluzman, in X1776. The plasmid was reisolated in this laboratory and introduced into DH-1 for routine experimental purposes (Litzkas et al., 1984). Purified DNA was kindly provided by Dr. K.K. Jha. All procedures involving recombinant DNA were performed in accordance with NIH guidelines.

2.4.3 Isolation of Cellular DNA for DNA analysis

Cells were grown to confluence in 100mm Petri dishes and refed 24 hours before harvesting. Monolayers on 4-5 Petri dishes were washed once with sterile PBS

and scraped with a rubber policeman into PBS (5 ml/Petri dish). Cells were pelleted by centrifugation for 5 min at 600 rpm in 15 ml conical polypropylene tubes. The PBS was decanted and the cells were resuspended in 2.5 ml of lysis buffer (10mM Tris, pH 7.9, 10mM Na EDTA, 100mM NaCl). SDS was added to a final concentration of 0.2% and tubes were immediately incubated at 65°C for 15 min to inactivate endogenous nucleases. Tubes were cooled to room temperature and proteinase K (Boehringer Mannheim, 5mg/ml stock) was added to a final concentration of 100 ug/ml, mixed by inversion and incubated at 37°C overnight. The cell lysate was extracted with an equal volume of buffer-saturated redistilled phenol; phases were separated by centrifugation at 2000 rpm for 15 min at room temperature. The aqueous phase was removed with a wide-bore pipette and re-extracted with phenol:chloroform:isoamyl alcohol (48:48:2) once, followed by two extractions with chloroform:isoamyl alcohol(24:1). The viscous aqueous phase was transferred to a glass corex tube; 2.5 volumes of ice cold 95% ethanol was added and the mixture was swirled gently to precipitate the DNA. The DNA precipitates were centrifuged at 2,000 rpm for 20 minutes. The pellet was washed in 70% ethanol and dried under vacuum. The pellet was dissolved in TE buffer plus 10 ug/ml RNAse A overnight at 37°C. It was then dialyzed

against TE buffer and the DNA concentration was determined by diluting the sample 1:50 in TE and reading the uv absorption at 260nm (1 O.D.is equivalent to 50 ug/ml double-stranded DNA).

2.4.4 Agarose gel electrophoresis (Southern, 1980)

For preparation of a 10 % agarose gels, the correct amount of powdered agarose (Seakem, ME) was added to a measured amount of Tris phosphate (TPE) electrophoresis buffer (0.08 M Tris-phosphate, 0.008M EDTA). Agarose was melted by brief autoclaving in a pressure cooker, allowed to cool to 50^{0}C, ethidium bromide was added to a final concentration of 0.5 ug/ml, and the mixture was poured into the agar-sealed bed of a horizontal electrophoresis apparatus. A well-forming comb was immediately set into position and the gel allowed to harden at room temperature, after which enough TPE (containing 0.5 ug/ml ethidium bromide) was added to the apparatus to cover the gel to a depth of 1mm. The well former was carefully removed and the gel was pre-run at 30 V (constant voltage) for at least 10 min. Cellular or plasmid DNA was digested overnight at 37°C with excess Eco R1 or Hind III restriction enzymes under conditions recommended by the supplier (Bethesda Research Laboratories). The reaction was

terminated by addition of EDTA to 10 mM. DNA samples were mixed with a vortex in 0.1 volume of 10X gel loading buffer (0.25% bromophenol blue, 10X TPE, 0.1% SDS, 25% Ficoll 400 [Pharmacia] in H_2O), heated at 65°C for 10 min, and applied to the slots of the submerged gel with a micropipettor. Ten micrograms of cellular DNA was loaded per slot. Electrophoresis was conducted at 40 V for 16 hours. Lambda phage DNA cleaved by the restriction enzyme Hind III was used as a molecular weight marker. DNA in the gel was visualized on an ultraviolet transilluminator light box (wave length is 302 nm) and photographed with a Polaroid camera using type 57 film.

2.4.5 Transfer of DNA from agarose gels to nitrocellulose (Southern, 1975)

After electrophoresis, gels were soaked in 500 ml of a solution of 0.5 M NaOH and 1.5 M NaCl for 1 hour at room temperature with constant shaking to denature the DNA. Gels were rinsed twice with distilled water and neutralized by soaking in 500 ml of a solution of 0.5 M Tris-HCl (pH 8.0) and 1.5M NaCl for 1 hour at room temperature with constant shaking. A stack of Whatman 3MM paper (9 X 11 inches) 12 layers thick in a plastic cafeteria tray was saturated with 20 X SSPE

(3.6 M NaCl, 200mM NaH_2PO_4, 20mM EDTA, pH 7.4). The gel was placed on top of the stack, carefully removing any air bubbles by rolling with a glass rod. A piece of nitrocellulose paper (BA, Schleicher and Schuell) cut to the size of the gel was pre-wet in water and placed with forceps on top of the gel, removing air bubbles as before. Two pieces of 3MM paper also cut to size and pre-wet in water, were placed on top of the nitrocellulose filter. A stack of paper towels (2-3 inches high) cut to the size of the gel was placed on top of the 3MM paper; a glass plate was put on top of the stack and weighed down with a 1 kg weight. Transfer of DNA was allowed to proceed for 16 hours. The stack was then disassembled; the nitrocellulose filter was peeled off the dehydrated gel and rinsed briefly in 2X SSPE. The wet filter was sandwiched between two pieces of 3MM paper and baked for 2 hours at 80°C under vacuum.

2.4.6 Nick translation of DNA

^{32}P-labeled DNA probes with a specific activity greater than 10 cpm/ug were prepared by the method of Rigby et al. (1977) as modified by Maniatis et al. (1982). The nick translation reaction was set up by the addition of the following reagents to a 1.5 ml

Eppendorf tube: 1 nmole of each ulabeled dNTP (generally dGTP and dTTP), 100 pmoles of each (α-P) dNTP (New England Nuclear, specific activity 800 Ci/mmole; generally dCTP and dATP), 1 ug of DNA, 5 ul of 10X nick translation buffer (0.5 M Tris-HCl, pH 7.2, 0.1 M $MgSO_4$, 1mM DTT, 500 ug/ml BSA), and deionized H_2O to a final volume of 47 ul. The mixture was chilled on ice for 5 min, after which time 1.5 ul of 100 ng/ml solution of DNase I (Worthington) in 50% glycerol was added and mixed by mixing thoroughly with a vortex. The nicking reaction was allowed to proceed on ice for 10 min, after which 1.5ul (10 units) of E. coli DNA polymerase I (Boehringer Mannheim) was added. The nick translation reaction was performed at 15°C for 60 min. The reaction was terminated by addition of 2 ul of a 0.5M EDTA and heating at 65°C for 5 min. The nick-translated DNA was separated from unincorporated dNTP's by "spun-column" chromatogrphy as described in Maniatis et al. (1982). The bottom of a 1 ml tuberculin syringe was plugged with a small amount of glass wool. The syringe was packed with Sephadex G-50 (pharmacia) previously swelled in STE (100 mM NaCl, 10mM Tris-HCl, pH 8.0, 1mM EDTA) by centrifugation in a 15 ml conical centrifuge tube at 1600 X g for 4 min until the packed column volume was 0.9 ml. One hundred microliters of STE was added to the column and recentrifuged under identical conditions. This step was repeated once. The nick

translation reaction, brought to a volume of 100 ul with STE, was applied to the column which was then centrifuged exactly as before, collecting the 100 ul of effluent from the syringe in a decapped Eppendorf tube. (The unincorporated [^{32}P]dNTP's remain in the syringe, which is discarded). The recovered probe DNA (mouse DHFR containing plasmid Chang et al. (1978) and large Pst I fragment of SV40 eluted from agarose gel), was diluted with TE to a final volume of 0.5 ml; a small aliquot was used to determine specific activity of the probe by liquid scintillation spectrometry.

2.4.7 Hybridization and autoradiography of Southern filters

The hybridization procedure described by Wahl et al. (1979) was followed with slight modification. The baked filter was immersed in 2x SSPE for 2 min, then placed into a "seal-a-meal"bag sealed on three sides. Ten ml of prehybridization solution (consisting of 5X SSPE, 5X Denhardt's reagent (0.1% Ficoll, o.1% polyvinyl pyrrolidone, 0.1% BSA), 0.5% SDS, and 100 ug/ml heat-denatured, sheared salmon testis DNA, (Calbiochem) was added to the bag, which was then sealed and incubated for at least 3 hours hours submerged in a water bath at 45°C. A corner of the bag was cut off and the prehybridization solution was

squeezed out. Five ml of hybridization solution (consisting of 50% formamide, 5X SSPE, 10% dextran sulfate (Pharmacia), 0.1% SDS, 100 ug/ml heat-denatured salmon testis DNA, and 5 X 10^6 cpm of heat-denatured P-labeled probe DNA) was added to the bag. Excess air was removed from the bag, which was then heat-sealed and incubated for 16 hours submerged in a water bath at 45 C. At the end of the hybridization, the filter was placed in a glass tray containing 2X SSPE and 0.5% SDS at room temperature and agitated for 5 min. The solution was decanted and a fresh 2X SSPE and 0.1% SDS was added at room temperature for 15 min with occasional shaking. It was finally washed with 0.1X SSPE and 0.1% SDS with gentle agitation at 50°C for 2 hours. The filter was blotted between 2 sheets of 3 MM paper and wrapped while still damp in Saran Wrap. In a darkroom, the filter was mounted in an x-ray film holder; a single sheet of Kodak X-Omat AR-2 film was placed between the filter and an intensifying screen (Cronex Lightning-Plus, Dupont) following the method of Laskey and Mills (1977). The film holder was wrapped in aluminum foil and placed at -70°C for the duration of exposure. The X-ray film was developed manually by conventional photographic processing methods as described previously.

2.4.8 Dot Blot Hybridization (Kafatos, 1979)

Appropriate dilutions of DNA for the purpose of quantitation are made in 1mM EDTA, 1mM Hepes, pH 7.5, and 0.3 N NaOH, in a total volume of 50 ul. Samples were incubated overnight at room temperature to hydrolyze the RNA. Before dotting, a precut nitrocellulose filter (BA85, Schleicher and Schuell) was soaked in H_2O for 15 minutes followed by 1M ammonium acetate for 15 minutes. The nitrocellulose filter was placed on a Filtration Manifold (Minifold, Schleicher and Schuell) on top of 2 pieces of Whatman type filter paper so that the sample would spread more evenly over the membrane surface within each well. Immediately before the DNA was added to the wells, an equal volume (50 ul) of 2M ammonium acetate was added to the samples. The DNA was added to the wells under vacuum. The filter was then removed and washed in 6 X SSC for 10 minutes and baked for 2-3 hours at 80°C under vacuum. Subsequent hybridization was performed as described for the Southern Procedure.

CHAPTER 3: RESULTS

3.1 Ad2 INFECTION OF CHINESE HAMSTER CELLS

This laboratory is engaged in the long term study of the regulation of mammalian cell DNA synthesis. Emphasis has been placed on genetic approaches such as the isolation of conditional-lethal, temperature-sensitive mutants in pathways directly relevant to DNA synthesis in established chinese hamster cell lines. Biochemical analysis of such mutants is often quite complicated. Virus infection frequently utilizes a wide-range of host functions. The virus-cell interaction has been successfully exploited, in many cases, as a model system for analyzing cell as well as viral biochemistry. The human adenoviruses are a case in point. This thesis was undertaken to characterize the parameters of virus-cell interaction of adenovirus and chinese hamster cells in culture, and determine whether such infection could be effective as such an approach.

It was reported by Longiaru and Horwitz (1981), shortly after the onset of this thesis research, that

human adenovirus type 2 (Ad2) was able to initiate infection of a subline of chinese hamster ovary cells (CHO) in suspension. The infection was, however, restrictive since there were 6000-fold less infectious virions produced than in a permissive cell line (HeLa). As the initial step in these studies, it was sought to determine whether these findings were applicable to infection performed in monolayer culture with this and other chinese hamster cell lines. Ad2 infection of a variety of cell lines was performed under standard experimental conditions, involving a 2-hour period for adsorption. Samples were harvested following adsorption, at 16-24 hrs post infection (pi) prior to the expected onset of production of progeny virus ("eclipse"), and 3 days and/or 5 days pi when progeny virus would have been expected to have accumulated. The results obtained with a wide-variety of chinese hamster cell lines are presented in Table 6. The origins of the cell lines and their relationships have been summarized in Tables 2 and 3 in the Materials and Methods. None of the cell lines showed significant levels over background (i.e. adsorption and/or eclipse values) although there was a suggestion of an increase in the case of the non-established chinese hamster line CHE. Even in that case, the average yield would be less than 0.001 infectious virion per cell. Three different human cell lines were included for

Table 6

GROWTH OF Ad2 IN CH CELLS[a]

CHINESE HAMSTER CELL LINES	PFU/CULTURE ADSORPTION	ECLIPSE	DAY 3
CHO-K1	2.1×10^3	2.7×10^3	3.5×10^3
TNT	1.0×10^3	7.5×10^2	5.0×10^2
DON	ND[b]	ND	2.5×10^2
V79	6.3×10^2	2.5×10^2	1.2×10^2
CHE	1.3×10^2	1.3×10^2	3.8×10^3
HUMAN CELL LINES			
HELA	ND	ND	1.8×10^9
HEK	ND	ND	6.5×10^8
HS74	5.0×10^3	3.8×10^3	1.9×10^6

a Cells were infected at MOI of 0.5 pfu/cell and assayed as described in Materials and Methods.

b Not Determined

comparison; an established cell line commonly used for Ad2 replication (HeLa), and early passage normal diploid embryonic kidney cells (HEK) typically used for viral plaque assays, and fetal bone marrow derived fibroblasts (HS74). All three were permissive although the levels of virus produced did vary (range: $1.9 \times 10^6 - 1.8 \times 10^9$). These studies were performed under conditions of relatively low multiplicity of infection (0.5 pfu/cell) to facilitate detection of even modest virus replication. Two representatives of established Chinese hamster cell lines were selected for more detailed study; TNT derived from CHO-K1 and E36-34 derived from V79. Both cell lines were retested at a 30-fold higher moi (15 pfu/cell) see Table 7. Again, no evidence for a significant increase was observed. (As expected, there was higher residual input or adsorbed virus consistent with the higher initial moi). Virus assayed at 3-5 days pi was within 2-fold of the minimal, eclipse, value.

Virus infection can be divided into a series of convenient stages: adsorption, penetration and uncoating of viral particle, expression of early genes, viral DNA synthesis, expression of late viral genes (after onset of DNA synthesis) and assembly of the viral particles. Given the fact that no virus was produced, the stage at which viral replication was blocked was then examined.

Table 7

GROWTH OF Ad2 IN CH CELLS AT 2 DIFFERENT MOI[a]
PFU/CULTURE

CELL LINE	ADSORPTION	ECLIPSE	DAY 3	DAY 5
MOI=0.5				
V79	3.8×10^3	2.5×10^3	ND[b]	5.0×10^3
CHO	6.3×10^3	2.5×10^3	5.0×10^3	1.3×10^3
MOI=15				
V79	3.0×10^4	5.0×10^4	2.1×10^5	ND
CHO	1.8×10^5	1.0×10^5	1.5×10^5	1.6×10^5

a procedures as in Table 6
b Not Determined

From data in the preceeding section there was a suggestion that virus adsorption and penetration were occurring since the titer of virus decreased with time post infection. This point was addressed more directly by determining the synthesis of early viral proteins (not dependent on DNA synthesis) by immunofluorescence (IF). The Ad DNA-binding protein of 72,000 daltons (72K), was selected as a representative early-protein. Coverslip cultures were infected with Ad2 at an MOI of 84 and harvested at different times post infection. Cells were fixed and stained (see Materials and Methods), the positively stained cells (green fluorescent nuclei) were counted, and the percent positive cells were calculated. The results are summarized in Table 8. 72K is produced in a significant proportion of the cells. The number is dependent on the multiplicity of infection (Data not shown). The highest values are obtained at 40 hrs pi (Data not shown). At comparable virus input, infection is less efficient than in HeLa cells; therefore, it was thought that there might be a block early in virus infection; for example, prior to or at the level of early gene expression. However, at the multiplicities of infection of 84, approximately 50% or greater of the cells were infected; more than enough to show virus production if this were the only problem. Consequently, it was concluded that the more critical block is subsequent to synthesis of 72K.

Table 8

PERCENT 72K POSITIVE CELLS

CELL LINE	72K
CHO-K1	38
TNT (CHO)	73
A23 (DON)	45
E36-34 (V79)	38
CHE	47
HELA	100

Chinese hamster cells infected at MOI of 84, fixed 36 hr pi.
HeLa infected at MOI of 17, fixed at 24 hr pi.

Ad2 DNA replication in CHO, specifically in TNT since it seemed most susceptible, was investigated to see whether it was taking place and, if so, whether it was altered. TNT cells were infected with Ad2 at an MOI of 75. HeLa cells were infected with Ad2 at an MOI of 6. At 20 hours pi for HeLa, and at 10, 24, 33, 47 hours pi for TNT, the cells were labeled with high specific activity ^{3}H-thymidine for one hour followed by a chase for 30 min with excess cold thymidine as described in Materials and Methods. The time course of Ad2 DNA replication was analyzed by alkaline sucrose gradients which determines the amount of unit length, single strand viral DNA. Cell DNA sediments to the bottom of the gradient; viral DNA is in mid gradient. The results are shown in Figure 4. Ad2 DNA replication in these cells occurs to an extent consistent with a normal level but is somewhat delayed. Viral DNA was not detected at 10 hr pi; however, appreciable levels were observed at 24 and 33 hr pi. Incorporation was significantly reduced at 47 hr pi. As expected, uninfected cells showed extensive incorporation into high molecular weight DNA which sedimented much more rapidly than viral DNA; there were minimal counts per minute in the region in which viral DNA would be expected. In a second experiment (Figure 5), it was observed that the level of viral synthesis in CHO at 32 hr pi was similar to that observed in permissive HeLa

Figure 4: Alkaline Sucrose Gradient Analysis of the time course of Ad2 DNA synthesis in CHO. TNT cultures in 60mm dishes were infected at an MOI of 75 pfu/cell and pulse labeled for 1 hr with a total of 25uci in 5ml ^{3}H TdR (79Ci/mM new England Nuclear) followed by a 30 min chase with 2 X 10^{-5} M thymidine. Two thirds of the culture were sedimented on a gradient as described in Materials and Methods. (+) is the sample pulsed at 12 hrs pi, (o) is the 24 hr sample, ($\triangle$) is the 33 hr sample, (X) is the 47 hr sample, and ($\Diamond$) is the uninfected control.

FIGURE 4

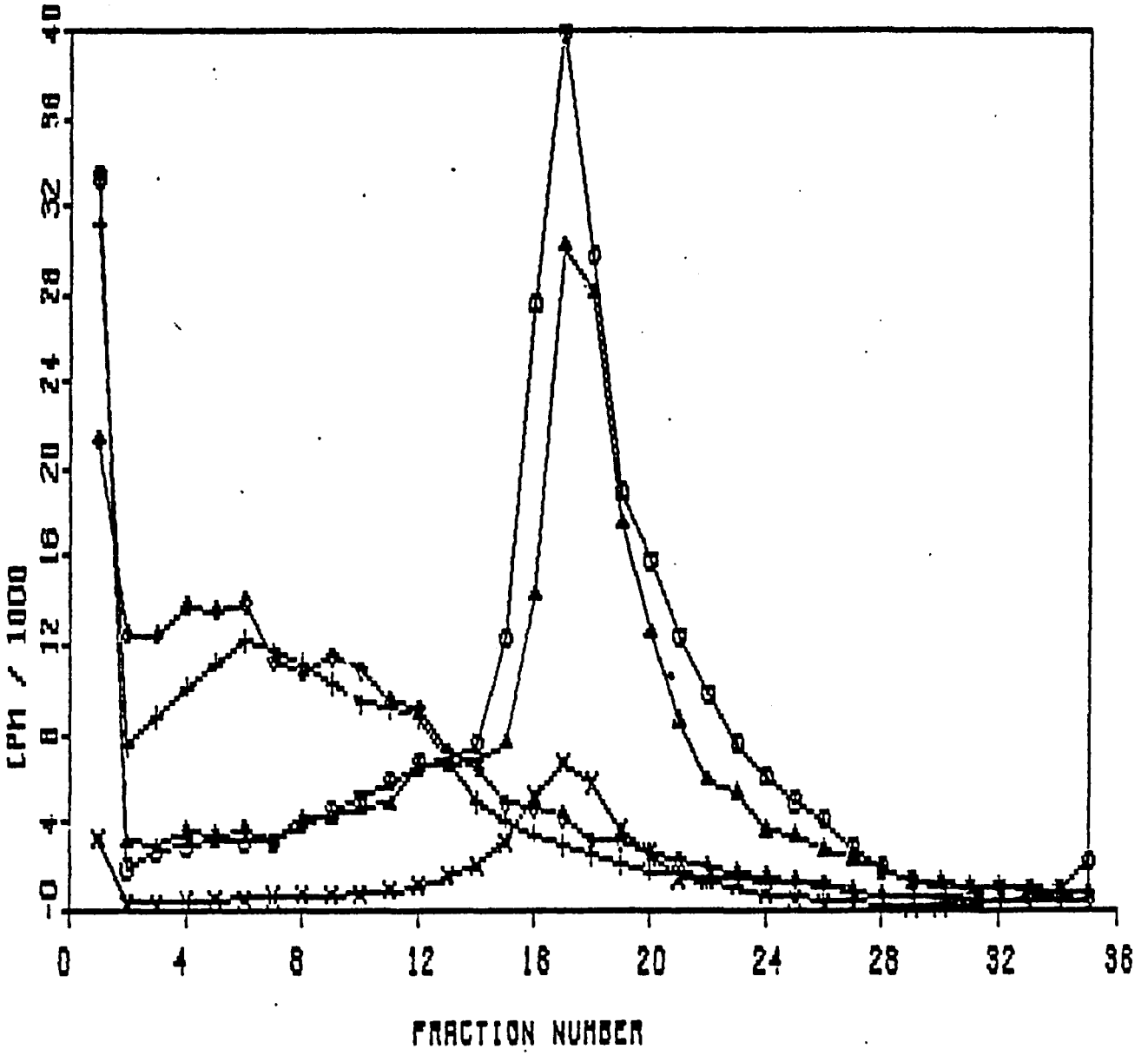


Figure 5: Comparison of Ad2 DNA synthesis in CHO and HeLa cells by Alkaline Sucrose Gradient Analysis. Two-thirds of cultures of TNT cells which had been mock-infected or virus- infected at an MOI of 75 and labeled at 32 hrs pi for 1 hr (panel A) and 1/3 of cultures of HeLa cells, mock-infected or virus-infected at an MOI of 6 and labelled 20 hrs pi for 1 hr (panel B) were processed as described in Materials and Methods and the legend to Figure 4. Infected samples (O), Uninfected samples (Δ).

FIGURE 6-A

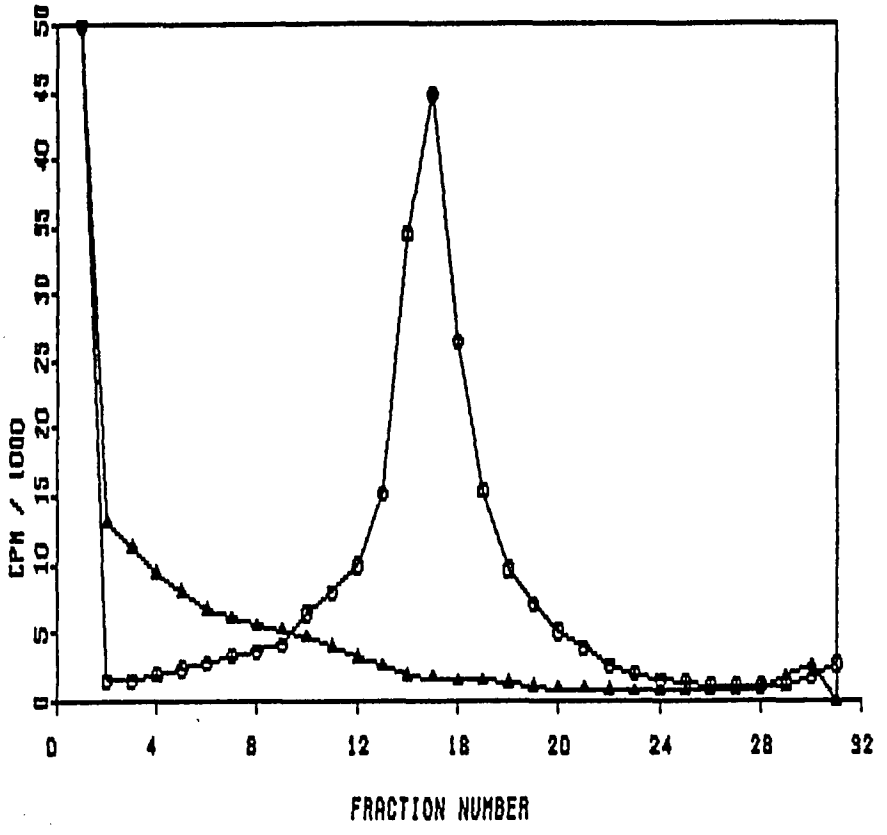
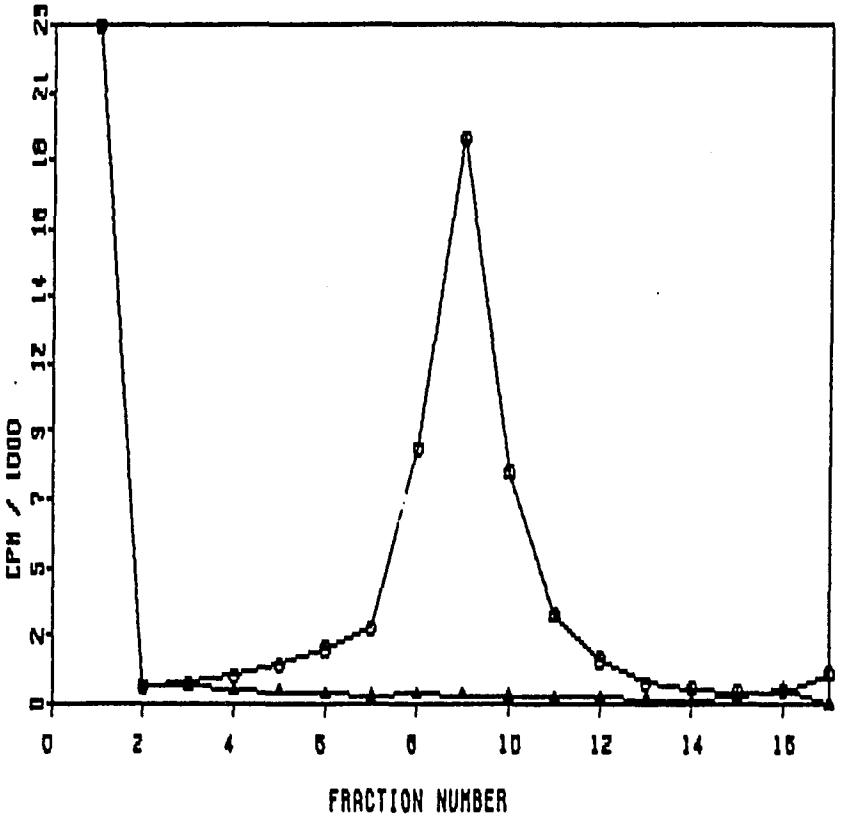


FIGURE 6-B



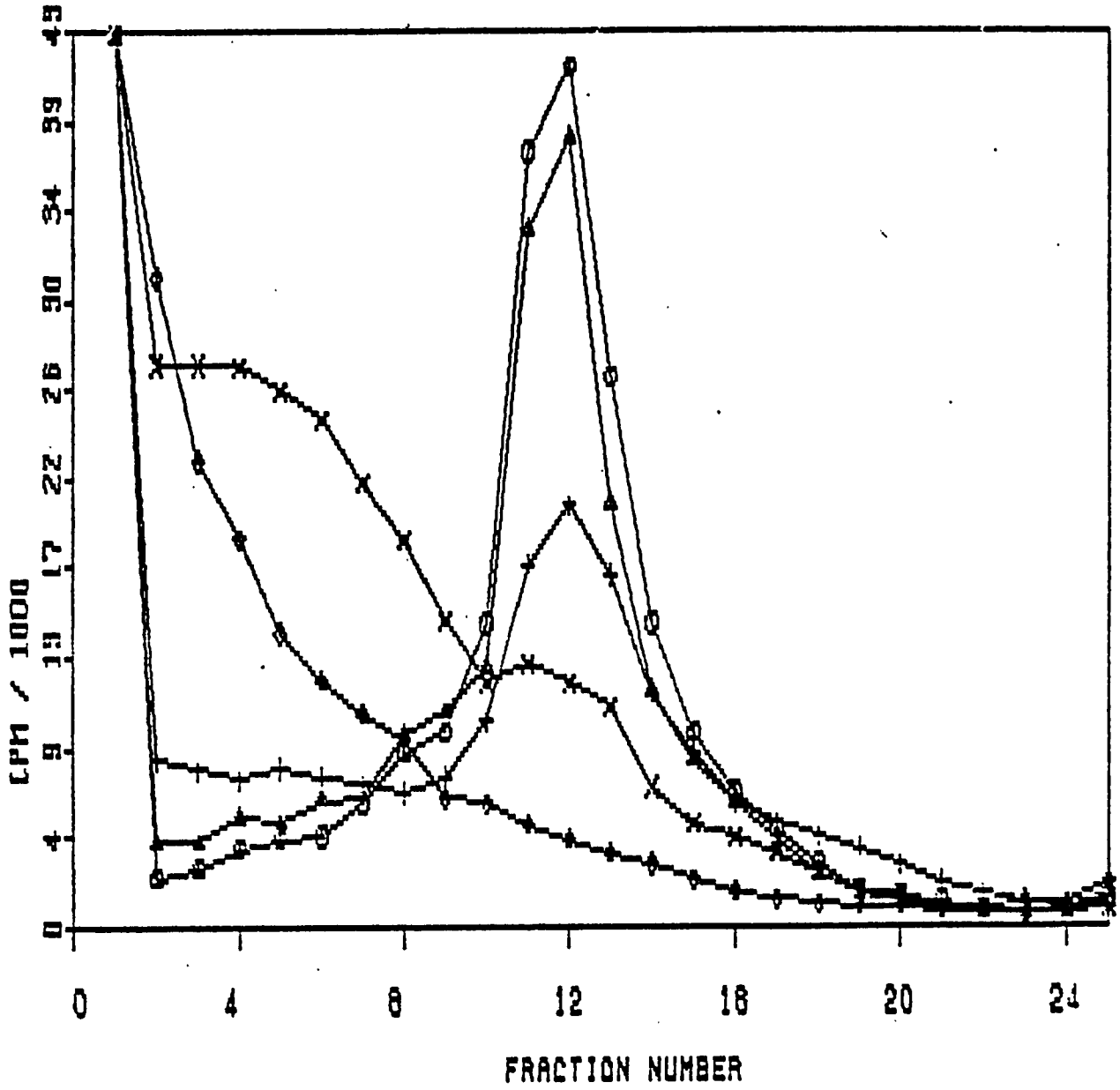
cells (20 hr pi). These results were consistent with those previously noted by Longiaru and Horwitz who concluded that peak rates of viral DNA synthesis in CHO were obtained at 33-36 hr pi and were comparable to that in permissive cells.

In another experiment, the relationship between virus MOI and DNA synthesis was examined. TNT cells were infected with several dilutions of stock Ad2 i.e., 1:50, 1:100, 1:250, and 1:1000 (estimated moi between 40 and 2 pfu/cell). Samples were labeled and harvested at 36 hr pi. Figure 6 shows the results. The amount of DNA synthesis taking place is dependent upon the amount of input virus. It can also be determined from these data that a 1:2 dilution of the standard dilution of 1:50 (used throughout most of this study) does not reduce the amount of DNA synthesis by 2-fold. This result is important to note because it indicates that the input virus is in excess.

The levels of 72K protein and DNA synthesis do not seem to explain the deficiency in virus production. Indeed these results are in perfect agreement with the findings of Longiaru and Horwitz. However, they reported an inability to detect late viral protein production. We consequently examined infected cells using the same IF methods as for the 72K with a variety of monoclonal antibodies to late proteins including 2 structural proteins (Hexon and Fiber) and a

Figure 6: Alkaline Sucrose Gradient Analysis of viral DNA synthesis as a function of different input MOI. Virus and mock infected cultured TNT cells were pulse labelled at 36 hr pi and processed as described in Materials and Methods and legend to Fig 4. Virus (1×10^{10} pfu/ml) was diluted in 1% FBS-DME and used for infection at 1:50, MOI=40 pfu/cell (o), 1:100, MOI=20 pfu/cell (Δ), 1:250, MOI=10 pfu/cell (+), and a 1:1000, MOI=2 pfu/cell (×) and mock-infected with diluent alone (◊).

FIGURE 6


CPM / 1000
0
4
9
13
17
22
26
30
34
39
43
0
4
8
12
16
20
24
FRACTION NUMBER

nonstructural protein (100K). Controls included HeLa infected with Ad2.

The results are summarized in Table 9. The coverslip cultures were fixed 40 hr pi. The monoclonal antibody directed against the 100K protein, the major late non-structural protein, is reactive with free 100K and 100K-hexon complex. The number of positive cells varied among the several hamster cell lines; in all cases, it can be seen that the 100K is somewhat reduced compared to the percent positive for 72K in the same experiment. (see Table 8)

The monoclonal antibody directed against the hexon, the major virion capsid protein, is reactive against hexon trimers and their association into ninemers but not to the monomer form of hexon. From the numbers in Table 8, a further reduction of percent positive cells is observed compared to 100K and 72K. The percent of cells positive for fiber, the spike like protein found at the vertices of the icosohedral structure, was even more drastically reduced. The monoclonal antibody is reactive with free and assembled fiber.

From these findings it was concluded that there is expression of at least some late proteins, confirminmg DNA synthesis. Some of the late proteins are expressed to a lesser degree than others, especially fiber protein. The failure to detect viral proteins at

Table 9

IMMUNOFLOURESCENT STAINING FOR LATE VIRAL PROTEINS
PERCENT POSITIVE CELLS

CELL LINE	100K	HEXON	FIBER
CHO-K1	19	5	< 0.3
TNT	14	3	< 0.3
A23	0	NT	0
V79	8	< 1	< 1
CHE	25	NT	3
HELA	100	100	100

Chinese hamster cells infected at MOI of 84, fixed at 40 hr pi.
HeLa cells infected at MOI of 17, fixed 24 hr pi.

normal levels was not due to poor quality of the immunoreagents since 100% of the HeLa cells were positive in all instances. A possible explanation for the reduced frequencies could be that expression and/or accumulation of these polypeptides in CHO were delayed, as suggested by the fact that DNA synthesis is delayed. When the stained cultures were looked at very late after infection (57 hrs pi), it was difficult to evaluate the coverslips because of alterations in cell morphology (rounding). Therefore, the amount of late proteins that were made could have been underestimated. To evaluate that possibility, it was decided to analyze infected cells using PAGE.

CHO cells were infected with Ad2 at a MOI of 150 pfu/cell. The cells were labelled for 24 hr ("long label") or 2 hr ("pulse label") with ^{35}S-Methionine at different times pi (as described in Materials and Methods). Figure 7 shows an autoradiograph of such a gel including extracts from HeLa as a permissive infection for comparison. Lane A contains uninfected HeLa lysate whereas lane B contains infected HeLa, readily demonstrating 5 major viral proteins (among others): the hexon polypeptide at 120K, 100K, Penton at 85K, the DNA binding protein at 72K, and Fiber at 65K. As expected, there is also a shut off of host protein synthesis. Lanes C to F contain the CHO lysates which were pulsed for 2 hrs; uninfected (lane C) or at 1, 2,

Figure 7: PAGE analysis of whole cell lysates:comparison of HeLa and CHO cell lines. Mock-infected or infected cells were labelled with ^{35}S-methionine at different times pi. HeLa cells were infected at a MOI of 5 pfu/cell and pulse-labelled from 24-26 hrs pi. CHO cells (TNT subline) were infected at a MOI of 150 pfu/cell and labelled for 2 hrs or for approximately 24 hrs as indicated for each lane. Whole cell lysates were prepared and analysed by PAGE and autoradiography as described in Materials and Methods. Equal amounts of TCA-precipitable cpm were loaded in each lane (2 X 10^5 cpm).

Lane A: HeLa, mock-infected pulse labelled 24-26 hr pi.

Lane B: HeLa, infected, 24-26 hr pi.

Lane C: CHO, mock-infected pulse labelled 19-21 pi.

Lane D: CHO, infected, 19-21 hr pi.

Lane E: CHO, infected, 43-45 hr pi.

Lane F: CHO, infected, 66-68 hr pi.

Lane G: CHO, mock-infected long labelled, 21-43 hr pi.

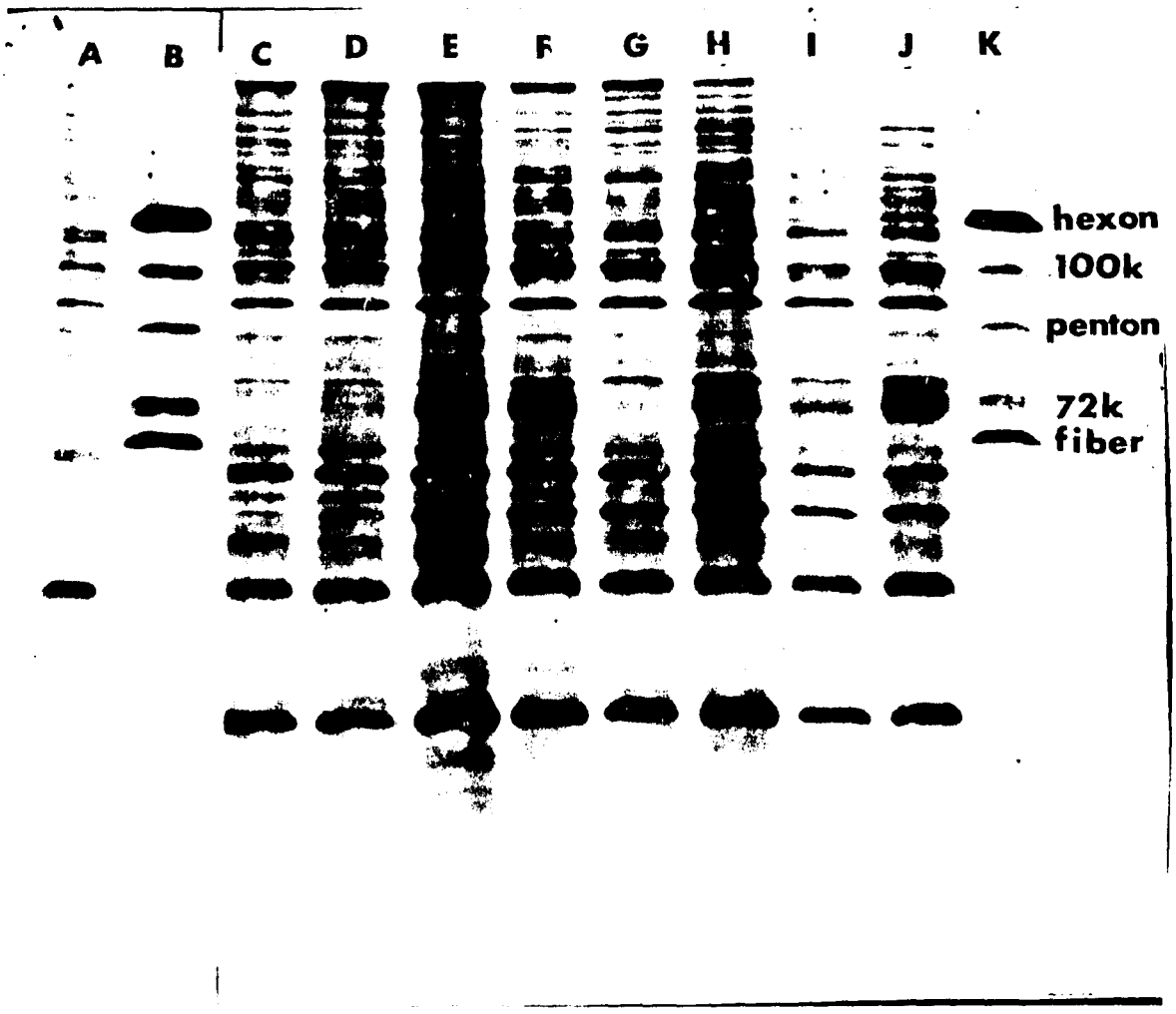
Lane H: CHO, infected, 21-43 hr pi.

Lane I: CHO, infected, 43-66 hr pi.

Lane J: CHO, infected, 66-90 hr pi.

Lane K: HeLa, as marker.

Figure 7



and 3 days pi (lanes D, E and F, respectively). Lane D is essentially not different from the uninfected lysate; however, one can see a 72K band in lanes E and F. Lanes G to J contain CHO lysates from cells which were labelled continuously for 24 hr intervals: uninfected (lane G) or from 1 to 2 days, from 2 to 3 days, or from 3 to 4 days pi (lanes H, I, and J respectively). The only viral band which is readily detectible is again the 72K. From the fluorescent antibody data, it was known that the 100K and hexon were being produced. The high background of cell proteins, most likely because of persistant host protein synthesis by the residual population of uninfected cells (25-50%), is presumably masking their detection. Immunoprecipitation on these lysates to look for these viral late proteins was performed.

Late viral proteins were looked for using immunoprecipitation in conjunction with PAGE. The antibodies used were the same as those in the IF studies unless otherwise noted. First, to confirm the finding for 72K, the samples were assayed with antibody prepared against purified 72K protein in rabbit. This polyvalent antisera should precipitate only the 72K protein. Immune complexes were adsorbed with bacteria bearing Staph A, washed extensively and eluted for PAGE. Figure 8 is the resultant autoradiograph of such an immunoprecipitation and electrophoresis. The

72K protein is clearly detectible in all the infected samples (lanes B, D, E, G-I). An immunoprecipitate from a small amount of lysate prepared from infected HeLa is included as molecular weight marker in the outside lane (J).

The late viral proteins were then looked for using immunoprecipitation. Equal volumes of samples were immunoprecipitated using monoclonal antibody directed against the 100K protein provided by Dr. C. Cepko. Figure 9 is the resultant autoradiograph of the appearance of 100K protein at different times pi. Lysate from infected CHO cells pulse labelled at day 1 (lane C) is not different than the uninfected control (lane B). This result is as expected. Since 100K is a late protein; its production begins after DNA synthesis has been initiated. The pulse time at 19-21 hrs pi is before the peak of DNA synthesis, estimated to be after 24 hrs pi for these cells. Lysates from infected cells pulse-labelled at day 2 (lane E) and day 3 (lane F) clearly show the presence of the 100K protein although a relatively long exposure is required, making contaminting cell proteins more evident. Samples prepared from infected cells labelled for long periods day 1-2 (lane H), day 2-3 (lane I), and day 3-4 (lane J) all show the presence of 100K protein

Another late protein that was not detected in the

Figure 8: PAGE analysis of ^{35}S-methionine labelled CHO cell lysates immunoprecipitated with antibody against 72K protein. CHO samples were infected and labeled as described in Materials and Methods and the legend to figure 7. Equal volumes of lysates (200 ul) were immunoprecipitated with 2 ul of rabbit anti 72K protein antibody. Half of the immunoprecipitated sample was loaded onto the gel.

Lane A: CHO, mock-infected, pulse labelled 19-21 hr pi. contained 3.8 X 10^7 TCA precipitable cpm.

Lane B: CHO, infected, 19-21 hr pi, 2.8 X 10^7 cpm.

Lane C: CHO, mock infected, 43-45 hr pi, 2.8 X 10^7 cpm.

Lane D: CHO, infected, 43-45 hr pi, 2.2 X 10^7 cpm.

Lane E: CHO, infected, 66-68 hr pi, 1.7 X 10^7 cpm.

Lane F: CHO, mock-infected, long labelled, 21-43 hr pi, 3.6 X 10^7 cpm.

Lane G: CHO, infected, 21-43 hr pi, 1.5 X 10^7 cpm.

Lane H: CHO, infected, 43-66 hr pi, 6.4 X 10^6 cpm.

Lane I: CHO, infected, 66-90 hr pi, 5.6 X 10^6 cpm.

Lane J: HeLa, infected, 19-21 hr pi as markers.

Figure 8

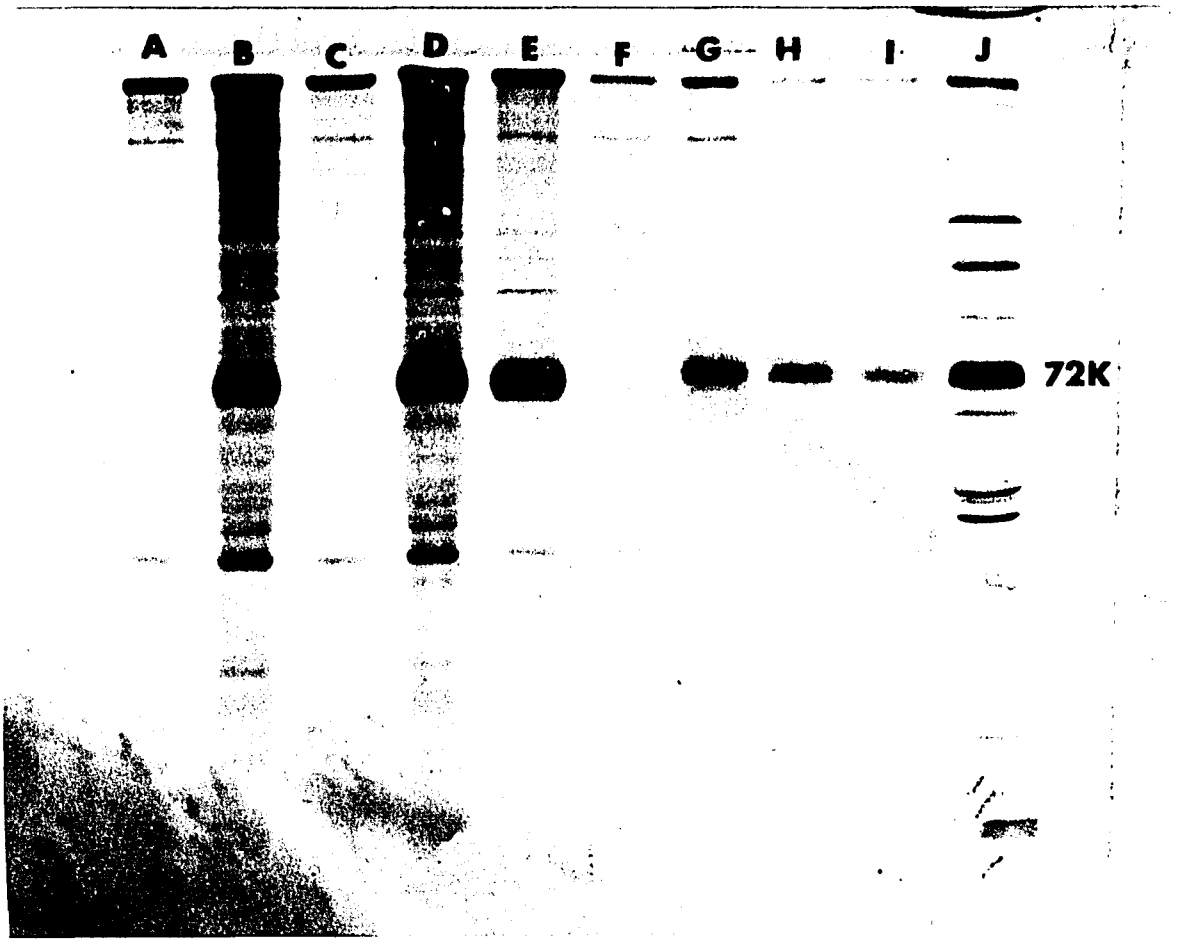


Figure 9: PAGE analysis of ^{35}S-methionine cell lysates immunoprecipitated with monoclonal antibody against 100K protein. CHO samples were infected, labeled and equal volumes were immunoprecipitated as described in the Materials and Methods section and in legend to figure 8. The HeLa immunoprecipitate was performed with 1/8 the volume of CHO.

Lane A: HeLa, infected, pulse labelled 19-21 hr pi.

Lane B: CHO, mock-infected, pulse labelled 19-21 hr pi.

Lane C: CHO, infected, labelled 19-21 hr pi.

Lane D: CHO, mock-infected, labelled 43-45 hr pi.

Lane E: CHO, infected, labelled 43-45 hr pi.

Lane F: CHO, infected, labelled 66-68 hr pi.

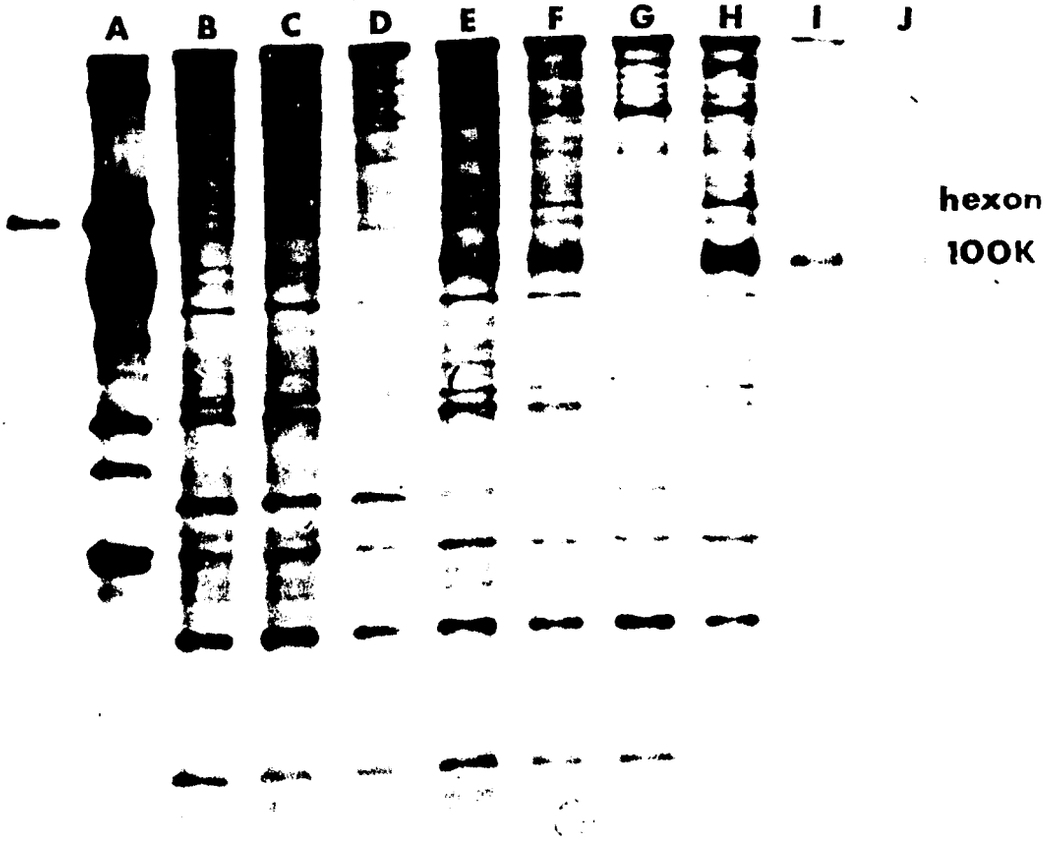
Lane G: CHO, mock-infected, labelled 21-43 hr pi.

Lane H: CHO, infected labelled 21-43 hr pi.

Lane I: CHO, infected labelled 43-66 hr pi.

Lane J: CHO, infected labelled 66-90 hr pi.

Figure 9



whole lysate gel was hexon. This protein has a molecular weight of 120K and is made in largest amounts in HeLa cells. The PAGE of the immunoprecipitation using monoclonal antibody directed against hexon trimers and ninemers is shown in Figure 10. Again the sample pulse labelled at day 1 (lane C) is not different than the corresponding uninfected lane (B). Comparison of samples pulse-labelled at day 2 (lane E) and day 3(lane F) with that of uninfected cells (lane D) appears to show a very slight band at the hexon region. The infected samples which were continuously labeled day 1-2 (lane H), day 2-3 (lane I), and day 3-4 (lane J) all show a hexon band. It seems to be reduced compared to the 100K. It should be emphasized that there is much more hexon than 100K in infected HeLa. However, the amount of hexon could be underestimated due to the fact that the monoclonal antibody is directed against hexon trimers and their association into hexon ninemers. It is not reactive against hexon monomers. Hence, this antibody will not rule out the possibility that hexon is produced but is not associated into trimers or ninemers.

Immunoprecipitation for fiber was performed next on these samples. Figure 11 presents a time course of samples immunoprecipitated with monoclonal antibody directed against fiber protein. None of the

Figure 10: PAGE analysis of ^{35}S-methionine cell lysates immunoprecipitated with monoclonal antibody against hexon protein. CHO cells were infected, labeled and equal volumes of lysates were immunoprecipitated as described in Materials and Methods and in the legend to figure 8.

Lane A: HeLa, infected, pulse labelled 19-21, as markers.*

Lane B: CHO, mock-infected, 19-21 hr pi.

Lane C: CHO, infected, 19-21 hr pi.

Lane D: CHO, mock-infected, 43-45 hr pi.

Lane E: CHO, infected, 43-45 hr pi.

Lane F: CHO, infected, 66-90 hr pi.

Lane G: CHO, mock-infected, long labelled 21-43 hr pi.

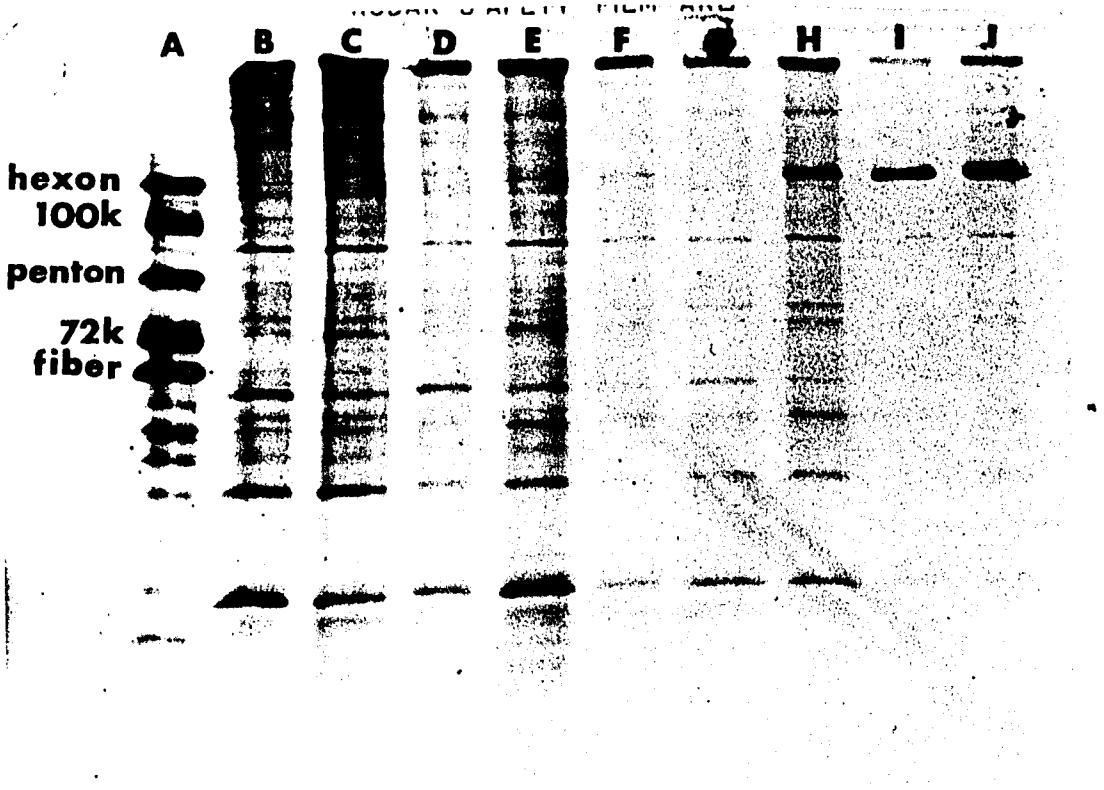
Lane H: CHO, infected, 21-43 hr pi.

Lane I: CHO, infected, 43-66 hr pi.

Lane J: CHO, infected, 66-90 hr pi.

* extract had been manipulated prior to analysis so that the distribution of viral polypeptides is not representative of such an extract (cf. Figure 7)

Figure 10

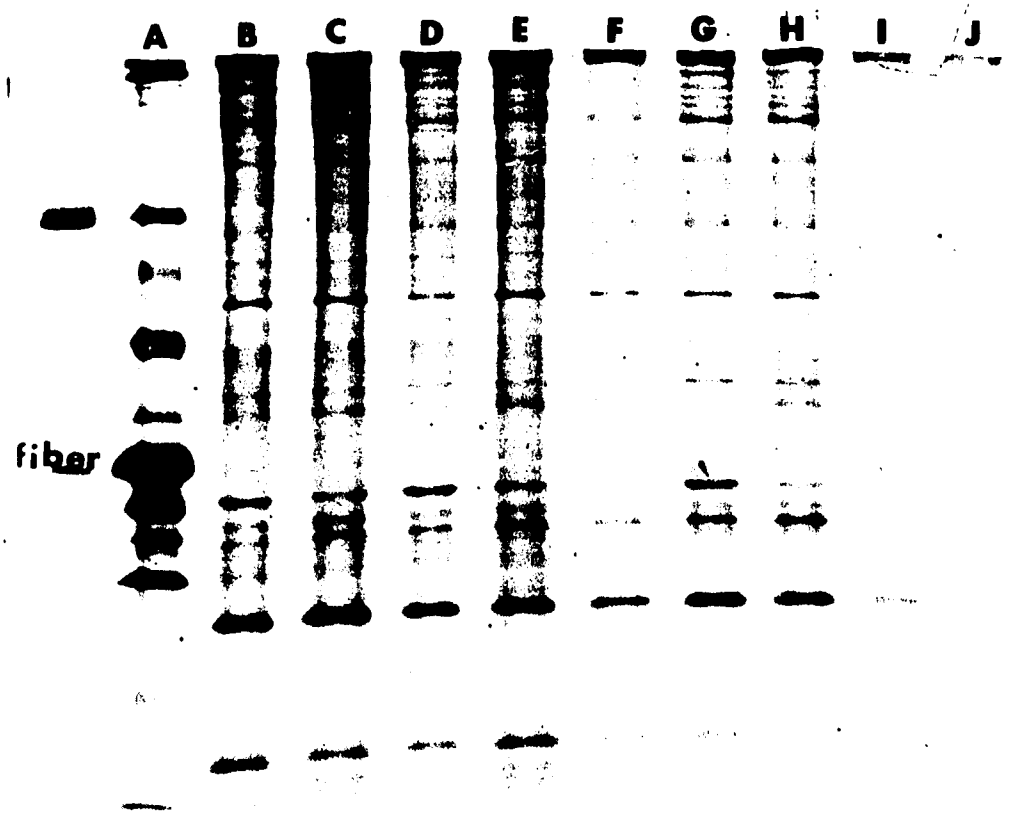


samples were positive for fiber and longer incubation of the autoradiograph failed to show an increase in the fiber band.

The amount of fiber in these samples is barely detectible and this was true regardless of the antibody used and at all times pi. To rule out the possibility that the monoclonal antibody was not recognizing the particular antigenic determinant, I repeated this immunoprecipitation for fiber on these same samples using polyvalent antibody raised in rabbit against purified fiber protein. Figure 12 lanes B and C are immunoprecipitates of infected HeLa lysates obtained with rabbit antiserum and mouse monoclonal antibody to fiber. Comparable amounts of fiber are observed in both cases; however, there are differences in the level of hexon precipitated. It would appear that the monoclonal antibody preferentially precipitated fiber which has not been assembled into virus particles, although it may be assembled into a subunit structure as in association with pentons (85K). Conversely, the rabbit antiserum reacts with all classes of fiber. Hexon (and penton) co-precipitated because of its association with fiber in virion-like particles. It is not possible to comment on the relative distribution of these forms of fiber in this particular extract since attempts were not made to optimize precipitation with the different antibody

Figure 11: PAGE analysis of ^{35}S-methionine labelled cell lysates immuneprecipitated with monoclonal antibody against Fiber protein. CHO cells were infected, labeled and equal volume of lysates were immunoprecipitated as described in Materials and Methods and in the legend to figure 8. The order of the samples in the lanes is as for figure 10.

Figure 11



specificities. It is known that excess fiber monomers are synthesized in infected HeLa cells (see INTRODUCTION).

The samples for CHO cells are shown in lanes G-K Uninfected cell extracts were incubated with the rabbit antiserum; no precipitation of fiber-like protein (65K) was observed with pulse labelled (lane G) or long labelled (lane I) lysates, as previously observed with the monoclonal antibody. Immunoprecipitation of the pulse labelled lysate (lane H) shows a faint band in the position of fiber and also hexon in the midst of a considerable background of cellular proteins. The samples with extracts prepared from cells long labelled at 2-3 days pi (lane J) and 3-4 days pi (lane K) similarly show a band at a position consistent with hexon. The background is much improved. More importantly, these samples show hexon coprecipitation. This can be interpreted to mean that some fiber is made in these samples and some virus assembly is taking place, by virtue of the fact that this rabbit antiserum is reactive against virion-assembled fiber and there is 20 times more hexon than fiber per virion. In conclusion, the amount of fiber synthesized is very much lower than hexon or 100K protein.

Concluding that there is a differential effect on late viral proteins and that this pattern of selective

Figure 12: Comparison of antifiber antibodies. Autoradiograph of PAGE analysis of ^{35}S-methionine labeled extracts where Cultures were infected, labelled immunoprecipitated and processed for PAGE as described in Materials and Methods and legend to Figure 8. HeLa cell extracts were immunoprecipitated with rabbit antiserum (lane A) and with monoclonal anti fiber antibody (lane B). In the case of CHO equal volumes of samples were immunoprecipitated using rabbit antiserum directed against fiber protein (lanes G-K).

Lane A: HeLa, infected, 7.0×10^5 cpm.

Lane B: HeLa, infected, 7.0×10^5 cpm.

Lanes C-F: removed from analysis, not relevant.

Lane G: CHO, mock-infected pulse, 2.7×10^7 cpm.

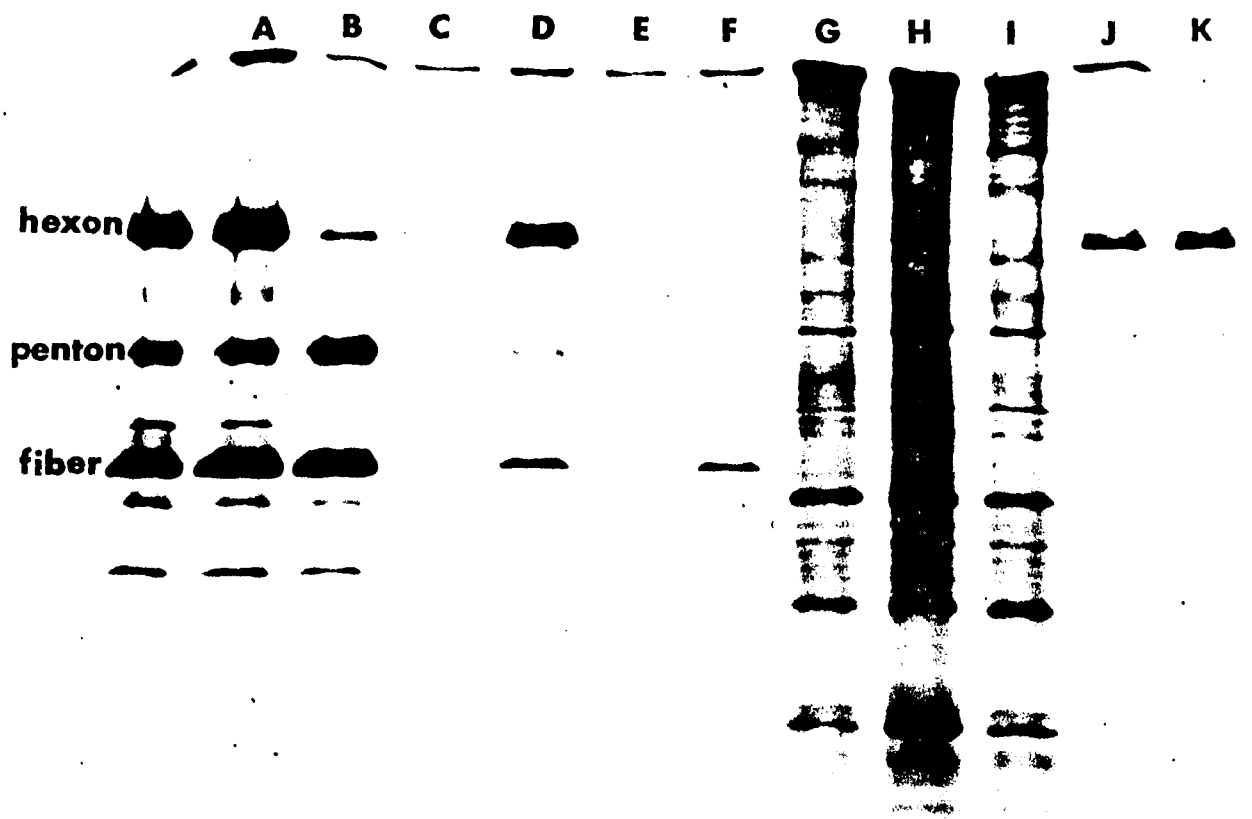
Lane H: CHO, infected, labelled 43-45 pi, 2.1×10^7 cpm.

Lane I: CHO, mock-infected, labelled 21-43 pi, 3.6×10^7 cpm.

Lane J: CHO, infected, labelled 43-66 pi, 6.5×10^6 cpm.

Lane K: CHO, infected, labelled 66-90 pi, 5.6×10^6 cpm.

Figure 12



inhibition is reminiscent of the block in monkey cells, I looked at the Ad2 infection of monkey cells for comparison.

Figure 13 shows the whole lysate infected profile of HeLa (lanes A, B), CV1 (lanes C, D) and CHO (lanes E, F). The patterns are different among the three infected cell lines with the CV-1 (lane D) quite similar to that of HeLa (lane B). However, this is mostly due to the marked degree of inhibition of host protein synthesis upon infection in the two primate cells (compare lanes B with A and D with C), as contrasted to CHO (lane F with uninfected lane E). More importantly, however, a fiber and a penton band is not detectible in the monkey lysate (lane D) fiber is not found in the CHO (lane F) lysate in contrast to the infected HeLa cells. Other viral-encoded proteins are apparent in both primate cell lines. Upon immunoprecipitation of these samples for fiber, Figure 14 shows that fiber protein is precipitable from the monkey cell lysate (lane C) and is barely detectible in the CHO sample (lane A), as previously noted. The densitometer tracing of the whole lysate gel of Ad2 infection of CV-1, CHO and HeLa is shown in Figure 15. The tracing also emphasizes the absence of a discrete peak in the position expected for fiber in CV-1 and CHO. In addition, it permits an approximate estimate of the level of other major viral proteins in the two systems since the

Figure 13: PAGE analysis of whole lysates: comparison of primate and CHO cell lines. Mock infected or infected cells were pulse- labelled with ^{35}S-methionine for 2 hours and analyzed as in figure 7. HeLa and CV-1 cells were infected at a MOI 1 pfu/cell and pulse-labelled at 24-26 hr pi, CHO cells were infected at a MOI of 15 pfu/cell and pulse labelled at 43-45 hr pi. The subline clone 41D-7 was employed, its designation and properties are described in chapter 3.3. Equivalent amounts of a culture were loaded for each cell line.

Lane A: HeLa, mock-infected, 9.8 X 10^5 cpm.

Lane B: HeLa, infected, 2.8 X 10^5 cpm.

Lane C: CV-1, mock-infected, 3.6 X 10^5 cpm.

Lane D: CV-1, infected 1.0 X 10^5 cpm.

Lane E: CHO, mock infected, 3.8 X 10^5 cpm.

Lane F: CHO, infected, 1.8 X 10^5 cpm.

Figure 13

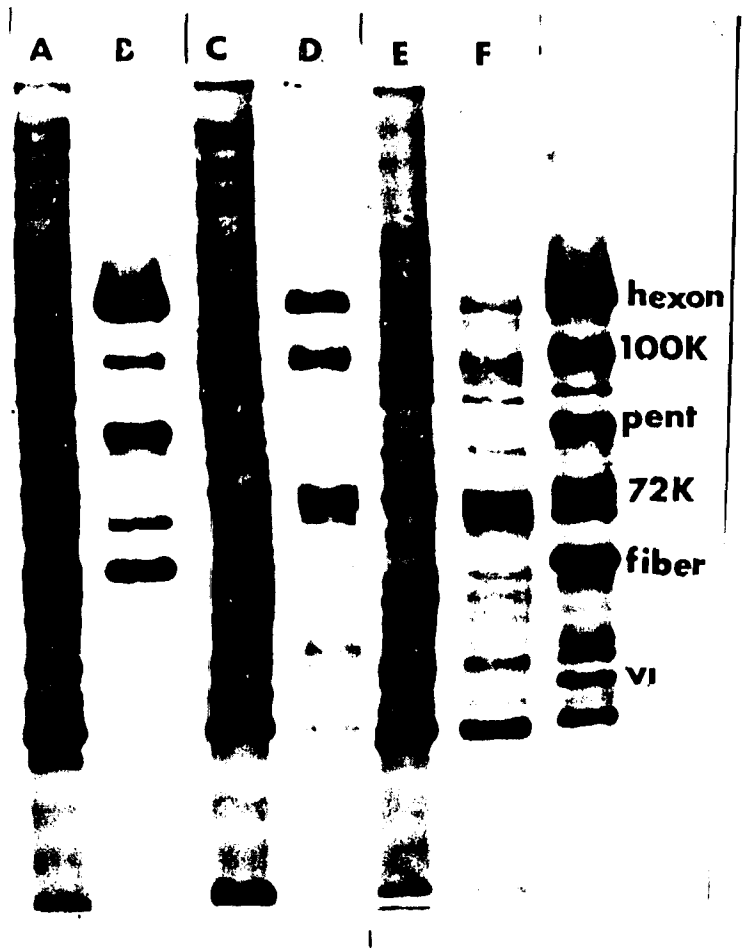


Figure 14: PAGE analysis of ^{35}S-methionine cell lysates immunoprecipitated with monoclonal antibody against fiber protein. Pulse- labelled infected lysates were prepared as for Figure 13 and approximately 4 X 10^6 cpm were immunoprecipitated per sample.

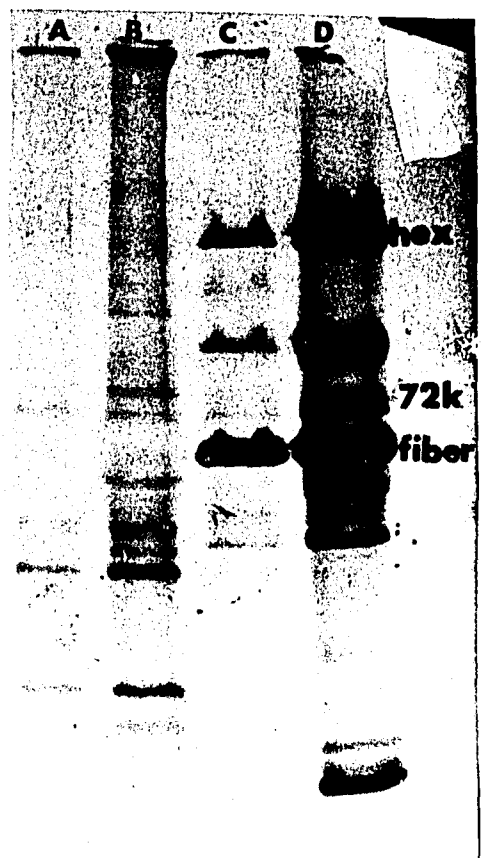
LANE A: CHO, TNT subline.

LANE B: CHO, uninfected.

LANE C: CV-1, infected.

LANE D: HeLa, markers.

Figure 14



viral proteins in CHO are visible on the gel from this experiment in contrast to a previous experiment (see figure 7). The area under the peak was determined by a planimeter. The outlines of the peak were indicated as dotted lines in the figure. This selection was made to minimize the contribution of the residual host proteins in CHO. The calculated data are presented in Table 10. The area in the region of 72K serves as a control and is roughly equivalent in the CV-1 and CHO. In HeLa cells, the late viral proteins are the predominant species with hexon in greatest excess. More specifically, hexon (and fiber) exceed 100K by a considerable amount. In CV-1, hexon is reduced to the level observed for 100K. In addition, the penton base and protein VI are indetectible. In CHO both hexon and 100K are reduced with hexon further reduced to a level less than 100K. These data suggest that the low level of hexon deduced from the immunoprecipitation data with the monoclonal antibody was not grossly misleading since no large pool of hexon monomers was detected. It further confirms that the level of fiber synthesis is most reduced since it is indetectibe (or barely detectible) when both 100K and hexon can be observed as in immunoprecipitates and PAGE.

How low is the amount of fiber produced in Ad2 infection of CHO? It is less than 100K. There is no reason to believe that the 100K protein is

Figure 15: A densitometer tracing of a PAGE analysis of Ad2 infected HeLa, CV-1 and CHO cells whole lysates from figure 13. (Planimeter Noris Instruments, Germany).

Figure 15

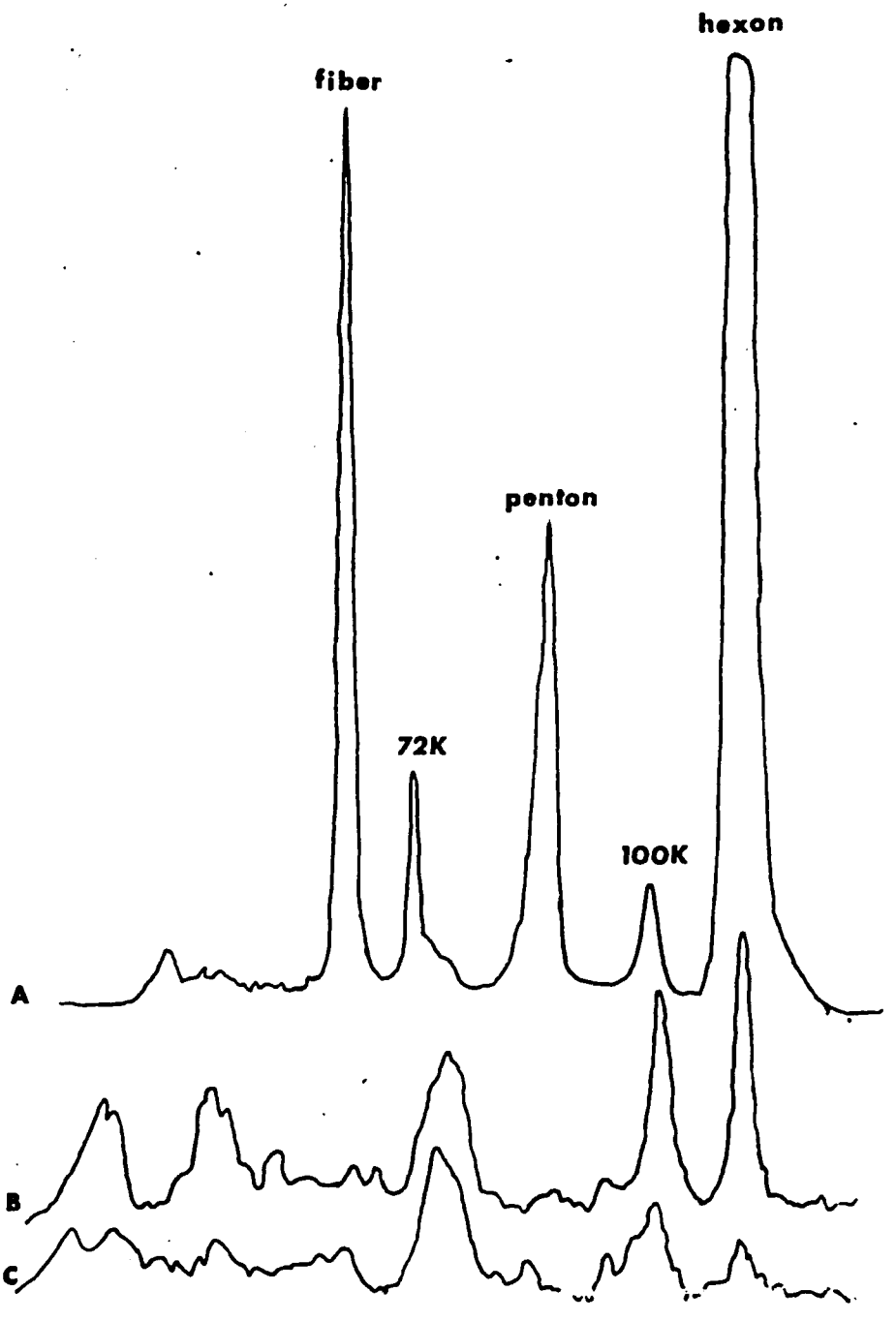


Table 10

Measurement of area under peaks representing some late viral proteins in Ad2 infected HeLa, CV-1 and CHO

cell line	Hexon	100K	Fiber
HeLa	>1266	54	566
CV-1	246	208	<51
CHO	62	91	ND

a planimetry were performed as described in text
b Not determined. The background in this area precluded estimation of the viral-specific protein.

overproduced in CHO. Less fiber is produced in CHO than in monkey cells based on the immunoprecipitation analysis. In monkey cells the 100K protein is synthesized at normal levels and the hexon is somewhat reduced. This decrease in fiber synthesis leads to a 1000 fold decrease in infectious virus as pfu (Klessig and Anderson, 1975). Since in CHO there is even less fiber, it follows that the pfu produced would be further reduced. Therefore, the fiber defect would be sufficient to explain the absence of infectious virus in CHO. Comparison of the level of 100K and fiber in immunoprecipitates prepared from the same cell extracts should, therefore, provide a convenient indicator of the extent of restriction in chinese hamster cells.

3.2 Ad2 INFECTION OF CHINESE HAMSTER AND HUMAN CELL HYBRIDS

The role of host factors in virus replication is readily demonstrable by observations as those described in the preceding chapter. Further insight can be obtained by use of cell hybrids between cells with different degrees of permissivity for adenovirus. As summarized in the INTRODUCTION, cell hybrids between semi-permissive monkey cell (fiber-defective) and permissive human cells restored fiber synthesis as assayed by immunofluorescence (Anderson, 1981). Such results indicated that monkey cells are deficient in a host factor rather than that they contain an inhibitor of expression of fiber proteins. The investigators were unable to take their genetic analysis further because of genetic and cytogenetic limitations in that cell system. We elected to ask whether the defect in fiber synthesis observed in Chinese hamster cells was similarly regulated; i.e. whether human genes would make Chinese hamster cells permissive for Ad2 expression and replication. We could examine this question more fully because of the favorable genetics

in our system. Stable cell hybrids exist between Chinese hamster and human cells so that we can perform experiments with more nearly homogeneous cell populations than possible with the mixed cell populations obtained soon after cell fusion. Furthermore, cell hybrids are also available with various combinations of human chromosomes such that we might be able to identify one (or a few) chromosomes which confer the required genes for permissivity. We initiated such studies in collaboration with Dr. Uta Francke (Yale Medical School) who provided the cell hybrids and verified their complement of human chromosomes.

We evaluated Chinese hamster X human cell hybrids to determine whether permissivity was expressed. In contrast to prior studies, we could determine whether all restrictions were corrected by assessing synthesis of progeny infectious virus. Two systems were examined: a CHO X human fibroblast hybrid cell line (UF11) which contains 19 human chromosomes and a V79 X human lymphocyte hybrid cell line (X7A) which contains the full complement of 23 human chromosomes (22 autosomes plus the X) in at least one copy each. In each case, the individual human chromosomes are present in at least 20% of the cell population. (The cell lines are identified more fully in the Materials and Methods, Table 4). A growth curve of Ad2 in these

cells was performed and as can be seen from Table 11 there was a marked increase of virus over the input, adsorbed or eclipsed virus. There was no increase in virus post infection in either chinese hamster parent alone as shown previously (Table 6) and confirmed in a parallel experiment with the cell hybrids (data not shown). In both cell hybrids, the virus yield at 3 days was approximately 500-fold greater than the eclipse value (16-24 hr pi). This is a particularly sensitive indicator since it corrects for differences due to improved virus adsorption due to possible cell surface receptors for adenovirus contributed by the human genes. Although the yield was significantly lower than that obtainable for highly permissive cells such as HeLa, it is quite consistent with yields obtained for human fibroblasts (HS74) such as those used in the CHO cell fusion. It was therefore concluded that permissivity was "dominant" and the study of which human chromosome or chromosomes was responsible for this aspect of permissivity to Ad2 was undertaken.

Dr. Francke provided this laboratory with a set of 8 previously-karyotyped hybrid cell lines involving V79 as the Chinese hamster parent. The cells contained different combination of human chromosomes (see Table 12). Two types of experiments in these cells were performed: a labelling experiment to assay for fiber production by immunoprecipitation and PAGE as well as a

Table 11

Ad2 Growth in X-7A and UF11[a]

Cell Line	Ad2 Virus Yield (pfu/culture)[b] Adsorbed	Eclipse	Day 3
X7A	1×10^3	0.8×10^3	3.9×10^5
UF11	ND[c]	7.5×10^3	4.8×10^6

a cells were infected at an moi of 1 pfu/cell as described in Materials and Methods.
b (pfu/0.2ml X 25 = pfu/culture)
c Not determined

Table 12

Karyotype of Chinese hamster X human cell hybrids

cell line	Human Chromosomes																						
	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21	22	X
UF1	-	+	P	+	+	-	-	+	-	-	+	+	-	+	+	+	+	-	+	+	+	+	+
UF2	+	-	-	-	-	-	+	-	-	-	+	+	-	P	-	-	-	-	-	-	-	-	+
UF3	+	-	+	+	-	+	-	+	P	-	-	+	+	+	+	+	-	+	+	L	+	+	+
UF4	-	-	-	-	+	-	-	-	-	-	-	-	-	+	-	+	-	-	-	-	-	+	+
UF5	+	+	+	+	+	+	+	-	+	+	+	+	+	+	+	+	-	+	+	+	+	+	+
UF6	P	+	+	+	+	+	+	-	-	+	-	+	+	+	+	+	-	+	+	+	+	+	-
UF7	+	+	+	P	+	P	-	+	+	-	P	+	-	+	+	+	+	+	+	+	+	+	+
UF8	P	+	P	+	+	P	-	+	+	-	+	+	+	+	+	+	-	-	+	+	+	+	+

a Partial chromosome is present
b the chromosome is present in < 20% of the cells.

virus growth curve. Not all the cells were ready at the same time, so a preliminary labelling experiment with ^{35}S-methionine was done with UF3, UF6 and UF7 in which the hybrids were pulse labelled at 48-51 hrs pi and continuously labeled between 27-51 and 51-76 hrs pi. This was done in an attempt to establish a good labelling protocol that would be most representative. Figure 16 shows the pattern of PAGE for a whole lysate of these samples. Equal volumes of lysates were applied and run on the gel. Lanes A and M are HeLa markers. Lanes C and D represent the pulse label of virus infected UF3 and UF6, respectively. Compared to the uninfected UF3 (lane B), one can clearly see a hexon band. Lanes F, I and K represent long label samples of UF3, UF6 and UF7, respectively. Again the hexon is clearly visible. The long label uninfected controls and the 51-76 hr pi label of the infected cells (UF3, UF6) had a 10-fold decrease in the TCA precipitable radioactivity and, therefore, were not detectible on the autoradiograph.

Since every band that was present in the infected pulse sample was also present in the infected long label sample, it was decided that all further labelling experiments will be done with long label. Such a protocol measures an accumulation of a protein rather than rate of synthesis and lessens the likelihood of inadvertently selecting a non-representative time pi

Figure 16: PAGE analysis of whole cell lysates of UF3, UF6 and UF7. Cells were infected with an MOI of 50-100 pfu/cell. Infected and mock infected cells were pulsed and continuously labelled with ^{35}S-methionine at different times pi. Whole cell lysates were prepared and analyzed by PAGE and autoradiography as described in Materials and Methods. Equal amounts of lysates were loaded in each lane.

LANE A: HeLa marker

LANE B: UF3, mock infected, 48-51 hr pi, 1.5 X 10^5 cpm.

LANE C: UF3, infected, 48-51 hr pi, 4.8 X 10^5 cpm.

LANE D: UF6, infected, 48-51 hr pi, 2.1 X 10^5 cpm.

LANE E: UF3, mock infected, 27-51 hr pi, 1.6 X 10^4 cpm.

LANE F: UF3, infected, 27-51 hr pi, 2.4 X 10^5 cpm.

LANE G: UF3, infected, 51-76 hr pi, 7.0 X 10^3 cpm.

LANE H: UF6, mock infected, 27-51 hr pi, 6.2 X 10^4 cpm.

LANE I: UF6, infected, 27-51 hr pi, 1.1 X 10^5 cpm.

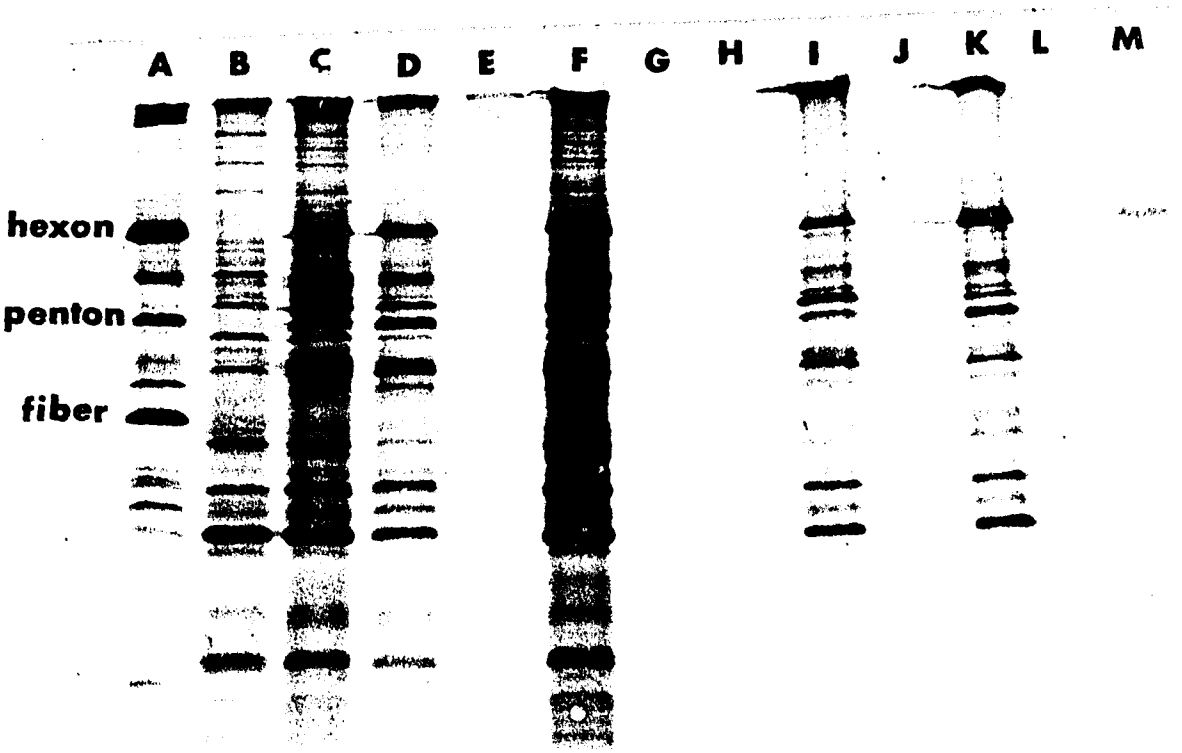
LANE J: UF6, infected, 51-76 hr pi, 8.9 X 10^3 cpm.

LANE K: UF7, infected, 51-76 hr pi, 9.5 X 10^5 cpm.

LANE L: Blank lane.

LANE M: Marker.

Figure 16



for analysis.

Equal volumes (which approximated equal TCA precipitable counts) were immunoprecipitated for fiber using rabbit and monoclonal anti fiber antibodies. Figure 17 shows an autoradiograph of the PAGE. All samples are clearly positive with samples immunoprecipitated with rabbit sera in lanes A-F showing slightly more fiber than in the monoclonal anti fiber in lanes G-L. Immunoprecipitation of extracts of UF7 brought down the most fiber in this experiment. Hexon and penton were also immunoprecipitated by both antibodies, demonstrating that the fiber was assembled into virus-like particles, as well.

When the rest of the hybrids grew out, UF1, UF2, UF4, UF5 and UF8, they were infected. UF7 was also retested to permit comparison with the previous experiment. Cultures were labelled at 42-66 hr pi, which became the standard labelling time for most of the cell hybrid experiments. Equal volumes of samples were immunoprecipitated with anti fiber antibody. Figure 18 shows the result of this experiment. In the cases of UF5 and UF7 (lanes F and H respectively), appreciable fiber was precipitated compared to the other samples of UF1, UF2, UF4 and UF8 (lanes B, C, D and I, respectively). It is further seen that hexon and penton are brought down with the fiber immunoprecipitate indicating that some assembly is

Figure 17: PAGE analysis of UF3, UF6, and UF7 lysates immunoprecipitated for fiber. Cells were infected and labelled as described in Materials and Methods and legend to Figure 16. Set of samples were matched for counts and immunoprecipitated using anti fiber monoclonal and polyvalent antibodies. One half of the immunoprecipitate was loaded on the gel. Lanes A-F are samples immunopreciptated with rabbit antibody and lanes G-L with monoclonal antibody.

LANE A: HeLa, infected, as marker.

LANE B: UF3, mock, 48-51 hr pi, 2.9 X 10^6 cpm.

LANE C: UF3, infected, 48-51 hr pi, 4.5 X 10^6 cpm.

LANE D: UF3, infected, 27-51 hr pi, 4.0 X 10^6 cpm.

LANE E: UF6, infected, 27-51 hr pi, 2.3 X 10^6 cpm.

LANE F: UF7, infected, 51-76 hr pi, 2.8 X 10^6 cpm.

LANE G: HeLa, infected, as marker.

LANE H: UF3, mock, 48-51 hr pi, 2.9 X 10^6 cpm.

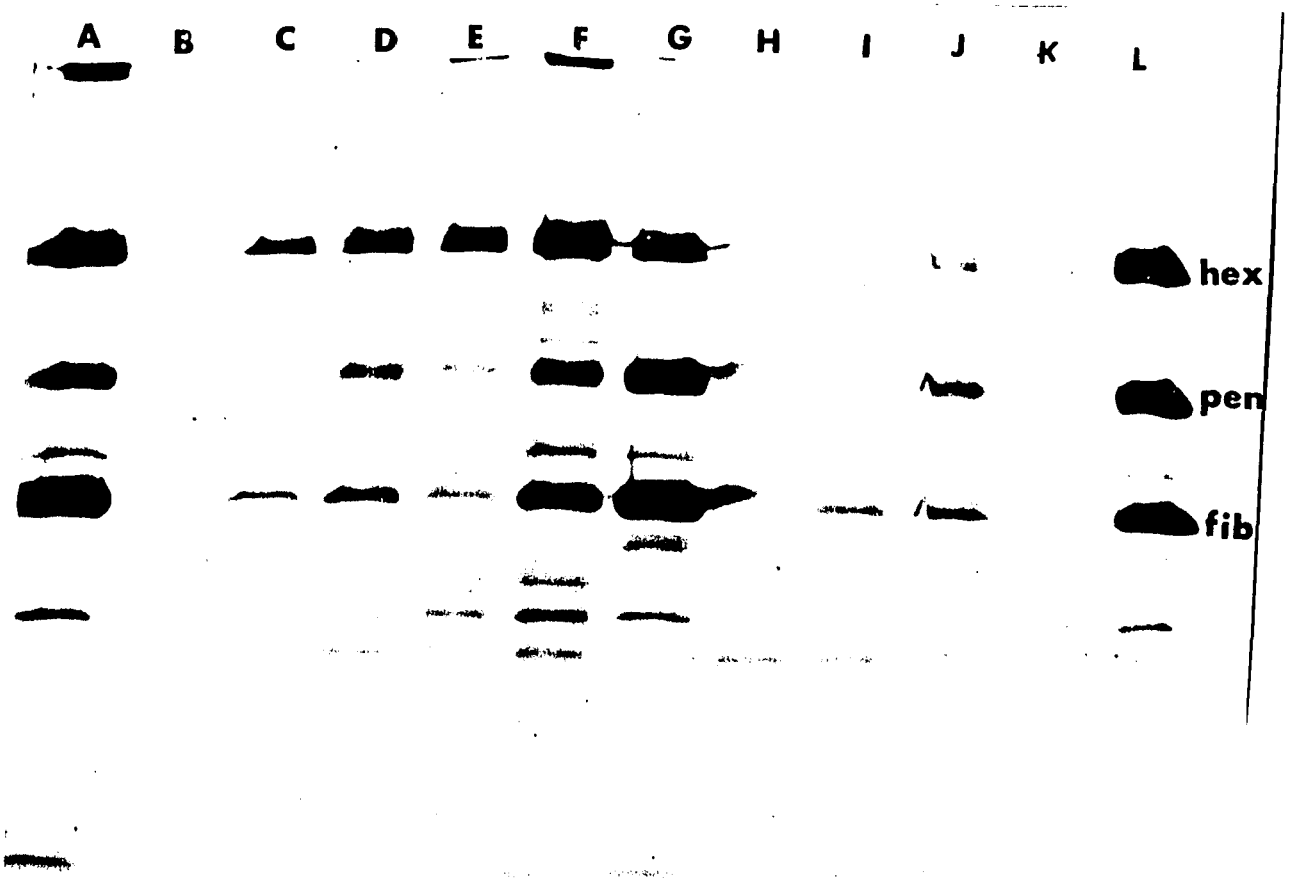
LANE I: UF3, infected, 48-51 hr pi, 4.5 X 10^6 cpm.

LANE J: UF3, infected, 27-51 hr pi, 4.0 X 10^6 cpm.

LANE K: UF6, infected, 27-51 hr pi, 2.3 X 10^6 cpm.

LANE L: UF7, infected, 51-76 hr pi, 2.8 X 10^6 cpm.

Figure 17



taking place. Lanes E and G are uninfected controls and are negative, as are lanes K (the long label) and lane L (the pulse label) of infected V79.

These same samples were then examined for immunoprecipitation of 100K protein to ensure that the quality of infection was comparable among all these cell lines. Figure 19 shows the result of such an immunoprecipitation where the same equal volumes of cell extracts were immunoprecipitated using monoclonal antibody against 100K protein. With the exception of UF2 (lane C) and V79 (lane K), all the cells seem to show a comparable infection. UF1, UF4, UF5, UF7 and UF8 (lanes B, D, F, H and I, respectively).

It was concluded that, by the criterion of fiber production, UF1, UF2 (a repeat experiment, data not shown) UF4, UF8 are negative and UF3, UF5, UF6 and UF7 are positive. Failure to see fiber where there was an adequate amount of 100K polypeptide precipitated indicates a block to fiber production rather than a poor infection.

Careful examination of the karyotype of these cells (Table 12) show that there is a complete concordance of fiber protein synthesis with human chromosomes 3,6 and 18. Hybrids UF3, UF5, UF6 and UF7 are all positive for fiber and all contain chromosomes 3, 6 and 18. The other hybrids are negative for fiber and all three chromosomes as in the case of UF2 and UF4. UF1 is missing chromosome 6 and 18 but contains a

Figure 18: Fiber immunoprecipitation and PAGE analysis of hybrid cells UF1, UF2, UF4, UF5, UF7 (repeat), UF8 and V79. Cells were infected and processed for PAGE and autoradiography as described in legend to figure 16. Samples were labelled at 42- 66 hr pi, and immunoprecipitated with monoclonal antibody directed against the fiber protein. Equal volumes of lysates were immunoprecipitated. Half the immunoprecipitate was loaded on the gel.

LANE A: HeLa, marker.

LANE B: UF1, infected, 2.1 X 10^6 cpm.

LANE C: UF2, infected, 2.4 X 10^6 cpm.

LANE D: UF4, infected, 3.2 X 10^6 cpm.

LANE E: UF5, mock infected, 1.3 X 10^5 cpm.

LANE F: UF5, infected, 2.9 X 10^6 cpm.

LANE G: UF7, mock infected, 1.4 X 10^6cpm.

LANE H: UF7, infected, 1.9 X 10^6 cpm.

LANE I: UF8, infected, 3.5 X 10^6 cpm.

LANE J: V79, Mock infected, 42-66 hr pi, 4.8 X 10^6cpm.

LANE K: V79, infected, cpm not determined.

LANE L: V79, mock infected, 43-46 hr pi, 1.7 X 10^6 cpm.

LANE M: V79, infected, 43-46 hr pi, cpm not determined.

Figure 18

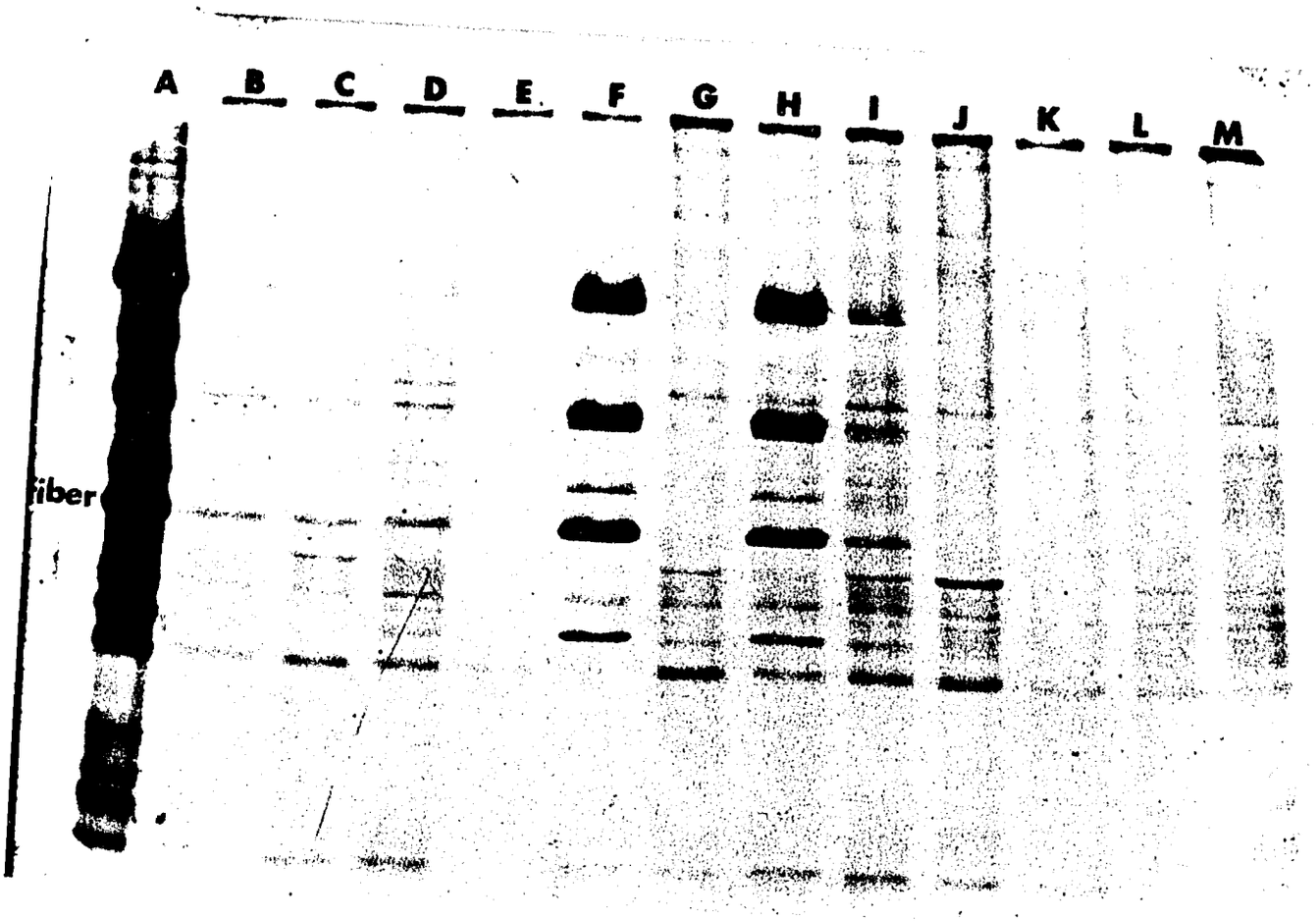
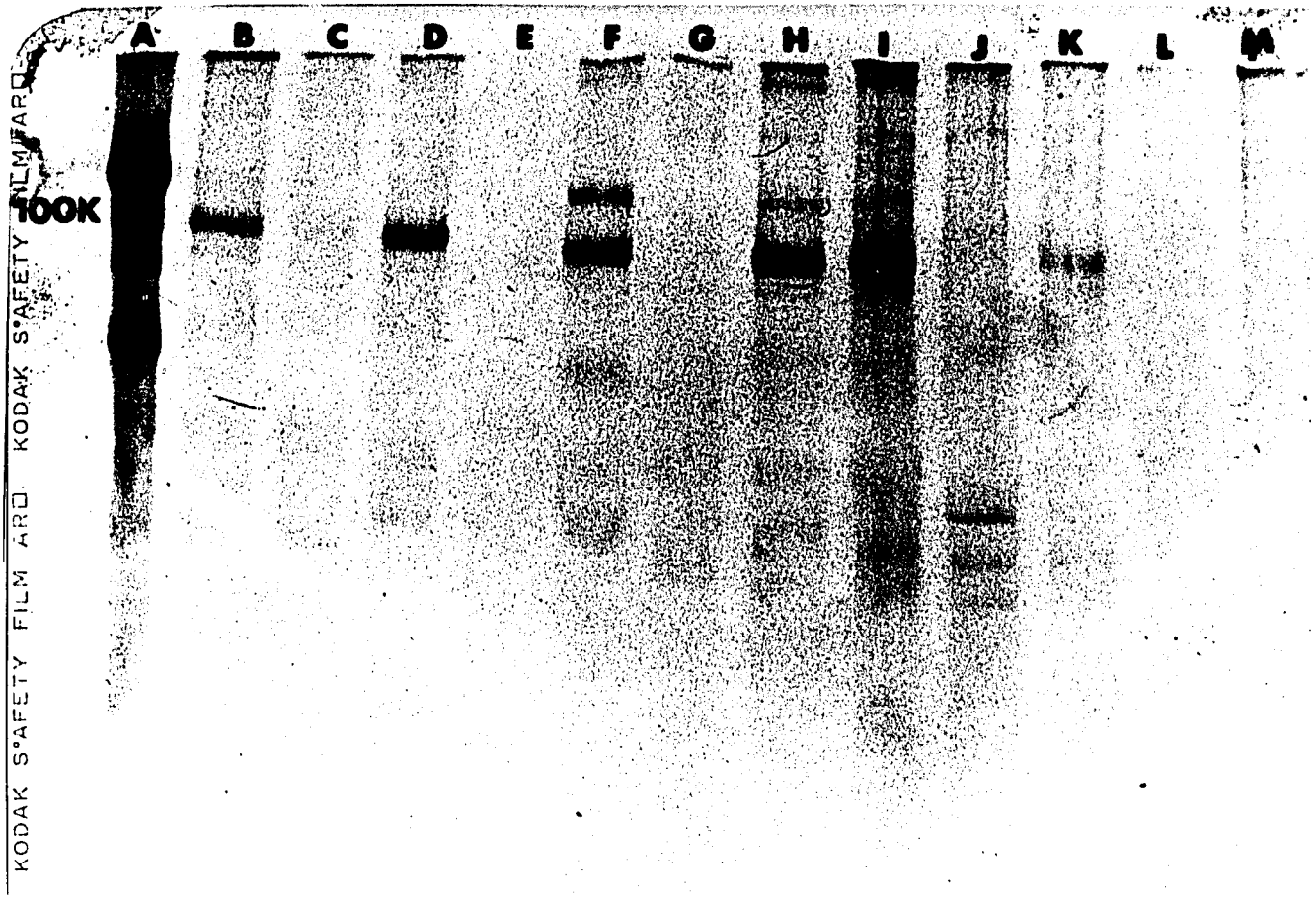


Figure 19: 100K immunoprecipitation and PAGE analysis of hybrid cells UF1, UF2, UF4, UF5, UF7, UF8 and V79. Cells were infected, labelled, immunoprecipitated, processed for PAGE and autoradiography, as described in legend to figure 18. Samples were immunoprecipitated with monoclonal antibody directed against the 100K protein. Samples are in the same order as those described for figure 18.

Figure 19


A
B
C
D
E
F
G
H
I
J
K
L
M
100K
KODAK SAFETY FILM ARO KODAK SAFETY FILM ARO

partial chromosome 3. UF8 is missing chromosome 18 and a part of chromosome 3 and 6.

These results indicated that a definite assignment of "permissivity" to a unique chromosome could be possible. It was a matter of sorting out among chromosomes 3,6 and 18.

As a further degree of analysis, a viral growth curve was performed on these hybrid cells as described in the Materials and Methods section. The results are summarized in Table 13. Times corresponding to adsorption, eclipse, 3 days pi and, in some cases 5 days pi were analyzed and the increase over the eclipse value was calculated. Cell hybrids which showed a good level of virus production (i.e. >50-100 fold increase over the eclipse value) were considered positive. These were UF3 and UF5, both of which were also positive for fiber production. From the table one can see that some fiber-positive hybrids were not able to synthesize appreciable levels of virus, e.g. UF6 which was negative for virus production and UF7 which is ambiguous since it seems to be variable and show generally borderline levels of virus yield. These results may reflect more subtle differences in the quantitative aspects of infection since UF5 was positive for virus production only at high MOI of infection. Possible interpretation of this

Table 13

GROWTH OF Ad2 IN HYBRID CELLS UF1-UF8

Cell Line	Input MOI	PFU/CULTURE Ads[a]	Eclipse[b]	day 3[c]	day 5
UF1	1	1×10^3	1.1×10^3	2.9×10^3	
UF2	1	7.5×10^2	3.8×10^2	1.2×10^2	
UF3	1	4.4×10^3	5.0×10^2	3.6×10^5	
UF4	1	5.0×10^3	7.5×10^3	2.5×10^3	
UF5	1	2.3×10^3	4.0×10^3	1.1×10^4	
	38	1.0×10^5		7.0×10^6	1.4×10^7
UF6	1	1.5×10^3	1.9×10^3	1.2×10^4	
UF7	1	5.0×10^3	1.5×10^3	2.7×10^4	
		1.6×10^4		3.8×10^5	6.0×10^5
			1.5×10^4	8.8×10^4	
	38	1.1×10^5		6.2×10^6	1.4×10^6
UF8	1	2.4×10^3	2.5×10^3	6.1×10^3	
	38	1.2×10^5	1.4×10^6	1.1×10^6	

a adsorption value harvested immediately following infection.
b eclipse value harvested at day 1.
c day 3 harvested three days pi.

discrepancy are presented more fully in the discussion section. It should be pointed out here that the cells for both radiolabelling experiments and the growth curve were subcultured and infected at the same time. The growth curve was harvested by freezing and storage at -20^{0} C until all the samples were collected; they were then plaque assayed together. This point is important because as these hybrid cells divide, it is possible that they might segregate human chromosomes and a loss in ability to support viral growth may be due to a loss of a particular chromosome.

To clarify further the possible roles of chromosomes 3 and 6, Dr. Francke sent another hybrid cell line made from a fusion of Chinese hamster cell and a human lymphocyte, designated UF9, which presumably contained only human chromosomes 3 and 6. Cells were infected and labelled from 48 to 72 hr pi in parallel with appropriate positive and negative controls. Figure 20 shows the resulting autoradiograph of a PAGE of equal volume samples immunoprecipitated for 100K protein using rabbit antiserum and fiber using monoclonal antibody. UF9 (in lane E) shows a greater amount of fiber protein than the negative control UF8 (in lane C) and a little less than the positive control UF7 (in lane F). Lanes B and D are uninfected UF8 and UF9, respectively. The immunoprecipitation for 100K protein (lanes G-K) shows a good level of production, indicating that the quality of

Figure 20: Fiber, 100K protein immunoprecipitation and PAGE analysis of UF9. Cells were infected at an MOI of 50-100, labelled at 48-72 hr pi and processed for PAGE and autoradiogrphy as described in Materials and Methods and legend to figure 16. Equal volumes of samples were immunoprecipitated with monoclonal anti fiber antibody (lanes B-F) and with rabbit antibody against 100K (lanes G-K). Half of the immunoprecipitate was loaded on the gel.

LANE A: HeLa, used as marker.

LANE B: UF8, mock infected, 6.4 X 10^6cpm.

LANE C: UF8, infected, 7.1 X 10^6 cpm.

LANE D: UF9, mock infected, 6.4 X 10^6cpm.

LANE E: UF9, infected, 1.9 X 10^6 cpm.

LANE F: UF7, infected, 3.9 X 10^6 cpm.

LANE G: UF8, mock infected, 6.4 X 10^6 cpm.

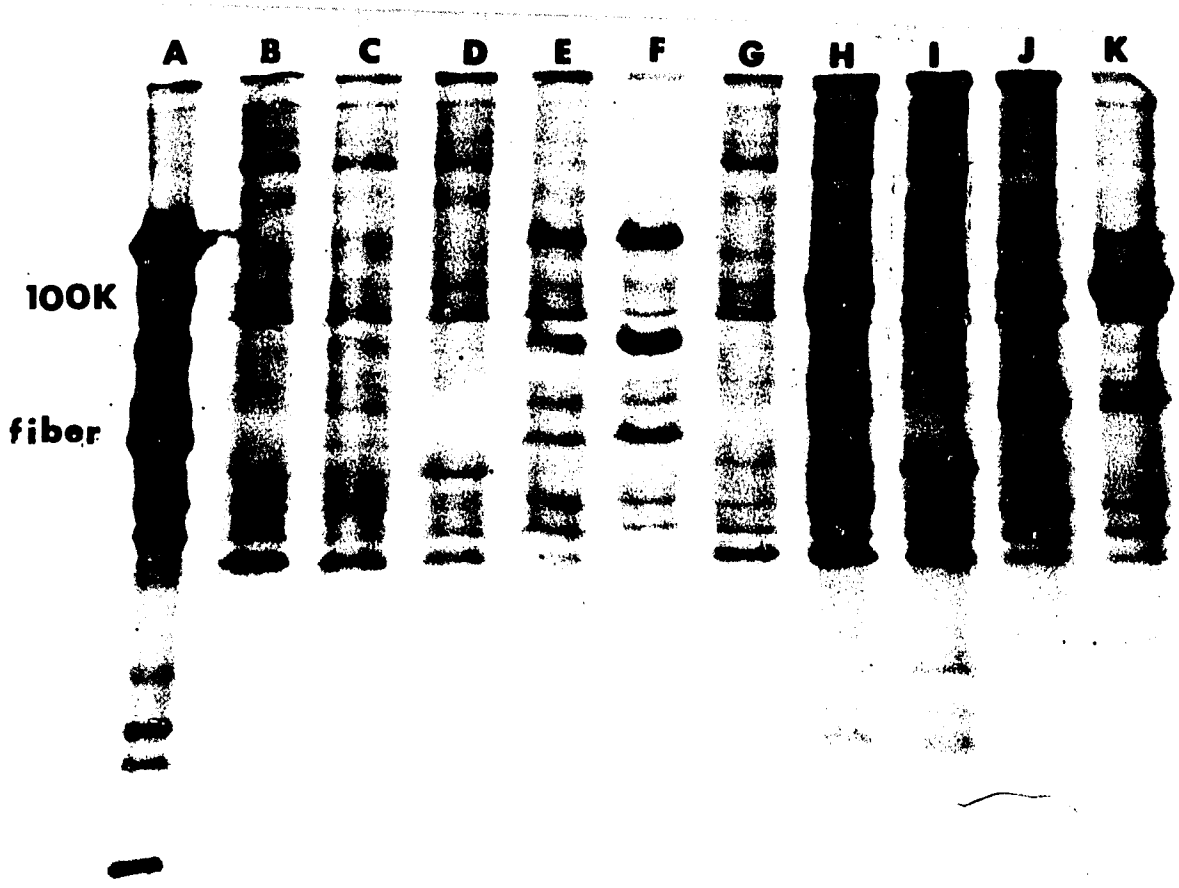
LANE H: UF8, infected, 7.1 X 10^6 cpm.

LANE I: UF9, mock infected, 6.4 X 10^6 cpm.

LANE J: UF9, infected, 1.9 X 10^6 cpm.

LANE K: UF7, infected, 3.9 X 10^6 cpm.

Figure 20



infection is comparable in these three cell lines.

Since UF9 was positive for fiber production, either chromosome 3 or 6 looked very promising. It was determined that only fifty percent of the cell population of UF9 contained chromosome 3. To further test the possibility that chromosome 6 is the one responsible for permissivity, it was reasoned that by seeding UF9 under cloning conditions and picking random clones, a collection of subclones would be obtained some of which would contain only chromosome 3 or 6, both 3 and 6, or possibly neither. Infection and testing of these clones for fiber production and then examination of the karyotypes should help in determining whether chromosome 3 and/or 6 is essential for permissivity. For example, if chromosome 6 is involved, subclones of UF9 containing chromosome 6 should be fiber positive and subclones of UF9 which do not contain chromosome 6 should be fiber negative. The same reasoning holds for the presence or absence of chromosome 3.

Seven subclones of UF9 were picked and were tested for their ability to support viral growth and ability to synthesize fiber. All seven subclones were infected and radiolabelled at 40-60 hr pi. Figure 21 is the result of an autoradiograph of PAGE of an immunoprecipitation of fiber using monoclonal antibody. Clones B1 and C1 in lanes E and G, respectively, were positive as was the positive control, UF9 in lane A.

Clones A2, A3, A4, B3 and C4 (in lanes B, C, D, F and H, respectively) were negative as was V79 in lane I. Lanes J and L are uninfected controls with UF9 and C4 respectively. As in other experiments, the 100K protein was immunoprecipitated to monitor the quality of infection and Figure 22 shows the result of this immunoprecipitation using the rabbit antiserum. With the exception of UF9, B1 and V79 (lanes A,E and I), all other samples showed a poor 100K protein production indicating that, in this particular experiment, Ad2 did not infect all the clones equally well. It was therfore, not valid to call these clones negative for fiber production. However, some statements can be made. B1 and C1 are fiber positive as both exhibited good fiber production. B1 was positive for 100K as well. C1 is positive by virtue of the fact that it made fiber despite a low 100K protein production. UF9 is positive as it was in previous experiments and V79 is negative (good 100K production, low fiber). The experiment was repeated under a slightly different protocol. Clones were infected with two different concentration of virus and labelled at different times pi. The clones were infected with virus at an MOI of 62 pfu/cell and labelled at 24-48 hr pi; and at an MOI of 6 pfu/cell and labelled at 40-60 hr pi. This was done to obviate the possibility that labelling at 40-60 hrs using the higher MOI is not efficient due to cell killing.

Figure 21: Fiber immunoprecipitation and PAGE analysis of UF9 subclones A2, A3, A4, B1, B3, C, C4 and V79. Cells were infected, labelled 40-60 hr pi., immunoprecipitated with monoclonal antibody and prepared for PAGE and autoradiography as described in legend to figure 16 and Materials and Methods. Half the immunoprecipitate was loaded on the gel.

LANE A: UF9, infected, 5.8×10^5 cpm.

LANE B: A2, infected, 3.4×10^5 cpm.

LANE C: A3, infected, 3.9×10^5 cpm.

LANE D: A4, infected, 1.4×10^5 cpm.

LANE E: B1, infected, 6.9×10^5 cpm.

LANE F: B3, infected, 1.8×10^5 cpm.

LANE G: C1, infected, 1.5×10^5 cpm.

LANE H: C4, infected, 2.0×10^4 cpm.

LANE I: V79, infected, 7.9×10^5 cpm.

LANE J: UF9, mock infected, 2.5×10^6 cpm.

LANE K: UF9, mock infected, 1/25 of sample.

LANE L: C4, mock infected, 2.2×10^6 cpm.

Figure 21

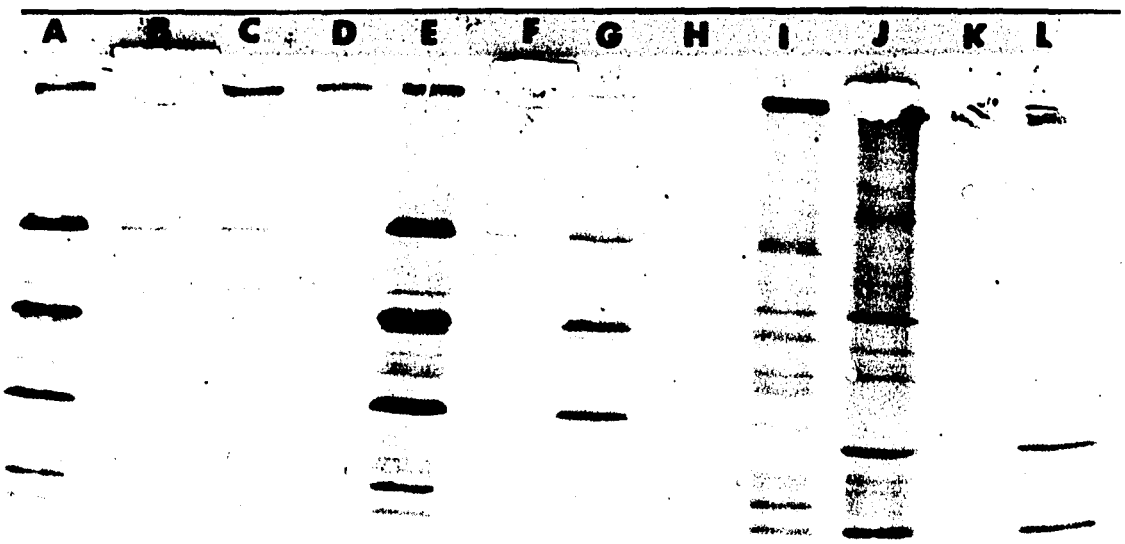
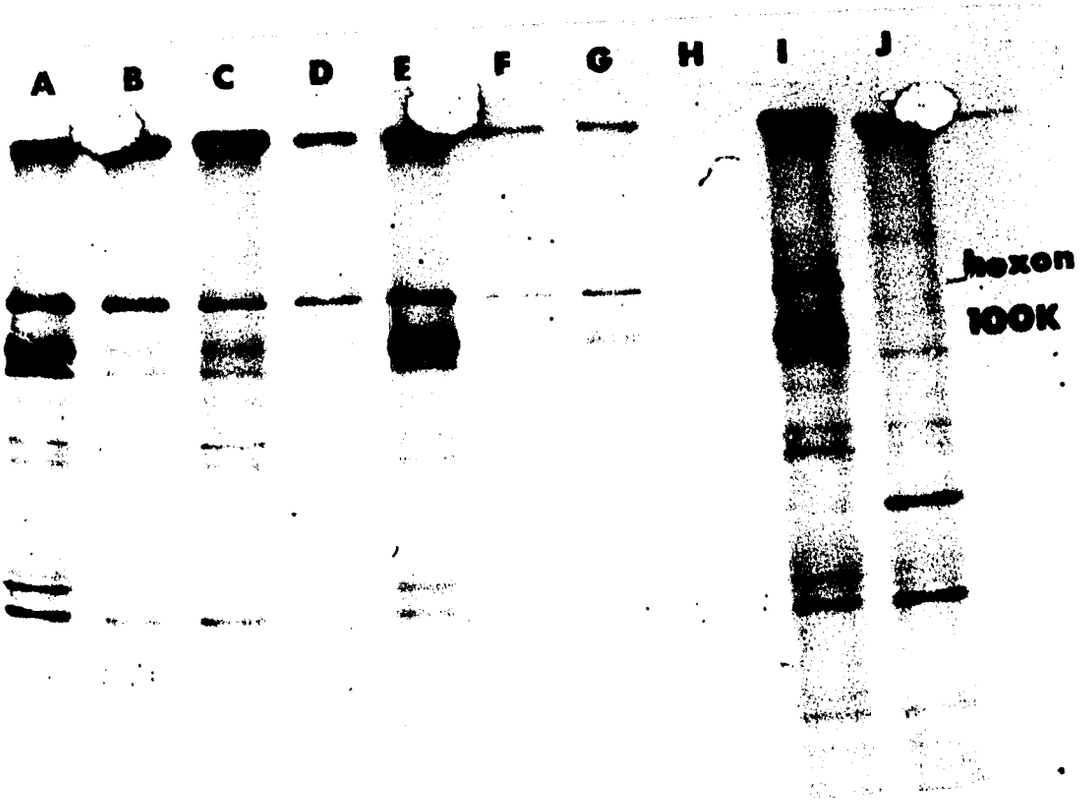


Figure 22: 100K protein Immunoprecipitation and PAGE analysis of UF9 subclones: A2, A3, A4, B1, B3, C1, C4 and V79. Cells were infected, labelled immunoprecipitated with rabbit anti 100K antibody, processed for PAGE as described in the legend to Figure 21 and the Materials and Methods. Samples are in the same order as they are for Figure 21.

Figure 22



The resultant immunoprecipitation for fiber of the infection with the higher MOI is shown in Figure 23. C1, C4, A3 (possibly) and UF9 (lanes F, G, C and H, respectively) are positive. C1 and C4 show more fiber than UF9; that is reasonable since C1 and C4 are a pure population as opposed to UF9. Clones A2, A4 and B3 (lanes B, D and E) are negative as is the negative control V79 (lane I). Examination of the companion gel with 100K protein immunoprecipitation with rabbit antiserum Figure 24, shows that the infection was much improved, in general. Most showed a comparable infection (A3, A4, B3, UF9 and V79 in lanes C, D, E, H, and I, respectively) with C1 and C4 in lanes F and G somewhat higher for 100K polypeptide. Clones A2 (lane B) remained uninterpretable in view of low 100K as well as fiber band.

Samples that were infected with 10-fold less virus were also tested and labelled at 40-60 hr pi (data not shown). The fiber immunoprecipitate was practically negative as very little fiber was detectible in all the samples including UF9, C1 and C4. Synthesis of 100K protein was similarly very low in all cases. From these experiments, it was concluded that clones C1, B1 and C4 were positive for fiber production, clone A3 exhibited a low level fiber production and clones B3 and A4 were negative. Data on clone A2 were inconclusive.

A viral growth curve was performed on A3, A4, B3 C1 and C4. Table 14 shows the result of the plaque assay.

Figure 23: Fiber immunoprecipitation and PAGE analysis of UF9 subclones. Cells were infected at MOI of 50-100 pfu/cell and labelled at 24-48 hr pi. Equal number of radioactive counts (3.0 X 10^6 cpm) were immunoprecipitated with monoclonal anti fiber antibody. The samples were prepared for PAGE as described in Materials and Methods.

LANE A: HeLa, marker.

LANE B: A2, infected.

LANE C: A3, infected.

LANE D: A4, infected.

LANE E: B3, infected.

LANE F: C1, infected.

LANE G: C4, infected.

LANE H: UF9, infected.

LANE I: V79, infected

LANE J: V79, uninfected.

Figure 23

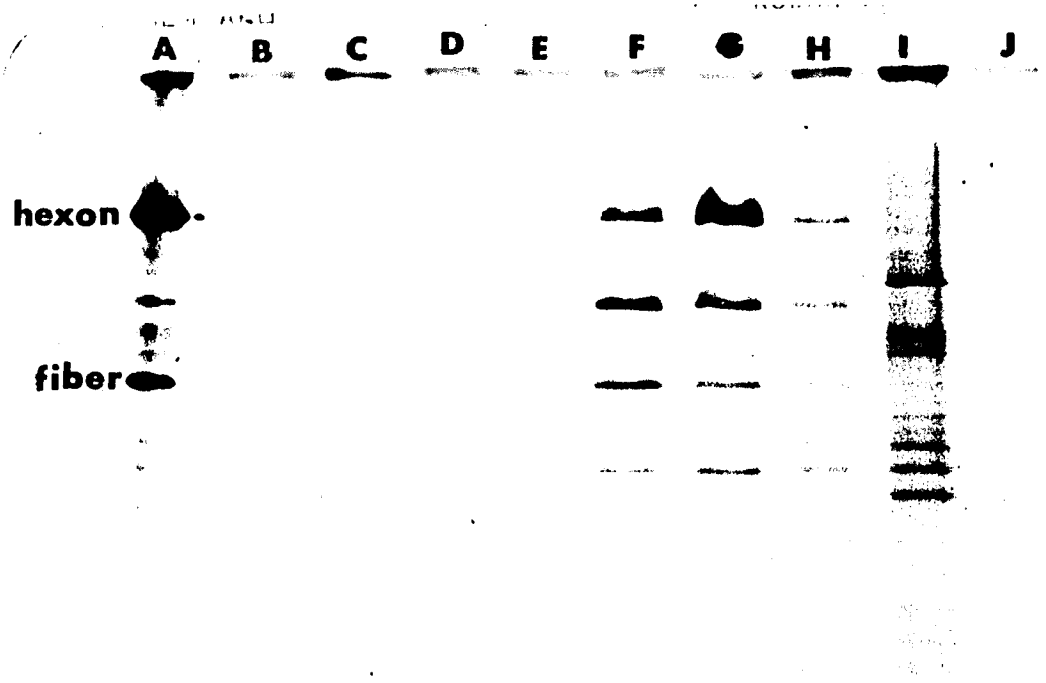
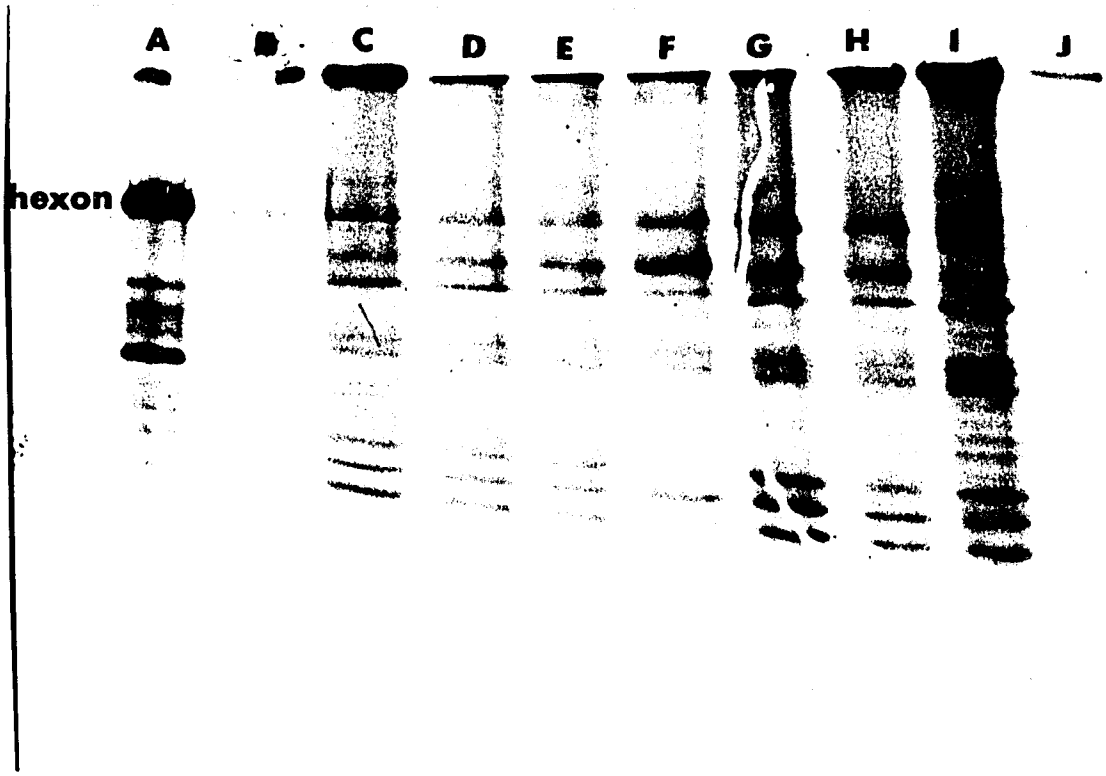


Figure 24: 100K immunoprecipitation and PAGE analysis of subclones of UF9. Cells infected and processed as described in the legend to figure 23. Rabbit anti 100K antibody was used for the immunoprecipitation. The samples are in the same order as for figure 23.

Figure 24



The result of this growth curve indicates that neither UF9 nor its segregants support viral growth even though some of the segregants do produce fiber. Selected clones were sent back to Dr. Francke for karyotyping. The results of the karyotype are shown in Table 15. This result was disappointing in that it failed to assign permissivity to either chromosome 3 or 6. However, it ruled out chromosome 6 as a likely candidate. The C1 result was curious. The C1 cell population did not seem to have a recognizable human chromosome. A plausible explanation is that one or more small translocations of human chromosomes to Chinese hamster chromosomes had occurred. This subclone was examined for the presence of human DNA sequences. A dot blot DNA hybridization was performed as described in Materials and Methods. The resultant autoradiograph did not demonstrate the presence of human repetitive sequences unambiguously (data not shown). If human sequences were present, they represented less than 0.01% of the total cellular DNA based on reconstruction experiments.

Results of the experiments on UF9 subclones eliminated chromosome 6 as the one relevant to permissivity. B3 which contained chromosome 6 and a partial chromosome 3 was negative for fiber. The only statement that can be made about C1 is that possibly a small human DNA sequence translocated onto a hamster chromosome allowing for the fiber positive nature of

Table 14

Ad2 Virus Yield in UF9 subclones[a]

Cell Line	Day 1 (pfu/culture)	Day 3 (pfu/culture)	Yield/Eclipse[b]
UF9	7.5×10^2	3.7×10^3	5.0
A3	1.5×10^4	1.0×10^5	6.0
A4	3.7×10^3	7.5×10^3	2.0
B3	1.5×10^4	1.4×10^4	0.9
C1	2.5×10^3	2.8×10^4	11.0
C4	1.5×10^4	9.3×10^4	6.2

a Cells were infected at a moi of 1 as described in the Materials and Methods.
b yield/eclipse is the ratio of progeny virus to input virus.

Table 15

Karyotype of UF9 Subclones

Hybrid	Fiber	Chromosome 3	Chromosome 6
C1	+	-	-
C4	+	-	+ (90%)
A4	-	Q^{a-}	-
B3	-	Q	+ (90%)

a short arm of the chromosome.

this subclone. Nothing could be said about the origin of this sequence so chromosomes 3 and 18 are not ruled out.

A set of hybrids resulting from a CHO X human cell fusion (UF11-UF13) were received next. These were actually received while the subclones of UF9 were being picked. These hybrids were selected for testing because they all contained chromosomes 3 and 18 and each contained a different fragment of chromosome 6. The karyotypes are in Table 16. The following experiment was actually done before the results of the subclones of UF9 were completed. It was of particular interest that chromosome 6 might be involved since chromosome 6 has many markers on it; this set of hybrids offered a possibility of actually mapping "permissiveness" to a distinct arm of the chromosome.

These cells were infected and labelled at 40-60 hr pi. Equal counts were immunoprecipitated for fiber and 100K protein and Figure 25 shows the results of this experiment. All these hybrids (UF7, UF10, UF11, UF12 and UF13) were positive for fiber production (lanes H, I, J, K, and L respectively). They all exhibited good 100K protein production (Lanes B, C, D, E and F). This added to the thesis that chromosome 6 is not relevant but these cell lines contained chromosomes 3 and 18. Hybrid UF10 which is the result of a fusion between V79 and human lymphocytes was also analyzed.

Table 16

Karyotype of Chinese hamster ovary X human cell hybrids

Cell Lines	Human Chromosomes																						
	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21	22	X
UF11	+	-	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	-	-	+	-	+
UF12	+	+	+	P	-	P	+	-	-	+	L	+	+	L	+	+	-	+	-	+	+	-	+
UF13	+	+	+	P	+	P	+	+	-	+	+	+	+	+	+	+	+	+	-	-	+	+	+

a partial chromosome is present.
b chromosme is present in less than 20% of the cells.

Figure 25: Immunoprecipitation and PAGE analysis of infected Chinese hamster ovary X human cell hybrid cells. Cells were infected at a MOI 50-100 pfu/cell and processed for PAGE as described in legend to figure 16. Cells were labelled 46-68 hr pi. Equivalent 4 X 10^5 cpm were immunoprecipitated with monoclonal antibody against fiber (lanes H-M) and rabbit antibody to 100K protein (lanes B-G).

LANE A: HeLa, marker.

LANE B: UF7, infected.

LANE C: UF10, infected.

LANE D: UF11, infected.

LANE E: UF12, infected.

LANE F: UF13, infected.

LANE G: UF7, infected, 1/5 as much as in lane B.

LANE H: UF7, infected.

LANE I: UF10, infected.

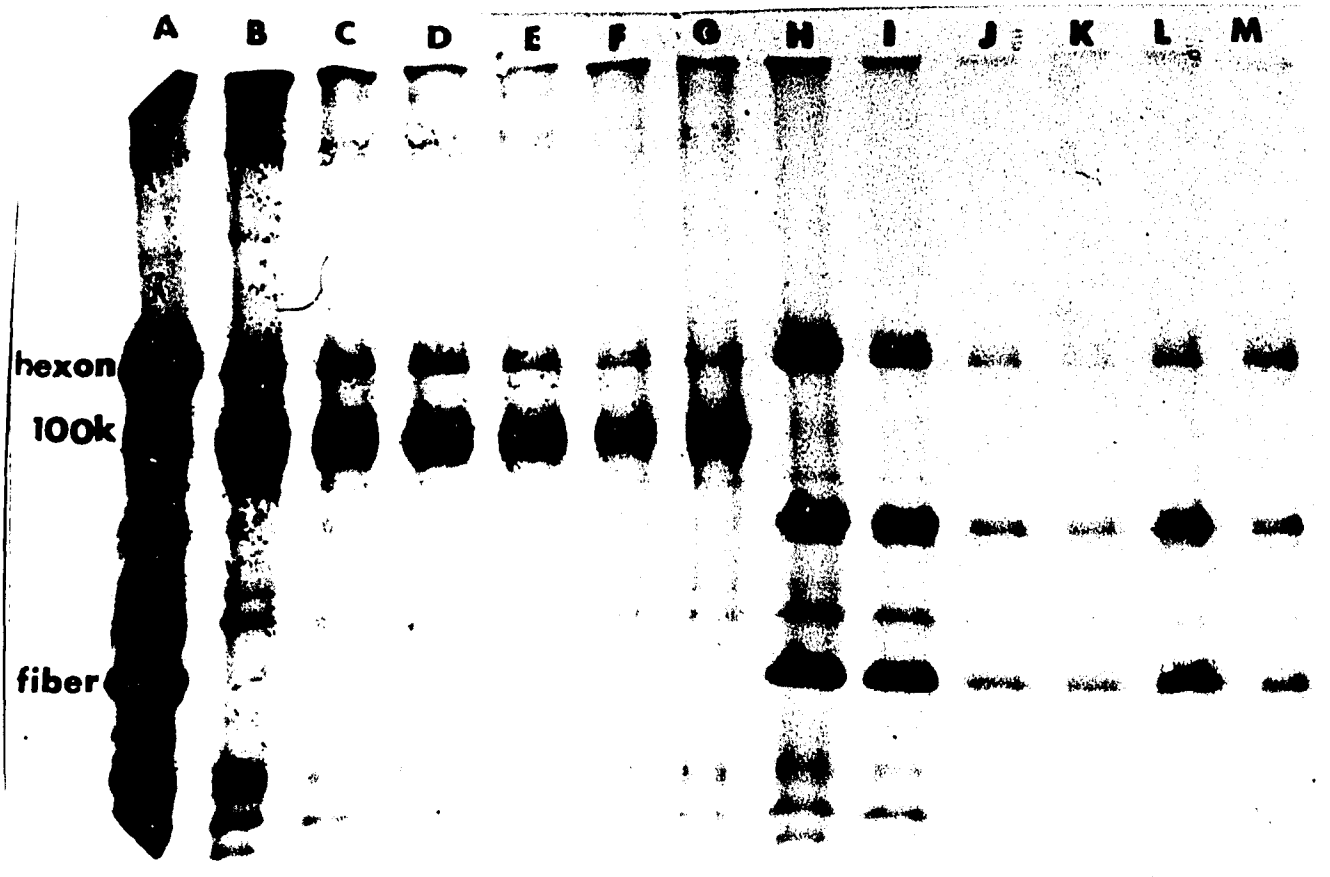
LANE J: UF11, infected.

LANE K: UF12, infected.

LANE L: UF13, infected.

LANE M: UF7, infected, 1/5 as much as in lane H.

Figure 25



It contains chromosomes 3 and 18 but is missing 6.

At the time that this experiment was performed, the finding that all the hybrids were positive for fiber production was disappointing, as this added evidence to the irrelevancy of chromosome 6. The fact that the Chinese hamster parent (CHO) in this set of hybrids was different than the Chinese hamster parent (V79) involved in the other fusion could not be the explanation since UF10 also demonstrated that chromosome 6 was not relevant. The findings with the subclones of UF9, described above, served to provide final documentation.

A viral growth curve was performed and the results are show in Table 17. UF10 is negative for viral growth but UF11, UF12 and UF13 show a marked increase over CHO.

The next set of hybrids were chosen by Dr. Francke as a blind experiment to try to sort out the involvement of chromosome 3 or 18. Hybrids designated as UF14, UF15, UF16 and UF17 were received. Virus growth and fiber production assays were performed at standard virus concentration and labelling protocol. Equal number of counts were immunoprecipitated with the two antibodies. Figure 26 shows the resulting autoradiograph of the PAGE, lanes A-E are samples immunoprecipitated for fiber and lanes F-K are the same samples immunoprecipitated for 100K protein. From this figure

Table 17

Ad2 yield in CHO X Human Cell Hybrids

(pfu/culture)

Hybrid	DAY 1	Day 3	Yield/eclipse
UF10	5×10^3	$<2.5 \times 10^4$	< 5
UF11	7.5×10^3	4.8×10^6	640
UF12	2.5×10^3	4.5×10^6	1800
UF13	6.3×10^3	4.0×10^6	634
TNT	2.1×10^3	$<2.5 \times 10^3$	< 1-2

a Data obtained as described in Table 14

it can be clearly seen that UF15 (lane C), UF16 which turned out to be the same hybrid as UF10 (blind study, lane D) and C1 (lane F) are clearly positive. UF15 is a result of a fusion with a different Chinese hamster parent A23 cells, so it is not appropriate to compare it with the other hybrids. C1 was redone at this point after the results of the karyotyping of the subclones of UF9 were known. It was still fiber positive. UF17 in lane D, though not seen too clearly on this gel, was unambiguously positive when rerun on a subsequent gel. The 100K protein immunoprecipitation shows a good infection with UF15 and UF16 (lanes I and J, respectively) producing more 100K protein than UF17 and C1 (lanes K and L, respectively). UF14 was determined to be negative for fiber production (data not shown). Though less 100K (data not shown) was immunoprecipitated from this hybrid than the other hybrids tested along with it, enough was made so that the fiber immunoprecipitation would obviously be positive if this hybrid had overcome the block to fiber production. The results of the growth curve are found in Table 18. Only UF16 is positive for virion production. It should be noted that UF16, is the same as UF10 but received at a different time. UF10 was, however, negative for virion production. This discrepancy could be possible because it is feasible that when the cells were thawed for the experiments, their genetic makeup was no longer

Figure 26: Fiber, 100K protein immunoprecipitation and PAGE analysis of UF15, UF16, UF17 and C1. Cells were infected, labelled from 40-60 hr pi immunoprecipitated and processed for PAGE as previously described in the legend to figure 16 and Materials and Methods. Equivalent counts were immunoprecipitated (1.5×10^5 cpm) with monoclonal anti fiber antibody (lanes A-F), and monoclonal anti 100K antibody (lanes G-L). Half of the immunoprecipitate was loaded on the gel.

LANE A: HeLa, 19-21 hr pi, cpm not determined.

LANE B: UF16, mock infected.

LANE C: UF15, infected.

LANE D: UF16, infected.

LANE E: UF17, infected.

LANE F: C1, infected.

LANE G: HeLa, 19-21 hr pi, cpm not determined.

LANE H: UF16, mock infected.

LANE I: UF15, infected.

LANE J: UF16, infcted.

LANE K: UF17, infcted.

LANE L: C1, infected.

Figure 26

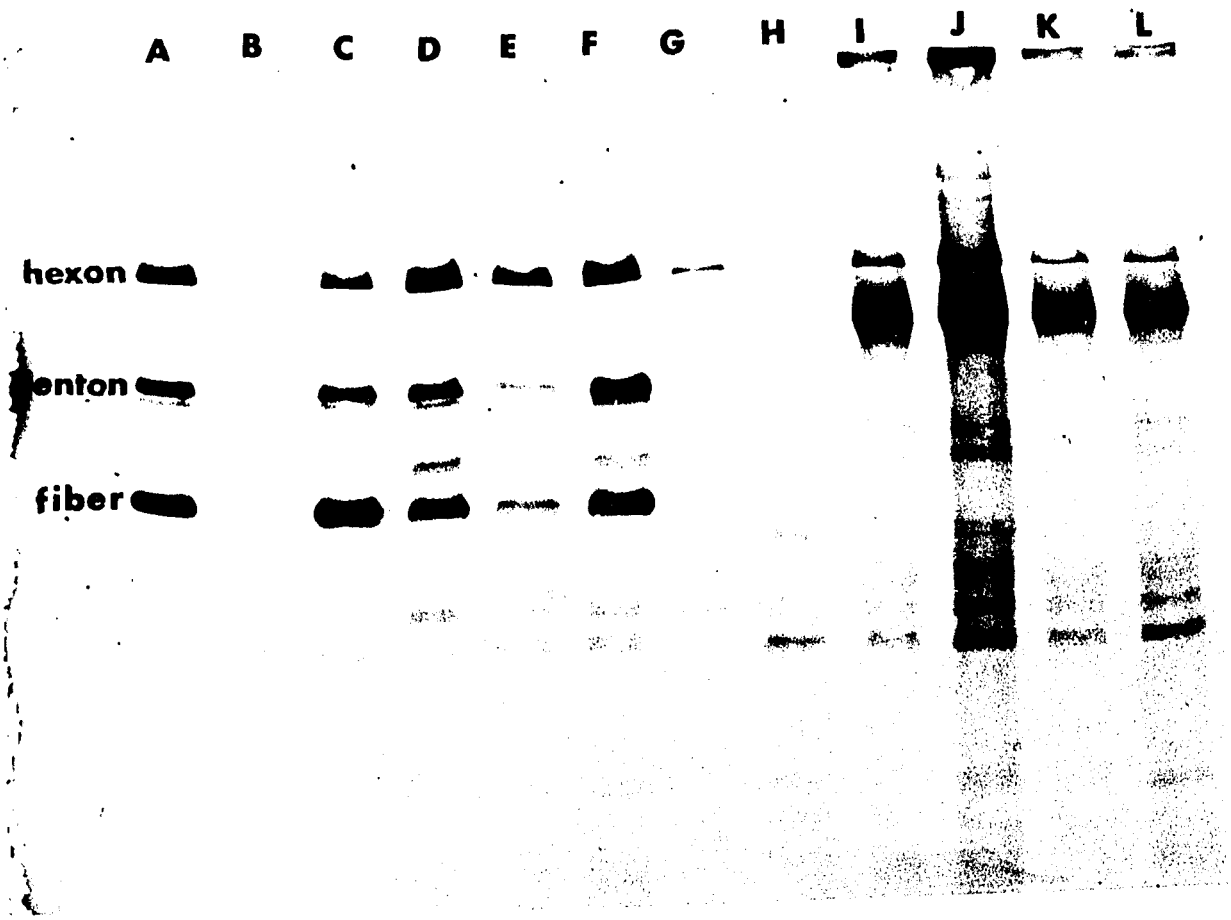


Table 18

Ad2 yield in hybrids UF14, UF15, UF16 and UF17[a]

(Pfu/Culture)

Hybrids	Day 1	Day 3	Yield/Eclipse
UF14	2.0×10^4	2.6×10^5	13
UF15	2.5×10^4	6.0×10^5	24
UF16	1.5×10^4	6.0×10^6	600
UF17	5.7×10^4	2.1×10^4	< 1

a Data obtained as described in Table 14

identical, thus giving conflicting results. These cells were never rekaryotyped. The karyotype of this new set of hybrids is shown in Table 19. The results ruled out chromosome 18 as relevant; UF14 which contained chromosome 18 was negative for fiber and UF17 which was positive for fiber did not have chromosome 18.

The possibility that "permissivity" might be uniquely assigned to chromosome 3 was examined. Again two hybrids were recieved in the hope to finally make a definite assignment. These hybrids were tested for fiber production. Figure 27 shows the results of immunoprecipitation with anti fiber monoclonal antibody. Both UF18 and UF19 are fiber positive. A growth curve was performed on these cells but not processed. The results were disappointing as one of these hybrids did not have chromosome 3 yet was fiber positive.

Therefore, it was not possible to make an unambiguous assignment of "permissivity" to a distinct human chromosome.

Table 19

Karyotype of Chinese hamster X human cell hybrids

Cell Line	Human Chromosomes																						
UF	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21	22	X
10	-	-	+	-	-	-	L[b]	+	-	-	+	-	+	P[a]	-	+	-	+	-	+	-	-	+
16																							
14	-	-	-	-	+	-	+	+	+	-	-	-	+	P	+	-	+	+	+	+	-	+	+
15	-	-	+	+	+	-	+	+	+	+	+	-	-	+	+	+	+	+	+	+	-	+	P
17	-	-	+	-	-	+	-	-	-	-	-	+	+	+	+	+	-	-	-	-	+	-	-
18	-	-	+	P	-	+	+	-	-	+	+	+	-	-	+	-	-	-	-	+	+	+	+
19	-	-	-	-	+	-	+	+	-	-	-	-	+	-	+	-	-	+	-	+	-	+	-

a partial chromosome is present.
b chromosome is present in less than 20% of the cells.

Figure 27: Anti fiber immunoprecipitation and PAGE analysis of selected hybrids and UF18 and UF19. Cell lines were infected at MOI of 25 pfu/cell and labelled at 24-48 hr pi. Samples were prepared for PAGE as described in Materials and Methods. Equivalent cpm were immunoprecipitated with the monoclonal antibody.

LANE A: HeLa, 19-21 hr pi, cpm not determined.

LANE B: V79

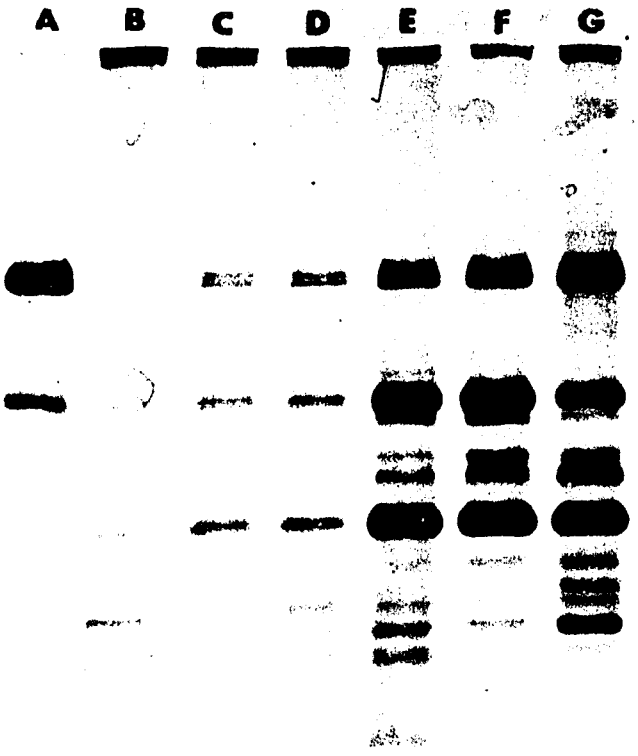
LANE C: UF3

LANE D: UF6

LANE E: UF10

LANE F: UF18

Figure 27



3.3: Role of SV40 T antigen in Adenovirus replication in CHO

Klessig and Anderson (1975) reported that when Ad2 infects CV1 cells, there is a drastic reduction in the production of fiber protein with a reduction to a lesser degree of other late proteins as compared to infection of permissive human cells. Some late proteins, such as 100K and IVa2, were made at normal levels. A similar response with Ad2 infection of Chinese hamster cells was observed except that the whole pattern of synthesis of late viral proteins was comparably reduced even further. Whereas it was possible to detect a fair amount of fiber in CV1 cells using immunoprecipitation, none or negligible amounts was detected in the Chinese hamster cells.

It was first reported by Rabson et al. (1964) that coinfection with SV40 enhances the replication of Ad2 in CV1 cells to a level comparable to that found in human cells as discussed in the INTRODUCTION. It seemed appropriate to ask whether T antigen would also enhance Ad replication in Chinese hamster cells. I was, however, unable to perform a standard coinfection

experiment with SV40 and Ad2 (as done originally for CV-1 cells) because Chinese hamster cells are resistant to infection with SV40 virions (La Bella et al., 1985). This block is at a level prior to viral gene expression since transfected SV40 DNA does express T antigen.

A family of nondefective hybrid adenoviruses (AdND) which are able to replicate on both monkey and human cells was isolated by Lewis et al. (1969;1973). The genomic structure of these viruses was determined and all the hybrids were found to contain a segment of SV40 DNA inserted at a site in region E3 (map position 0.85) from which Ad2 sequences had been deleted. The inserted SV40 sequences begin at the SV40 map position 0.11 and extend for various lengths into the SV40 early region. The SV40 sequences in Ad2ND1 is from SV40 map position 0.28-0.11. A fusion polypeptide is synthesized. The fact that the carboxyl end of the SV40 T protein provided a helper function for Ad2 infection of CV1 cells led to to the question of whether this protein (although not an intact T antigen sequence) can provide the factor necessary for Ad2 infection of Chinese hamster cells.

The infection of CHO with Ad2ND1 was analyzed. Two stocks of virus were used: Pool 1 was provided by T. Grodzicker, Pool 2 was prepared in this laboratory by infection of CV-1 cells with pool 1 as described in Materials and Methods. Table 20 shows the data which were used to calculate the titer of infectious virus in

pool 2. Two types of experiments were performed to assess whether CHO cells were permissive: virus yield and immunoprecipitation for fiber with PAGE. A growth curve of Ad2ND1 was performed in CHO in parallel with HeLa and CV-1 cells. The results are presented in Table 21. There is greater than a 1000-fold decrease in virus (as pfu) in CHO cells as compared to primate cells. Clearly, therefore, CHO are not permissive for AdND1 Although the values obtained in CHO are all within experimental error, there is a suggestive pattern of a progressive increase over the 3-day period (5-fold). The radiolabelling studies are discussed later in the context of clone 41 (p168).

The possibility that T antigen was not being made in sufficient amounts to act effectively as helper was considered. Perhaps not enough T antigen (as an Ad-SV40 fusion protein (Grodzicker, et al., 1976)) is being made in the murine environment of the Chinese hamster cells. In an attempt to investigate whether the T antigen would act as helper if it were made in sufficient quantities, I undertook the construction of a Chinese hamster cell line which would be producing large amounts of SV40 T protein such that an infection of these cells with Ad2 might mimic levels attainable in coinfection of SV40 and Ad2 in CV-1 cells.

An SV40 genome was introduced into CHO by co-transfection with the coding sequence from the gene

Table 20

TITRATION OF Ad2ND1 IN CV-1 CELLS

Virus prep	Dilution used for infection	Cells positive for 72K (%)[a]	Infectious Virus (units)[b]	Calculated virus conc.[c]
Pool 2	undiluted	100	$> 10^7$	-
	1 : 2	99	$> 10^7$	-
	1 : 10	63	5.2×10^6	5.2×10^7
	1 : 50	30	8.0×10^5	4.0×10^7
Pool 1	undiluted	95	$> 10^7$	-

a Coverslip cultures of subconfluent CV-1 were infected with virus as described in Materials and Methods. Twenty-four hours after infections, cultures were fixed and processed for immunofluorescence with rabbit antibody to Ad2 72K protein.

b The number of infectious virions was determined form the standard curve comparing infectious virus (as pfu) and percent viral infected cells, see Figure 1 in Materials and Methods.

c Number of infectious virions multiplied by dilution of virus used for infection.

Table 21

GROWTH CURVE OF Ad2ND1[a]

Hours post Infection	Cell Line (pfu/culture) HeLa	CV-1	CHO[b]
2	1.6×10^5	1.3×10^5	1.0×10^5
6	2.9×10^5	8.5×10^4	5.0×10^4
24	3.5×10^7	1.9×10^7	6.6×10^4
48	1.0×10^9	7.8×10^8	1.6×10^5
72	7.8×10^8	1.4×10^9	2.6×10^5

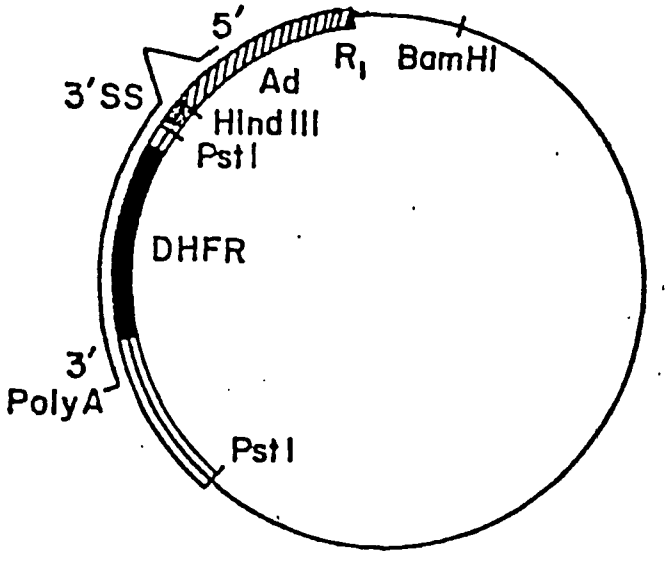
a Subconfluent cultures were infected with Ad2ND1 (pool 2, undiluted) and harvested for virus at different times post infection. Virus yield was determined as plaque forming units on HEK cells. Procedures were as described in Materials and Methods.

b The TNT subline of CHO was used.

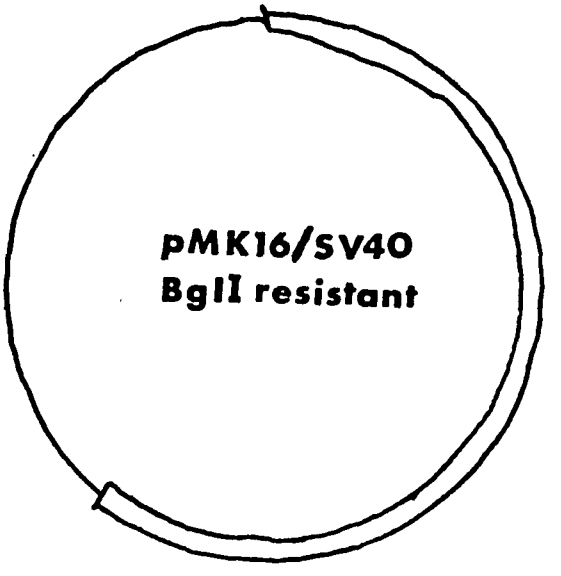
for dihydrofolate reducatase (DHFR, EC 1.5.1.3). The two recombinant DNAs are diagrammed in Figure 28. This was done for two reasons; first CHO are already "transformed" for growth control so that direct selection for transfectants involving SV40 is not convenient; second, amplification of the dhfr locus by subsequent treatment of cells with methotrexate (MTX) could result in co-amplification of the SV40 early region were it to become linked as is often observed with DNA co-transfer techniques (Wigler et al., 1980). As SV40 DNA, the origin defective mutant was used, pMK SV40 (Bgl I-R) isolated by Gluzman (1979). The origin defective mutant was chosen because it was reasoned that efficient expression of T could promote replication of a resident competent SV40 genome with possible loss of sequences and/or cell death. (Low levels of SV40 DNA replication have been found to occur in CHO cells, La Bella and Ozer, 1985). This possibility would serve as a counterselection to the goal of isolating a cell producing a high level of T antigen. As a source of DHFR sequences, the recombinant pAdD26SV(A)#1 constructed by Kaufman and Sharp (1982) was used. It contains the mouse cDNA for DHFR under control of the Ad2 late promotor. They have shown that this recombinant (or its relative pAdD26SV(A)#3 which contains an intact SV40 origin and early region) can transform DHFR negative CHO (DUK cells isolated by L. Chasin, 1980) to

Figure 28: Schematic diagram of plasmids used. An Ad2 major late promoter DHFR cDNA recombinant pAdD26SV(A) # 1 (A). A SV40 origin deficient recombinant pMK16/SV40 BglI resistant, (B). SV40 genome map with relevant restriction sites (C).

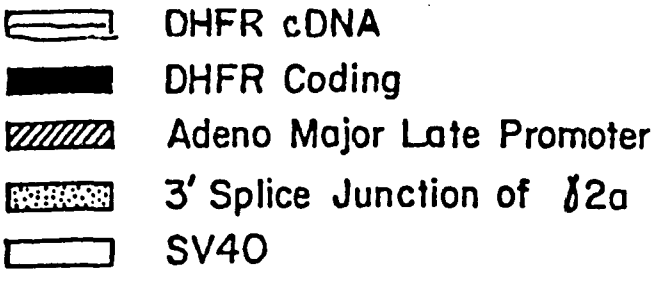
FIGURE 28

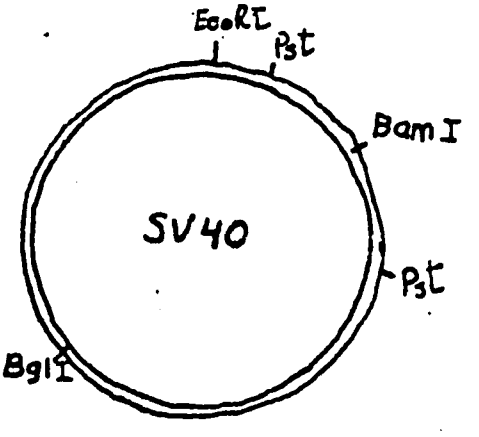


A



B





C

DHFR positive and can become amplified in MTX (Kaufman and Sharp, 1982). They did not, however, observe persistence of T antigen in any amplificant of pAdD26SV(A)#3. It was thought that because they were using a plasmid with an intact SV40 origin that they were possibly excising the integrated viral genome(s) and causing cell death. If that were the case, these experiments should succeed by using the origin defective mutant. The DUK cells were cotransfected with the two DNAs by the Ca-P co-precipitation method in the absence of carrier to maximize intracellular linkage, probably through homologous recombination involving the respective plasmid sequences. DHFR positive cells were selected in DME medium with dialyzed serum supplemented with proline (CHO-K1, the parent of DUK, is a proline auxotroph). Two weeks after the transfection, 54 colonies were picked and propagated in two parallel cultures; one containing coverslips so the cells could be screened for the presence of SV40 T antigen by immunofluorescence and the other for passage of cells for further experiments and long term storage. The colonies were assayed for T antigen using monoclonal antibodies provided by E.T. Gurney (1980): pAb 101 directed against the carboxyl terminal end and pAb 108 directed against the amino terminal end. Most of the colonies were positive for T when reacted with pAb101 but gave a negative reaction with pAb108. Clone 41

was picked for further experiments because it gave positive reactions with both antibodies though the reaction was somewhat stronger with the antibody directed against the carboxyl terminal region. Eventually clone 41 also lost its ability to react with the antibody directed against the amino terminal region. Since the T helper function was mapped to the carboxyl end of the protein, the pursuing of these experiments further was justified. Clone 41 was amplified as follows: 5 X 10^5 cells were seeded per dish and incubated with DME medium containing 0.01uM MTX (Calbiochem) supplemented with 10% dialyzed FBS and proline. After two weeks, involving repeated changes of medium, colonies resistant to this concentration of MTX were picked, screened for the presence of T antigen by immunofluorescence, and grown for the next step in amplification which was growth in 0.02 uM MTX. This procedure was repeated until colonies resistant to 0.01, 0.02, 0.1, 1.0 and 5.0 uM referred to as 41A, 41B, 41C, 41D and 41E respectively were collected. At all stages of this selection, colonies were screened for the presence of T antigen and frozen down for future studies.

After isolation of these putative amplificants, clones 41, 41A, 41D and 41D-7 (a clone of 41D) were infected with Ad2 and Ad2ND1. The results of the virus growth curve is shown in Table 22. Infection with these viruses

differed among the cell lines but in no case was there a significant increase over the eclipse value. As previously shown in Table 21 the yield of AdZND1 in permissive cells is approximately, 1 X 10^9 pfu/culture under comparable conditions.

Cells were also infected and labelled with ^{35}S-methionine from 43-68 hr pi. Ad2 and AdZND1 were examined in parallel in clone 41 D-7, CV-1 and HeLa. PAGE analysis of the whole lysate is shown in Figure 29. Comparison of the uninfected (lane A) and infected (lanes B and C) lysates of clone 41 shows evidence of infection by the presence of a broad band in the position of 72K and a weaker band in the position of hexon. Samples prepared from both infected cultures were quite similar. No fiber polypeptide is evident. The other samples verify the expected results for AdZND1 (and Ad2). Infection of HeLa cells (lanes H and I) shows prominent viral polypeptides. Although the levels are somewhat different with the two virus infections. Infection of CV-1 with Ad2 (lane E) shows the same viral polypeptides with selective reduction of penton,fiber and a polypeptide of 24 kd (protein VI or hexon-associated protein I); a faint band in the position of fiber is evident on longer exposure. Infection with AdZND1 (lane F) shows restored synthesis of both polypeptides to levels comparable to those observed in HeLa cells. Hence, the AdZND1 was functioning

Table 22

Ad2 infection of clone 41

Cell line	Virus	Virus Yield (PFU/Culture)[a] Day 1	Day 3	Day 5
DUK	Ad2	$<2.5 \times 10^3$	1.2×10^3	$<2.5 \times 10^3$
Clone 41A	Ad2	1×10^5	1.8×10^5	ND[c]
clone 41D-7	Ad2	2.6×10^5	6.3×10^5	ND
Clone 41D-7	ND1	8.8×10^5[b] 2.8×10^5	1.9×10^6[b] ND	ND ND

---

a Virus infection was performed as described in materials and methods, using Ad2, pool 2 and Ad2ND1, pool 1. Virus yield was determined as plaque forming units on HEK monolayers.

b Virus yield was determined on CV-1 monolayers. Plaque assay of Ad2ND1 on HEK and CV-1 indicated a 2-4 fold higher titer on the latter cell line.

c Not determined.

Figure 29: Whole lysates of CHO (subline 41D-7), CV-1 and HeLa, infected with Ad2 and Ad2ND1. CHO were labelled at 43-67 hr pi. HeLa and CV-1 were labelled 24-48 hr pi. 10 ul volumes of extracts were analyzed by PAGE.

LANE A: clone 41D-7, uninfected 1.9 X 10^5 cpm

LANE B: Clone 41D-7, Ad2 infected, 9.3 X 10^4 cpm

LANE C: Clone 41D-7, Ad2ND1 infected, 1.7 X 10^5 cpm

LANE D: CV-1 uninfected, 1.8 X 10^5 cpm

LANE E: CV-1, Ad2 infected, 5.0 X 10^4

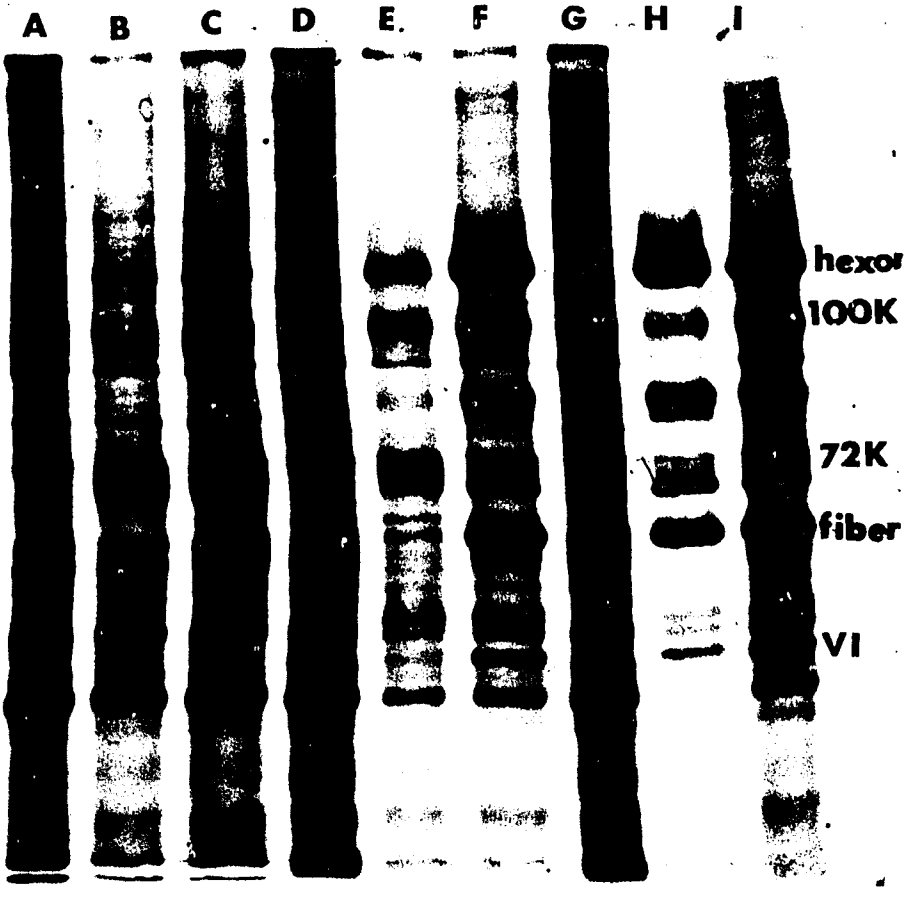
LANE F: CV-1, Ad2ND1 infected, 2.0 X 10^5 cpm

LANE G: HeLa, uninfected, 4.9 X 10^5 cpm

LANE H: HeLa, Ad2 infected, 1.4 X 10^5 cpm

LANE I: HeLa, Ad2ND1 infected, 4.5 X 10^5 cpm

Figure 29



consistent with previously published data (Lewis, 1973).

Immunoprecipitation studies were performed on these samples (and others) and the result of PAGE are shown in Figure 30. Lane L shows an immunoprecipitation of CV-1 infected with Ad2 which demonstrates that considerable fiber is present in extracts from these cells. Much lower levels are present in the CHO and clone 41 extracts. There appears to be an increase in the amount of fiber made in the Ad2ND1 infection of CHO cells (lane I) compared to Ad2 infection of CHO (lane J). Although equal amount of radioactivity were used for immunoprecipitation in both lanes, this result cannot be interpreted as a real increase in fiber production. This reservation is due to the data from the immunoprecipitation for 100K protein as shown in Figure 31. Comparison of immunoprecipitation for 100K of Ad2ND1 infected CHO (lane I) and Ad2 infected CHO (lane J) shows that the overall Ad2 infection was not equivalent. Consequently, one should expect immunoprecipitation for fiber to be poorer. In any case, the pattern of infection with this virus in these cells was the best for demonstrating virus-specific proteins (compare figures 7, and 29). A densitometry tracing of the Ad2ND1 infected lanes of the PAGE analysis is shown in Figure 32. (The

Figure 30: Monoclonal antifiber immunoprecipitation and PAGE analysis of CHO subclones, CV-1 and HeLa. Cells were infected with Ad2 and ND1 at comparable MOI. CHO subclones were labelled 40-60 hr pi. Hela and CV-1 were labelled 24-48 hr pi.

LANE A: DUK, uninfected, 2.6 X 10^6 cpm.

LANE B: DUK, Ad2 infected, 2.4 X 10^6 cpm.

LANE C: clone 41D-7, Ad2 infected, 2.3 X 10^6 cpm.

LANE D: clone 41D-7, Ad2ND1 infected, 2.3 X 10^6 cpm.

LANE E: clone 41, Ad2 infected, 1.8 X 10^7 cpm.

LANE F: clone 41A, Ad2 infected, 1.8 X 10^7 cpm.

LANE G: clone 41D, Ad2 infected, 1.8 X 10^7 cpm.

LANE H: clone 41D-7, uninfected, 1.8 X 10^7 cpm.

LANE I: TNT, Ad2ND1 infected, 1.4 X 10^7 cpm.

LANE J: TNT, Ad2 infected, 3.7 X 10^6 cpm.

LANE K: TNT, uninfected, 1.5 X 10^7 cpm.

LANE L: CV-1, Ad2 infected, 4.1 X 10^6 cpm.

LANE M: HeLa, Ad2 infected, 3.7 X 10^6 cpm.

Figure 30

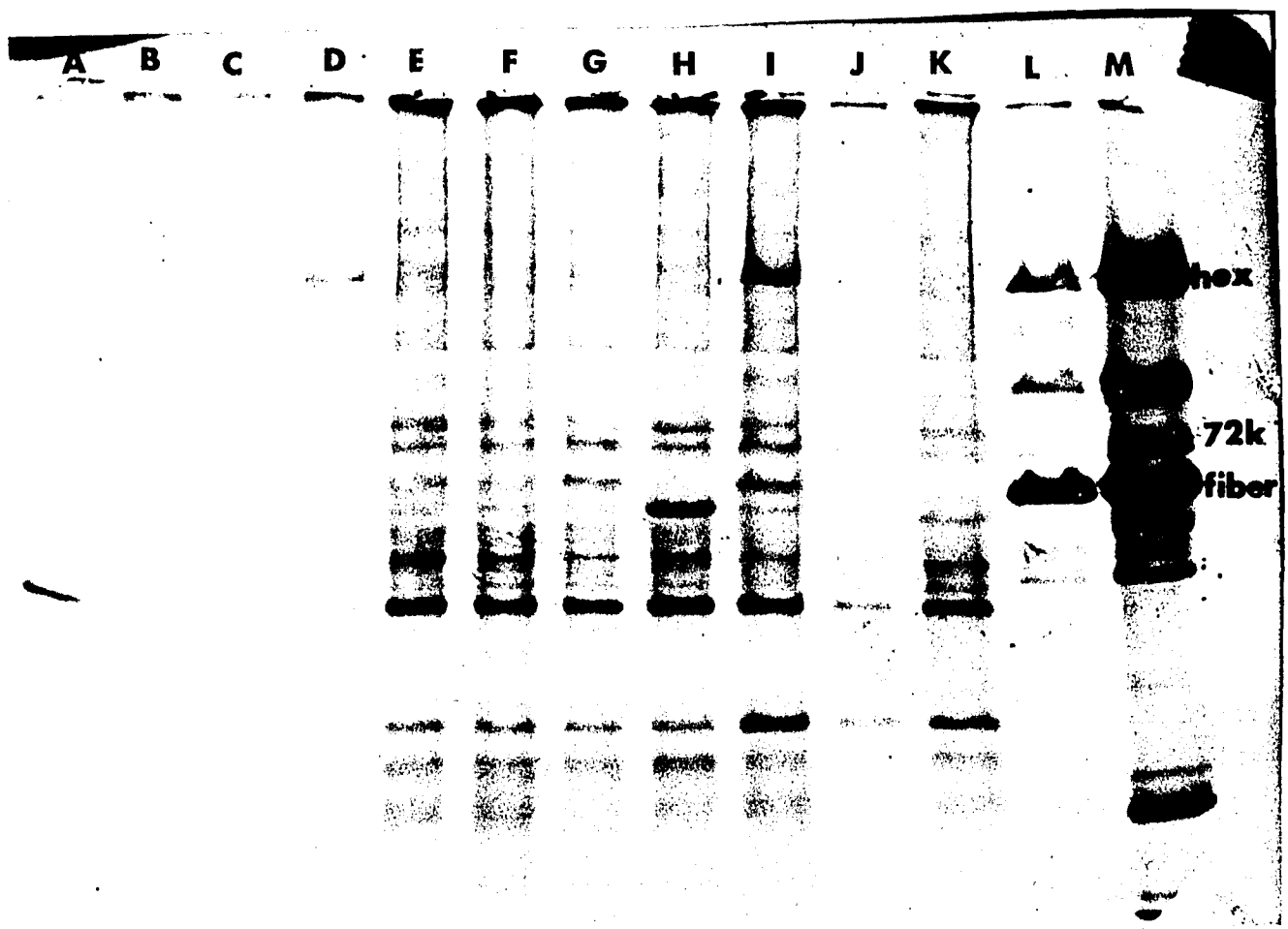
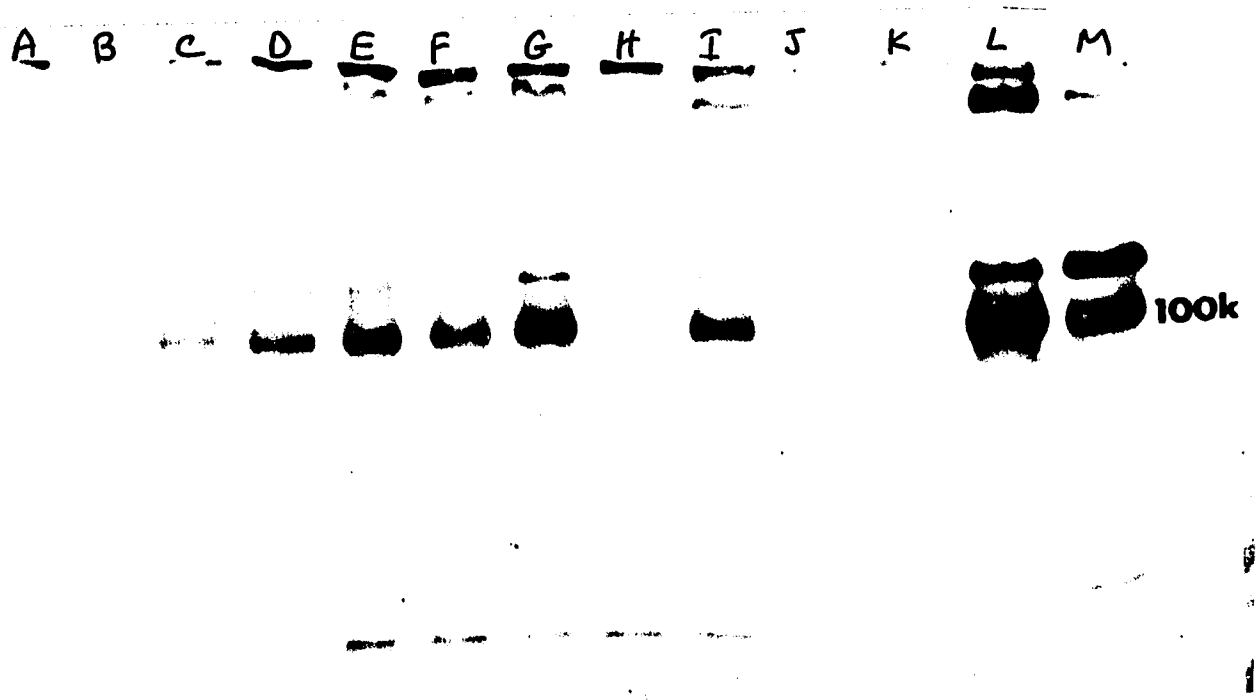


Figure 31: 100K immunoprecipitation with samples described in legend to figure 30 order of the sample is the same as in figure 30.

Figure 31

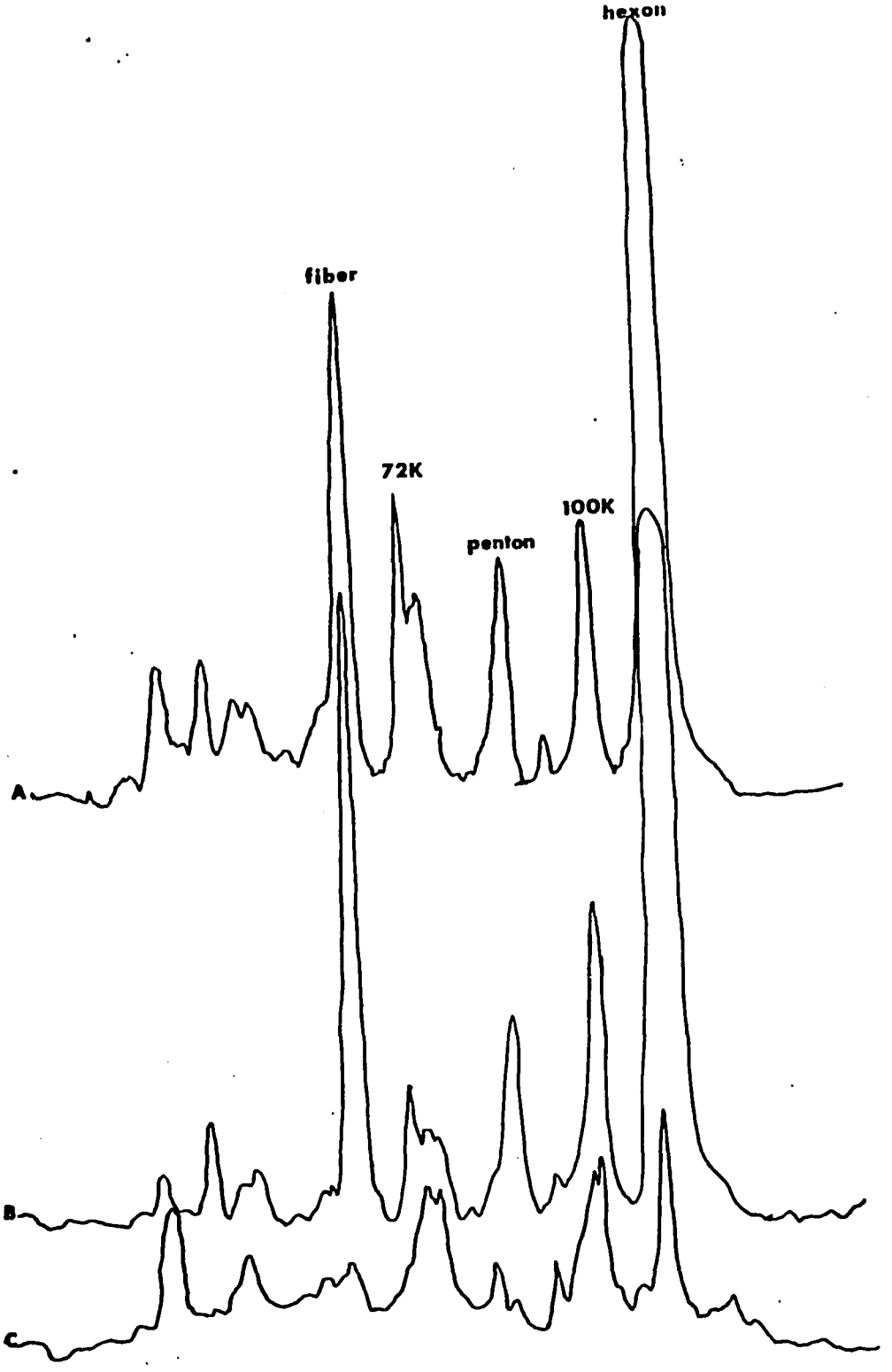


densitometry tracing of the Ad-infected lanes was previously presented as figure 15).

The amount of fiber produced in these cell lines was not very much and, at best, at the same level as Ad2ND1 infection of TNT. A possible explanation for the failure to see enhancement due to T helper is that in isolation of the MTX resistant clones the T antigen sequences did not get amplified as was expected. Therefore, verification of amplification of the DHFR sequences and the presence of amplified SV40 sequences as well in the MTX treated derivatives of clone 41 was sought. DNA was prepared from 3 cell lines (clone 41, 41-CC, and 41-DD), digested with Hind III, and analysed by the Southern procedure. Figure 33 panel A was obtained by hybridization of the Southern blot with nick-translated recombinant DNA containing only the mouse cDNA for DHFR in a plasmid (i.e. different from either original recombinant DNA used in the transfection see Materials and Methods). After the autoradiograph was obtained, the nitrocellulose filter was dehybridized, verified to be free of residual radioactivity, and rehybridized to detect SV40 sequences (Figure 33 panel B). Since pAdD26SV(A)#1 contains approximately 800 base pairs of SV40 as a source of the poly A addition sequences (see Figure 28), it was important to use a probe for rehybridization which is directed against the relevant SV40 sequences but does not include these 800 base

Figure 32: A densitometer tracing of a PAGE analysis of Ad2ND1 infected Hela (A), CV-1 (B), and CHO (C) cells whole lysates from figure 13.

Figure 32



pairs found in the pAd plasmid. Therefore, the large Pst I fragment was prepared from SV40 DNA (isolated from purified virions) and used to make the second nick-translated probe.

Hybridization in part A was evident with both MTX-resistant cell lines (lanes 2 and 3) but not clone 41 itself (lane 1), demonstrating at least some increase in copy number consistent with amplification of the donor DHFR sequence. It is furthermore likely that the DHFR sequences are present at a higher molecular weight than the comparably digested transfected DNA containing DHFR sequences (compare lanes 2 or 3 with lane 7).

Hybridization for the SV40 sequences demonstrated the same result; namely, the MTX-amplified cell lines were more reactive with this probe, indicating amplification of SV40 sequences as well. Moreover, the SV40 sequences were organized in an interesting fashion. The Hind III digested cellular DNA contained sequences of a higher molecular weight than the input pMKSV40 (compare lanes 2 or 3 with lane 5). Furthermore, these high molecular weight sequences were superimposable with those in part A, suggesting that the SV40 and DHFR sequences were in the same DNA fragment. Finally several of the other amplified SV40 sequences co- migrated with the SV40 sequences in the input DNA, indicating that the SV40 sequences were

Figure 33: Southern analysis of CHO (DHFR) that were co-transfected with pAdD26SV(A)#1 and pSVori- and selected for expression of DHFR. A clone was isolated (clone 41) and further selected for increasing resistance to MTX. 41-CC amd 41-DD are pooled populations resistant to 0.1 uM and 1.0 uM MTX, respectively. DNA was prepared from the 3 cell lines, digested with HindIII, and analysed. Panel A was obtained by hybridization of the gel with nick-translated DNA containing plasmid and dhfr (mouse cDNA). After the autoradiograph was obtained, the nitrocellulose filter was dehybridized, verified free of residual radioacitvity, and rehybridized with the large PstI fragment from SV40 isolated from purified virions (panel B).

LANE 1: Clone 41.

LANE 2: Clone 41-CC.

LANE 3: Clone 41-DD.

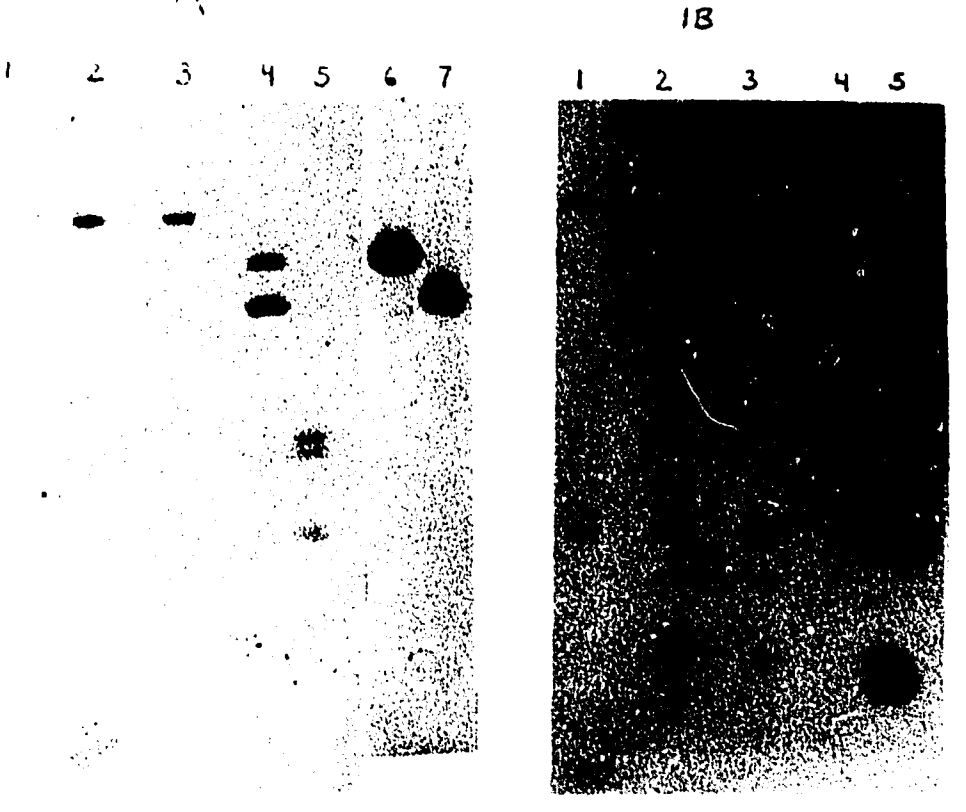
LANE 4: pMK SV40 digested with EcoR1.

LANE 5: pMK SV40 digested with HindIII.

LANE 6: pAdD26SV(A)#1 digested with EcoR1.

LANE 7: pAdD26SV(A)#1 digested with HindIII.

Figure 33



unlikely to be grossly rearranged consistent with functional expression. It should also be noted that the "SV40 sequences" being detected were authentic since the probe did not react with the pAdD26SV(A)#1 (Data not shown).

Although the SV40 sequences seem to be increasingly amplified in the MTX resistant cell lines, pulse labelled cell extracts which were immunoprecipitated with antibody against T antigen did not demonstrate increasing amount of T antigen by PAGE and autoradiography (C. Prives, personal communication). To verify this result, a Western blot was performed as described in Materials and Methods to assess the steady-state level of T antigen. Figure 34 shows that clone 41 and its MTX resistant derivates have similar amounts of immunoreactive T antigen (lanes b-e). This level is consistent with that observed in a monkey cell line (COS 7) containing a single integrated copy of pMKSV40BglIR (lane a). Moreover, the T antigen is approximately of the same size. T antigen in COS 7 is biochemically functional in all respects (Gluzman, 1981).

As a final approach, whether the mutation in the Ad DBP gene which corrected the restriction for Ad in CV-1 would function similarly in Chinese hamster cells was examined. These host range (hr) mutants replicate as efficiently on CV-1 as HeLa cells, in the absence of

Figure 34: Western Blot analysis of clone 41 and its MTX resistant derived clones. Confluent monolayers were extracted with HIP buffer (.14M NaCl, .01M Hepes, .001M $MgCl_2$, .5% NP40 and PMSF). The cells were scraped and centrifuged in a microfuge for 15 minutes. 15 ul of the supernatant were loaded on a gel. Transfer of proteins onto a nitrocellulose filter and subsequent immunoblottibg were carried out as described in Materials and Methods. Samples were immunoblotted with monoclonal antibody against SV40 T antigen (lanes a-g). Samples in lanes (h-K) were reacted with normal serum.

LANE a: COS cells, positive control.

LANE b: clone 41.

LANE c: clone 41 C.

LANE d: clone 41 D

LANE e: clone 41 E-1.

LANE f: clone 41 E-2.

LANE g: CHO (TNT).

LANE h: COS.

LANE i: clone 41 E-1.

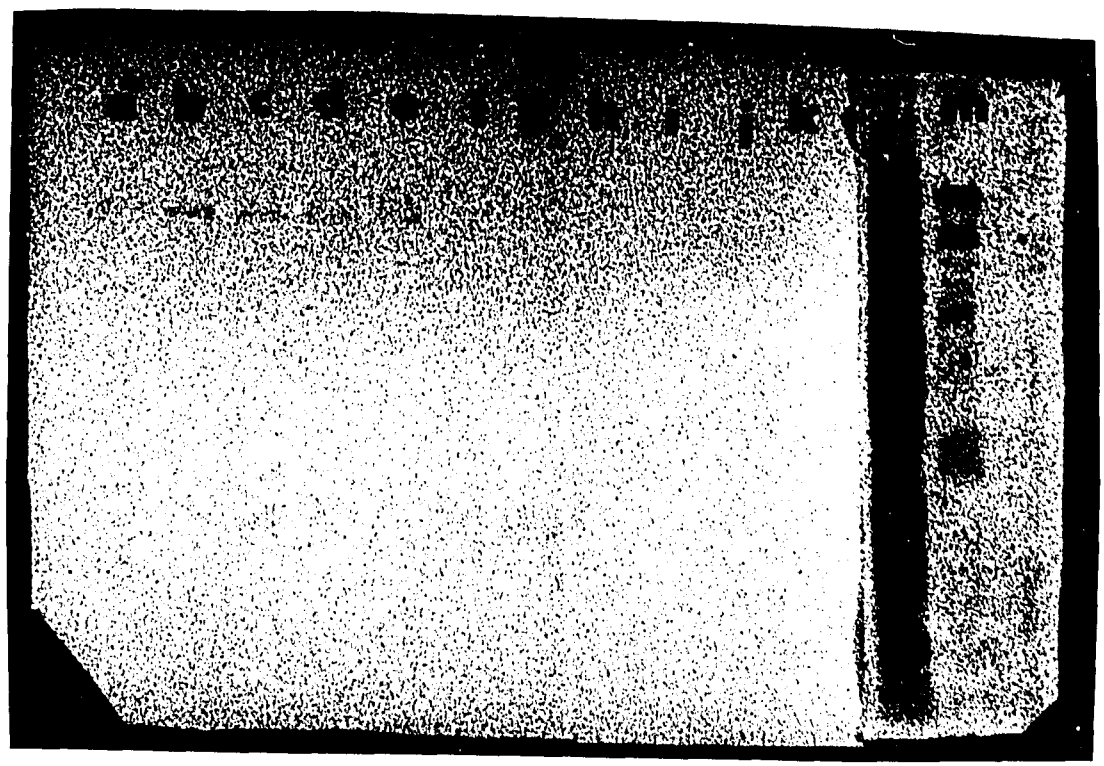
LANE j: clone 41 E-2.

LANE k: CHO (TNT).

LANE l: amido black stained to verify protein transfer.

LANE m: molecular weight marker.

Figure 34



any SV40 sequences. The data on virus production with Ad2hr400 and Ad2hr401 are shown in Table 23. The infection is restricted for these viral mutants as well. Both viruses replicated in control cell lines as expected with virus yields between 10^8 - 10^9 in CV-1 (and HeLa). This is not unexpected since the mutations presumably select for an improved interaction between the Ad DBP and cell proteins. The change appropriate for monkey cells need not be relevant to cells from unrelated species.

Table 23

Infection of CHO by Ad2 Host Range Mutants

	(Virus Yield (pfu/culture)		
Hours PI	Ad2	Ad2hr400	Ad2hr401
2 hr	$< 2.5 \times 10^3$	$< 2.5 \times 10^3$	7.5×10^4
6 hr	$< 2.5 \times 10^3$	1.3×10^3	6.3×10^4
24 hr	$< 2.5 \times 10^3$	1.5×10^3	3.9×10^3
48 hr	$< 2.5 \times 10^2$	7.5×10^2	7.3×10^3
72 hr	$< 2.5 \times 10^2$	1.5×10^3	8.3×10^3

a CHO cells (TNT subline) were infected as described in Materials and Methods. Virus yield was determined by plaque assay on HEK monolayers.

CHAPTER 4: DISCUSSION

Chinese hamster cell lines such as CHO and V79 offer considerable advantages for genetic and biochemical studies. It is remarkable that little is known about viral replication in these cells. In fact, the available literature indicates that no DNA viruses replicate efficiently in these cells. Because one of the major interests of our laboratory is host cell factors in virus-cell interaction, the characterization of the infection of Chinese hamster cells with adenovirus was undertaken. This problem was examined because of two long term considerations. First, analysis of viral replication in mutant or wild type cells may be restricted in some manner. A study of this restriction should provide insight into cell functions needed by the virus in different aspects of the virus-cell interaction. Second, since Ad2 is rather well characterized in terms of its genome structure and molecular biology, it provides an elegant probe or model for different aspects of cellular macromolecular biosynthesis since it uses host functions extensively.

In characterizing the infection of Chinese hamster

cells by Ad2 it was found that there is a block in virus replication. Production of infectious progeny virus is markedly inhibited in several Chinese hamster cell lines, including CHO-K1 and V79. One subline derived from CHO-K1 (lab designation TNT) was selected for more detailed study. Virus enters the cell and early viral genes are expressed. Viral DNA synthesis occurs although both the onset and maximal rate of viral DNA synthesis is somewhat delayed in CHO (e.g. at 24-36 hr pi compared to prior to 24 hr in Hela). A MOI of 1-10 infects virtually all primate cells (HeLa, CV-1) with Ad2, as shown by IF assay for the 72K protein. On the other hand, approximately 38-73 % of CHO were infected at a MOI of 84. However, such a reduction should have only a marginal effect on virus yield. Longiaru and Horwitz (1981), examined viral DNA synthesis in vivo in greater detail and concluded that viral DNA replication was essentially normal except for its delayed peak of synthesis. Hence, in all these areas, the efficiency of infection is reduced but only to a limited extent.

On the other hand, a marked reduction of several late viral structural proteins was observed. This finding was made in three ways: immunofluorescence for individual viral proteins; polyacrylamide gel electrophoresis in SDS (PAGE) of ^{35}S-labelled cell sonicates, and immunoprecipitation of cell extracts

followed by PAGE.

Immunofluorescence studies showed that even under conditions in which most CHO cells are infected (positive for 72K protein), a minority of cells synthesize hexon and virtually none synthesize fiber. These studies were limited by the requirement to examine infected cells within the first 2-3 days post infection, i.e. before changes in cell morphology complicated the microscopic examination. Since viral DNA synthesis is somewhat delayed, it is conceivable that there is insufficient time for accumulation of these late proteins. This interpretation is supported by the finding that another class of late protein (100K) was present in more cells, although the incidence was less than that positive for 72K.

Consequently the emphasis was shifted to radiolabelled cell extracts. However, basically the same pattern of results were observed. Of the major viral proteins (i.e. 72K, hexon, penton, and fiber), only 72K was consistently evident against the background of persistent cell synthesis. Immunoprecipitation of cell extracts followed by PAGE markedly improved the ability to detect viral proteins, as expected. Conditions were determined for effectively immunoprecipitating 72K, hexon (two subclasses), 100K and fiber from infected HeLa cells, using a variety of rabbit antisera and/or mouse

monoclonal antibodies (as ascites) from different laboratories and staph A (pansorbin). When Ad2 infected CHO were examined, a differential pattern of expression was observed. First, 100K was readily detectible. Second, hexon was detectible but in much reduced amounts. A partial explanation is provided by the fact that the monoclonal antibody used has a restricted reactivity (trimers and ninemers, but not monomers or assembled virus particles). In one series of experiments, a weak band of hexon was evident in direct PAGE of labelled CHO cell extracts (see Figure 13) Its level was consistent with significant inhibition, i.e. less than 72K and equivalent to 100K in the same extracts. In a permissive infection much more hexon than 100K is synthesized. Third, and most dramatic, fiber was virtually undetectible. These results were observed over a variety of experimental conditions including different times after infection (up to 4 day pi), short labelling (2 hrs) in low methionine containing medium or long labelling (16-24 hr) regimens in reduced methionine-containing medium, and a range of MOI with purified virus (with and without prior dialysis) or unpurified virus.

The restriction to Ad replication in Chinese hamster cells was due to an apparent deficiency state since cell hybrids between CHO and human fibroblasts or V79 and human leukocytes or fibroblasts were

permissive, yielding 500-fold more virus than either Chinese hamster parent. This result was a novel finding since there were no prior reports on permissivity of propagated cell hybrids involving cells restrictive to Ad. (Prior studies had reported that heterokaryons or 50-100 cell hybrid clones involving human cells and restrictive cells (non-Chinese hamster) also restored permissivity.) As expected, there was also an increase in fiber production. The correction in fiber synthesis or virus production was still below the level observed in permissive cells, although readily distinguishable from that of the Chinese hamster cell parent.

Taken together, this pattern of restriction was reminiscent of that previously reported for Ad infection of monkey cell lines. As discussed in the INTRODUCTION, there is a range of restriction depending, at least in part, on which monkey cell lines are examined. Nonetheless, there is a clear pattern of reduced fiber synthesis and decreased virus production. Furthermore, this restriction is corrected in short-term cell fusion studies. Therefore a comparison of Ad2 infection of CV-1 to that of CHO was made in a limited fashion.

Klessig and Anderson (1975) reported a depression in late viral protein synthesis in unenhanced Ad2 infection of monkey cells. They also reported that

some late proteins such as the 100K and IVa2 protein were made at a normal level. In our hands, Ad2 infection of CV-1 and PAGE analysis of ^{35}S-methionine pulse labelled extracts confirmed previous results. Due to efficient suppression of host synthesis, the major viral proteins including 72K, 100K, and hexon were readily apparent. Penton and fiber were barely visible. The amount of fiber protein synthesized in the infected Chinese hamster cells is much lower than the amount made during Ad2 infection of monkey cells, however. A considerable amount of fiber protein on PAGE was observed when immunoprecipitation of fiber from infected monkey cells was performed, whereas there was difficulty in detecting fiber immunoprecipitated from infected Chinese hamster cells (see Figure 30, chapter 3.3).

Several attempts were made to quantify the results. First, direct densitometry measurements of autoradiographs were performed and the areas under the peaks calculated. Although patterns were readily interpretable for Ad2- infected HeLa and CV-1 cells, the level of viral proteins were generally too low in Ad infected CHO. In one experiment, densitometry analysis was possible, (see figures 15 and 32). Even though the cells had been infected for 2 to 3 days (labelled 40-60 hr pi), 72K represented the major viral protein. Comparison of the levels of the other viral

proteins to 72K in that cell line vs the primate cell lines readily demonstrates the discrepent patterns. Due to differences in labelling conditions among cell systems, it is not possible to make further direct comparisons with confidence. Furthermore, because of the high background of cellular proteins in the CHO gel, it was also difficult to quantitate the relevant viral proteins. It is apparent, however, that the level of hexon in CHO is reduced since it is comparable to 100K in contrast to their respective levels in HeLa cells in this figure. It should also be noted that Ad2 infection of CV-1 can show reduction of hexon synthesis to the level of 100K polypeptide (Klessig and Anderson, 1975). As a second approach, I rehydrated the dried gel and cut out the region corresponding to the relevent band on the autoradiograph. Samples were counted by scintillation spectrometry. However, the counts were variable and, because of the high background on the gel due to the presence of labelled cellular proteins, were not consistent.

Taken together, the marked reduction in the levels of the major viral structural proteins would appear to be a sufficient basis for the failure to observe production of progeny virus. The level of fiber in monkey cells has been suggested to explain the 1000-fold reduction in virus yield as compared to HeLa cells. The level of fiber in Ad2 infected CHO is

considerably more than an order of magnitude lower than in Ad2 infected CV-1, consistent with the further reduction in virus yield. Ad infection of CV-1 is considered a semi-permissive infection since detectible, although low, levels of virus are produced. If the restriction in CHO represented a further quantitative defect of the same sort, one would be tempted to consider this interaction as semi-permissive as well. Strictly speaking, however, we were unable to demonstrate any significant increase in virus yield over a 5-day period, more consistent with a non-permissive virus infection. A low but significant level of virus prodution was observed in one experiment with early passage Chinese hamster fibroblasts.

Since the restriction in fiber synthesis was the most dramatic, it was emphasized in these investigations of CHO (and the Chinese hamster X human cell hybrids to be discussed later), although it cannot be concluded that such is the sole basis of the restriction. Several possibilities for the failure to detect fiber were considered. First, it was possible that the monoclonal antibody that was routinely used had restricted specificity. Fiber exists in multiple structural forms including monomer, trimer, in association with penton base as penton, and in association with hexon in virus particles. The monoclonal antibody immunoprecipitated predominantly

free fiber from infected HeLa extracts (see figure 12). Low levels of penton and hexon were also in the immune precipitate. It could not be concluded unequivocally that this represented precipitation of associated hexon (although this is likely to be the case) since it is the major labelled protein in the extract and could non-specifically contaminate the immunoprecipitate. For example, it is also present in the immunoprecipitate with rabbit anti 72K. Therefore, I also utilized a polyvalent rabbit antiserum prepared against purified fiber. It precipitated a significantly higher amount of assembled fiber as well as unassembled fiber as evidenced by the hexon and penton upon PAGE. When infected CHO (figure 11 and 12) and selective cell hybrids (figure 17) were examined with both of the antibodies, corresponding levels of fiber were immunoprecipitated. In addition, that monoclonal antibody co-precipitated hexon from several hybrids in which hexon was not the major labelled protein in the extract. Therefore, there is no evidence for a defect solely in a subclass of fiber being responsible. A second possibility involves glycosylation of fiber, which this study did not evaluate. Chee-Sheung and Ginsberg (1982) reported that a ts mutant in the fiber gene synthesizes a polypeptide which is not immuno-precipitable with their antiserum, presumably due to a defect in the glycosylation of fiber. This study did not

evaluate whether the antibodies which were utilised would only recognize glycosylated fiber. It was not possible to evaluate glycosylation of fiber in CHO or V79 directly since there would be insufficient levels to follow the polypeptide in the absence of antibody (e.g. analysis of whole cell extracts as in figure 7).

A third possibility which is modelled on the restriction of Ad in monkey cells is favored; namely, at a stage prior to translation as discussed in the INTRODUCTION. Although there is controversy as to the specific step most responsible, cell studies concur that the fiber polypeptide is synthesised in reduced amounts. It was observed by Fox and Baum (1972) that adenovirus mRNA failed to associate with monkey cells polyribosomes and it was deduced from this that the monkey cell protein synthesis machinery could not translate these particular viral mRNA causing a reduction in fiber synthesized. It should be noted that the viral late proteins are all transcribed under control of a common major late promotor. It is then curious why there should be a differential reduction in the synthesis of these late proteins; that is, the 100K protein is synthesized in a normal amount, hexon is reduced (variably in different cell lines) and fiber is even further reduced. It was then reported by Anderson and Klessig (1983) that monkey cell ribosomes were able to translate mRNA from Ad2 infected cells as efficiently

as human cells in a cell free system. A more plausible explanation was put forth by Klessig and Chow (1980) who showed, using electron microscopy, that approximately 15% of the fiber message in infected CV1 cells contain long segments of RNA between the tripartite leader and the fiber encoding portion of the message. These are normally removed by splicing in a permissive infection. These data suggest a defect in the fiber message splicing mechanism in monkey cells. Anderson and Klessig (1984) examined the 5' ends of fiber messages isolated from permissive and semipermissive infected cells. They particularly looked at the "x", "y" and "z" ancillary leaders on fiber messages. This was of interest because these leaders are encoded by sequences within early region 3 and do not occur in any other late messages except the fiber mRNA. They reported a direct correlation between the synthesis of the fiber polypeptide in vivo and the presence of the "x" and/or "y" ancillary leaders on messages encoding the fiber polypeptide. It is only the fiber RNA that can contain the "x" and "y" leaders, and the fiber protein is the only late protein which is glycosylated. They postulate that these ancillary leaders play a role in the synthesis of this glycoprotein, as well as that of the Ad2 19K glycoprotein encoded by early region 3 whose mRNA also contains the "x" and "y" leaders. Whether the block to Ad2 replication in Chinese hamster

cells is the same as the block to Ad2 replication in monkey cells is not known. Such detailed studies into the nature of the block in Ad2 infection of Chinese hamster cells have not been performed.
One experiment that would be interesting to perform is the infection of monkey X Chinese hamster cell heterokaryons to determine whether they complemented each other as assayed by fiber synthesis using immunofluorescence.

In view of this model of Ad in monkey cells, I examined three approaches which were effective in correcting the host restriction in that system;namely, the role of SV40 T antigen as a helper in an acute infection (Rabson, 1964) or in a cell system expressing an integrated copy of that gene (Shiroki and Shimojo,1971) and the role of host range mutations in the Ad DNA binding protein (hr400 and hr 401, Klessig and Grodzicker, 1979). None supported efficient replication of infectious virus in the Chinese hamster cell. However, negative results can frequently be interpreted in multiple ways and each experimental approach has its own potential limitation.

Coinfection of monkey cells with adenovirus and SV40 leads to an enhanced infection. The factor responsible for this enhancement resides in the carboxyl terminal region of SV40 T antigen. The fact that the SV40 T antigen functions in the role of helper can be rationalized by making the observation that

SV40 T antigen, a protein synthesized during an infection of monkey cells by a simian virus, is adept at interacting with monkey cellular proteins which might be needed by the virus to replicate (Tornow et al.,1985). Adenovirus needs host factors to complete its replication, some of which may be shared with SV40. Perhaps the SV40 T antigen which is better suited for interaction with monkey host cell proteins facilitates a viral-host cell protein interaction which is needed for the replication of adenovirus. Given that the SV40 T antigen acts as a helper, the question was asked whether it could function in that same capacity in CHO. A mixed infection such as the one that was done in the enhanced infection of monkey cells could not be performed because SV40 does not enter CHO well. Initially the non-defective hybrid virus Ad2ND1 was exploited. When this approach was ineffective, the development of a CHO cell line which would constitutively produce high levels of T antigen was undertaken by co-transfecting the SV40 T antigen gene (on a pMK plasmid) with another plasmid containing a mouse cDNA DHFR gene under the control of the adenovirus major late promotor (see Figure 28). This latter plasmid was chosen because the gene amplification phenomenon by MTX selection could be exploited to manipulate the amount of T antigen which is being made in the transfected cell (Wigler, 1980)

It was observed that a number of specific genes can undergo amplification when mammalian cells are placed under selective conditions (Schimke, 1984). N^5, N^{10}-methylenetetrahydrofolate is a coenzyme that serves as an electron donor and as a one-carbon donor in the methylation reaction of dUMP —> dTMP and in steps in de novo purine biosynthesis. Tetrahydrofolate is regenerated through the reduction of dihydrofolate by the enzyme dihydrofolate reductase (EC 1.5.1.3). Analogs of dihydrofolate such as aminopterin and amithopterin (methotrexate) are potent inhibitors of this enzyme. Mammalian cells selected in MTX can become resistant to this drug. MTX resistance can come about as a result of three mechanisms. These include altered transport of MTX (Sirotnak et al., 1981), reduced affinity of DHFR for MTX (Flintoff et al., 1976) and overproduction of DHFR (Alt et al., 1976). Step-wise selection greatly favors the last mechanism. I performed step-wise MTX selection on clone 41 which was isolated from DHFR-deficient DUK cells after cotransfection with the plasmids described above. I selected clones in up to 5 uM MTX (clone 41 E). A southern blot analysis of the DNA probed with the Pst I large fragment indicated that the SV40 sequences which were integrated in the CHO were indeed amplified. A western blot was performed to assess whether the T antigen protein was overproduced. However, there was

no difference between clone 41 and its amplified derivatives, suggesting that regulation of the level of this protein was occurring. It was not higher than a monkey cell line (COS) which has a single integrated copy of the T antigen gene, similarly derived from the origin-defective SV40 pMK recombinant, and expresses T antigen constitutively. Infection of unamplified and amplified cells with Ad2 (or Ad2ND1) failed to produce fiber protein upon immunoprecipitation and PAGE.

There are several explanations for the negative result. First, it could be because not enough SV40 T was being synthesized. T protein is autoregulated due to binding of the protein to two sites on the SV40 genome (Di Mayo and Nathans, 1982; Rio and Tijian, 1983). One site had been mutated due to the 6-base pair deletion at the origin; however, the other (site II) would be expected to be functional although this was not directly verified. Alternately, a mutation in cellular factors could have occurred. T antigen has effects on cellular gene expression and chronic high levels may not be compatible with good cell growth. This interpretation would also be consistent with the failure of Kaufman and Sharp to find even a single intact copy of the gene for T antigen in the amplified cell lines that they derived (Kaufman and Sharp, 1982). A second possibility is that the T antigen could not function properly in the environment of the hamster cell.

Although there is no direct information on this point, it is known that T is a multifuntional protein and different monoclonal antibodies recognize different subsets of T (Prives, 1983). Clone 41 did show differential reactivity with two monoclonal antibodies directed against T antigen. A third possibility is that the SV40 T antigen was made in a sufficient amount but was not functional. A single experiment to assess the functionality of T antigen in supporting DNA replication was inconclusive and was not pursued (data not shown).

One of the genetic advantages of Chinese hamster cells is the availability of stable interspecific hybrids, especially those with human cells. Upon infection of a Chinese hamster cell (V79) X human cell hybrid (Hybrid X-7A) and assay for the production of mature virion by plaquing on HEK cells, an increase in the amount of infectious virions made in this cell line over that made in the Chinese hamster parent of this hybrid was found. This hybrid culture was a mixed population of cells where every one of the human chromosomes were present in at least 20% of the culture. Since the hybrid cells were positive for Ad2 replication, the availability of karyotyped Chinese hamster X human cell hybrids presented the opportunity to identify the human genes which are involved. Two criteria were employed: (1) an increase in progeny

infectious virus and/or (2) an increase in fiber synthesis. In the case of the former, the criteria were relatively clear since viral production in V79 was minimal and X-7A showed over a 500-fold increase. It was, however, plausible that the restoration of virus synthesis involved more han one human gene. For example, it was found that the Ad 72K protein was involved in virus assembly in a host-dependent fashion in different human cells (Nicolas et al.,1983). One might expect this to involve a function separate from efficient synthesis of viral structural proteins. Even if both were mediated by 72K, as might be suggested by Ad hr mutants, one might expect them to involve interactions with different cell functions or different domains of 72K. Alternatively, there could be deficiencies in the synthesis of other viral proteins than the viral structural proteins which have been evaluated. Therefore, fiber synthesis was also examined. This approach, although conceptually simpler, suffered from greater experimental complexities. First, it was difficult to assess synthesis of the viral proteins quantitatively even after immunoprecipitation and PAGE, as discussed earlier. This problem was frequently true, even in cell hybrids. Second, infection and labelling of multiple different cell lines would be required, making comparisons more difficult. Third, the kinetics of viral

synthesis might differ. Finally, the presence of the appropriate gene product in less than 100% of the cells might be associated with incomplete restoration of synthesis of fiber as might fluctuating levels of the putative function due to uncontrolled variables. It was not feasible to do detailed examinations of each cell hybrid as if they were independent experimental systems. As a practical compromise, fiber synthesis was evaluated at a "standard" time post infection which had yielded reproducible results in the Chinese hamster parents (40-60 hr pi). By utilising a long labelling regimen, the minimization of those variables was anticipated involving variations in the time course of infection. Most important, the control for the quality of infection and labelling by normalization of fiber by an internal control; namely, synthesis of 100K in the same cell extracts was attempted. This protein was selected because it was unaffected in the Ad2 restriction in CV-1 and was synthesised in readily detectible levels in infected V79 and CHO cells. Two antibodies were available. The rabbit antiserum was more effective in immunoprecipitation that the monoclonal and was used in all but the initial experiments.

After Ad2 infection of 15 different Chinese hamster X human cell hybrids and 3 different CHO X human cell hybrids, a unique assignment of permissivity to a single human chromosome could not be made.

Experiments were performed with positive and negative controls included in each analysed series. These results were quite disappointing in view of the initial suggestion that such an assignment might be possible, as discussed in the relevant chapter. The composite data for virus production and fiber synthesis are summarised in Tables 24 and 25 respectively as concordant or discordant. (Concordant means the chromosome and function are either both present or both absent.) These analyses were performed to minimize the impact of any single hybrid. In the case of virus production, it can be seen that an unexpected correlation with chromosome 1 is evident since 14 of 17 are concordant with the presence of the long arm of that chromosome. 12 that are concordant have the entire chromosome and 2 that are discordant have only the short arm (p) on that chromosome. Two other discordant hybrids are also interesting. Hybrid UF7 gave equivocal results in that some increase in virus was often observed. Hybrid UF2 was clearly negative but had few human chromosomes (only 5 <u>in toto</u> including chromosome 1). This latter finding suggested the possibility that chromosome 1 might be necessary but not sufficient. Unfortunately, there were relatively few hybrids which were permissive and were concordant for chromosome 1. Examination of their karyotypes indicated that a large number of chromosomes (including chromosome 3) were shared by these 5 hybrids

Table 24

Summary of Concordance and Discordance
of Selected Chromosomes for Virus production

Cell lines	Virus	Human Chromosomes 1		3		6		8		12		14		18		X	
		C	D	C	D	C	D	C	D	C	D	C	D	C	D	C	D
UF1	-	+			P	+			+		+		+	+			+
UF2	-		+	+		+		+			+		P	+			+
UF3	+	+		+		+		+		+		+		+		+	
UF4	-	+		+			+	+		+			+	+			+
UF5	+	+		+		+			+	+		+		+		+	
UF6	-		P		+		+	+			+		+		+	+	
UF7	-		+		P		P		+		+		+		+		+
UF8	-		P		P		P		+		+		+	+			+
UF9	-	+			+		+	+		+		+		+		+	
UF10	-	+			+	+			+	+			P		+		+
UF16	+		+														
UF14	-	+		+		+			+	+			P		+		+
UF15	-	+			+		+		+	+			+		+		P
UF17	-	+		+			+	+			+		+	+		+	
UF18	NT																
UF19	NT																
UF11	+	+		+		+		+		+		+		+		+	
UF12	+	+		+		+			+	+			P	+		+	
UF13	+	+		+		+		+		+		+		+		+	
C/Total		14/16		10/16		12/16		8/16		10/16		6/16		11/16		8/16	

a concordant
b discordant
c partial chromosome present
d Not Tested

Table 25

Summary of Concordance and Discordance of Selected Chromosomes for Fiber

Human Chromosomes

Cell lines	Fiber	1		3		6		8		12		14		18		X	
		C	D	C	D	C	D	C	D	C	D	C	D	C	D	C	D
UF1	-	+			P	+			+		+		+	+			+
UF2	-		+	+		+		+			+		P	+			+
UF3	+	+		+		+		+		+		+		+		+	
UF4	-	+		+			+	+		+			+	+			+
UF5	+	+		+		+			+	+		+		+		+	
UF6	+	+		+		+			+	+		+		+		+	
UF7	+	+		+		+		+		+		+		+		+	
UF8	-		P		P		P		+		+		+	+			+
UF9	+		+	+		+			+		+		+		+		+
UF10	+		+	+			+	+			+	+		+		+	
UF16																	
UF14	-	+		+		+			+	+			P		+		+
UF15	+		+	+		+		+		+		+		+		+	
UF17	+		+	+		+			+	+		+			+		+
UF18	+	+		+		+			+	+			+		+	+	
UF19	+		+		+		+	+			+		+	+			+
UF11	+	+		+		+		+		+		+		+		+	
UF12	+	+		+		+			+	+			P	+		+	
UF13	+	+		+		+		+		+		+		+		+	
		10/18		17/18		8/18		9/18		11/18		9/18		14/18		10/18	

a summary of data for concordance with fiber. Analysis of chromosomes not listed (2, 4, 5, 7, 9, 10, 11, 13, 15, 16, 17, 19, 20, 21, 22) showed more discordant than concordant.

and absent from hybrid UF2. There are additional limitations to be considered. First a single hybrid was obtained and tested independently on two separate occasions, yielding two different results. It is, of course, plausible that a minor component of the population had chromosome 1 and was not evident in UF16. Secondly, only two of the positive hybrids involved V79 whereas all three hybrids involving CHO supported virus. This could represent a bias of an indeterminate sort.

The results for restoration of fiber synthesis were even less conclusive, which might be attribute to the experimental difficulties cited above. As expected from the original analysis, only a single hybrid (UF19) was formally discordant for the long arm of chromosome 3. (No other had less than 4 discordant hybrid cell lines.) As noted in the text, there was a reluctance to assign the putative human gene solely to that chromosome, however. First, UF19 did not have the chromosome, yet restored fiber synthesis. Secondly, although UF9 was concordant, two subclones were not since both C1 and C4 had no evidence for any large segment of chromosome 3. It is possible that a segment of that chromosome had become translocated to a Chinese hamster chromosome. Dr. Francke examined these hybrids with detailed Giemsa 11 banding (McKay, 1973) but was unable to recognize a human segment. In the case of C1, I

attempted to demonstrate human repetitive sequences but was unsuccessful; less than 0.01% of human equivalents could have been missed. More sensitive Southern analysis was not performed. Consequently, the two exceptions are particularly compelling. It should be emphasized that expression of complex function can be particularly difficult to assess in cell hybrid (Davis and Adelberg, 1973). In addition, examples are known in which genes which are present may not be expressed. This suppression has been most clearly demonstrated for human ribosomal sequences in human X mouse hybrids (Croce et al. 1977). Unfortunately, that is the converse of what was observed. Table 26 considers the possibility that chromosome 3 is necessary but not sufficient in the relevant hybrid cell lines. I omitted UF9 (and its subclones) since it would be patently incompatible and must be explained on other grounds, in any case. UF19 will be considered separately in this model. UF10 and UF16 are considered as a single hybrid since they both supported fiber synthesis. It is evident that several other chromosomes are present in virtually all cases. Two chromosomes (14 and 16) are present in all cases. Six chromosomes are present (6, 12, 13, 15, 21, and X) in all but one case. If UF19 were considered to have an inapparent segment from chromosome 3, it may be interpretated as an independent assessment. It

Table 26

Concordance of other chromosomes with chromosome 3 and Fiber production

Cell lines	Human Chromosomes																					
	1	2	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21	22	X
UF3	c	-	c	-	c	-	c	c	-	-	c	c	c	c	c	-	c	c	c	c	c	c
UF5	c	c	c	c	c	c	-	c	c	c	c	c	c	c	c	-	c	c	c	c	c	c
UF6	c	c	c	c	c	c	-	-	c	-	c	c	c	c	c	-	c	c	c	c	c	c
UF7	c	c	c	c	c	-	c	c	-	c	c	-	c	c	c	c	c	c	c	c	c	c
UF10	-	-	-	-	-	c	c	-	-	c	-	c	c	-	c	-	c	-	c	-	-	c
UF11	c	-	c	c	c	c	c	c	c	c	c	c	c	c	c	c	c	-	-	c	-	c
UF12	c	c	c	-	c	c	-	-	c	c	c	c	c	c	c	-	c	-	-	c	-	c
UF13	c	c	c	c	c	c	c	-	c	c	c	c	c	c	c	c	c	-	-	c	c	c
UF17	-	-	-	-	c	-	-	-	-	-	c	c	c	c	c	-	-	-	c	c	-	-
C	7	5	7	5	8	6	5	4	5	6	8	8	9	8	9	3	8	4	6	8	5	8
chromosome present in UF19					N						N	Y	N	Y	N					N		N

c: concordant
-: discordant
N: not found
Y: present
C: total number of times a particular chromosome is concordant

contains only 13 and 15 of the relevant chromosomes. Hence no other chromosome and 3 are invariably present, although several are compatible in the general sense (single exceptions). The caveats cited in the preceeding paragraph apply to these analyses as well.

In conclusion, it was not possible to make the unique assignment sought in the cell hybrid experiments. The involvement of multiple unlinked genes was not ruled out. It was entirely possible, however, that experimental variability was the overiding consideration in this complex virological and biochemical phenomenon. In any case, it was possible to delineate successfully the basic parameters of Ad2 infection of Chinese hamster cells and narrow the focus for future, more directed analysis.

BIBLIOGRAPHY

Alt, F.W., Kellems, R.E., and Schimke, R.T. (1976). Synthesis and degradation of folate reductase in sensitive and methotrexate resistant lines of S-180 cells. J.Biol Chem 251:3063-3074.

Anderson, C.W., Baum, P.R., and Gesteland, R.F. (1973). Processing of Adenovirus 2 Induced Proteins. J. Virol. 12:241-252.

Anderson, C.W. (1981). Spontaneous Mutants of the Adenovirus-Simian Virus 40 hybrid, Ad2+ND3, That Grow Efficiently in Monkey Cells. Virology 111:263-269.

Anderson, K.P., and Klessig, D.F. (1983). Posttranscriptional Block to Synthesis of a Human Adenovirus Capsid Protein in Abortively Infected Monkey Cells. J. Mol. Appl. Gen. 2:31-43.

Anderson, K.P., and Klessig, D.F. (1984). Altered mRNA Splicing in Monkey cells Abortively Infected with Human Adenovirus May be Responsible for Inefficient Synthesis of the Virion Fiber Polypeptide. Proc. Natl. Acad. Sci. USA 81:4023-4027.

Bellett, A.J.D., and Younghusband, H.B. (1972). Replication of the DNA of Chick Embryo Lethal Orphan Virus. J.Mol. Biol. 72:3171.

Berget, S.M., Berk, A., Harrison, T., and Sharp, P.A. (1978). Spliced Segments at the 5' Termini of Adenovirus 2 Late mRNA: A Role for Heterogenous Nuclear RNA in Mammalian Cells. Cold Spring Harbor Symp. Quant. Biol., 42:523.

Berk, A.J., and Sharp, P.A. (1978). Structure of the Adenovirus 2 Early mRNAs. Cell 14:695-711.

Boulanger, P.A. (1975). Adenovirus Assembly: Self-Assembly of Parially Digested Hexons. J. Virol. 16:1678.

Brown, S., Oie, H.K., Gazdar, A.F., Minna, J.D., and Francke, U. (1979). Requirement of Human Chromosomes 19, 6 and Possibly 3 for Infection of Hamster X Human Hybrid Cells with Baboon M7 type C Virus. Cell 18:135-143.

Burlingham, B.T.,Brown, D.T., and Doerfler, W. (1974). Incomplete Particles of Adenovirus. I. Characteristics of The DNA Associated with Incomplete Adenovirions of Types 2 and 12. Virology 60:419-430.

Carstens, E.B., and Weber, J. (1977). Genetic Analysis of Adenovirus Type 2. Pleiotropic Effects in an Assembly Mutant. J. Gen Virol. 37:453-474.

Cepko, C.L., Changelian, P.S., and Sharp P.A. (1981). Immunoprecipitation with Two-Dimensional Pools as a Hybridoma Screening Technique: Production And Characterization of Monoclonal Antibodies Against Adenovirus 2 Proteins. Virol. 110:385-401.

Challberg, and Kelly, T. (1979). Adenovirus DNA Replication In Vitro. Proc. Natl. Acad. Sci. USA 76:655.

Chang, A.C.Y., Nunberg, J.H., Kaufman, R.J., Erlich, H.A., Schimke, R.T., and Cohen, S.N. (1978). Phenotypic Expression in E. Coli of a DNA Sequence Coding for Mouse Dihydrofolate Reductase. Nature, 275:617-624.

Chardonnet, Y., and Dales, S. (1970a). Early Events in the Interaction Between Adenoviruses and HeLa Cells. I. Penetration of Type 5 and Intracellular Release of The DNA Genome. Virology, 40:462-477.

Chardonnet, Y., and Dales, S. (1970b). Early Events in the Interaction Between Adenoviruses and HeLa Cells. II. Comparative Observation on the Penetration of Types 1, 5, 7, and 12. Virology, 40:478-485.

Chardonnet, Y., and Dales, S. (1972). Early Event in the Interaction of Adenoviruses with HeLa Cells. III. Relationship Between an ATPase Activity in Nuclear Envelopes and Transfer of Core Material: A Hypothesis. Virology, 48:342-359.

Chee-Sheung, C., and Ginsberg, H., (1982). Characterization of a Temperature-Sensitive Fiber Mutant of Type 5 Adenovirus and Effect of the Mutation on Virion Assembly. J. Virol., 42:932-950.

Chow, L.T., Gelinas, R.E., Broker, T.R., and Roberts, R.J. (1977). An Amazing Sequence Arrangement at the 5' Ends of Adenovirus 2 Messenger RNA. Cell, 12:1.

Croce, C.M., Talavera, A., Basilico, C., and Miller, O.J. (1977). Suppression of Production of Mouse 28S Ribosomal RNA in Mouse- Human Segregating Mouse Chromosomes. Proc. Natl. Acad. Sce. USA 74:694-697.

Daniell, E., (1976). Genome Structure of Incomplete Particles of Adenovirus. J. Virol., 19:685-708.

Davis, F.M., and Adleberg, E.A. (1973). Use of Somatic Cell Hybrids for Analysis of The Differentiated State. Bact. Review 37:197-214.

DiMaio, D., and Nathans, D. (1982). Regulatory Mutants of SV40: Effect of Mutation at a T Antigen Binding Site on DNA Replication and Expression of Viral Genes. J. Mol. Biol. 156:531-548.

Doerfler, W., (1968). The Fate of the DNA of Adenovirus 12 in Baby Hamster Kidney Cells. Proc. Natl. Acad. Sci. USA 60:636-643.

Dorsch-Hasler, K., Fisher, P.B., Weinstein, I.B., and Ginsberg, H.S. (1980). Patterns of Viral DNA Integration in Cells Transformed by Wild-Type or DNA-Binding Proteins Mutants of Adenovirus Type 5 and Effect of Chemical Carinogens on Integration. J. Virol. 34:305-314.

Dorsett, P.H., and Ginsberg, H. S.(1975). Characterization of the Type 5 Adenovirus Fiber Proteins. J.Virol. 15:208-216.

Fanning, E., Schick, J. and Doerfler, W. (1978). Studies on the Occurence of High Molecular Weight Adenovirus Type 2 DNA in Productively Infected Cells. Virol. 88:186-190.

Feldman, L.A., Butel,J.S. and Rapp, F. (1966). Interaction of a Simian Papovavirus and Adenoviruses. I. Induction of Adenovirus Tumor Antigen During Abortive Infection of Simian Cells. J. Bacteriol., 91:813-818.

Flintoff, W.F., Davidson, S.V., and Sminovitch, L. (1976). Isolation and Partial Characterization of Three Methotrexate-Resistant Phenotypes From Chinese Hamster Ovary Cells. Somat. Cell Genet. 2:245-261.

Fox, R.I., and Baum, S.G. (1972). Synthesis of Viral RNA During Restricted Adenovirus Infection. J. Virol. 10:220.

Francke, U., and Francke, B. (1981). Requirement of the Human Chromosome 11 Long Arm for Replication of Herpes Simplex Virus type 1 in Nonpermissive Chinese Hamster X Human Diploid Fibroblast Hybrids. Somatic Cell Genetics, 7:171-191.

Friedman, M.P., Lyons, M.J., and Ginsberg, H.S. (1970). Biochemical Consequences of Type 2 Adenovirus and Simian Virus 40 Double Infections of African Green Monkey Kidney Cells. J. Virol 5:586-597.

Gallimore, P.H., Sharp, P.A., and Sambrook, J. (1974). Viral

DNA in Transformed Cells. II. A Study of the Sequences of Adenovirus 2 DNA in Nine Lines of Transformed Rat Cells Using Specific Fragments of the Viral Genome. J. Mol. Biol. 89:49.

Garon, C. F., Berry, K.W., and Rose J.A. (1972). A Unique Form of Terminal Redundancy in Adenovirus DNA Molecules. Proc. Natl. Acad. Sci.USA 69:2391-2395.

Garver,J.J., Pearson, P.L., Abrahams, P.J., Van der Eb, A.J. (1980). Control of SV40 Replication by a Single Chromosome in Monkey Hamster Cell Hybrids. Somatic Cell Genetics 6:443-453.

Gluzman,Y., Frisque, R.J., and Sambrook, J. (1979). Origin Defective Mutants of SV40. Cold Spring Harbor Symp. Quant. Biol. 44:293-300.

Gluzman, Y., (1981). SV40-Transformed Simian Cells Support The Replication of Early SV40 Mutants. Cell 23:175-182.

Graham, F.L. (1984). Covalently Closed Circles of Human Adenovirus DNA are Infectious. EMBO J., 3:2917-2922.

Green, M., and Daesch, G.E. (1961). Biochemical Studies on Adenovirus Multiplication. I. Kinetics of Nucleic Acod and Protein Synthesis in Suspension Cultures. Virol. 13:169.

Green, M., Pina, M., Kimes, R., Wensink, P.C., Machattie, L.A., and Thomas, C.A. (1967). Adenovirus DNA I. Molecular Weight and Conformation. Proc. Natl. Acad. Sci. 57:1302.

Grodzicker, T., Lewis, J.B., and Anderson, C.W. (1976). Conditional Lethal mutants of Adenovirus Type 2-Simian Virus 40 Hybrids. II. Ad2+ND1 Host-Range Mutants That Synthesize Fragments of the Ad2+ND1 30K protein. J. Virol. 19:559.

Gurney, E.G., Harrison, R.O., and Fenno, J. (1980). Monoclonal Antibodies Against SV40 T Antigens: Evidence for Distinct Subclass of Large T Antigen and for Similarities Among Non Viral T Antigen. J. Virol. 34752:763.

Horne, R.W., Brenner, S., Waterson, A.P., and Wildy, P.(1959). The Icosahedral Form of an Adenovirus. J. Mol. Biol. 1:84.

Houweling, A., Van Den Elsen, P.J., and Van der Eb, A.J. (1980). Partial Transformation of Primary Rat Cells by the Leftmost 4.5% Fragment of Adenovirus 5 DNA. Virology 105:537-550.

Ishibashi, M., and Maizel, J.V.,Jr. (1974). The polypeptides of Adenovirus. V. Young Virions, Structural Intermediate Between Top Components and Mature Virions. Virology 57:409.

Kafatos, F.C., Jones, C.W. and Efstratiadis, A. (1979).

Determination of Nucleic Acid Sequence Homologies and Relative Concentrations by a Dot Hybridization Procedure. Nucl. Acids Res. 7:1541-1552.

Kaufman, R.J., and Sharp, P.A. (1982). Construction of a Modular Dihydrofolate Reductase cDNA Gene: Analysis of Signals Utilized for Efficient Expression. Molec. Cell. Biol. 2:1304-1319.

Kaufman, R.J., and Sharp, P.A. (1982). Amplification and Expression of Sequences Cotransfected With a Modular Dihydrofolate Reductase cDNA Gene. J. Mol. Biol. 159:601-621.

Klessig, D.F., and Anderson, C.W. (1975). Block to Multiplication of Adenovirus Serotype 2 in Monkey Cells. J. Virol. 16:1650-1668.

Klessig, D.F. (1977). Isolation of a Variant of Human Adenovirus Serotype 2 That Multiplies Efficiently on Monkey Cells. J. Virol. 16:1650.

Klessig, D.F., and Grodzicker, T. (1979) Mutations That Allow Human Ad2 and Ad5 to Express Late Genes in Monkey Cells Map in the Viral Gene Encoding the 72K DNA Binding Protein. Cell 17: 957-966.

Klessig, D.F., and Chow, L.T. (1980). Incomplete Splicing and Deficient Accumulation of the Fiber Messenger RNA in Monkey Cells Infected by Human Adenovirus Type 2. J. Mol. Biol 139:221-242.

Kruijer,W., van Schark, F.M.A, and Sussenbach, J.S., (1981). Structure and Organization of the Gene Coding for The DNA Binding Protein of Adenovirus Type 5. Nucl. Acid Res. 9:4439-4457.

La Bella, F., and Ozer, H.L. (1985). Differential Replication of SV40 and Polyoma DNAs in Chinese Hamster Ovary Cells. Virus Research, in press.

Laemmli, U.K. (1970). Cleavage of Structural Proteins During The Assembly of The Head of Bacteriophage T4. Nature (London) 227:680-685.

Lemons, R.S., O'Brien, S.J., and Sherr, C.J., (1977). A New Genetic Locus, Bevi, on Human Chromosome 6 Which controls the Replication of Baboon Type C Virus in Human Cells. Cell 12:251-262.

Lewis, A.M.,Jr., Levine, M.J., Wiese, W.H., Crumpacker, C.S. and Henry, P.H. (1969). A Nonedefective (Competent) Adenovirus-SV40 Hybrid Isolated From the Ad2-SV40 Hybrid Polulation. Proc. Natl. Acad. Sci. USA. 63:1128-1135.

Lewis, A.M., Levine,A.S., Crumpacker, C.S., Levine, M,J., Samaha, R.J., and Henry, P.H. (1973). Studies of Undefective Adenovirus 2- SV40 Hybrid Viruses. V. Isolation of Additional Hybrids Which Differ in Their SV40-Specific Biological Properties. J. Virol. 11:655.

Litzkas, P., Jha, K.K., and Ozer, H.L.(1984). Efficient Transfer of Cloned DNA into Human Diploid Cell: Protoplast Fusion in suspension. Molec. and Cell. Biol. 4:2549-2552.

Lonberg-Holm, K., and Philipson, L. (1969). Early Events of Virus-Cell Interactions in an Adenovirus System. J. Virol., 4:323-338.

Longiaru, M., and Horwitz, M.S. (1981). Chinese Hamster Ovary Cells Replicate Adenovirus Deoxyribonucleic Acid. Mol. Cell. Biol., 1:208-215.

McKay, R.D.G.(1973). The Mechanism of G and C Banding in Mammalian Metaphase Chromosomes. Chromosoma 44:1-14.

Maniatis, T., Fritsch, E.F., and Sambrook, J. (1982). Molecular Cloning: A Laboratory Manual, Cold Spring Harbor Laboratory, New York.

Mayer, A.J., and Ginsberg, H.S. (1977). Persistence of Type 5 Adenovirus DNA in Cells transformed by a Temperature Sensitive Mutant, H5ts125. Proc. Natl. Acad. Sci USA 74:785-788.

Miller, D.A., Miller, O.J.,Dev, V.G., Hashmi, S., Tantravahl, R., Medrano, L., and Green, H. (1974). Human Chromosome 19 Carries a Poliovirus Receptor Gene. Cell, 1:167-173.

Nakajima, K., Ishitsuka, H., and Oda, K. (1974). An SV40 Induced Initiation Factor For Protein Synthesis Concerned with Regulation Of Permissiveness. Nature (London) 252:649-653.

Nakajima, K., and Oda, K. (1975). The Alteration of Ribosomes for mRNA Selection Concerned with Adenovirus Growth in SV40-Infected Simian Vells. Virology 67:85-93.

Nicolas, J.C., Sarnoe, P., Girard,M., and Levine, A.J., (1983). Host Range Temperature-Conditional Mutants in the Adenovirus DNA Binding Protein Are Defective in the Assembly of Infectious Virus. Virol. 126:228-239.

Nunberg, J.H., Kaufman R.J., Schimke, R.T., Urlaub, G., and Chasin L.A. (1978). Amplified Dihydrofolate Reductase Genes are Localized to a Homogeneously Staining Region of a Single Chromosome in a Methotrxate-Resistant Chinese Hamster Ovary Cell Line. Proc. Natl. Acad. Sci. 75:5553-5556.

O'Brien, S.J.,Editor. (1984). Genetic Maps: A Compilation of Linkage and Restriction Maps of Genetically Studied Organisms. Volume 3, Cold Spring Harbor Laboratory.

Oie, H.K.,Gazdar, A.F., Lalley, P.A., Russell, E.K., Minna, J.D., DeLarco, J., Todaro, G.J., and Francke, U. (1978). Mouse Chromosome 5 Codes fo Ecotropic Murine Leukaemia Virus Cell- Surface Receptor. Nature 274:60-62.

Oosterom-Dragon, E.A., and Ginsberg H.S., (1980). Purification and Preliminary Immunological Characterization of the Type 5 Adenovirus, Nonstructural 100,000-Dalton Protein. J. Virol 33:1203-1207.

Ozer, H.L., and Jha, K.K. (1977). Malignancy and Transformation: Expression in Somatic Cell Hybrids and Variants. Adv. in Ca. Res. 25:53-93.

Pellicer, A., Wigler, M., Axel, R., and Silverstein, S. (1978). The Transfer and Stable Integration of The HSV Thymidine Kinase Gene Into Mouse Cells. Cell 14:133-141.

Philipson, L., Pettersson, U., Lindber, U., Tibbetts, C., Vennstrom, B., and Persson, T. (1975). RNA Synthesis and Processing in Adenovirus Infected Cells. Cold Spring Harbor Symp. Quant. Biol., 39: 447-456.

Prives, C.,Covey, L., Scheller, A. and Gluzman, Y. (1983). DNA Binding Properties of SV40 T-Antigen Mutants Defective in Viral DNA Replication. Molec. and Cell Biol. 3:1958-1966.

Rabson, A.S., O'Connor, G.T., Berezesky, I.K., and Paul, F.J.(1964). Enhancement of Adenovirus Growth in African Gren Monkey Kidney Cells by SV40. Proc. Soc. Exp. Biol. Med. 116:187.

Rekosh, D.M.K., Russel, W.C., Bellet, A.J.D., and Robinson, A.J. (1977). Identification of a Protein Linked to the Ends of Adenovirus DNA. Cell, 11:283.

Rigby, P.W.J., Dieckmann, M., Rhodes, C. and Berg, P. (1977). Labeling Deoxyribonucleic Acid to High Specific Activity in Vitro by Nick Translation with DNA Polymerase I. J. Mol. Biol. 113:237.

Rio, D.C., Tjian, R. (1983). SV40 T Antigen Binding Site Mutations that Affect Autoregulation. Cell 32:1227-1240.

Robinson, A.J.,Younghusband, H.B., and Bellett, A.J.D.,(1973). A Circular DNA Protein Complex From Adenoviruses. Virology, 56:54-69.

Sakaguchi, A.Y., Shows, T.B. (1982). Coronavirus 229E Susceptibility in Man-Mouse Hybrids Is Located on Human Chromosome 15. Somatic Cell Genetics 1:83-94.

Sambrook, J.,Botchan, M., Gallimore, P., Ozanne, B., Pettersson, U., Williams, J.F., and Sharp, P.A. (1975). Viral DNA Sequences in Cells Transformed by Simian Virus 40 Adenovirus Type 2 and Adenovirus Type 5. Cold Spring Harbor Symp. Quant Biol. 39:615.

Schimke, R.T. (1984). Gene Amplification in Cultured Animal Cells. Cell 37:705-713.

Sharp, P.A., Pettersson, U., and Sambrook, J. (1974). Viral DNA in Transformed Cells. II. A Study of the Sequences of Adenovirus 2 DNA in a Line of Transformed Rat Cells Using Specific Fragments of the Viral Genome. J. Mol. Biol. 86:709.

Sharp, P.A., Gallimore, P.H., and Flint, S.J. (1975). Mapping of Adenovirus 2 RNA Sequences in Lytically Infected Cells and Transformed Cell Lines. Cold Spring Harbor Symp. Quant. Biol., 39:457-474.

Shiroki, K., and Shimojo, H. (1971). Transformation of Green Monkey Kidney Cells by SV40 Genome: The Establishment of Transformed Cell Lines and The replication of Human Adenoviruses and SV40 in Transformed Cells. Virology 45:163.

Silverstein, G., and Strohl, W.A. (198). Interaction of Adenovirus Type 2 With Mouse Balb-3T3 Cells In Vitro. Arch. of Virol. Submitted for publication.

Sirotnak, F.M., Moccio, D.M., Kelleher, L.E., and Goutas, L.J. (1981). Relative Frequency and Kinetic Properties of Transport-Defective Phenotypes Among Methotrexate-Resistant L1210 Clonal Cell Lines Derived In Vivo. Cancer Res. 41:4447-4452.

Small, M.B., Gluzman, Y., and Ozer, H.L. (1982). Enhanced Transformation of Human Fibroblasts by Origin-Defective Simian Virus 40. Nature, 296:671-672.

Solomon, F., Magendantz, M., and Salzman, A. (1979). Identification with Cellular Microtubules of One of The Co-Assembling Microtubule-Associated Proteins. Cell 18:431-439.

Southern, E. (1975). Detection of Specific Sequences Among DNA Fragments Separated by Gel Electrophoresis. J. Mol. Biol. 98:503.

Southern, E. (1980). Gel Electrophoresis of Restriction Fragments Methods Enzymol. 69:152.

Takahashi, M. (1972). Isolation of Conditional Lethal Mutants (Temperature-sensitive and Host-range mutants) of Ad5. Virology, 49:815-817.

Tornow, J., Polvin-Bodnar, M., Santangelo, G., and Cole, C.N. (1985). Two Separable Functional Domains of Simian Virus 40 Large T Antigen Carboxyl-Terminal Region of Simian Virus 40 Large T Antigen Is Required for Efficient Capsid Protein Synthesis. J. Virol 53:415-424.

Towbin, H., Staehlin, T., and Gordan, J. (1979). Electrophoretic Transfer of Proteins From Polyacrylamide Gels to Nitrocellulose: Procedures and Some Applications. Proc.Natl. Acad. Sci. USA 76:4350-4355.

Tooze, J., DNA Tumor Viruses, Molecular Biology of Tumor Viruses, Second Edition Part 2. (1980), Cold Spring Harbor Laboratory. Cold Spring, New-York.

Urlaub, G., and Chasin, L.A. (1980). Isolation of Chinese Hamster Cell Mutants Deficient in Dihydrofolate Reductase Activity. Proc. Natl. Acad. Sci. U.S.A. 77:4216-4220.

van der Vliet, P.C., and Sussenbach, J.S.(1975). An Adenovirus Type 5 Gene Function Required for Initiation of Viral DNA Replication. Virology 67:415.

van der Vliet, P.C., Landberg, J., and Jansz, H.S. (1977). Evidence for a Funtion of the Adenovirus DNA-Binding Protein in Initiation of DNA Synthesis as Well as in Elongation on Nascent DNA Chains. Virology, 80:98.

Van Doren, K., Hanaltan, D., and Gluzman, Y. (1984). Infection of Eucaryotic Cells by Helper-Independent Recombinant Adenoviruses: Early Region 1 is not Obligatory for Integration of Viral DNA. J.of Virol. 50:606-614.

Wahl, G.M. Stern, M., and Stark, G.R. (1979). Efficient Transfer of Large DNA Fragments From Agarose Gels to Diazobenzyloxymethyl- Paper and RApid Hybridization by Using Dextran Sulfate.Proc. Natl. Acad. Sci. U.S.A. 76:3683-3687.

Weber, J.M., Dery, C.V., Mirza, M.A., and Horvath J. (1985). Adenovirus DNA Synthesis is Coupled to Virus Assembly. Virology 140:351-359.

Weber, J.M., and Stich, H.F., (1969). Response of Somatic Cell Hybrids to Exposure to Adenovirus Type 2 and 12. Exper. Cell Res. 56:319-325.

Wigand, R., Bartha, A., Dreizin, R.S.,Esche, H., Ginsberg, H.S., Green, M., Hierholzer,J.C., Kalter, S.S., McFerran, J.B., Pettersson, U., Russell, W.C., Wadell, G. (1982). Adenoviridae:Second Report. Intervirol. 18:169-176.

Wigler, M., Pellicer, A., Silverstein, S., Axel, R.(1978). Biochemical Transfer of Single-Copy Eukaryotic Genes Using Total Cellular DNA as Donor. Cell 14:725-731.

Wigler,M., Perucho, M., Kurtz, D., Dana, S., Pellicer, A., Axel, R., and Silverstein, S. (1980). Transformation of Mammalian Cells With an Amplifiable Dominant-Acting Gene. Proc. Natl. Acad. Sci USA 77:3567-3570.

Wolfson, J., and Dressler, D. (1972). Adenovirus DNA Contains an Inverted Terminal. Proc. Natl. Acad. Sci. U.S.A., 69:3045-3057.

Younghusband, H.B., Tyndall, C., and Bellett, A.J.D. (1979). Replication and Interaction of Virus DNA and Cellular DNA in Mouse Cells Infected by a Human Adenovirus. J. Gen Virol. 45:455-467.

Zorn, G.A., and Anderson, C.W. (1981). Adenovirus type 2 Expresses Fiber in Monkey-Human Hybrids and Reconstructed Cells. J. Virol 37:759-769.