STUDIES ON AD5 EARLY GENE EXPRESSION

EFFECTS OF INHIBITION OF PROTEIN SYNTHESIS ON ADENOVIRUS 5 EARLY GENE EXPRESSION

By

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ABSTRACT

Previous studies have shown that a functional Ad 5 E1A protein from the 13 S mRNA is required during lytic infection for activation of early viral gene expression (Jones and Shenk, 1979b; Berk et al, 1979; Montell et al, 1982). The mechanism by which E1A exerts this regulatory function is still unclear and has been the subject of intense investigation (Persson et al, 1981a; Katze et al, 1981, 1983; Nevins, 1981; Shaw and Ziff, 1982). Most of these recent investigations have relied on the use of metabolic inhibitors such as cycloheximide to eliminate protein synthesis as a means of determining the role of viral and host proteins in the regulatory process. The results from these studies have been inconsistent. The purpose of this research project has been to re-examine the regulation of Ad 5 early gene expression without the use of drug inhibitors. In this study tsH1 cells, a mutant CHO cell line which at temperatures above 37^{o}C are inhibited in protein synthesis, were used. At critical times during the course of wild type or host-range 1 infection of tsH1 cells, protein synthesis was inhibited and Early Region 4 expression was examined. In every case, efficient E4 expression was dependent on functional E1A protein and this requirement could not be replaced by simply inhibiting protein synthesis. The results are discussed in relation to models proposed to explain the regulation of Ad 5 Early Gene Expression.

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LIST OF ABBREVIATIONS

Ad 5	adenovirus type 5
α-MEM	alpha-minimum essential medium
DNA	deoxyribonucleic acid
EDTA	ethylenediaminetetra-acetic acid
hr-1	Ad 5 group I host range mutant
h pi	hours post infection
Joklik-MEM	Joklik-minimum essential medium
kb	Kilobase
kd	kilodalton
l-strand	leftward transcribed strand
mRNA	messenger ribonucleic acid
M	molar
mu	map unit
moi	multiplicity of infection
PFU	plaque forming unit
PBS	phosphate buffered saline
r-strand	rightward transcribed strand
tRNA	transfer ribonucleic acid

INTRODUCTION

I Discovery of Adenovirus

In 1953 Rowe and co-workers identified cytogenic agents which caused the degeneration of cultured adenoidal tissue. The following year a similar degenerating agent was isolated from military recruits at Fort Leonard Wood in the United States (Hilleman and Werner, 1954). Subsequent complement fixation and neutralization tests indicated that the degenerating agents were practically identical in both cases (Hilleman, op. cit.). Investigators later determined that these agents were viruses and were prevalent in other host species and clinical situations (Enders, 1956). By 1958 it had been generally agreed that these viral agents be termed Adenovirus as a reminder of the source of the original prototype strain identified by Rowe and co-workers (reviewed by Flint, 1980a).

The most significant discovery in early adenovirus research was made in 1962. At that time a vaccine was developed against a strain of adenovirus which was known to be responsible for an epidemic of mild respiratory illness (Trentin et al, 1962). While testing the vaccine Trentin (1962) discovered that some strains of adenovirus were capable of inducing malignant tumours in newborn hamsters. The Trentin discovery provided a model system in which researchers could begin to investigate the molecular basis of mammalian cell transformation and tumorigenesis. In the time since the Trentin discovery none of the known adenoviruses have been isolated as the causative agent in human tumors but the

knowledge obtained from investigations into the molecular biology of these viruses has provided insights into the carcinogenesis phenomenon and eukaryotic gene expression.

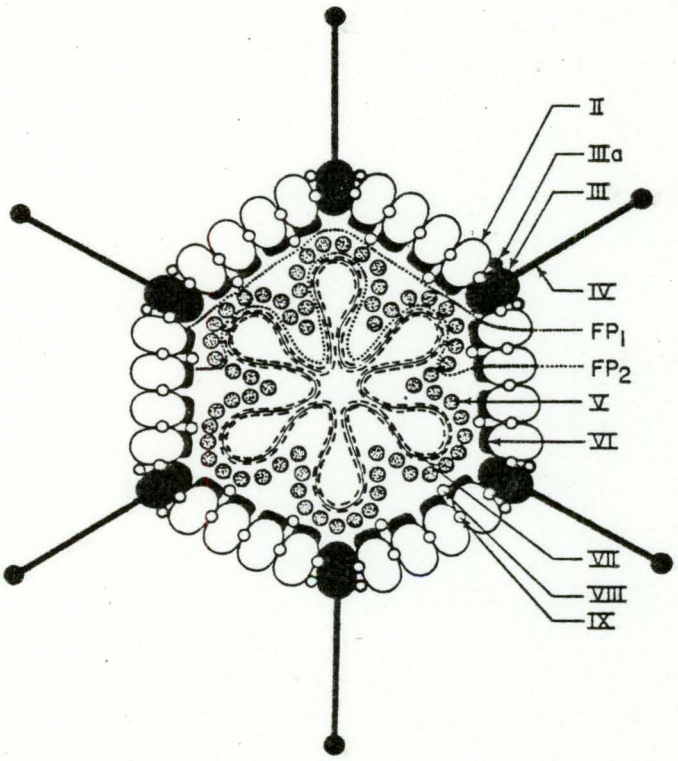
There are at least 31 known serotypes of human adenoviruses and these have been classified into four subgoups (Beladi, 1972). Members within each subgroup have the same degree of oncogenicity, and share nucleic acid homology and immunological properties (reviewed by Flint, 1980a). The most oncogenic adenoviruses belong to subgroup A and are represented by type 12, while members in subgroup B, such as type 7, are only weakly oncogenic. The non-oncogenic adenoviruses are classified into subgroups C or D. The best characterized of all serotypes are the closely related types 2 and 5 of subgroup C. They share many biological and molecular properties including 99% homology in their DNA sequences.

Figure 1 is a diagramatic representation of the structure and composition of an adenovirion. The outer capsid of the virus measures approximately 65-80 nm in diameter, is nonenveloped and icosahedral. The outer shell is composed of 252 capsomers, 240 of which form the faces and edges of the 20 triangular facets. Each of the 240 capsomers are surrounded by six other similar capsomers termed hexon. The remaining 12 capsomers form the corners, have 5 neighbours, and are called pentons. Projecting out from each penton is an antenna like structure termed a fiber (reviewed by Phillipson et al, 1975; Flint, 1980a).

The inner core of the virus contains approximately 18-20% of the total protein of the virion and a single copy of the genome (Laver et al, 1967). Electron microscope studies have revealed that the core particles

Figure 1: Structure and Composition of an Adenovirion.

Taken from Flint (1980a). The positions of the structural polypeptides, indicated by Roman numerals, are based on the studies described in the introduction.


II
IIIa
III
IV
FP1
FP2
V
VI
VII
VIII
IX

are arranged in 8-10 spherical subunits, each 21.6 nm in diameter (Brown et al, 1975). When packed the core structure may resemble eukaryotic chromatin (Corden et al, 1976). Despite these findings the exact structure of the core, packaging constraints and assembly mechanisms remain uncertain.

The genome of Ad 5 is linear duplex DNA molecule with a molecular weight of $2.0\text{-}2.5 \times 10^6$ daltons containing approximately 36,000 nucleotide pairs (Flint, 1980a; van Ormondt, 1980; Gingeras et al, 1982). Interesting features of these genomes include a 55 kd protein which is covalently bound to the 5' termini of the viral DNA strands and the presence of a 102 nucleotide long inverted terminal repeat (ITR) at each end of the genomic DNA (Steenburgh et al, 1977; Arrand and Roberts, 1979; Flint, 1980a). The end proteins and ITRs may be important in replication of the genome (Rekosh, 1977; Sharp et al, 1976; Robinson and Bellet, 1975).

Replication of adenoviral DNA is semiconservative (Pearson and Hanawalt, 1971; Bellet and Younghusband, 1972). There is a termination site and an origin of replication at or near each end of the viral genome (Sussenbach and Kuijk, 1977). The site of viral DNA replication remains in doubt but available evidence seems to suggest that replication occurs in complexes either in random or specific association with the nuclear membrane (Vlak et al, 1975a). Viral DNA integration into the host chromosome does not appear to be a necessary first step in viral DNA replication (Flint, 1980a).

II Infection of Cells by Adenovirus

(a) Productive cycle

Adenoviruses, like other viruses, are obligate intracellular parasites. In order to produce progeny these viruses are dependent on a permissive host. The productive cycle begins with the adsorption of the virus to the host cell. With the use of the structural fiber the virus is able to attach to receptors in the host's cellular membrane. Entry into the cell is achieved through pinocytosis or by direct penetration of the membrane (Chardonnet and Dales, 1970a; Morgan et al, 1969). Once in the cytoplasm the viral pentons are lost and the virion becomes associated with pores in the nuclear membrane (Chardonnet and Dales, 1970a; Dales and Chardonnet, 1973). The core is somehow liberated from the virion and gains entry to the nucleus. The final uncoating of the cores results in naked viral DNA (Chardonnet and Dales, 1972; Philipson et al, 1968).

Transcription of viral mRNA, translation of proteins and replication of the parental DNA occur in a series of patterned events. The productive cycle is divided by convention into early and late phases with the onset of viral DNA replication being regarded as the delineation point. Viral mRNAs which have been transcribed before viral DNA replication are considered early while those transcribed following viral DNA replication are termed late. Viral proteins are similarly classified. As the infection proceeds from early to late times the host cell macromolecular machinery becomes almost entirely directed towards

the production of viral progeny (Green and Daesch, 1961). Studies have shown that between 4×10^3 and 1×10^4 new viral particles are produced per infected cell (Green and Daesch, 1961). The typical length of this cycle is 48 hours in permissive Hela cells at 37^oC but is prolonged in primary cultures of human cells (Ledinko, 1970).

(b) Events early in the productive cycle

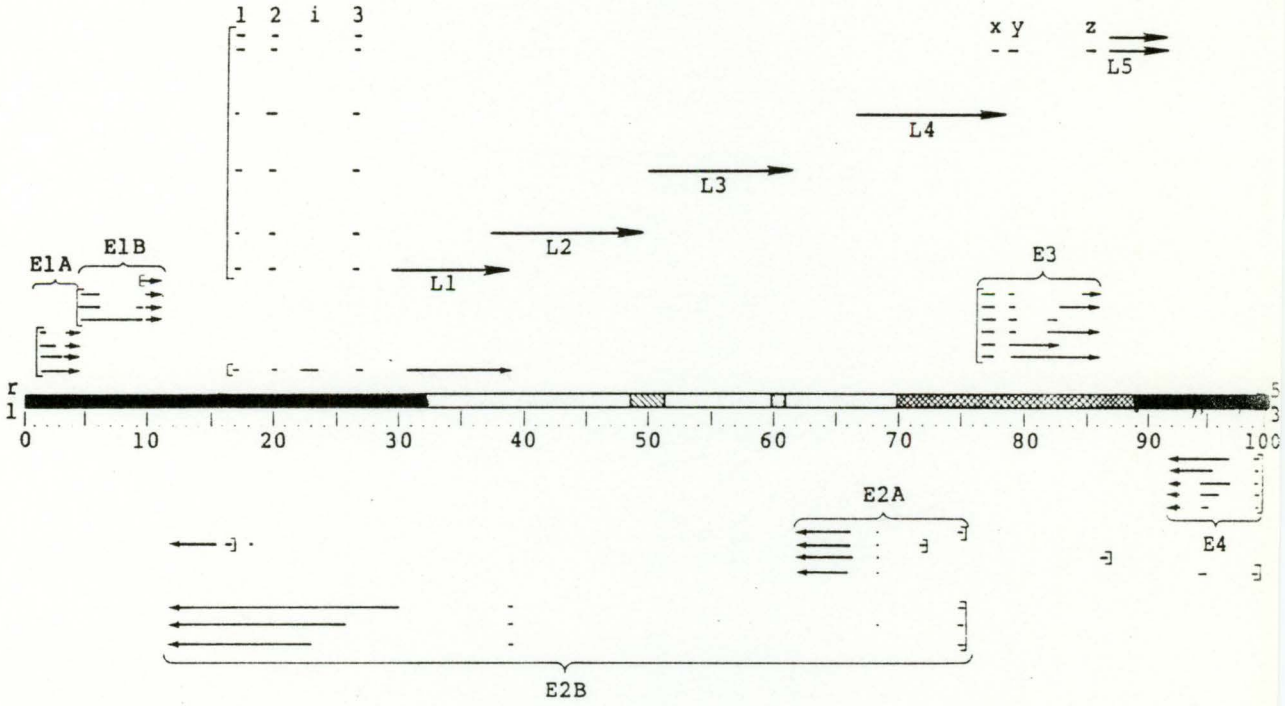
At early times only a fraction of the information encoded in the viral genome is expressed as mRNA (Fujinaga et al, 1968; Green et al, 1971). The fraction of viral mRNA to cellular mRNA is small and there are only a few viral proteins being synthesized. The host continues to manufacture cellular proteins but division is inhibited and the cell begins to increase in mass (Green and Daesch, 1961; Ginsberg, 1967; Pina and Green, 1969).

With the use of S1 nuclease, R-Loop and UV Ray promoter mapping techniques it has been determined that there are six distinct coding regions being expressed at early times. These coding regions are diagrammed in figure 2. On the rightward reading strand (r-strand) are early regions 1A (E1A), 1B (E1B), and 3(E3). On the leftward reading strand (1-strand) are early regions 2A (E2A), 2B (E2B) and 4 (E4). In addition to the six early genes the major late promoter, on the r-strand, is active at early times (Chow et al, 1977a; Neuwald et al, 1977; Kitchingman et al, 1977; Berk and Sharp, 1978; Chow et al, 1979; Gingeras et al, 1982).

A map co-ordinate system for the Ad5 genome has been developed in which the genome is assigned the length of 100 map units so that 1 map

Figure 2: Transcriptional Mpa of the AD2 Genome; Modified from Gingeras et al. (1982)

The solid and cross-hatced areas indicate regions of the genome for which DNA sequences are available. The six early regions are labelled E1-E5 and EL1. The five families of late RNAs are labelled L1-L5 and the leaders present on the transcripts made from the major late promoter are designated 1, 2, i, 3, x, y and z. Ad 5 is closely related to Ad 2 and has an identical transcriptional map.


1 2 i 3
x y
z
L5
L4
L3
L2
L1
E1A
E1B
E3
r
1
5
3
0
10
20
30
40
50
60
70
80
90
100
E2A
E4
E2B

unit (mu) corresponds to approximately 360 nucleotides.

The E1A promoter is located at 1.28 mu and the polyadenylation site at 4.46 mu (van Ormondt et al, 1980). The primary transcript from E1A is processed post transcriptionally into two mature mRNAs of 1100 and 900 nucleotides in length having sedimentation values of 13 S and 12 S respectively. At late times in lytic infection a 9 S mRNA is produced from the E1A promoter. The 13 S, 12 S, and 9 S mRNAs differ only in the size of the internal splice removed from the primary transcript and have identical 5' and 3' ends (Montell et al, 1982).

Identification of the E1A in vivo translation products has been difficult because of the low quantities in which they are synthesized and because antitumour sera appear to have a low avidity for them (Harter and Lewis, 1978; Lassam et al, 1979b; Schrier et al, 1979). Recently Yee et al (1983) used a synthetic peptide the sequence of which corresponded to the predicted common carboxy terminus of the E1A gene products. In productively infected KB cells antisera, raised against this peptide, precipitated four major proteins of 52, 50, 48.5, and 45 kd, as well as two minor proteins of 37.5 and 35 kd. It has been determined that the 52 and 48.5 kd proteins are translated from the 13 S mRNA while the 50 and 45 kd proteins are assigned to the 12 S mRNA (Rowe et al, 1983; Montell et al, 1982). It is not known if the 9 S mRNA codes for a protein in vivo (Yee et al, 1983). It should be noted that the molecular weights of these proteins were estimated with the use of SDS gels and the values are higher than the 32 kd and 26 kd products predicted from the sequences of the 13 S and 12 S mRNAs (Perricaudet et al, 1979). It is possible that

phosphorylation of the E1A proteins or the high proline content, which would alter mobility in SDS gels, of these proteins may account for these discrepancies (Perricaudet et al, 1979; Harter and Lewis, 1978; Yee et al, 1983).

Early Region 1B has two promoters. The first is located at 4.57 mu and the second at 9.70 mu. There is one common polyadenylation site at 11.12 mu (Bos et al, 1981). Three mature mRNA products are transcribed from E1B. One transcript, initiated from the first promoter, is processed into two mature mRNAs of 2200 and 1000 nucleotides in length having sedimentation values of 22 S and 13 S respectively. They have common 5' and 3' ends but differ in the size of the intron removed during splicing (Perricaudet et al, 1980; van Ormondt et al, 1980). The other transcript is initiated from the second promoter at late times after infection and extends to the polyadenylation site yielding an unspliced mature mRNA of approximately 750 nucleotides in length with a sedimentation value of 9 S (Alestrom et al, 1980).

The 13 S mRNA codes for a 19 kd polypeptide which is initiated from the first AUG codon on the transcript (Bos et al, 1981). The 22 S mRNA codes for a 58 kd protein unrelated to the 19 kd protein as initiation is from a second AUG codon in a different reading frame (Bos et al, 1981). It has been observed that <u>in vitro</u> translation of the 22 S mRNA initiation can begin at the first AUG codon to give a 19 kd polypeptide identical to the 19 kd protein derived from the 13 S mRNA (Esche et al, 1980; Lupker et al, 1981; Bos et al, 1981). <u>In vitro</u> synthesis of the 58 kd polypeptide also occurs from the 22 S mRNA but is

reduced compared to the 19 kd product although *in vivo* the 58kd is present in large amounts (Bos et al, 1981). This result suggests that viral factors not present in cell free translation may be assisting initiation from the second AUG codon on the 22 S mRNA.

The second transcriptional unit, which produces the 9 S mRNA, codes for polypeptide IX at late times in productive infection (Alestrom et al, 1980).

Early Region 2A encodes a primary transcript initiated from the promoter located at 75.2 mu through the coding sequence to the polyadenylation site at 61.5 mu. The major cytoplasmic mRNAs transcribed from this region are spliced from the primary transcript by removal of an intron from 75.0 mu to 66.6 mu. A number of minor mRNA species can also be observed at early times which apparently do not code for functional protein products (Chow et al, 1979; Berk and Sharp, 1978). At early times a polypeptide of 72,000 daltons is the only product of the mature mRNA. This protein has been identified as the DNA binding protein, essential in adenoviral DNA replication (Wold and Green, 1979; Ross et al, 1980). Following DNA replication the 72kd protein is still produced but the pattern of expression of the primary mRNA is different, reflecting the change in gene regulation which accompanies the early to late switch (Chow et al, 1979). Early Region 2B initiates from the same promoter as E2A but the transcripts are coded from sequences between 30 and 10 mu. The E2B transcripts are prevalent at advanced stages of the early phase.

Early Region 3 have three primary transcripts all initiating from

the promoter at 76.6 mu but terminating at different polyadenylation sites located at 82.7, 84.7 and 86.0 mu (Herisse et al, 1980; Herisse and Galibert, 1981). The primary transcript is further processed by splicing in up to five different ways, giving rise to a complicated pattern of at least eight early mRNA species (Chow et al, 1979). The assignment of all the E3 translation products to mRNAs is not yet clear.

The primary transcript from Early Region 4 stretches from the promoter at 99.17 mu to a polyadenylation site at 91.27 (Chow et al, 1979). Tigges and Raskas (1982) have identifed up to 9 mature mRNA species from E4. These mRNAs have the same 5' and 3' terminals but differ in the size of the intron removed during splicing (Herisse and Galibert, 1981; Gingeras et al, 1982; Matsuo et al, 1982a) and they range in size from 2.5 to 0.75 kb (Tigges and Raskas, 1984). There is a definite temporal pattern in the expression of E4 mRNA as not all the species are observed at all times during early infection (Tigges and Raskas, op.cit.). The precise number and function of E4 proteins has not yet been defined.

(c) Events late in the productive cycle

The late phase of adenovirus 5 infection begins with the onset of viral DNA replication. In a fully permissive system at 37^{o}C viral DNA synthesis usually begins at approximately 6 to 8 h pi (Ginsberg et al, 1967; Pina and Green, 1969; Flint, 1980a). DNA replication requires prior synthesis of early viral proteins but the process becomes independent of continuing protein synthesis once progeny DNA are present (Horwitz et al, 1973). At about the same time that viral DNA is being

replicated synthesis of host proteins and cellular DNA replication ceases (Ginsberg et al, 1967). Furthermore, most of the cellular mRNA in polysomes is replaced by mRNA of viral origin. During this phase viral RNA accounts for 20-40% of the total RNA in the infected cell but only 10-20% of the newly synthesized RNA is viral and host mRNA is stable. The mechanism by which the viral mRNA is preferentially translated is not clear (Ginsberg et al, 1967; Greenberg, 1972; Singer and Penman, 1972; Philipson et al, 1975; Flint, 1980a). The late viral genes code for most of the structural polypeptides of the virus (Evans et al, 1977; Goldberg et al, 1977; Ziff and Evans, 1978). The initiation site and the promoter for the late genes is located at map co-ordinate 16.45. Transcription is directed in the rightward direction (from the r-strand) with termination at or very near the end of the genome at 99 mu. The precursor late mRNA extends from the promoter to 99 mu (Fraser et al, 1979).

The precursor mRNA is processed by a complicated mechanism of differential poly(A^+) site selection at the 3' end and splicing at the 5' end to result in the formation of 20 mature mRNAs (Nevins and Wilson, 1981; Shaw and Ziff, 1980). The mature products of this process are arranged in five families, L1-L5, with all members within a family being 3' co-terminal. There are five polyadenylation sites in the precursor molecule. The post-transcriptional selection of one of these polyadenylation sites determines the 3' end of the mature mRNA. The poly (A+) sites are located at 37, 45, 63, 77 and 93 mu for families L1 to L5 respectively. The mechanism which governs the differential selection is unknown (Akusjarvi and Persson, 1981; Nevins and Wilson, 1981; Shaw and

Ziff, 1980; Chow et al, 1979).

The precursor mRNA is spliced from a location 5' to the family polyadenylation site to a common tripartite leader sequence encoded from co-ordinates 16.6, 19.6 and 26.0. Each of the 20 late mRNAs share this common feature (Chow et al, 1977a; Berget et al, 1977). Members within a family are not all spliced from the same location 5' to the polyadenylation site so that members within a family can differ in the length of their coding sequences. Adjacent late families do not have overlapping coding sequences (Akusjarvi and Persson, 1981).

The late mRNAs range in size from 1000 to 4000 nucleotides in length (Chow et al, 1979; Akusjarvi and Persson, 1981). There have been six mRNAs assigned to each of L2 and L4 while 2 have been assigned to both L3 and L5. The best characterized of the late mRNAs is the L1 family which at late times has 3 transcripts of 4.3, 3.8 and 2.3 kb in length. There are at least three proteins of 55, 52 and 14 kd assigned to L1. Assignment of other late proteins to their mRNAs is not yet clear (Akusjarvi and Persson, 1981; Shaw and Ziff, 1980; Nevins, 1981).

Reports have suggested that mRNA initiated from the major late promoter can be detected during early times in a productive infection (Chow et al, 1979; Kitchingman and Westphal, 1980; Lewis and Matthews, 1980). At early times transcripts initiate from the major late promoter but the pattern of mature transcripts is substantially different than at late times (Ziff and Evans, 1978; Fraser et al, 1979; Nevins and Wilson, 1981; Akusjarvi and Persson, 1981). Nevins and Wilson (1981) using the UV irradiation technique have shown that at early times transcripts

originating from the major late promoter terminate at the 3' end of L3 at 63 mu. A similar study by Akusjarvi and Persson (1981) claims that transcription terminates in the coding sequences of L2 at 40 mu. The explanation for this observed differences isn't clear but it seems certain that at early times the late transcript precursor terminates near the middle of the genome.

At early times nuclear L1 mRNA is in greater abundance than either L2 or L3 mRNA by approximately 2:1 (Nevins and Wilson, 1981). There is preferential transport of the L1 mRNA to the cytoplasm as roughly 80% of the mRNA originating from the late promoter originating mRNA and accumulating in the cytoplasm at early times is L1 (Nevins and Wilson, 1981; Akusjarvi and Persson, 1981). Transcription of L4 and L5 are blocked until times after DNA replication, by an unknown mechanism (Thomas and Matthews, 1980; Crossland and Raskas, 1983).

The L1 mRNA at early times has an additional leader sequence termed the "i" leader. It is positioned between the number 2 and 3 leader in the tripartite sequence and is coded from sequences around 23 mu. The "i" leader affects the translation of the L1 mRNA. At late times L1 encodes primarily the 55 and 52 kd polypeptides. With the "i" leader the 14 kd product, which is unrelated to the other two species, predominates (Akusjarvi and Persson, 1981).

At early and late times during infection there are two species of virus associated RNA, VA-RNA_1 and VA-RNA_2. The VA-RNAs are encoded by two genes located closely together around 29 mu. The VA-RNA genes are transcribed by the cellular RNA polymerase III (Weinman et al, 1974) and

contain intragenic transcriptional control regions (Fowlkes and Shenk, 1980). Both VA-RNAs can be detected in the same relative amounts at early times during infection but with the onset of DNA replication, synthesis of VA-RNA_1 increases and is made in larger amounts than VA-RNA_2 by approximately 40:1 (Schneider et al, 1984). Studies with two Ad 5 mutants defective for the production of either of the VA-RNAs have been used to determine that only VA-RNA_1 is required for efficient viral growth in a productive infection (Schneider et al, 1984). Schneider et al attributed the reduced growth potential to inefficient translation of late viral mRNAs. The formation of a stable 48 S preinitiation complex of mRNA and tRNA is a required step in translation and is dependent on the interaction between a 43 S preinitiation complex and the mRNA which is to be translated. It appears that the VA-RNA facilitates the interaction between the 43 S complex and the mRNA. The mechanism whereby VA-RNA, facilitiates this event is still uncertain (Schneider et al, 1984).

A number of proteins are synthesized at late times in a productive infection (Reviewed by Flint, 1980a). Among these are a major non-structional protein of 100 kd which has an unknown function; minor non-structural proteins of 95, 80, 50, 14 and 11 kd also of unknown function; structural proteins of 120 kd (protein II-hexon), 62 kd (protein IV-fiber), and 12 kd (protein IX-hexon associated).

The infection process in permissive cells culminates with the release of progeny virus (Green and Daesch, 1961). Progeny tend to remain in or around the nucleus of the infected cells and are observed as

large intranuclear eosinophilic inclusions (Green and Daesch, op.cit.). The end stage of this process begins with the death and subsequent lysis of the host cell resulting in the release of progeny virus.

(d) Non-productive infection

Ad 5 infection of a susceptible host leads to either of two cellular responses. If the host cell is permissive, as Hela or other human cells, infection will proceed through the viral replicative cycle resulting in cell death and release of progeny virus. During infection by Ad 5 of a semi or non-premissive host some stage of viral replication is blocked, usually one or more of the late functions (Williams, 1973, Ginsberg et al, 1974; Williams et al, 1974). Few if any new virions are released and a small fraction of the cells infected with the virus may become transformed. The transformants are similar to cells explanted from adenovirus induced tumours (reviewed by Flint, 1980a).

Researchers investigating transformation of non-permissive cells by adenovirus have discovered that adenoviral DNA from at least the left 14% of the genome was integrated into random sites in cellular DNA in every transformant analyzed (Sharp et al, 1974; Gallimore et al, 1974). The precise identification of the adenovirus transforming gene was greatly facilitated with the advent of DNA transfer via the calcium phosphate transfection technique (Graham and van der Eb, 1973). This transfection assay made it possible to transfer naked, defined and purified restriction fragments of Ad 5 viral DNA to cells. In this way it was determined that the E1 region was necessary for complete morphological transformation of non-permissive rodent cells (Graham et

al, 1974; van der Eb et al, 1979).

Morphological transformation is possible with less than the full complement of E1 gene products (Schrier et al, 1979). In vitro transformation by the transfection technique requires an intact E1A plus the region of E1B encoding 19 kd and the amino terminal half of 58 kd (Schrier et al, op.cit.). This result suggests that the full 58 kd protein is not required for morphological transformation by DNA. Cells transformed by E1A alone are immortalized but they tend, unlike fully transformed cells, to have fibroblastic appearance, grow more slowly and to lower saturation densities (Houweling et al, 1980). The immortalization of cells by E1A and expression of an E1B function appear to be required for transformation.

III Regulation of Adenovirus 5 Early Gene Expression During Lytic Infection

Studies of early transcription performed by Nevins et al (1979) confirmed that expression of the six early transcription units was controlled and patterned with regards to both the onset and continuation of transcription. When Hela cells were infected with Ad 5 the results indicated that E1A, E3 and E4 were transcribed first and reached a maximal rate of transcription at about 3h pi. E1A continued to be expressed at about 90% of its maximal rate through 9h pi. E3 transcription decreased steadily to 55% of its maximal rate and the expression of E4 dropped sharply to about 25% of its maximal rate by 9h pi. The kinetics of expression of E1B and E2 were in sharp contrast to

the other regions. E2 and E1B reached their maximal rates at 7h pi and remained constant to 9h pi (Nevins et al, 1979). The synthesis of the various early viral proteins is temporally regulated in a pattern similar to that observed at the transcriptional level (Rowe et al, 1984; Neuwald et al, 1977). In KB cells infected with Ad 5, E1A proteins were detected at 2h pi, reached a peak in the next hour and declined to very low levels by 7h pi. Synthesis of E2, E3 and E4 proteins began at 3h pi, was maximal by 6h pi and thereafter declined sharply. The E1B proteins were first detected around 3h pi, reached maximal levels by 8h pi and declined by 12h pi (Rowe et al, 1984; Neuwald et al, 1977).

There are several lines of evidence which have implicated the viral early gene products as regulators of this complex expression pattern. The precise effects of E1B products on regulation at early times have yet to be fully defined. Lassam et al (1979b) working with hr-6, an E1B mutant defective in 58 kd production, reported that expression of the 72 kd protein from E2 was produced at wild type levels at early times but the level of late mRNA was reduced suggesting that 58 kd protein may serve as a positive effector of late gene expression. In studies with hr-7 (phenotypically similar to hr-6) Ross et al (1980) reported the over production of E2 mRNA, a result which suggested that the 58 kd protein was a negative effector for E2 transcription. Rowe et al (1984) reported that the E1A 44 kd protein in hr-6 infected cells peaked at 6h pi but at lower levels than in wt infected cells. Synthesis of the 44 kd protein reached a minimum at 8h pi but increased again over the next four hours. This pattern was in contrast to wild type infection

which continues to decline after reaching its first peak at 6h pi. These results suggest that the E1B 58 kd protein can act as either a positive or negative regulator of E1A expression. The mechanism by which E1B exerts these pleiotropic effects its unknown.

The E2 72 kd DNA binding protein has two primary regulatory effects. A repressor effect has been demonstrated in studies of early *in vivo* transcription with the Ad 5 E2A mutant, ts 125, which at the restrictive temperature, is defective in production of the 72 kd protein. Cells infected with this mutant have repressed E4 transcription at 6h pi at the permissive temperature but not at the non-permissive temperature (Nevins and Winkler, 1980). Handa et al (1983) reported that in an *in vitro* transcription system the E2 72 kd protein specifically repressed transcription from the E4 promoter. The results from these two studies suggested that the 72 kd protein may be exerting its effect by binding to the E4 promoter causing the RNA polymerase II to be sterically blocked or alternatively may act by terminating E4 transcription at a post initiation stage (Handa et al, 1983). In other studies with ts 125 it has been demonstrated that at the non-permissive temperature the stability of E1 mRNA is three to five times greater than at the permissive temperature, a result which suggest that 72 kd has a destabilizing effect on cytoplasmic mRNA (Babich and Nevins, 1981).

The study of early gene expression was greatly facilitated with the development of 293 cells. These cells were derived from a human embryonic kidney (HEK) cell transformed with an intact fragment of E1 DNA. 293 cells express E1 specific mRNA and proteins and because of this

property can be used to grow mutant virus which because of defects in either E1A or E1B are unable to grow in Hela or KB cells (Graham et al, 1977; Aiello et al, 1979). With the use of these mutant viruses it has been shown that expression of E1B, E2, E3 and E4 is dependent upon the expression of a functional E1A product (Berk et al, 1979; Jones and Shenk, 1979b; Lewis and Matthews, 1980; Nevins, 1981; Persson et al, 1981a, b; Katze et al, 1981; Katze et al, 1983; Shaw and Ziff, 1982; Cross and Darnell, 1983; Gaynor and Berk, 1983). To study gene regulation Jones and Shenk (1979b) constructed three relevant mutants of Ad 5: dl 311 has a 150 bp deletion from 3.5 to 4.5 mu at the 3' end of E1A; dl 312 has 1030 nucleotides removed from 1.5 to 3.5 mu which entirely deletes the E1A structional gene; virus dl 313 has a large deletion from map co-ordinate 3.5 to 9.5 which removes the 3' part of E1A and most of E1B. These mutants were isolated on the basis of reduced growth on Hela cells and on their ability to replicate at nearly wild type levels on 293 cells.

When Hela cells were infected at low multiplicities (MOI) with dl 311 or dl 313 viral RNA from E1, E2, E3 and E4 was found in the cytoplasm of the cells but when infected with dl 312 there was no evidence of any viral RNA in the cytoplasm (Jones and Shenk, 1979b). When dl 312 or dl 313 were used alone to infect Hela cells the production of mature virions was reduced by a factor of at least 10^3 but when co-infected the result was the production of nearly wild type levels of both viruses (Jones and Shenk, 1979b; Persson et al, 1981b). This result suggested that the E1A function absent from dl 312 was provided, in trans, by dl 313.

Conversely the E1B function missing from dl 313 was provided by dl 312. Jones and Shenk (1979b) concluded that a functional E1A product was required for the efficient expression of the other early genes and that E1B was necessary for a complete productive cycle.

Harrison et al (1977) generated a series of mutants in both E1A and E1B. Those mutants defective in E1A were classified in complementation group I while those defective in E1B were assigned to group II. Members from both groups grew efficiently on 293 cells but not in Hela (Graham et al, 1978). Hr-1 virus, belonging to group I, has a single base deletion in E1A at nucleotide 1085 in the exon of the 13 S mRNA. This point deletion causes premature termination which results in the production of non-functional truncated proteins from the 13 S mRNA (Berk et al, 1979; Ricciardi et al, 1981; Shaw and Ziff, 1982). Hr-7 belongs to group II and has an unidentified mutation in the coding sequences of E1B which results in the underproduction of 58 kd protein (Berk et al, 1979). The presence of viral mRNA corresponding to E2, E3 and E4 in the nuclear and cytoplasmic fractions of Hela cells infected with hr-7 but not with hr-1 was discovered by Berk et al (1979). In agreement with Jones and Shenk (1979a, b) this result suggested that an E1A protein is required to initiate transcription or stablize early viral mRNA.

A regulatory role for E1A in the control of L1 expression, at early times in lytic infection, appears certain. In Hela cells infected with dl 312, Lewis and Matthews (1980) reported the appearance of a small translatable mRNA initiated from the major late promoter which codes for

a 13.5 kd protein. This mRNA was synthesized at 1/10 the rate in dl 312 infection as was found in Hela cells infected with wild type virus (Lewis and Matthews, 1980). Akusjarvi and Persson (1981) reported the appearance of a 2900 nucleotide mRNA in dl 312 infected cells but all other transcripts originating from the major late promoter were severely reduced compared to wild type infection. Nevins (1981) probed for labelled nuclear RNA with DNA sequences corresponding to the L1 coding region, 31-37 mu, in Hela cells infected with hr-1 and found little or no activity from the major late promoter compared to wild type infection. From these results it appears that transcripts initiating from the major later promoter at early times, are dependent on a functional E1A product. The significance of transcription from the major late promoter at early times isn't yet clear.

Montell et al (1982) constructed an Ad 5 mutant, pm 975, which has a single transversion in the second base of the 12 S mRNA intron that does not alter the proteins encoded by the 13 S mRNA but does prevent splicing of the 12 S mRNA. Studies with pm 975 demonstrated that the 13 S mRNA encodes all of the E1A functions required for normal early gene regulation and is phenotypically wild type in permissive cells in vitro (Montell et al, 1982; Gaynor et al, 1982). A requirement for the 12 S mRNA proteins in growth arrested cells has been demonstrated (Braithwaite et al, 1983; Montell et al, 1984).

The dependence of early gene expression on the E1A regulatory product(s) is not absolute. At late times in a low moi dl 312 infection of Hela cells there was detectable expression of early region mRNA but

the level of this expression was reduced compared to wild type (Nevins, 1981; Nevins et al, 1979). The temporal expression of early mRNA in dl 312 infection does not resemble that found in wild type. Furthermore it has been shown that dl 312 viral progeny can be produced, at reduced titres, from permissive cells if the period of incubation is longer than the normal wild type incubation (Berk et al, 1979; Nevins, 1981; Gaynor and Berk, 1983). In infection with dl 312 or hr-1 at moi greater than 200 pfu/cell the E1A independent expression of early mRNA is more readily observed but these infections are still not as efficient as wild type (Nevins, 1981; Gaynor and Berk, 1983). It is apparent that a functional product from the E1A 13 S message is required for the efficient and timely expression of early region genes (Jones and Shenk, 1979b; Berk et al, 1979; Nevins, 1981; Persson et al, 1981a,b; Katze et al, 1983; Cross and Darnell, 1983; Gaynor and Berk, 1983).

Results from the E1A mutant studies predicted that if protein synthesis were inhibited at early times in a wild type infection there would be little or no expression from the early regions (Jones and Shenk, 1979b; Berk et al, 1979). To test this prediction and to understand the pleiotropic effects of E1A, studies were carried out using anisomycin or cycloheximide to inhibit protein synthesis (Lewis and Matthews, 1980; Nevins, 1981; Persson et al, 1981a,b; Katze et al, 1981, 1983; Shaw and Ziff, 1982; Cross and Darnell, 1983). Conclusions from these studies have been inconsistent from one investigator to another and have failed to clearly define how the E1A regulatory protein(s) function.

Lewis and Matthews (1980) reported that the expression of E1A was

dependent on products from a novel family of pre-early mRNAs, initiating from the major late promoter, which code for a 13.5 kd protein distinct from the recognized 14 kd protein from L1. These conclusions were based on evidence obtained from wt-Ad 5 infected Hela cells which had been pretreated with 100 uM anisomycin that reduced protein synthesis by 99.6%. The evidence indicated that translatable mRNAs from all the early regions, including E1A, were severely reduced compared to less stringently inhibited controls. The only species of mRNA which were observed were those mRNAs originating from the late promoter. In agreement with this result was the discovery that in dl 312 infected cells, pre-treated with 10 uM anisomycin, only the pre-early mRNA species from the late promoter were observed.

The conclusions presented by Lewis and Matthews (1980) have not been supported elsewhere. In their own work they reported that under more stringent conditions (500 uM anisomysin pretreatment) production of the pre-early mRNAs was reduced or absent suggesting a dependence on some viral protein factor. In a subsequent study by Nevins (1981) it was clearly shown that activity from the L1 promoter was dependent on a functional E1A product(s). Temperature sensitive mutants mapping to the region described by Lewis and Matthews are not defective in the accumulation of early viral cytoplasmic mRNA (Berget et al, 1976). Other than the original Lewis and Matthews report there is no supporting evidence which would suggest that E1A expression is dependent on pre-early activity from the late promoter.

In a series of published accounts it has been hypothesized that

the primary function of the E1A regulatory product(s) may be to inactivate a cellular protein which, when present, would destabilize viral mRNA (Persson et al, 1981a; Katze et al, 1981, 1983; Persson et al 1981b). In support of this hypothesis it was found that in Hela cells infected with Ad 2 and treated at 1h pi with 10 uM anisomycin, accumulation of the early mRNA was enhanced as compared to untreated controls (Persson et al, 1981a). The addition of inhibitors of protein synthesis before infection decreased the accumulation of early viral mRNA emphasizing that viral proteins were required for accumulation of early viral mRNA (Persson et al, 1981a). In 293 cells infected with Ad 2 and treated with 100 uM anisomycin at 0.5 hours before infection there was an enhanced accumulation of viral mRNA from E4 and decreased accumulation of E2 and E3 mRNA compared to drug free controls (Persson et al, 1981b). This result suggested that E4 was most sensitive to E1A control and that additional factors were required to control the accummulation of mRNA from E2 and E3 (Persson et al, 1981b; Katze et al, 1981). Despite this finding, Katze et al (1983) demonstrated that the E2 72 kd protein was expressed in dl 312 or hr-1 infected Hela cells when the cells were treated at 1h pi with 100 uM anisomycin. Inhibition of protein synthesis before infection resulted in expression of E2 and E3 proteins in dl 312 infected cells but not in wild type infected cells. These results were interpreted to mean that the block in gene expression in dl 312 and hr-1 infected Hela cells could be overcome by inactivation of a cellular gene product (Katze et al, 1983).

The results presented in these series of papers are difficult to

interpret. In joint experiments the Persson group has reported the enhancement of expression of E2 and E3 products in wild type or E1A mutant infection of Hela cells pretreated with anisomycin. Under similar experimental conditions Shaw and Ziff (1982) showed that E2 and E3 expression was reduced when wild type infected cells were pretreated with anisomycin. The different findings between the groups suggest that the effects of anisomycin on early gene expression are not consistent so that results from studies using this drug should be interpreted with caution.

It is difficult to explain the difference between dl 312 and wt infection based on the intrepretation presented by the authors. When protein synthesis is inhibited before infection there should be no effective difference between a wild type or an E1A mutant infection. In both cases no E1A proteins will be synthesized and the net effect should be an equivalent lack of expression from the other early regions. The results from these studies have shown that dl 312/hr-1 and wild type behave differently with regard to early gene expression under conditions of protein synthesis inhibition. Because of this contradiction it is difficult to conclude that E1A acts primarily at the mRNA stabilization level.

In 1981 Nevins proposed that the E1A regulatory product served primarily to inactive a cellular factor which prevented transcription from the other early viral promoters (Nevins, 1981). Nevins assumed that the repressor was unstable. From this assumption it was predicted that if protein synthesis were inhibited during a wild type infection, transcription from early regions would proceed, since the inhibition of

protein synthesis would mimic the E1A function by preventing the production of the cellular repressor. To test this prediction Hela cells were pretreated at 0.5 hours before infection with 100 uM anisomycin and infected with Ad 5. Labelled nuclear mRNA from the infected cells indicated that transcription from E2 and E3 was reduced. These results, which were duplicated by Shaw and Ziff (1982), were consistent with the Nevins' prediction.

If a cellular repressor did exist then inhibition of protein synthesis during a dl 312 infection should be able to avoid the requirement for E1A protein in the efficient expression of the other early regions. In Hela cells pretreated with 25 ug cycloheximide at 0.75 hours before infection with dl 312, Nevins found that expression from E4 was at wild type levels whereas untreated dl 312 infected cells had a very low level of E4 expression (Nevins, 1981). In treated cells transcription from E1A, E1B, E2 and E3 were less than 20% of their wt expression and were not substantially increased compared to untreated dl 312 infected controls.

The Nevins' results are difficult to interpret. Under conditions of protein synthesis inhibition, infection with dl 312 or wt Ad 5 should be equivalent as in both cases there are no E1A proteins or putative cellular repressors. The results clearly showed that dl 312 and wt were not equivalent, especially in E2 and E3 expression.

In the dl 312 experiment it is not clear why Nevins chose cycloheximide instead of anisomycin to inhibit protein synthesis. The choice of cycloheximide makes comparisons difficult as these drugs may be

having different effects on viral gene expression. The finding that E4 expression was enhanced partially supports the Nevins prediction but it has been shown that the various species of E4 mRNA are differentially enhanced by cycloheximide treatment (Tigges and Raskas, 1982, 1984). This result suggests that cycloheximide may have an effect on E4 transcription not related to the ability to inhibit protein synthesis. Attempts to duplicate Nevin's results have failed to show enhanced levels of E4 transcription in dl 312 infected cells pretreated with cycloheximide (Cross and Darnell, 1983; Gaynor and Berk, 1983). On this basis it is difficult to conclude that the primary function of E1A is to inactivate a cellular repressor.

IV Description of Research Studies

As described above many previous studies have shown that a functional E1A protein from the 13 S mRNA is required during lytic infection for activation of early viral gene expression (Jones and Shenk 1979b; Berk et al, 1979; Montell et al, 1982). The mechanism by which E1A exerts this regulatory function is still unclear. If the E1A regulatory protein were required for direct activation of the other early genes then inhibition of protein synthesis, either before or during a wild type infection, should abolish transcription from these genes. Results from the Persson group and Nevins' studies have shown that E4 mRNA can be expressed from infected cells pretreated with anisomycin or cycloheximide. On the basis of these results it has been proposed that expression of the early regions of Ad 5 are controlled by a cellular

protein that either represses or destablizes viral transcription and that inhibition of protein synthesis removes this cellular protein. The function of the E1A protein under normal circumstances is to eliminate the cellular protein (Katze et al, 1981, 1983; Nevins 1981, Persson et al, 1981a,b).

There are a number of reasons which justify a re-examination of the proposal described above. The first of these reasons is that inhibition of protein synthesis does not affect expression from all of the early regions equally. Among the early genes only E4 has been found to be consistently enhanced by inhibition of protein synthesis. The effects on E1B, E2 and E3 are less certain as results have differed from one investigator to another. Secondly, these results have been inconsistent from one investigator to another. Thirdly the use of anisomycin or cycloheximide may not be appropriate. Both of these drugs have wide and many undefined effects on the metabolism of the host cell. The interaction of these drugs with the cells will result in a range of side effects which make it difficult to clearly differentiate between properties of the virus, cell or drug. Under these conditions it has been very difficult to clearly describe the role of the E1A regulatory protein.

In an attempt to clarify this situation we have re-examined the effects of protein synthesis inhibition on early gene expression, in both wt and hr-1 infection without the use of cycloheximide, anisomycin or other drug inhibitors. A convenient system in which this is possible is the tsH1 cell line. These cells are temperature sensitive mutants of a

Chinese Hamster Ovary (CHO) cell line, which at temperatures above 37^{o}C, undergo a conformational change to the leucyl-tRNA synthetase (Thompson et al, 1973). This change prevents the loading of leucine on to the $tRNA^{leu}$ resulting in the inhibition of protein synthesis. The primary advantage of this system is that protein synthesis can be inhibited in a specific and defined manner without drug induced side effects.

The approach in the following series of experiments has been to inhibit protein synthesis at critical times during early infection. By strategically inhibiting protein synthesis during this period it has been possible to examine the roles of viral and cellular proteins on early viral gene expression.

METHODS

I Cell Lines

(a) Chinese Hamster Ovary (CHO)-Wild Type

The wild type CHO cells used throughout this study were originally provided by Dr. Cliff Stanners, McGill University, Montreal, Canada. These wild type cells have a stable karyotype consisting of twenty-one chromosomes with little cell to cell variation (Thompson et al, 1973; Puck et al, 1958).

(b) Temperature Sensitive Chinese Hamster Ovary (tsH1)

The tsH1 cells used extensively throughout this study were originally provided by Dr. C.P. Stanners, McGill University, Montreal, Canada. The mutant was isolated from a culture of wild type CHO cells by a procedure described by Thompson et al (1973). A wild type culture of CHO cells was treated with N-methyl-N'-nitro-n-nitrosoquandine (MNNG) at 34^{o}C for seven days. The culture was then incubated at 38.5^{o}C for 24 hours at which time tritiated thymine (3H-dT) was added for 48 hours to kill cells capable of DNA synthesis. The survivors were cloned at 34^{o}C and tested for growth at 38.5^{o}C (Thompson et al, 1973; Stanners, 1974; Thompson et al, 1975; Stanners et al, 1978; Pollard et al, 1979).

One of the mutants isolated was tsH1. At 34^{o}C the leucyl-tRNA synthetase is able to load leucine normally on to $tRNA^{leu}$ but at temperatures above 37^{o}C an unknown permanent conformational change occurs in the enzyme. The net effect is that at 38.5^{o}C or higher, leucine is no longer added to $tRNA^{leu}$ and protein synthesis is inhibited (Thompson et

al, 1973; C.P. Stanners, pers. comm.). The temperature sensitive phenotype is stable. Cultures have been grown for 3 months and remain fully temperature sensitive with reversion frequency of approximately 1×10^{-5} (Thompson et al, 1973).

(c) Human KB Cells

The KB cells used in this study were drawn from our regular laboratory stocks. The line was originally isolated from an epithelial carcinoma of the cheek from someone with the initials KB (Eagle, 1955) and was brought to the department by Dr. S. Mak from the lab of Dr. M. Green, St. Louis, Missouri.

(d) 293 Cells

The 293 cells used in this study were provided in starter aliquots from Dr. F. L. Graham, McMaster University, Hamilton, Canada. 293 cells are Human Embryonic Kidney cells (HEK) transformed with E1 fragment (XhoIC) from Ad 5 and express viral mRNA and proteins (Graham et al, 1977; Aiello et al, 1979).

II Tissue Culture

(a) Monolayer Culture

Cells were grown on 150 mm NUNC tissue dishes in alpha minimal essential medium (α-MEM) supplemented with the appropriate mammalian serum and 1%(v/v) penicillin-streptoymcin solution to inhibit bacterial contamination. CHO and tsH1 cells were supplemented with 10%(v/v) fetal calf serum (Gibco) while KB cells were supplemented with 7.5%(v/v) calf serum (Gibco). 293 cells were grown in Jokliks modified alpha-MEM

supplemented with 5%(v/v) horse serum (Gibco). All cell lines were grown at 37°C in a 5% CO_2 humid incubator except tsH1 cells which were grown at 34°C.

Subculturing of confluent cells was accomplished by withdrawing the culture medium, rinsing with 3 mls/plate of Tissue Culture Trypsinizing (TCT) solution and removing attached cells by agitation with 2 mls of TCT solution. The cells removed by this process were diluted into prewarmed medium and seeded into new culture dishes. The subculturing procedure for the 293 cells was essentially the same except that a 1 x Versene solution was used in place of the TCT solution.

(b) Suspension Culture

Suspension cultures of tsH1 and CHO cells were grown in alpha MEM supplemented with 10%(v/v) fetal calf serum in Bellco Culture flasks and were stirred continuously with a suspended teflon coated magnetic stirring bar. Cultures were diluted with prewarmed medium as necessary to maintain cell densities of between 5×10^5 and 7×10^5 cells/ml.

Suspension cultures of KB cells were grown in Jokliks modified MEM supplemented with 5%(v/v) horse serum at 37°C in modified reagent bottles and were stirred continuously with a teflon coated magnetic stirring bar. Cultures were diluted with prewarmed medium to maintain cell densities of between 5×10^5 and 7×10^5 cells/ml.

III Virus

(a) Source

Adenovirus type 5 and the group I mutant, hr-1, were used

extensively throughout this study. Original stocks of the virus were provided by Dr. F.L. Graham, McMaster University, Hamilton, Canada.

(b) Growth of Virus

Both forms of the virus were grown into the stock supplies from the starter aliquots. The method used for growth of the wild stocks was as described by Downey (1983). Suspension cultures of KB cells were grown to volumes of about two litres and their densities estimated, by direct count with a haemocytometer, immediately before use. Cells were collected by centrifugation for five minutes at 1500 RPM at room temperature. The cell pellet was resuspended in approximately 50 mls of fresh Joklik-MEM and Ad 5 was added to the suspension at 1-5 PFU/cell. The virus was allowed to adsorb for ninety minutes with constant mixing in a 37^{o}C warm room. The infected cells were resuspended in the same volume of culture medium as the original but made up of one half conditioned reserved medium and one half fresh Joklik-MEM. These cultures were maintained at 37^{o}C with 2.5%(v/v) horse serum until harvesting. Infected cells were harvest at 45h pi by centrifuging the infected cells as decribed above, at 4^{o}C. The cell pellet was washed three times with Tris-saline and resuspended in a suitable volume of 1% Tris-saline and stored at 70^{o}C.

The purification of the viral preparation was a modification of the procedure described by Lawrence and Ginsberg (1967). The infected cells were fractured by freeze thawing and lysed by sonication for approximately thirty seconds on ice (setting 30 on a BIOSONIC III). Sodium deoxycholate was added to the lysate to a concentration of

1.21×10^{-4} M and incubated for thirty seconds at room temperature. The lysate was sonicated again to obtain an even suspension and adjusted to a density of 1.34 g/ml with the addition of solid cesium chloride (CsCl). This mixture was centrifuged on a Beckman preparative ultra centrifuge for approximately 16 hours at 35,000 RPM at $4^{o}C$. The resulting viral band was collected and rebanded in 1.34 g/cc CsCl. The final band was collected in a laminar flow hood with sterile technique, diluted five fold with sterile 1.25 x tris buffered saline (TBS) and stored at $-70^{o}C$. The virus titre was determined by plaque assay on Hela cells by Mrs. Carole Evelegh.

Stocks of mutant hr-1 virus were prepared by infecting monolayers of 293 cells which were 70-80% confluent. The total number of 293 cells to be infected was estimated as 3.0×10^{7} cells/dish. The culture medium was removed and the monolayer rinsed with 2 mls of phosphate buffered saline^{++} (PBS^{++}). To each monolayer was added 2 mls of infection solution which consisted of crude hr-1 extract and PBS^{++} in suitable volumes to provide a multiplicity of infection (moi) of about 1-5 PFU/cell. The plates were incubated at room temperature for sixty minutes with constant agitation to ensure even distribution. The cells were refed with 10 mls of fresh Joklik medium supplemented with 1%(v/v) penicillin-streptomycin and 5%(v/v) horse serum. At 48h pi the cells were harvested by scraping the surface of each dish with a sterile rubber policeman. Each dish was rinsed and the infected cells collected and combined. The final pellet was resuspended with 1-2 ml of sterile PBS^{++} and 10%(v/v) glycerol/plate. The final suspension was sonicated three

times then fractured by freeze-thawing three times. The final smooth suspension was aliquoted in 5 ml sterile plastic test tubes and stored at -70°C.

The titre of the virus suspension was determined by plaque assay on 293 cells by Mrs. Carole Evelegh.

IV Preparation of mRNA Extracts from Suspension Cultures

(a) Infection Conditions

Suspension cultures of either tsH1, CHO or KB cells were grown to appropriate densities, harvested and resuspended in a small volume of fresh prewarmed unsupplemented alpha-MEM to yield a cell density of approximately 1×10^7 cells/ml. After allowing fifteen minutes for stablization, with gentle stirring at appropriate temperatures, the virus was added in the amount of about 25-50 PFU/cell. The virus was allowed to adsorb at 34°C or 37°C for 90 minutes at which time the infected cells were resuspended in Bellco spinner flasks with supplemented alpha-MEM. TsH1 cells were always infected at 34°C while CHO and KB cells were infected at 37°C.

(b) Extraction of total RNA

Extraction of RNA from infected cells was essentially as described by Downey (1983). At the desired time after infection the infected cells were collected and the pellets washed three times with 15 mls of cold sterile Tris-Saline and resuspended in 15 x volume of a 1%(w/v) solution of napthalene disulfonic acid (NDS). Following a 10 minute incubation on ice a 1/10 volume of a 10%(v/v) solution of

Nonidet-P 40 (NP40) was added and mixed gently in to the cell suspension. The suspension was centrifuged at 2000 RPM in the International IEC centrifuge at 4°C for ten minutes. The supernatant, which contains the soluble fraction of the cytoplasmic lysate, was withdrawn into a suitable sterile container to which was added 1/30 volumes of 15%(w/v) solution of sodium docedyl sulphate (SDS) and 3 mg/ml polyvinyl sulfonic acid (PVS). The lysate was extracted three times with saturated phenol:chloroform:isoamyl alcohol (50:50:1) and once with chloroform:isoamyl alcohol (50:1). Cytoplasmic RNA was precipitated from this extract at -20°C in 70% Ethanol with 1/100 volumes of 2M potassium acetate. Following precipitation the RNA was collected in 50 ml plastic Sorval centrifuge tubes which had been pretreated with 2 mls of 10% hydrogen peroxide to destory RNAases and three washes of cold sterile H_2O. The RNA was pelleted in a Sorval Superspeed centrifuge at 10,000 RPM in an HB4 swinging bucket rotor, at -20°C for fifteen minutes. The pellet was resuspended and washed three times with a solution of 95% ethanol:0.2m NaCl (2:1). The final pellet was dissolved in approximately 3 mls of cold sterile double distilled water. The UV absorption spectrum was obtained for each preparation on a Pye Unicam spectrophotometer. The absorption value at the 260mm wavelength was used to estimate the amount of mRNA in the preparation.

(c) Isolation of Poly(A^+) mRNA

Cytoplasmic poly(A^+) mRNA was isolated from total cytoplasmic RNA by affinity binding to PolyU-sepharose 4B. An equal volume of 2 x High Salt Buffer I was added to an RNA preparation which was then loaded onto

a PolyU-sepharose column. The eluate was collected, reloaded and the column washed with 20 mls of cold High Salt Buffer I. The column was freed of non poly(A^+) mRNA by washing with 30 mls of cold High Salt Buffer II. The mRNA was eluted from the column with Elution Buffer. A total of three fractions of 2 mls were collected into a 5 ml polyallomer Beckman centrifuge tubes. The mRNA was precipitated in 60% ethanol with 0.2 mls of 4M ammonium acetate. The mRNA was sedimented at 4°C in a Beckman preparative ultracentrifuge for sixteen hours at 35,000 RPM in a SW50.1 swinging bucket rotor. The sedimented mRNA was collected and resuspended with 2 mls of sterile double distilled H_2O and reprecipitated as described above. The final pellet was dissolved in sterile double distilled H_2O at 50 ug mRNA/ul H_2O and stored at -70°C.

V Agarose Gel Electrophoresis and Northern Blot Analysis

(a) Gel Electrophoresis

The mRNA samples were prepared for electrophoresis on horizontal gels by suspending approximately 50 ug (usually 1 ul) of mRNA in 30 ul of sample electrophoresis buffer. The mRNA was denatured by heating to 65°C for five minutes.

A 1% denaturing agarose gel was prepared by boiling a mixture of 1.7 g of agarose and 145 ml of a 1 x M.O.P.S. buffer. After cooling, 25 mls of 100% formaldehyde were added and the gel was poured into a 17 x 20 mm gel box. The mRNA preparation was added into wells in the gel of approximately 50 ul in volume. The gel box was then filled with running buffer. The mRNA was electrophoretically separated by applying

35 volts to the gel for a period of ten hours. The gel was removed and soaked in 400 mls of 0.5M ammonium acetate in the presence of a few drops of 5 mg/ml ethidium bromide for approximately three hours. The gel was washed twice in 0.05M NaOH for twenty minutes and equilibrated in 0.5M Tris (pH 7.4):3M NaCl twice for thirty minutes.

The mRNA was transferred to nitrocellulose filter paper by placing the gel face down on 3MM chromatography paper which had been presoaked in 20 x SSC buffer. The nitrocellulose filter was overlayed on the gel. The mRNA was drawn out of the gel matrix by overlaying the filter with dampened 3MM chromatography paper and dry paper towelling. The sandwich was allowed to stand for approximately sixteen hours at room temperature to complete the transfer. To fully immobilize the mRNA the nitrocellulose filter was baked at 75°C for three hours.

(b) In Vitro Labelling of Viral DNA with ^{32}P-CTP (NICK TRANSLATION)

Specific restriction fragments of Ad 5 DNA, corresponding to the early region genes, have been previously isolated by Dr. F.L. Graham and cloned into bacterial plasmid, pBR322. A stock of plasmid with inserts of restriction fragments corresponding to early regions E1a, E1b, E2, E3 and E4 was maintained in our laboratory. Viral DNA was labelled by the procedure described by Rigby et al (1977) and Redfield (1978). Approximately 1.0 ug of plasmid containing the viral insert was incubated for ten minutes at 37°C in 50 ul of nick translation buffer in the presence of 0.25 ng of DNAse I and 10 uM dATP, TTP and GTP. This mixture was then combined with 100 uCi of ^{32}P-α-dCTP (NEN) and 6 units of Kornberg's DNA polymerase I and incubated at 12°C. The reaction was

stopped with the addition of 50 ul of 0.2M EDTA. Plasmid which had incorporated the ^{32}P-CTP label was isolated from other components in the reaction mixture by separation through Sephadex G-50. The total volume of the reaction mixture was phenol extracted to remove protein and then applied to a 10 ml Sephadex G-50 fine column. The column was washed several times with 1 ml applications of cold column buffer. The path of radioactive material was traced down the column with a hand held laboratory Geiger counter. The first peak of radioactivity to be eluted from the column contained labelled plasmid and was collected. Incorporation of the labelled nucleotide into the plasmid varied from 50-70% of input radioactivity, resulting in a specific activity of approximately 2 x 10^8CPM/ug DNA. The total volume of labelled plasmid in column buffer was adjusted to 0.15M NaCl. To this solution was added 15 ug/ml of salmon sperm DNA and 2.5 volumes of cold 95% ethanol. The plasmid solution was stored at -20^{o}C for 24 hours to precipitate DNA.

(c) Hybridization of Labelled Viral DNA to mRNA Bound to Nitrocellulose (Northern Blot Analysis)

The labelled plasmid was hybridized to mRNA bound to nitrocellulose filters according to the procedure developed by Southern (1975) and Nevins and Darnell (1978a). The nitrocellulose filter was preincubated in 10 mls of prehybridization buffer for at least one hour at 42^{o}C in a sealed plastic bag. The labelled plasmid was suspended in 10 mls of hybridization buffer. The preincubation buffer was removed from the filter bag and the hybridization buffer containing the plasmid DNA was added. Incubation was for sixteen hours at 42^{o}C with constant

agitation. Following incubation the buffer was removed and the filter was washed twice with 250 mls of 2 x SSC +0.1% SDS at room temperature and twice with 250 mls of 0.1 x SSC +0.1% SDS at 50^{o}C to remove DNA that had hybridized nonspecifically. The filter was dried at 75^{o}C for thirty minutes and exposed at room temperature to Kodak XRP or XAR-5 X-Ray film in light sealed cassettes with intensifier screens.

VI DNA-RNA Filter Hybridization

(a) In-Vivo Labelling of RNA

TsH1 cells were infected with adenovirus 5 under standard conditions. At the appropriate time after infection approximately 1 x 10^{8} cells were resuspended in 5 mls of fresh alpha MEM prewarmed to either 34^{o}C or 38.5^{o}C. To the suspension was added between 100-200 uCi of ^{3}H-Uridine (Amersham). The labelled suspension was incubated for 15 minutes at the appropriate temperature at which time the cells were harvested and total cyctoplasmic RNA extracted and purified as before.

(b) Immbolization of Viral DNA on Nitrocellulose Filters

Plasmid, pBR322, with inserts of specific restriction fragments corresponding to the Ad 5 early genes, at a concentration of 3 ug/ml in 0.1 x SSC was denatured by the addition of 1N NaOH in 2 x SSC with the addition of an appropriate volume of 20 x SSC. Presoaked nitrocellulose filters, 25 mm in diameter, were washed with 10 ml of 2 x SSC. The DNA solution was applied to the filters by suction. Each filter was then washed with 50 mls of 2 x SSC and placed in autoclaved glass scintillation vials to dry at room temperature and baked at 75^{o}C for two

hours.

(c) DNA-RNA Hybridization

Approximately 20 A_{260} units of total labelled cytoplasmic RNA was applied to each filter in a 1 ml hybridization solution of 2 x SSC + 0.1%(v/v) SDS and incubated at 64°C for 24 hours in a constant temperature water bath. Following incubation the filters were rinsed once with 300 mls of 2 x SSC, were washed twice by suction with 100 mls of 2 x SSC and were then incubated for one hour in DNAase inactivated pancreatic RNAase (20 ug/ml, 5 x Crystalline in 2 x SSC). The filters were again rinsed with 300 mls of 2 x SSC, washed twice by suction with 100 mls of 2 x SSC and dried at 75°C for thirty minutes. The dried filters were placed in plastic biovials and immersed in omnifluor cocktail solution. Total hybridized counts were measured in a Beckman Liquid Scintillation Counter.

VII Rate of ^{35}S-Methionine and ^{3}H-Uridine Incorporation

To measure the rates of protein synthesis and transcription in tsH1 cells at various temperatures the following procedure was used. Uninfected tsH1 or CHO cells were grown in suspension. Aliquots of 1.5×10^6 cells (approximately 3 mls of culture) were removed into sterile 15 ml centrifuge tubes to which were added 50 uCi/ml of ^{35}S-Methionine (NEN) or 20 uCi/ml of ^{3}H-Uridine. Aliquots were incubated at 34, 38.5 or 40°C for fifteen minutes with frequent agitation. The incorporation of the label was halted by the addition of 2 mls of ice cold Tris-saline. The cells were harvested in the usual manner and

washed three times with cold Tris-saline. The final cell pellet was resuspended with 0.2 ml of RIPA buffer and incubated on ice for 2 minutes at which time 2 mls of 10% TCA was added and incubated for a further ten minutes. The precipitate was collected on millipore filters under suction and washed with 10 mls of cold 5% TCA and dried at $75^{o}C$ for 15 minutes. The total amount of incorporated ^{35}S-Methionine or ^{3}H-Uridine was measured in the Beckman Scintillation Counter in omnifluor cocktail solution.

VIII Sodium Dodecyl Sulphate Polyacrylamide Gel Electrophoresis (SDS-PAGE)

Discontinuous SDS-PAGE was performed as described by Downey (1983). The resolving portion of the gel was 16 cm in length and 0.1 cm thick. The stacking gel was 1.5 cm long. The resolving gel contained 15% (w/v) acrylamide, 0.386 M Tris-HCL (pH 8.8) and 3.47 mM SDS while the stacking gel contained 4.5% (w/v) acrylamide, 0.127 M Tris-HCL (pH 6.8) and 0.014 M SDS. Gels were polymerized with ammonium persulfate and N,N,N^{1}, N^{1}-tetramethyl-ethylenediamine to concentrations of 1.18x mM and 0.03% (v/v) respectively. Samples were loaded in preformed wells about 0.75 cm in width. The samples were electrophoretically separated by applying 50 volts to the gel for 24 hours. Following electrophoresis the gel was fixed in staining solution, cleared in destaining solution and dried.

MATERIALS AND CHEMICALS

Some brands of materials and chemicals were used exclusively throughout the course of this research work. These are listed below:

alpha-MEM	Grand Island Biological Co. (GIBCO.)
Calf Serum	GIBCO
DNAase I	Worthington
DNA polymerase I (Kornberg's)	Boehringer-Mannheim Ltd.
Fetal calf serum	GIBCO
Horse serum	GIBCO
Joklik-MEM	GIBCO
M.O.P.S.	Sigma
Napthalene disulphonate	Eastman Kodak Co.
Nitrocellulose filter paper	Spectrex Ltd.
Nonidet P-40	Bethesda Research Labs (BRL)
Nucleotide triphosphates (ATP, GTP, TTP)	Pharmacia
NUNC tissue culture dishes	GIBCO
Omnifluor	NEW ENGLAND NUCLEAR (NEN)
Penicillin-Streptomycin Solution	GIBCO
Poly(U)-Sepharose 4B	Pharmacia
Polyvinylsulphate (K+salt)	Eastman Kodak Co.

^{32}P-CTP, ^{35}S-Methionine	NEN
^{3}H-Uridine	Amersham
Sephadex G-50 fine	Pharmacia
Sodium dodecyl sulphate (SDS)	Serva Feinbiochemica
Tris-HCl	Sigma
Trypsin Stock Solution (10 X)	GIBCO

Nick Translation Buffer

50 mM Tris pH 7.9

5 mM $MgCl_2$

10 mM B-mercaptoethanal

100 ug/ml B.S.A

Pre-incubation Buffer

50% deionized formamide

5 x SSC

5 x Denherdt's

250 ug/ml salmon sperm DNA

50 mM sodium phosphate buffer pH 6.5

Hybridization Buffer

50% deionized formamide

5 x SSC

1 x Denherdt's

20 mM $NaPO_4$ buffer pH 6.5

100 ug/ml salmon sperm DNA

10% Na dextram sulphate 500 (Pharmacia)

50 x Denherdt's

1% (w/v) Bovine Serum Albumin (BSA)

1% (w/v) Polyvinyl Pyrrolidine (PVP)

1% (w/v) Ficoll

Column Buffer

10 mM Tris-HCl pH 8.0

10 mM NaCl

2 mM EDTA

0.01% (w/v) SDS

Running Buffer

2 M Formaldehyde

0.1 M M.O.P.S. pH 7.0

5 mM sodium acetate

1 mM EDTA pH 7.6

Sample Electrophoresis Buffer

2 M Formaldehyde

0.1 M M.O.P.S. pH 7.0

5 mM Na acetate

1 mM EDTA pH 7.6

60% (v/v) deionized formamide

Napthalene Disulphonate (NDS) Buffer

2.00×10^{-2} M Tris-HCl (pH 7.6)

1.00×10^{-1} M NaCl

1.00×10^{-3} M EDTA (pH 7.6)

1% (w/v) napthalene disulphonate

High Salt Buffer I

7.00×10^{-1} M NaCl

1.00×10^{-2} M EDTA (pH 7.6)

5.00×10^{-2} M Tris-HCl (pH 7.6)

25% (v/v) formamide

High Salt Buffer II

5.00×10^{-1} M NaCl

1.00×10^{-2} M EDTA (pH 7.6)

5.00×10^{-2} M Tris-HCl (pH 7.6)

50% (v/v) formamide

mRNA Elution Buffer

1.00×10^{-2} M EDTA (pH 7.6)

1.00×10^{-2} M Tris-HCl (pH 7.6)

6.93×10^{-3} M SDS

90% (v/v) formamide

Radioimmune Precipitation Assay (RIPA) Buffer

2.41×10^{-2} M sodium deoxycholate

3.47×10^{-3} M SDS

1.50×10^{-1} M NaCl

5.08×10^{-2} M Tris-HCl (pH 7.4)

1% (v/v) Trinton X-100

20 x SSC

3 M NaCl

0.3 M Na Citrate

Sodium Phosphate Buffer pH 6.5

1 M dibasic phosphate buffer

1 M monobasic phosphate buffer

10X Versene

6.84×10^{-3} M EDTA

1.37 M NaCl

2.68×10^{-2} M KCl

8.10×10^{-2} M Na_2HPO_4

1.47×10^{-2} M KH_2PO_4

1.11×10^{-2} M D-glucose

Before use, this solution was sterilized at 115°C for 10 minutes

Tissue Culture Trypsinizing (TCT) Solution

10% (v/v) 10X Versene

10% (v/v) Trypsin Stock Solution (purchased)

Tris Saline

1.37×10^{-1} M NaCl

5.10×10^{-3} M KCl

7.00×10^{-4} M Na_2HPO_4

5.55×10^{-3} M D-glucose

2.48×10^{-2} M Tris-HCl (pH 7.4)

1.25X Tris Buffered Saline (1.25X TBS)

1.71×10^{-1} M NaCl

6.37×10^{-3} M KCl

8.80×10^{-4} M Na_2HPO_4

3.76×10^{-2} M Tris-HCl ([H 7.4)

6.94×10^{-3} M D-glucose

2.5% (v/v) glycerol

Before use, this solution was sterilized at 115°C for 10 minutes

PBS^{++}

8% (w/v) NaCl

0.2% (w/v) KCl

1.15% (w/v) Na_2HPO_4

0.2% (w/v) KH_2PO_4

1% (w/v) $CaCl_2$

1% (w/v) $MgCl_2.6\ H_2O$

Electrophoresis Buffer for SDS-PAGE

5.22×10^{-2} M Tris-HCl (pH 9.0)

5.33×10^{-2} M glycine

3.47×10^{-3} M SDS

Staining Solution

0.05% (W/v) Coomassie Brilliant Blue

25% (v/v) isopropanol

6.25% (v/v) glacial acetic acid

Destaining Solution

7.5% (v/v) glacial acetic acid

25% methanol

RESULTS

I Suitability of the CHO Cell System for the Study of Adenovirus 5 Early Gene Expression

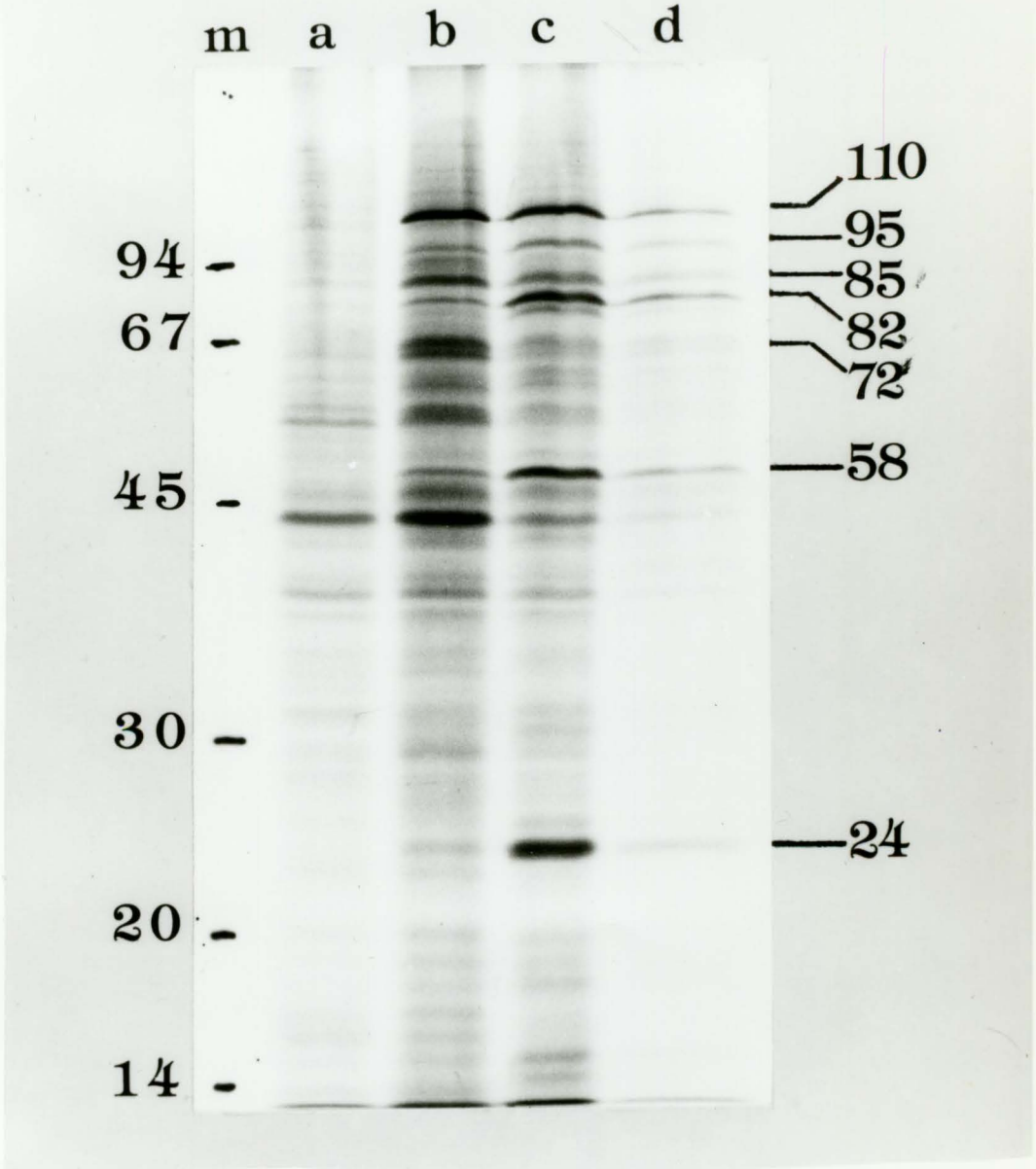
(a) Evidence of viral infection at late times

The CHO cell system is not normally used in the study of the adenovirus life cycle. Although generally regarded as a favorable system CHO cells appear to be only semi-permissive to adenovirus infection. Recently some unpublished reports have suggested that CHO cells are permissive at early times in the infection cycle and are blocked at some point at late times (Feldman, 1982 unpublished; Rainbow personal communication). To confirm these reports wt-CHO cells were grown in monolayer and infected with Adenovirus 5 at a multiplicity of 5 PFU/cell. Infected cells were harvested at 23, 47 and 70h pi and incubated at 37^{o}C for one hour in the presence of ^{35}S-Methionine. Protein from the infected cells was extracted and analysed by SDS-polyacrylamide gel electrophoresis.

Figure 3 is an autoradiogram of the SDS-polyacrylamide gel. There is clear evidence of viral infection in the CHO-wt cells. On the autoradiograph there are bands in the 24, 48 and 71h pi lanes (lanes b, c, and d respectively) which do not appear in the mock infected control (lane a). This suggests that Ad 5 late proteins were being synthesized in the infected cells.

At 24h pi (lane b) proteins with calculated molecular weights of 110, 95, 85, 82, 72 and 58 kd were observed. It is possible that the

Figure 3: CHO-wt cells were grown in monolayer to 75% confluency and infected with Ad 5 at 5 PFU/cell (approximately 5×10^7 cells/dish). At 24, 48 and 71h pi the cells were labelled with 10uCi/dish of 35 S-Methionine for 1 hour. The cells were harvested and total cellular protein was extracted and analysed on SDS-PAGE. Lane a, mock infected; lane b, 24h pi; lane c, 48h pi; lane d, 71h pi; lane M is the marker (a combination of purified non-viral proteins of known molecular weights). Values measured in Kilodaltons.

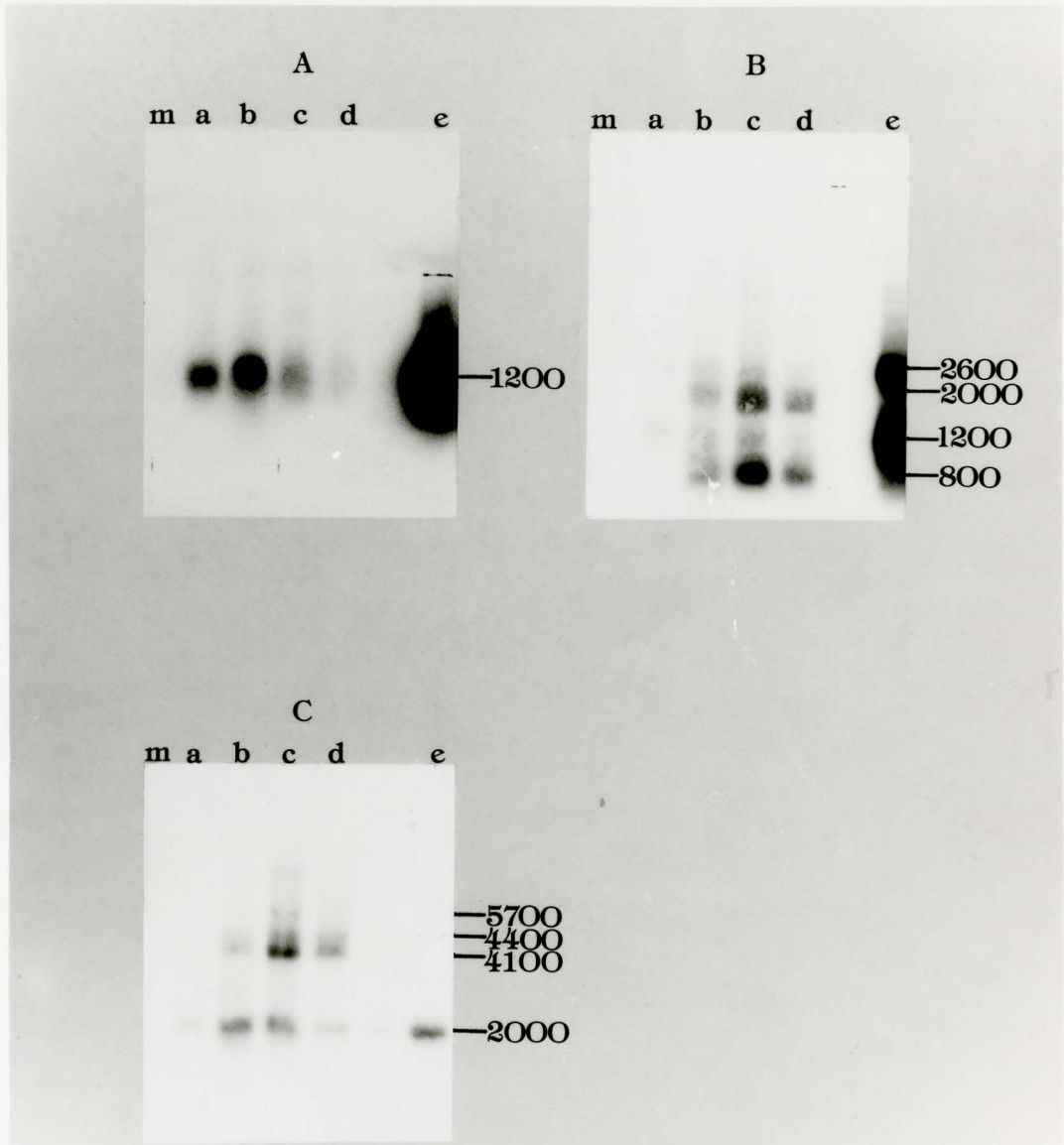

m
a
b
c
d
94
67
45
30
20
14
110
95
85
82
72
58
24

methods. The poly(A^+) mRNA was electrophorectically separated in a denaturing agarose gel and transferred to a nitrocellulose filter. The mRNA was immobilized on the nitrocellulose and hybridized to a nick translated probe labelled with alpha-^{32}P-CTP, (refer to Methods).

Figure 4A is an autoradiogram of a Northern Blot with a probe corresponding to the E1A region (described in legend to figure 4A). At 8, 12, 18 and 24h pi (lanes a,b,c and d respectively) the hybridization pattern reveals the presence of a 1200 nucleotide mRNA which probably corresponds to comigrating 12 and 13S E1A products. The lengths of the mRNA species include the poly (A^+) tail. Values reported from studies where this tail has been removed will be less than those observed in this study. There was no hybridization in the mock infected control (lane m). To make comparisons with a permissive infection poly(A^+) mRNA was isolated at 8h pi from KB cells which had been infected at 10 PFU/cell with Ad 5-wt at 37^{o}C. Unlike the CHO-wt the KB cells had been incubated in the presence of 25 ug/ml of cycloheximide from 2 to 8h pi to enhance yield of mRNA. In lane e there is E1A mRNA of similar length as in the infected CHO (compare lane e to lanes a,b,c, and d).

Figure 4B is an autoradiogram of a Northern Blot of the same mRNA preparations as in Figure 4A but with an E1 probe (XhoIc 0.0 to 14.2%). The results suggest the presence of at least four species of mRNA in the infected cells (lanes a,b,c and d) which are not present in the mock infected controls (lane m). At 8h pi (lane a) there are two species of viral mRNA of 1200 and 2600 nucleotides in length. The 1200 nucleotide mRNA probably corresponds to the 1200 nucleotide mRNA from E1A or E1B

Figure 4: Northern Blot analysis of mRNA isolated Ad5 infected CHO-wt cells. CHO-wt cells were infected at 100 PFU with wt-AD 5 at 37^{o}. Poly(A^{+}) mRNA isolated from infected cells at 8, 12, 18 and 24 h pi was analysed by Northern Blot analysis. Lanes a, 8h pi; b, 12h pi; c, 18h pi; d, 24h pi; M, mock infected CHO-wt; e, poly(A^{+}) mRNA isolated from Ad 5 infected KB cells at 8h pi. The mRNA was hybridized to nick translated alpha ^{32}P-CTP labelled probe DNA; plasmid pBR322 into which was cloned specific restriction fragments of Ad 5 DNA corresponding to the early genes: Figure A, E1A (HpaIE 0.0 to 4.5) probed; B, E1 (XhoIc, 0.0 to 15.9) probed; C, E2 (Hind III A 75.2 - 61.5) probed. The length of the mRNAs are given in nucleotides. Samples of ribosomal RNA from KB cells (1750 and 4800 nucleotides in length) and E. coli (1600 and 3100) were run in the outside lanes of each gel. The distance travelled through the gel was measured and plotted against the log lengths in nucleotide numbers to obtain a calibration curve. The length of the mRNA species was determined by measuring the distance travelled through the gel and extrapolating from the calibration curve into length in nucleotides. The length of individual mRNA species was reproducible to between 50 and 100 necleotides.


A
m a b c d e
1200
B
m a b c d e
2600
2000
1200
800
C
m a b c d e
5700
4400
4100
2000

whereas the 2600 nucleotide is probably the E1B 22 S species. At later times, starting at 12h pi (lane b), mRNA of 800 and 2000 nucleotides in length were observed in addition to those described at 8h pi. The 800 nucleotide mRNA may correspond to the 9 S late E1B species. At 18h pi (lane c) the same mRNA species as found at 12h pi are observed but there is an increased abundance of the 800 and 2000 nucleotide mRNAs. By 24h pi (lane d) the 800 and 2000 nucleotide mRNAs predominate. In the KB cells at 8h pi (lane e) the E1A 1200 nucleotide and the E1B 2600 nucleotide mRNAs predominate. This compares with the CHO-wt cells at 8h pi (lane a) as in figure 4A.

Figure 4C is an autoradiogram of a Northern Blot of the same mRNA preparations as in Figure 4A but with an E2 probe (Hind IIIA, 75.2 - 61.5 mu). At 8h pi (lane a) there was a 2000 nucleotide length mRNA in the infected CHO cells which was absent from the mock infected control (lane m). An mRNA species of equal length was observed in the KB control (compare lanes a and e). At 12, 18 and 24h pi (lanes b,c and d) mRNAs of 2000 and 4100 nucleotides were observed. A 4400 nucleotide mRNA was present at 18 and 24h pi (lanes c and d) whereas a 5700 nucleotide mRNA was observed only at 18h pi (lane c). These large hybridizing species were not observed in the KB controls (lane e). The transcriptional map of Ad 5 shows that there are no E2A transcripts of more than 2100 nucleotides (refer to Figure 2 in the introduction). It is possible that the 4100, 4400 and 5700 transcripts from E2B are long mRNAs of 4000 nucleotides or more. They are initiated from the E2A promoters at advanced times in the early phase and at their 5' ends share

common sequences with E2A transcripts (Chow et al, 1979; Gingeras et al, 1982). It is possible that the probe hybridized to the E2A sequences in the 5' end of the E2B mRNAs.

These results show that E1A, E1 and E2 are transcribed in Ad 5 infected CHO-wt cells. Transcription from each of the three gene regions resulted in mRNAs of lengths equal to those found in the permissive KB cells. The timed pattern of expression from E1A, E1 and E2 in CHO-wt cells was similar to the pattern observed in permissive cells.

II Properties of the tsH1 Cells at 34°C, 38.5°C and 40°C

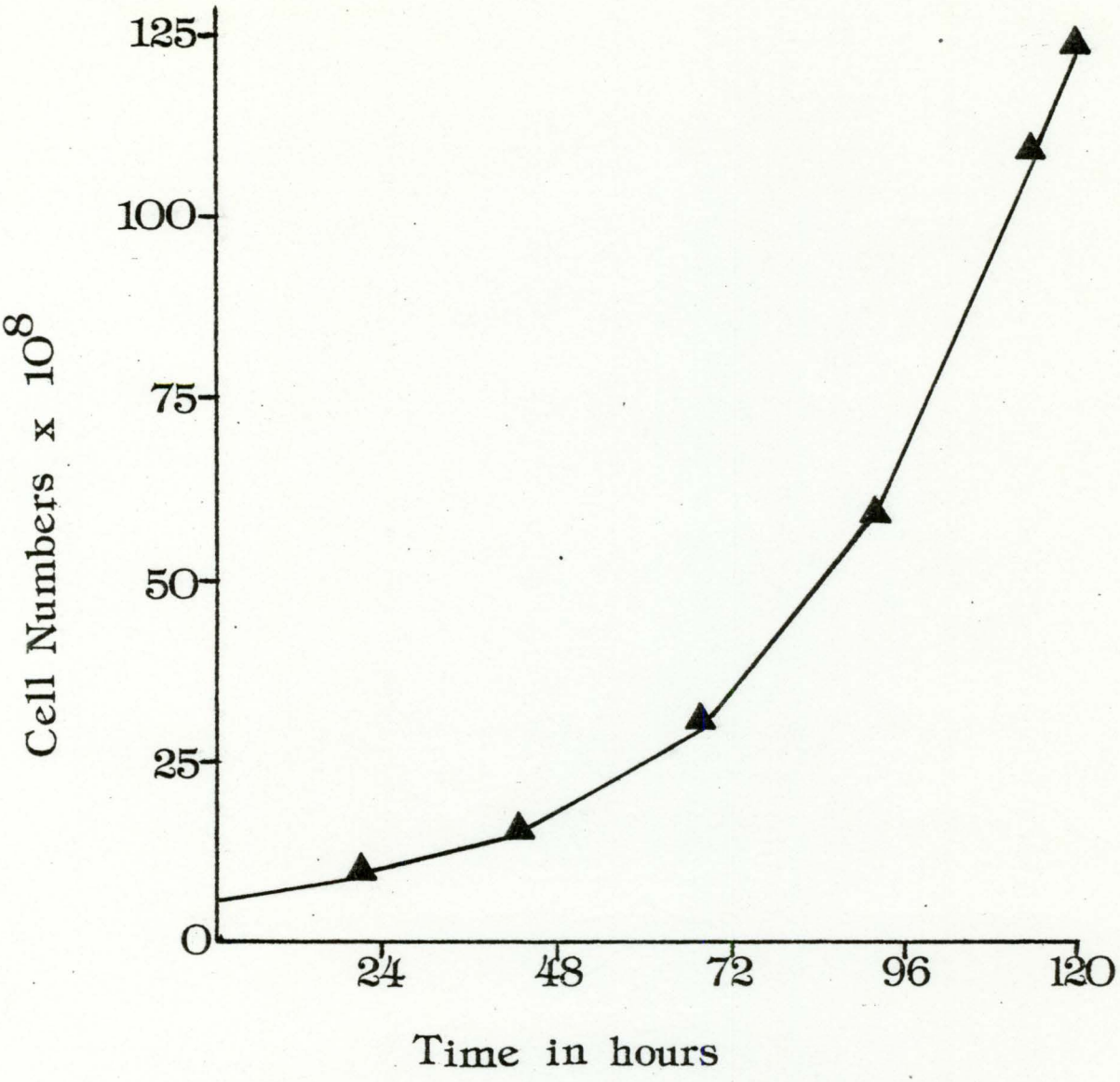
(a) Growth Characteristics of tsH1 at 34°C, 38.5°C and 40°C

To evaluate the growth potential of this mutant tsH1 cells were grown on tissue culture dishes at 34°C in alpha-MEM supplemented with 10% fetal calf serum. Under these conditions the tsH1 cells grew readily and formed even confluent monolayers. Once established the tsH1 cells would reach confluency from a 4:1 split in approximately three days. This compares with 2 days for wild type CHO and KB cells at the same temperature.

The doubling time and growth curve of the tsH1 cells were estimated in suspension culture by cell counting with the use of a haemocytometer. Figure 5 shows that growth in continuous culture increased geometrically with a doubling time of approximately 22 hours. The growth properties and doubling time compare well with the values cited by Thompson et al (1973). Under standard conditions these cells

Figure 5: Growth of tsH1 Cells at $34^{o}C$

Approximately 5.0×10^{8} tsH1 cells were suspended in 500 mls of alpha-MEM (+10% FCS + 1% PEN-STREP) at $34^{o}C$. At 24 hour intervals a 5 ml aliquot of cells was removed and the density estimated with a Neubauer haemocytometer. The suspension culture was maintained at cell densities from5×10^{5} to 7×10^{5} cells/ml. The total number of cells was calculted and plotted.


125
100
75
50
25
0
Cell Numbers x 10^8
24
48
72
96
120
Time in hours

were easily manipulated.

When incubated at temperatures above 37°C tsH1 cells undergo a permanent conformational change to the leucyl-tRNA synthetase (described in methods). At the elevated temperature the cells rapidly cease dividing and their viability is lost if they are incubated above 37°C for extended period (Stanners, personal communication). To test for viability, aliquots of tsH1 cells from suspension cultures grown at 34°C were incubated at either 38.5°C for 8 hours or at 40°C for 4 hours. Following incubation the cells were plated in tissue culture dishes and incubated at 34°C.

When first plated, the tsH1 cells which had been incubated at 38.5°C or 40°C demonstrated a small rounded appearance compared to cells incubated at 34°C alone. After 24 hours incubation at 34°C the plating efficiencies of those cells incubated either at 34°C, 38.5°C or 40°C were comparable and there were no distinguishable morphological differences between them. These qualitative observations show that under these defined circumstances, tsH1 cells remained viable following incubation at temperatures above 34°C.

(b) Incorporation of ^{35}S-Methionine by tsH1 cells at restrictive and non-restrictive temperatures

To determine the amount of protein synthesis in tsH1 cells at restrictive temperatures, the incorporation of ^{35}S-Methionine into cellular protein was measured. Uninfected tsH1 cells were grown in suspension culture at 34°C. The culture was divided and incubated at 38.5°C, 40°C or maintained at 34°C. Aliquots from the culture incubated

at 38.5°C were removed at 1, 3, 4, 5, 7 and 8 hours after the temperature shift. The aliquoted cells were maintained at 38.5°C in the presence of 50 uCi/ml of ^{35}S-Methionine for 15 minutes. The cells were harvested and the soluble fraction was extracted as described in methods. Cold tricholoroacetic acid (TCA) insoluble material was precipitated from the extracts and collected on nitrocellulose filters and the amount of incorporated radioactivity was measured. Similar experiments for 34°C and 40°C were performed as described in the legend of figure 6.

The amount of ^{35}S-Methionine incorporation from cells incubated either at 34°C, 38.5°C or 40°C are expressed as percentages of the zero time value at 34°C. At zero time the amount of incorporated ^{35}S-Methionine was 19,460 counts per minute (100%). Protein synthesis in cells incubated at 34°C increased gradually at 4 and 8 hours. The increase was probably the result of an increase in cell numbers. In cells incubated at 40°C there was a rapid decline of ^{35}S-Methionine incorporation to about 7% of the initial 34°C value after 30 minutes. Incorporation of the label after four hours at 40°C was less than 4%. After one hour at 38.5°C incorporation of the label was 20% and after 8 hours incubation only 2%. The results at 38.5°C and 40°C have been duplicated in repeated experiments and are consistent with those of Thompson et al (1973).

To evaluate how quickly protein synthesis would recover in tsH1 cells after being incubated at 40°C the following experiment was performed. A suspension culture of tsH1 cells was incubated at 40°C for four hours at which point the temperature was shifted down to 34°C.

Figure 6: Incorporation of ^{35}S-Mehtionine by tsH1 cells at restrictive and non-restrictive temperatures.

TsH1 cells were grown in suspension at 34^{o} and the temperature was increased to 38.5^{o}C (■) or 40^{o}C (▲) or maintained at 34^{o}C (●). At regular intervals approximately 1.5 x 10.^{6} cells were removed and incubated at the same tempeature in the presence of 50uCi/ml ^{35}S-Methionine for 15 minutes. Protein was TCA precipitated, collected on nitrocellulose filters and the amount of incorporated radioactivity was measured. The 100% value at zero time at 34^{o}C was 19,460 CPM.

Aliquots of cells were taken just before the shift up to 40°C and at 1, 2, 3 and 4 hours after the shift up. Aliquots were taken at 1, 2 and 3 hours after the shift down to 34°C.

Figure 7 shows that the level of protein synthesis in tsH1 declined rapidly at 40°C, consistent with those values established in figure 6. The recovery of protein synthesis after the return to 34°C was initially slow. Even after three hours, inhibition was still greater than 90%.

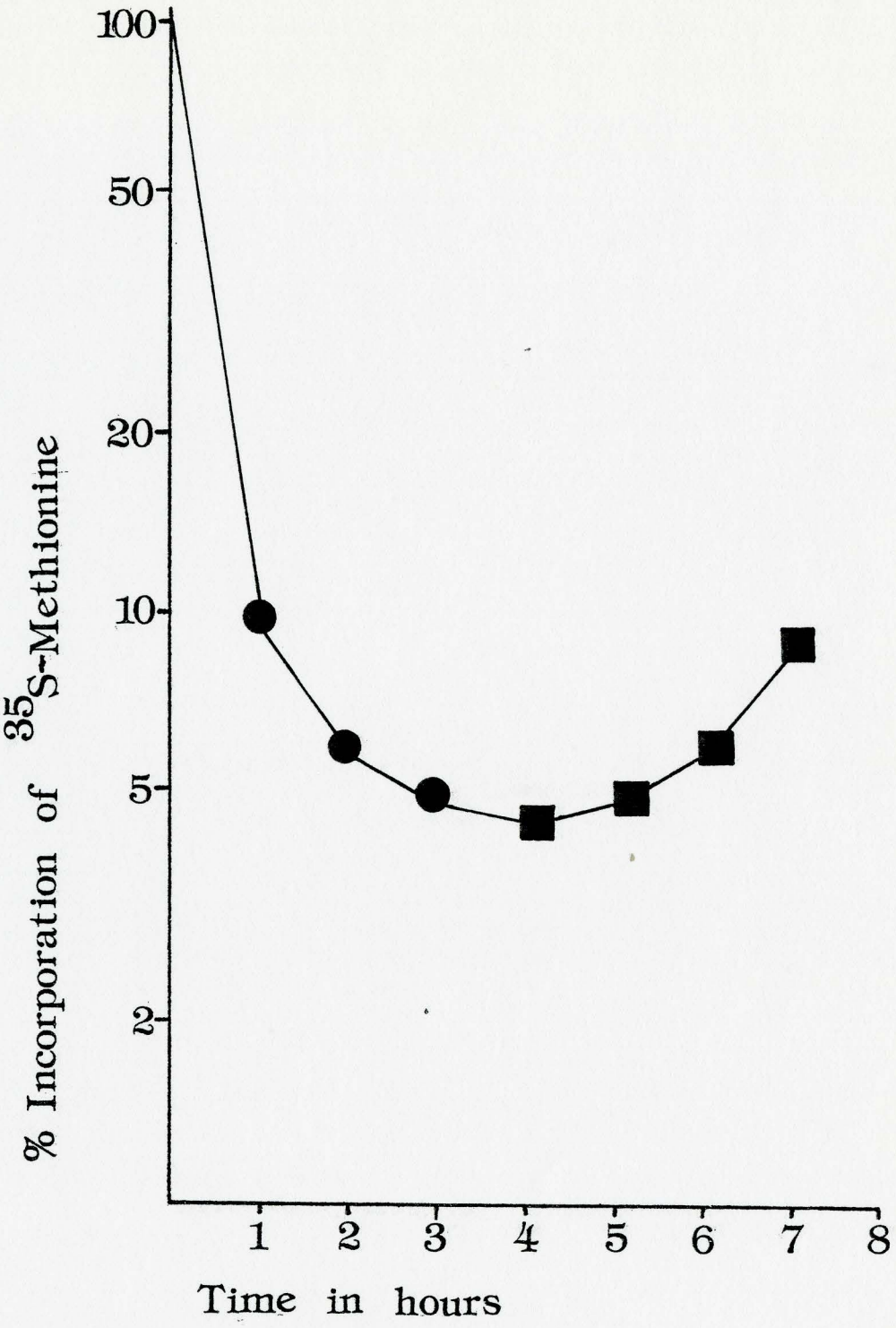
(c) Incorporation of ^{3}H-Uridine by tsH1 cells at restrictive and non-restrictive temperatures

To evaluate the effect of restrictive temperatures on transcription the incorporation of ^{3}H-Uridine into cellular mRNA was measured. Uninfected tsH1 cells were grown in suspension culture at 34°C. The culture was split in half, with one half being incubated at 38.5°C and the other maintained at 34°C. Aliquots from both cultures were removed at 2, 4, 6 and 8 hours after the cultures were split and were maintained at their respective incubation temperatures in the presence of 20 uCi/ml of ^{3}H-uridine for 15 minutes as described in methods. The aliquoted cells were harvested and the soluble fraction was extracted. Cold TCA insoluble material was precipitated from the extracts and collected on nitrocellulose filters and the amount of incorporated radioactivity was measured.

The incorporation of ^{3}H-Uridine was measured at 34°C just before the culture was divided. This was the zero hour aliquot at 34°C. The amount of ^{3}H-Uridine incorporation at other times from cells incubated

Figure 7: Incorporation of ^{35}S-Methionine by tsH1 cells after incubation of 40^{o}C.

TsH1 cells were grown in suspension culture at 34^{o}C and the temperature was increased to 40^{o}C (●). After four hours the temperature was lowered to 34^{o}C (▲). At regular intervals approximately 1.5 x 10^{6} cells were removed and incubated at the same temperature in the presence of 50uCi/ml of ^{35}S-Methionine for 15 minutes. Protein was TCA precipitated, collected on nitrocellulose filters and the amount of incorporated radioactivity was measured. The 100% value at zero time 34^{o}C wa 42,470 CPM.


100
50
20
10
5
2
% Incorporation of ^{35}S-Methionine
1
2
3
4
5
6
7
8
Time in hours

either at 34°C or 38.5°C are expressed as percentages of this initial value. At zero time the amount of incorporated ^{3}H-uridine was 4,140 counts per minute (100%). Figure 8 shows that after 8 hours of incubation at 34°C transcription had gradually increased to 117% of the initial 34°C value. This increase was probably the result of an increase in cell numbers. In cells incubated at 38.5°C there was a gentle decline of transcription to about 33% of the initial 34°C value after 8 hours in incubation. This decline was probably due to the inability of the cells to replace protein factors required during transcription (Thompson et al, 1973; Stanners personal communication). The results at 38.5°C have been duplicated in repeated experiments.

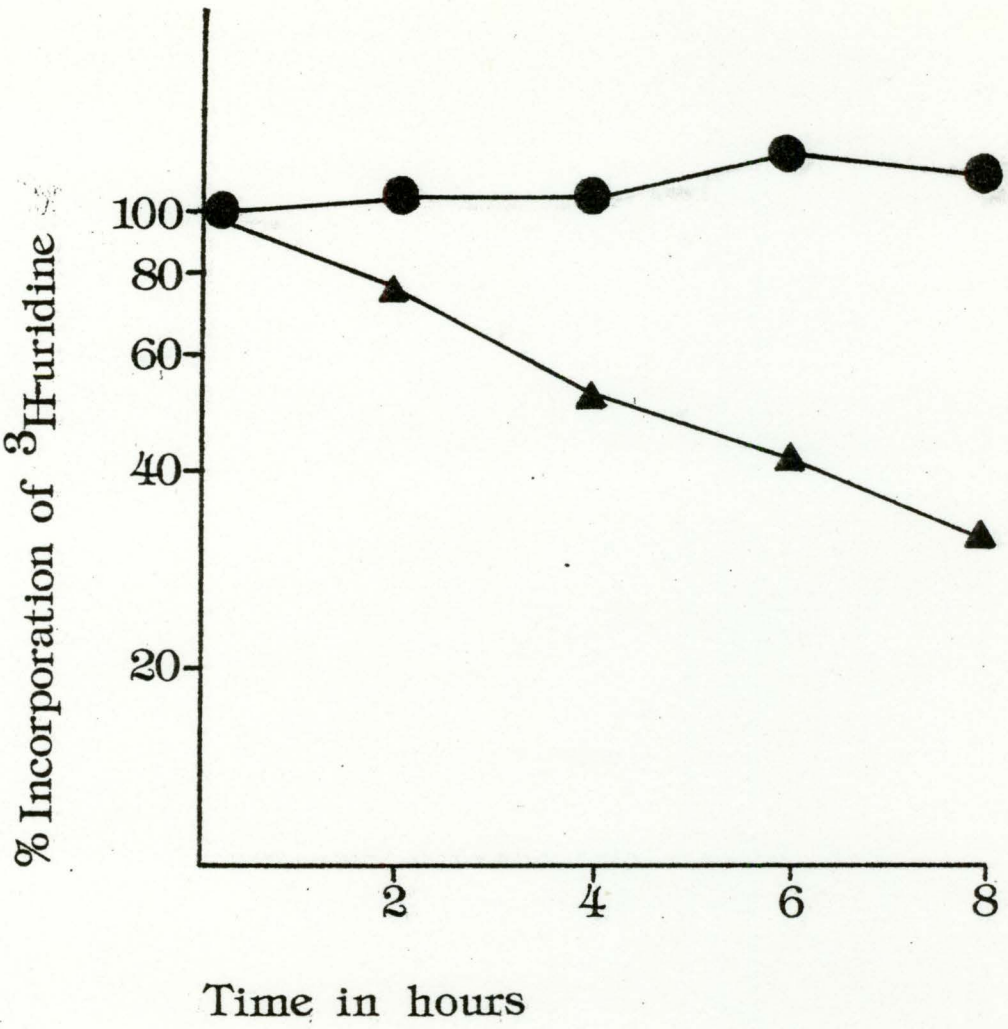
Thompson et al (1973) suggest that the decline of transcription in tsH1 cells is independent of the degree to which protein synthesis has been inhibited. The results presented here confirm that report. Figure 8 clearly shows that transcription in tsH1 cells after 2 hours at 38.5°C was reduced to approximately 78% of the initial 34°C value. In figure 6 it was shown that protein synthesis, after the same time at 38.5°C, declined to less than 15% of the initial 34°C value. The results show that incubation at restrictive temperatures under these defined conditions, reduces transcription but does not eliminate it.

III Expression of the Early Regions in tsH1 Cells Infected with Wild Type Adenovirus 5

To establish a time frame for expression of the early gene regions at 34°C, tsH1 cells were infected at 100 PFU with Ad 5. Poly(A^{+})

Figure 8: Incorporation of ^{3}H-Uridine by tsH1 cells at restrictive and non-restrictive temperatures.

TsH1 cells were grown in suspension at 34^{o}C and the temperature was increased to 38.5^{o}C (▲) or maintained at 34^{o}C (●). At regular intervals approximately 1.5 x 10^{6} cells were removed and incubated at the same temperature in the presence of 20uCi/ml ^{3}H-Uridine for 15 minutes. Total cellular RNA was TCA precipitated, collected on nitrocellulose filters and the amount of incorporated radioactivity was measured. The 100% value at zero time at 34^{o}C was 4,140 CPM.


% Incorporation of ^{3}H-uridine
100
80
60
40
20
2
4
6
8
Time in hours

mRNA was isolated from the cells at 8, 12, 18 and 24h pi and then subjected to Northern Blot analysis, described in methods.

Figure 9A is an autoradiogram of a Northern Blot with an E1A probe. At 8h pi (lane a) the hybridization pattern indicates the presence of a 1200 nucleotide mRNA which probably corresponds to comigrating 12 and 13S E1A products. The intensity of the band is weak suggesting that E1A was transcribed only at low levels but at 12h pi (lane b) the intensity of the band was much stronger. By 18h pi (lane c) the 1200 nucleotide mRNA was more strongly expressed. At 24h pi (lane d), there was marked expression of the 1200 nucleotide mRNA but in addition an 800 nucleotide mRNA appeared which probably corresponds to the 9 S late E1A mRNA. Transcription of E1A in tsH1 at 12h pi (lane b) resembled that at 8h pi in CHO-wt cells at 37^{o}C (lane e) suggesting that E1A transcription was delayed by at least 4 hours in tsH1 cells at 34^{o}C relative to wt-CHO cells at 37^{o}C.

Figure 9B is an autoradiogram of a Northern Blot of the same mRNA preparations as in figure 9A but with an E1 probe. There was no E1 expression detected at 8h pi (lane a). At 12h pi (lane b) there was expression of a 1200 nucleotide mRNA probably corresponding to 12 and 13 S E1A or E1B products. This result is consistent with previous findings (in figure 9A). At 18h pi (lane c) there are four mRNA species of 800, 1200, 2000 and 2600 nucleotides in length. The 800, 2000 and 2600 nucleotide species are probably transcribed from E1B. The 1200 nucleotide mRNA from E1B probably comigrated with the E1A 1200 nucleotide

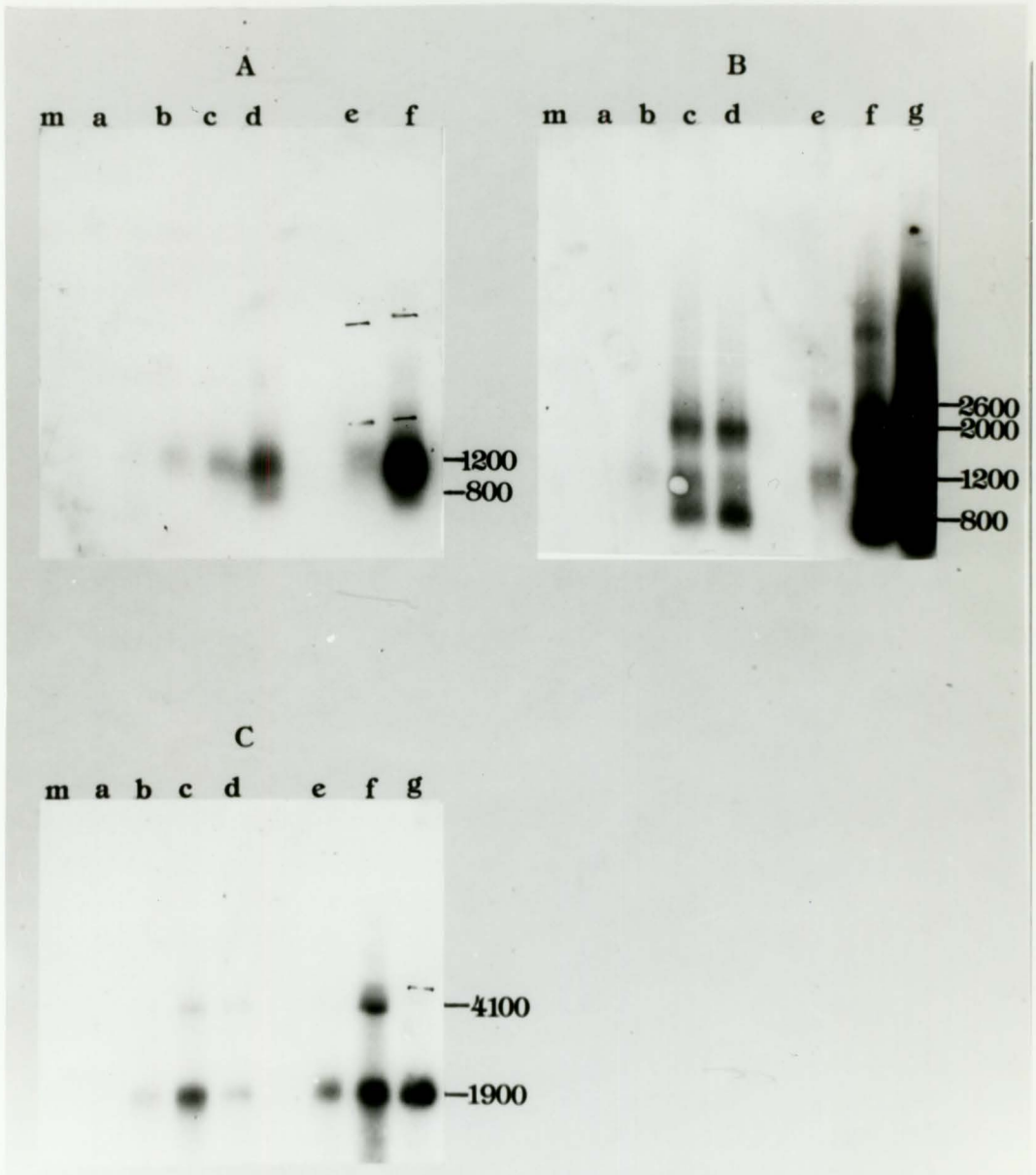

A
m a b c d e f
1200
800
B
m a b c d e f g
2600
2000
1200
800
C
m a b c d e f g
4100
1900

Figure 9: Northern Blot of mRNA isolated from wt-Ad 5 infected tsH1 cells at 34°C. TsH1 cells were infected at 100 PFU/cell with wt-Ad 5 at 34°C. Poly(A^+) mRNA isolated from these infected cells was analysed by Northern Blot analysis. Figure A, lane m, mock infected; a, 8h pi; b, s12h pi; c, 18h pi; d, 24h pi; e, 8h pi poly(A^+) mRNA from Ad 5 infected CHO-wt cells at 37°C; f, 8h pi poly(A^+) mRNA from KB cells infected with wt-Ad5 at 37°C. E1A probed (HpaIE; 0.0 to 4.5%). Figures B and C, lane m, mock infected; a, 8h pi; b, 12h pi; c, 18h pi; d, 24h pi; e, as in figure A lane e; f, 12h pi poly(A^+) mRNA from Ad 5 infected KB cells at 37°C; g, 8h pi poly(A^+) from Ad 5 infected KB cells at 37°C. KB cells ar 37°C; g, 8h pi (poly A^+) mRNA from Ad 5 infected KB cells at 37°C. Figure B, E1 probed (XhoIC, 0.0 to 15.9%). Figure C, E2 probed (Hind III A, 75.2 to 61.5%). Length of mRNAs given nucleotides.

species. At 24h pi (lane d) the same pattern as at 18h pi The expression of the E1B 2600 nucleotide mRNA began at 18h pi in the tsH1 at 34°C (lane c) but in CHO cells at 37°C it was observed at 12h pi (lane f). This result suggests that transcription of E1B was delayed by about 6 hours in tsH1 at 34°C relative to wt-CHO cells at 37°C.

Figure 9C is a Northern Blot using an E2 probe. There was no E2 expression detected at 8h pi (lane a). At 12h pi (lane b) there was evidence of a 1900 nucleotide mRNA which likely corresponds to the previously described E2 mRNA of 2000 nucleotides reported in CHO cells (figure 4C, lanes a to d). By 18h pi (lane c) the amount of the 1900 nucleotide mRNA increased and a 4100 nucleotide mRNA was observed. By 24h pi there was decreased transcription of both mRNAs compared to 18h pi (compare lanes c and d). Expression of the 1900 nucleotide mRNA began at 12h pi in tsH1 at 34°C but in CHO at 37°C it was observed at 8h pi (compare lanes b and f). This result suggests that E2 transcription was delayed in tsH1 at 34°C, relative to CHO-wt at 37°C.

Figure 10A is an autoradiogram of a Northern Blot using the same mRNA preparations as described in figure 9A but with an E3 probe. At 8h pi (lane a) no expression of E3 mRNA was detected. At 12h pi (lane b) three species of mRNA of 1025, 1900 and 3000 nucleotides were observed. The pattern at 12h pi persisted at 18h and 24h pi (lanes c and d). Expression of E3 mRNA at 12h pi in tsH1 at 34°C matched that found in CHO-wt at 8h pi at 37°C (compare lane b and e). This result suggests that E3 transcription is delayed by at least 4 hours in tsH1 at 34°C.

Figure 10: Northern Blot of mRNA isloated from Ad 5 infected tsH1 cells. TsH1 cells were infected at 100 PFU/cell with wt-Ad 5 at 34°C. Poly(A^+) mRNA was isolated from the infected cell at 8, 12, 18 and 24h pi and analysed by Northern Blot analysis. Lanes M, mock infected; a, 8h pi; b, 12h pi; c, 18h pi; d, 24h pi; e, poly(A^+) mRNA isolated at 8h pi from wt- Ad 5 infected CHO-wt cells at 37°C; f, poly(A^+) mRNA isolated at 8h pi; from wt-AD 5 KB cells at 37°C. Figures A, E3 (Hind III-B; 73 to 89.2%) probed; B, E4 (Hind III F; 89.2 to 97%). Length of mRNAs given in nucleotides.

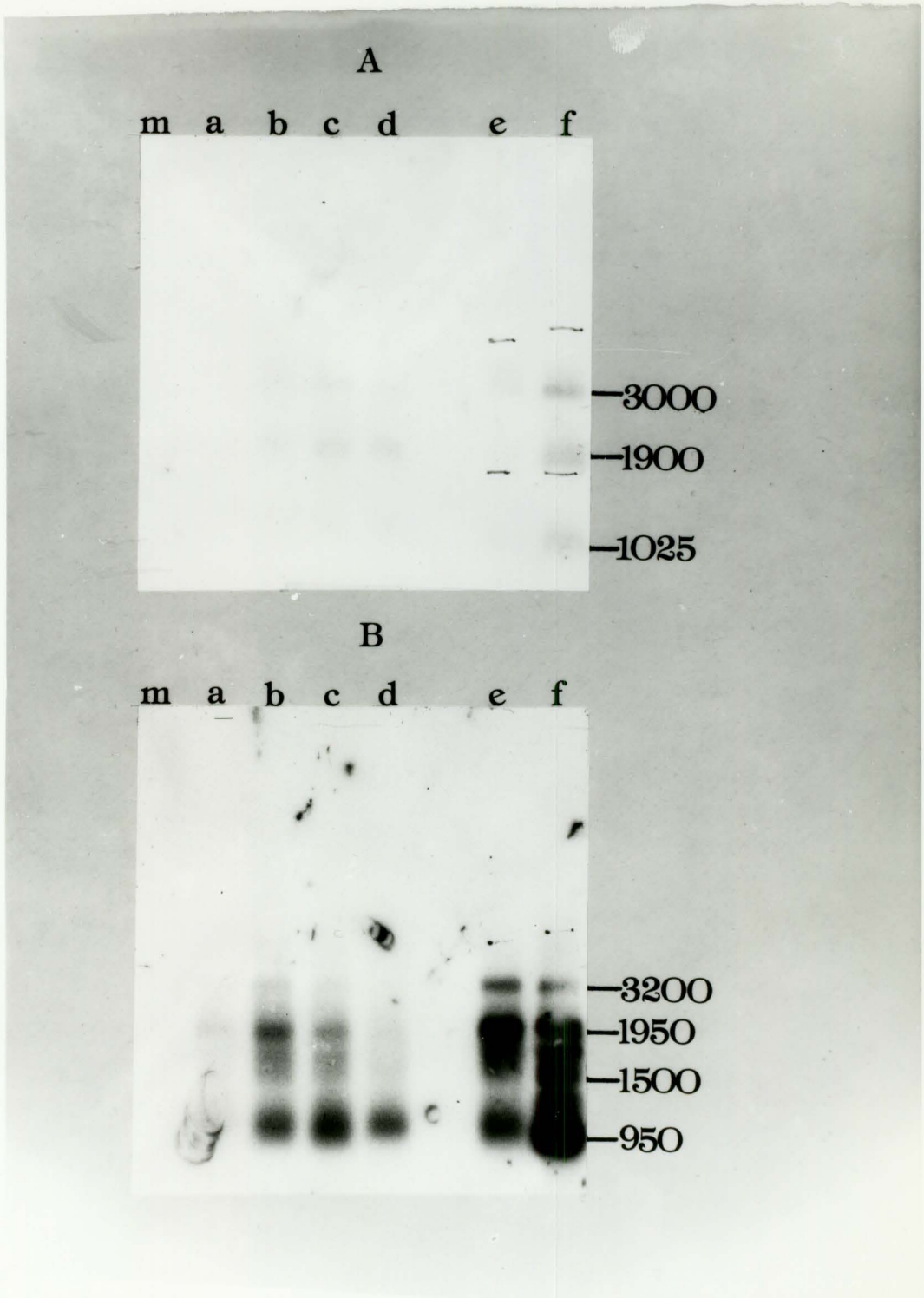

A
m a b c d e f
3000
1900
1025
B
m a b c d e f
3200
1950
1500
950

Figure 10B is an autoradiogram of a Northern Blot of the same mRNA preparations described in figure 9A but with an E4 probe. At 8h pi (lane a) there was expression of three species of mRNA of 950, 1950 and 3000 nucleotides in length. The length of these E4 mRNAs are consistent with the values cited by Raskas et al (1982), as described in the Introduction. At 12h pi (lane b) expression of the three mRNAs increased and a fourth species of 1500 nucleotides was observed (compare lanes a and b). At 18h pi (lane c) all four species were observed but have declined in intensity except for the 950 nucleotide mRNA. This observation is consistent with Raskas et al (1984) who report strong expression of the smallest E4 mRNAs at advanced times in the early phase. At 24h pi only the 950 nucleotide mRNA was strongly expressed (lane d). Expression of E4 mRNA in tsH1 at 34^{o}C at 12h pi resembles that found in CHO-wt at 8h pi (compare lanes b and e), consistent with a delayed onset of transcription.

Thus in tsH1 cells grown at 34^{o}C and infected with wt-Ad 5, it was found that E1A, E1B, E2, E3 and E4 were all transcribed. The lengths of the individual mRNA species from each of the early genes and their time of appearance during the course of early infection are summarized in table 1. From the results in figures 9 and 10 it can be concluded that early gene transcripts synthesized in tsH1 cells were of practically the same length as those from infected CHO-wt or KB cells. The ordered expression of the Ad 5 early regions in tsH1 exhibited a timed pattern similar to that found in CHO-wt but was delayed by about 4 hours. These results are consistent with the ordered expression pattern established by

	8h pi	12h pi	18h pi	24h pi
E1A	1200	1200	1200	1200 800
E1	-	1200	2600 2000 1200 800	2600 2000 1200 800
E2	-	1900	4100 1900	4100 1900
E3	-	3000 1900 1025	3000 1900 1025	3000 1900 1025
E4	3200 1950 950	3200 1950 1500 950	3200 1950 1500 950	950

Table 1: Lengths of mRNA species from each of the early gene regions and their times of appearance, observed from tsH1 cells infected with wt-Ad 5 at 34^{o}C. The values are a summary of data from figures 9 and 10.

Nevins et al (1979).

IV Expression of the Early Regions of Adenovirus 5 in tsH1 Cells at Restrictive and Non-restrictive Temperatures with Wild Type or Host Range Virus

(a) Blocking protein synthesis at 40^{o}C from 1.5 to 5.5h pi in wild type

The approach in the following series of experiments has been to inhibit protein synthesis at critical early times during the infection cycle. In Ad 5 infected tsH1 cells these critical times are from the time of infection to 12-18h pi when most of the early regions are strongly expressed (figure 9). By strategically inhibiting protein synthesis during this period it should be possible to examine the roles of viral and cellular proteins on early viral gene expression.

As outlined in the introduction it has been reported by several investigators that expression of E4 mRNA in cells infected with group I mutant or wt virus was enhanced up to 10 fold when protein synthesis was inhibited with cycloheximide or anisomycin (Nevins, 1981; Persson et al, 1981b; Katze et al, 1981; Tigges and Raskas, 1982). Under the same experimental conditions the effects of these drugs on expression from the other early genes have not been clearly established (Persson et al, 1981a; Katze et al, 1983; Nevins, 1981; Shaw and Ziff, 1982). On this basis E4 expression was chosen as a representative marker of Ad 5 early gene expression.

The effects of protein synthesis inhibition from 1.5 to 5.5h pi on E4 expression were evaluated. TsH1 cells which had been grown in suspension culture at 34^{o}C were infected with either hr-1, a group I

312, another group I mutant, is that the non functional E1A mRNA synthesized during an hr-1 infection serves as a positive marker for viral infection. To inhibit protein synthesis the cells were incubated at 40°C from 1.5 to 5.5h pi. Incubation at 40°C for longer than 4 hours reduces the recovery and viability of the cells. At 5.5h pi the incubation temperature was lowered to 34°C. Poly(A^+) mRNA was isolated from the infected cells at 18h pi and then subjected to Northern Blot analysis as described in methods.

Figure 11A is an autoradiogram of a Northern Blot with an E1 probe. In the wt infected cells at 18h pi (lane a) 900, 1200, 2000 and 2600 nucleotide mRNAs were produced. This evidence is consistent with expression of both E1A and E1B (figure 9B). In the hr-1 infected cells at 18h pi (lane c) the 1200 nucleotide mRNA was observed. The notable absence of the 2600 nucleotide mRNA strongly suggests that E1A and not E1B was expressed . The results show that the tsH1 cells were infected by hr-1 and were capable of transcribing viral genes but that the inhibition of protein synthesis failed to enhance E1B expression to wild type levels (compare lanes a and c).

Figure 11B is an autoradiogram of a Northern Blot using the same mRNA preparations as in figure 11A but with an E4 probe. In the wt infected cells (lane a) there was expression of at least three species of E4 mRNA, namely 950, 1950 and 3200 nucleotide species. In the hr-1 infected cells (lane c) no E4 mRNA was detected. This result shows that E4 transcription was not enhanced by inhibition of protein synthesis from

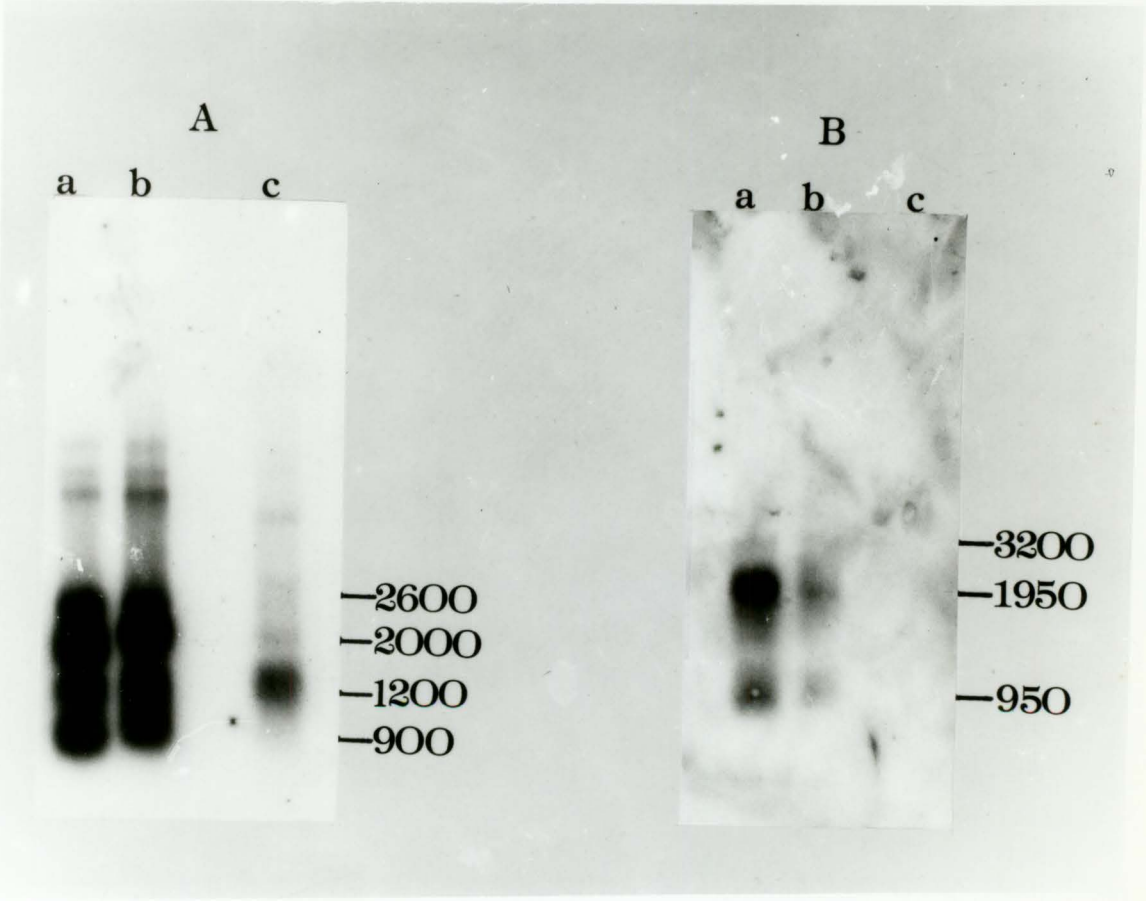

A
a
b
c
2600
2000
1200
900
B
a
b
c
3200
1950
950

Figure 11: Northern Blot of mRNA isolated from tsH1 cells which were infected at 50 PFU/cell with either wt-Ad 5 or hr-1 virus at 34^{o}C. The infected cells were incubated at 40^{o}C from 1.5 to 5.5h pi when the incubation temperature was lowered to 34^{o}C. Poly(A^{+}) mRNA was isolated and analysed by Northern Blot analysis. Lanes a, wt-Ad 5; 18h pi; lane b wt-Ad5, 24h pi; lane c, hr-1 18h pi. Figure A, E1 (XhoIC) probed; B, E4 (Hind III-F) probed.

transcription was not enhanced by inhibition of protein synthesis from 1.5 to 5.5h pi.

Incubation of tsH1 cells at 40°C reduced protein synthesis to about 7% within the first hour and to less than 4% after four hours (figure 6) and there was very little recovery within the first three hours after the return to 34°C (figure 7). Therefore the total time that protein synthesis was 10% or less was approximately 7 hours (1.5 to 8.5h pi). In wild type infected cells it is possible that the low level of translation of the E1A RNA, particularly during the recovery period after 8.5h pi, was sufficient to initiate E4 transcription. The result from the hr-1 infected cells suggests that inhibition of protein synthesis did not remove the necessity of functional E1A protein for the expression of E4 mRNA.

(b) Blocking protein synthesis at 40°C from 8.0 to 12.0h pi in cells infected with wt-Ad 5

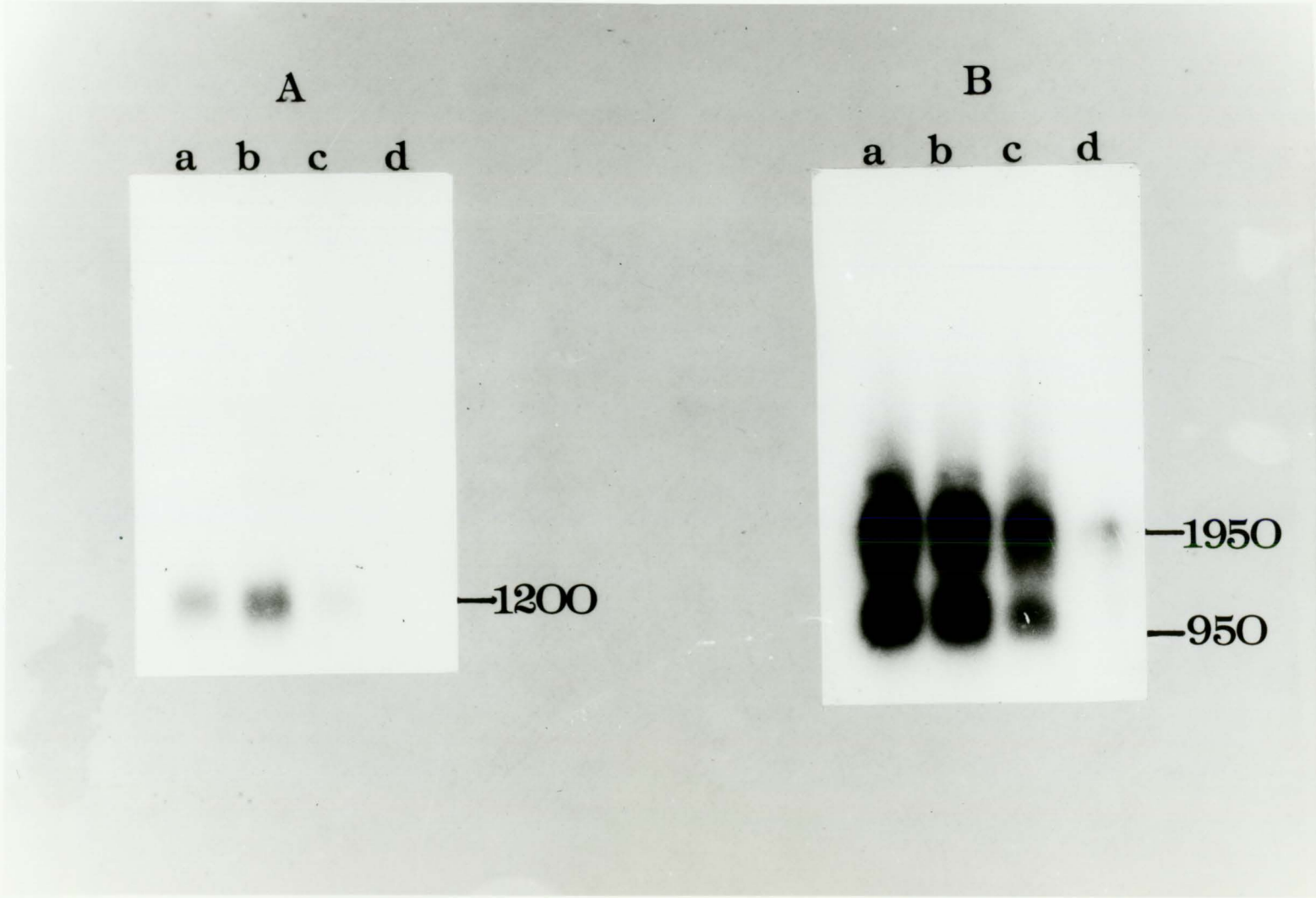
A possible explanation for the previous result in figure 11 is that the E1A requirement can only be avoided if the inhibition of protein synthesis is most stringent during some early critical stage of E4 expression. From the results in figure 10B expression of E4 begins at around 8h pi so that a logical time to inhibit protein synthesis would be from 8 to 12h pi. An added advantage of this time is that it eliminates the possibility of inhibition of E4 transcription by E2 since the small amount of E2 mRNA synthesized between 8 and 12h pi (figure 9C) would probably not be translated in sufficient amounts so as to repress E4 transcription. TsH1 cells, grown in suspension culture at 34°C, were

infected at 50 PFU/cell with wt-Ad 5, and maintained for 8h pi at 34°C when the culture was divided in half. Protein synthesis was inhibited by increasing the incubation temperature to 40°C for one half of the culture. At 12h pi Poly(A^+) mRNA was isolated from the infected cells and subjected to Northern Blot analysis as described in methods.

Figure 12A is an autoradiogram of the Northern Blot with an E1A probe. In tsH1 cells incubated at 34°C (lane c) there was expression of an E1A 1200 nucleotide mRNA. This band did not reproduce on the film but was present on the autoradiograph. This is consistent with previous results (figure 9A). In tsH1 cells incubated at 40°C from 8 to 12h pi (lane d) the expression of E1A mRNA was severely reduced (compare lanes c and d). In the absence of functional E1A protein, E1A transcription is reduced (Nevins, 1981; Lewis and Matthews, 1980). The transcription of sequences continues at 40°C. Expression from infected CHO-wt cells incubated either at 34°C (lane a) or 40°C (lane b) produced large amounts of the E1A 1200 nucleotide mRNA. These results suggest that the reduction of E1A expression in tsH1 cells incubated at 40°C was the result of inhibition of protein synthesis.

Figure 12B is an autoradiogram of a Northern Blot using the same mRNA preparations as in 12A, but with an E4 probe. In CHO-wt cells incubated at either 34°C (lane a) or 40°C (lane b) there was full expression of E4 mRNA, showing that transcription of E4 was normal at the increased temperature in wild type cells. Expression of E4 in tsH1 cells incubated at 34°C was also normal (lane c) but in cells incubated from 8 to 12h pi at 40°C E4 mRNA was severely reduced (lane d). These results,

Figure 12: Northern Blot of mRNA isolated from tsH1 and CHO cells which were infected at 50 PFU/cell at 34°C with wt-Ad 5. At 8h pi the two separate cultures were split in half. Half of the CHO and tsH1 cells were incubated at 34°C while the remaining halves were incubated at 40°C. At 12h pi the cells were harvested and poly(A^+) mRNA was isolated and analysed by Northern Blot analysis. Lane a, CHO at 34°C; b, CHO at 40°C from 8-12h pi; c, tsH1 at 34°C; d, tsH1 at 40°C from 8-12h pi. Figure A, E1A (HpaIE) probed; B, E4 (Hind III-F) probed.


A
a b c d
1200
B
a b c d
1950
950

which show that E4 transcription was normal at 40°C in CHO-wt but reduced in tsH1, indicate that expression of E4 mRNA is dependent on protein synthesis.

(c) Blocking protein synthesis at 38.5°C from 4.0 to 12.0h pi in cells infected with wt-Ad 5 or hr-1

If a destabilizing or repressor protein had a long half life, the four hour block at 40°C, in part b above, may not have been long enough to remove the requirement for E1A proteins in E4 expression. It was of interest to extend the period of inhibition in the tsH1 cells but incubation at 40°C for longer than 4 hours results in decreased cell viability (refer to section IIa). A convenient incubation temperature was 38.5°C. At this temperature the decline of protein synthesis is slower than at 40°C but inhibition is greater than 90% for the first six hours and the level at 8 hours is about 97% (figure 6). The block can be held for eight hours without reducing cell viability. The level of transcription after eight hours is about 32% (figure 8). By increasing the temperature to 38.5°C at 4h pi protein synthesis was inhibited before any early region mRNA can be detected (figure 9).

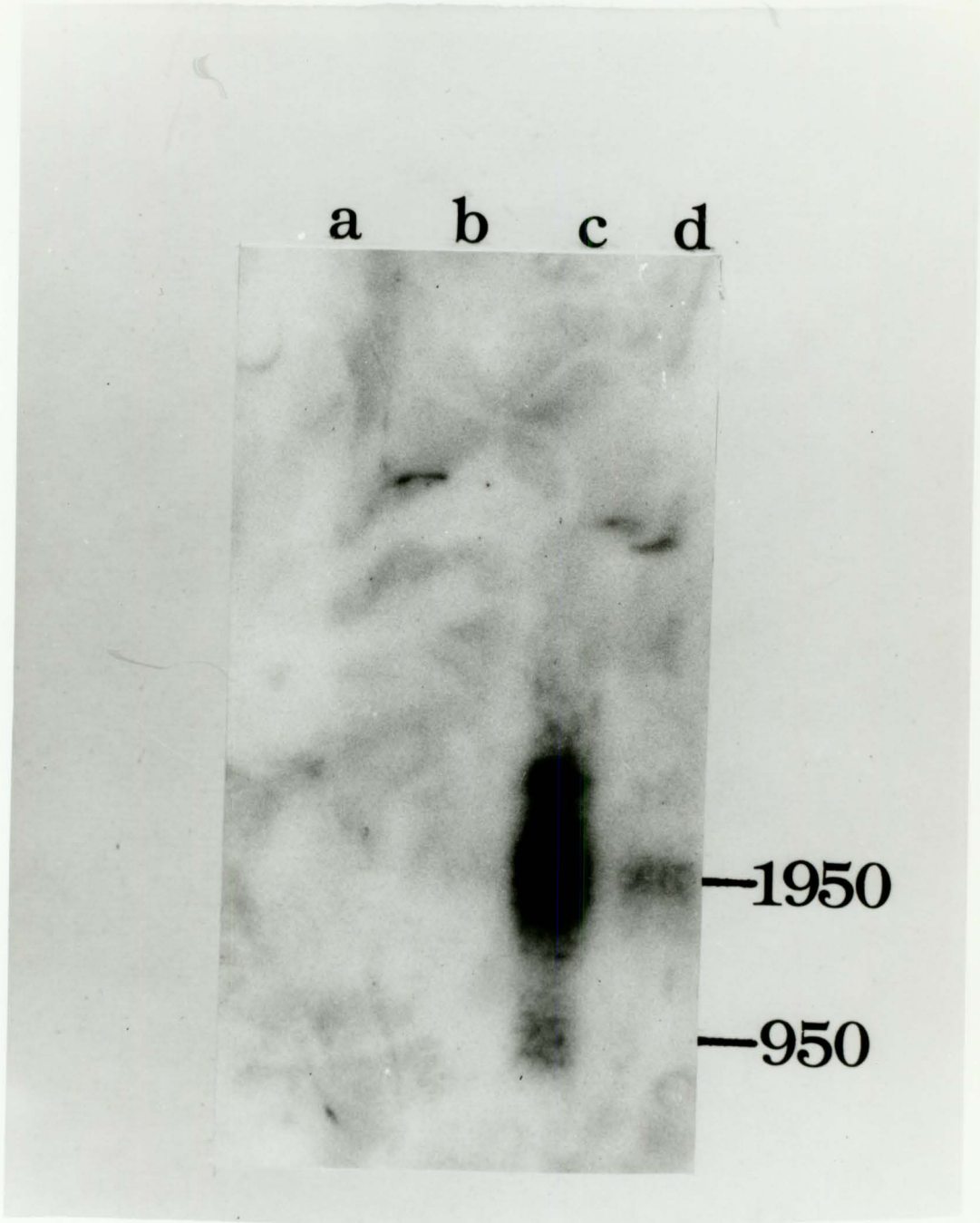
A suspension culture of tsH1 cells grown at 34°C was split in two; one half was infected at 50 PFU/cell with hr-1 and the other half with wt-Ad 5. The infected cells were incubated at 34°C from 0 to 4.0h pi when the cultures were split again. Half of the hr-1 infected cells and half of the wt-Ad 5 infected cells were maintained at 34°C until 12h pi. The remaining halves of these cultures were incubated at 38.5°C from 4.0 to 12.0h pi. Poly(A^+) mRNA was isolated from all of the

infected cells at 12h pi and analysed by Northern Blot analysis.

Figure 13 is an autoradiogram of the Northern Blot using an E4 probe. In wt infected cells incubated at 34^{o}C the normal pattern of E4 expression was observed (lane c). In wt infected cells incubated at 38.5^{o}C (lane d) the amount of the E4 expression was severely reduced (compare lanes c and d). This result is consistent with the previous finding (figure 12) that E4 expression is reduced during protein synthesis inhibition in a wt infection. In hr-1 infected cells incubated at 34^{o}C there was no E4 expression (lane a). In hr-1 infected cells incubated at 38.5^{o}C no expression of E4 mRNA was observed (lane b). This result agrees with the finding in figure 11. These results have been repeated under identical experimental conditions. In each experiment E4 mRNA could not be detected from the hr-1 infected cells at either temperature. These results suggest that after prolonged inhibition of protein synthesis the requirement for functional E1A protein in the expression of E4 was not removed in either hr-1 or wt-Ad 5 infected cells.

The experiments described above was a measure of the total accumulated amount of E4 mRNA present in the cytoplasmm of the infected cells. To measure the rate of E4 transcription under the same experimental conditions, tsH1 cells were infected with either hr-1 or wt-Ad 5 as described above except that at 11.75h pi the cells were incubated, at 34^{o}C or 38.5^{o}C, in the presence of ^{3}H-Uridine for 15 minutes. At 12h pi total labelled cytoplasmic RNA was isolated from the infected cells and hybridized to unlabelled E4 probe DNA which had been

Figure 13: Northern Blot of mRNA isolated from tsH1 cells infected at 50 PFU/cell with either hr-1 (lanes a and b) or wt-Ad 5 (lanes c and d) at 34°C. At 4h pi half of the tsH1 cells were incubated at 38.5°C and the other half was maintained at 34°C. At 12h pi the cells were harvested and poly(A^+) mRNA isolated and analysed by Northern Blot. Lane a, hr-1 infected tsH1 cells at 34°C; lane b, hr-1 infected tsH1 cells incubated at 38.5°C from 4-12h pi; lane c, Ad 5 infected tsH1 cells at 34°C; lane d, Ad 5 infected tsH1 cells incubated at 38.5°C. E4 (Hind III-F) probed.


a
b
c
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1950
950

bound to nitrocellulose filters. The total number of counts bound to the filter was considered a measure of the rate of E4 transcription.

Table 2 shows the results of a typical hybridization experiment. These results indicate that the rate of E4 transcription was highest in wt infected tsH1 cells incubated at 34°C (2847 CPM 100%). The rate was reduced substantially (19%) when wt-infected tsH1 cells were incubated at 38.5°C from 4 to 12h pi. In the hr-1 infected cells incubated at 34°C there was little E4 transcription (18%) while those incubated at 38.5°C had the lowest rate (8%). The rates of E4 synthesis in cells incubated at 38.5°C were reduced to levels lower than can be explained by a generalized reduction of transcription resulting from the prolonged period of protein synthesis inhibition. After 8 hours at 38.5°C total transcription was reduced to about 32% of the pre-inhibition level (figure 8). These results suggest that the reduced rate of E4 transcription in these cells was directly attributable to protein synthesis inhibition and not the reduced rate of transcription in tsH1 cells at 38.5°C. The reduced rate of E4 transcription in hr-1 infected cells at 34°C was the result of the lack of functional protein translated from the 13 S mRNA. Hr-1 E1A mRNA does not translate into functional protein. Repeats of this experiment have produced qualitatively similar results and are consistent with results from the Northern Blots described in figure 13. These results strongly suggest that the requirement for E1A protein in the expression of E4 mRNA can not be removed by simply inhibiting protein synthesis.

	Hr-1 (34°C)	Hr-1 (34°C/38.5°C)	wt (34°C)	wt (34°C/38.5°C)
Counts/Min (CPM)	513	229	2847	542
% wt	18	8	100	19

Table 2: Rate of Incorporation of ^{3}H-Uridine by Infected tshl Cells.

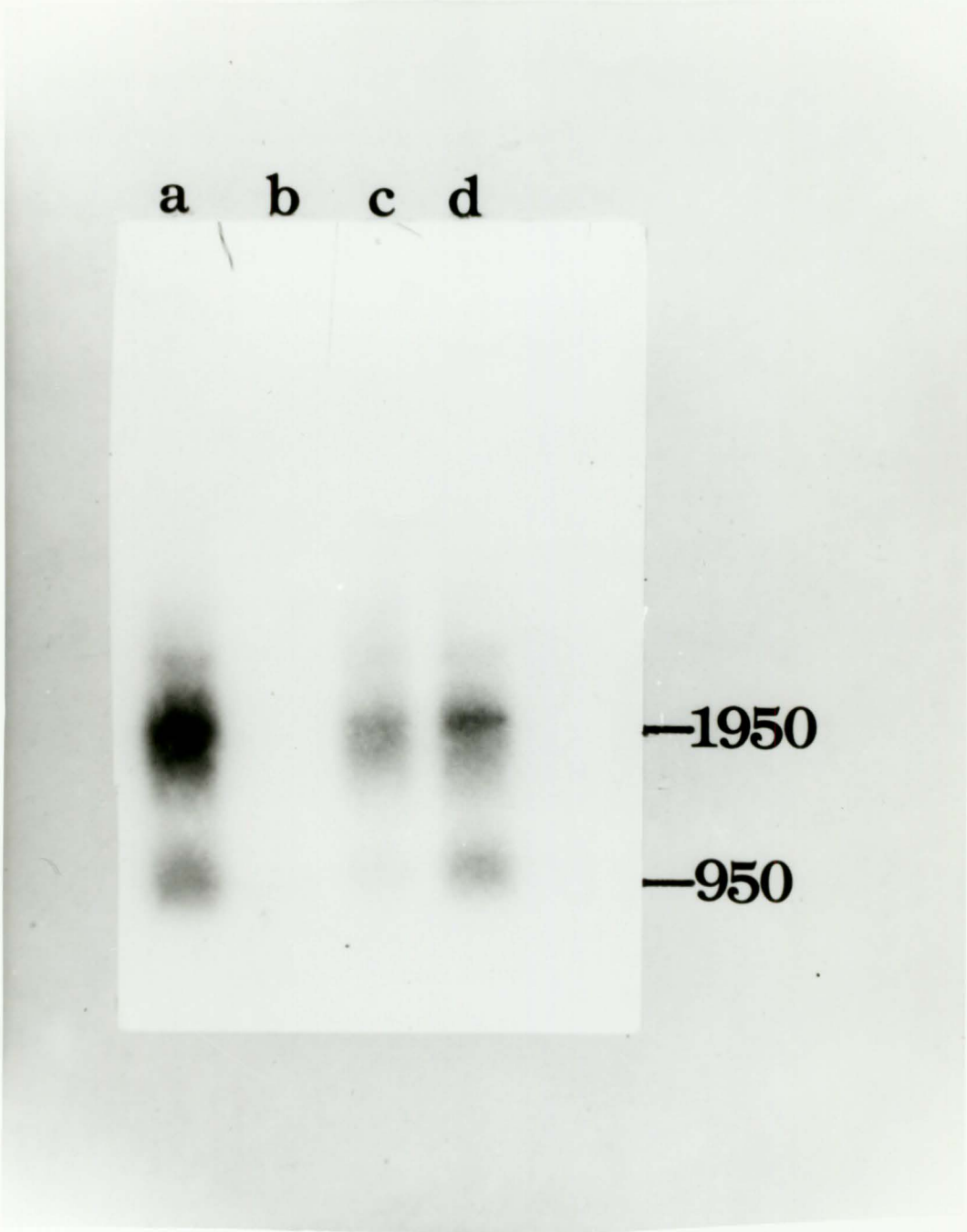
TsHl cells were infected at 34°C with wt or hr-1 as described in figure 13. At 4h pi the incubation temperature was increased to 38.5°C in half of the tsHl cells. At 11.75h pi approximately 1.0 x 10^{8} cells were removed and incubated in the presence of 200 uCi of ^{3}H-Uridine for 15 minutes. Total cytoplasmic RNA was extracted and hybridized to filters bearing plasmid DNA containing early region 4 (Hind III-F fragment). The total number of counts bound to the filter was considered a measure of the rate of E4 transcription.

(d) Blocking protein synthesis at 40°C from four hours before infection until time of infection.

It has been reported that inhibition of protein synthesis with drugs prior to infection with hr-1 or wt-Ad 5 results in the enhancement of E4 transcription (Nevins, 1981; Persson et al, 1981b; Katze et al, 1981; Shaw and Ziff, 1982). It was of interest therefore to examine E4 transcription from infected tsH1 cells which had been incubated at 40°C prior to infection. A suspension culture of tsH1 cells was incubated at 40°C for four hours. The incubation temperature was lowered to 34°C and the culture was split in two with one half being infected at 50 PFU/cell with hr-1 and the other half with wt-Ad 5. The infected cells were incubated at 34°C until 12h pi, when poly(A^+) mRNA was isolated and analysed by Northern Blot analysis.

Figure 14 is a Northern Blot of the isolated mRNA hybridized to an E4 probe. In tsH1 cells infected with wt-Ad 5 (lane a) there was normal expression of E4 mRNA. This result suggests that the prior incubation of protein synthesis did not alter E4 transcription by 12h pi. It is likely that recovery of protein synthesis, during the incubation at 34°C, was sufficient to allow E1A protein synthesis resulting in E4 transcription. In hr-1 infected tsH1 cells there was no expression of E4 mRNA (lane b). This result suggests that prior inhibition of protein synthesis does not replace the requirement for functional E1A protein. Lanes c and d are controls not incubated at 40°C prior to infection.

Figure 14: Northern Blot of mRNA isolated from tsH1 cells which were incubated at 40°C from 4 hours before infection to 0h pi when the temperature was lowered to 34°C and the cells were infected at 50 PFU/cell with either hr-1 or wt-Ad 5. The cells were incubated at 34°C until harvested at 12h pi when poly(A^+) mRNA was isolated and analysed by Northern Blot. Lane a, wt infected; lane b, hr-1 infected; lane c, tsH1 cell not incubated at 40°C before infection with wt at 15h pi; lane d, tsH1 cells not incubated at 40°C before infection with wt 12h pi. E4 (Hind III-F) probed.


a
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DISCUSSION

I Overview

In this study I have re-examined the effects of protein synthesis inhibition on Adenovirus 5 early gene expression without the use of cycloheximide, anisomycin or other drug inhibitors. At various times during the course of wt or hr-1 infection of tsH1 cells, protein synthesis was inhibited and E4 expression examined. In every case the results strongly suggested that efficient E4 expression was dependent on the presence of functional E1A protein and that this requirement could not be replaced by simply inhibiting protein synthesis.

There are two major limitations inherent in this study. First, the evidence obtained from protein synthesis inhibition studies such as this one, or others, can not provide direct insights into the actual mechanism by which E1A exerts its regulatory effect. Nevertheless, objective conclusions based on the data are valuable tools in either refuting or supporting a given model of E1A regulation. Second, is the question of whether protein synthesis inhibition in the tsH1 cell system is sufficiently stringent to permit definitive conclusions concerning the E1A regulatory mechanism. The residual amounts of protein synthesis, in tsH1 cells incubated at restrictive temperatures, are in the 5% range. It is not possible to definitively rule out that transcriptionally important proteins are synthesized in this residual amount. This will remain an uncertainty. The real value of this study is in relation to the three general models which have been proposed to explain the E1A

regulatory mechanism. The implication that my results have on these models are discussed below.

II E1A Inactivation of a Cellular Factor which Destablizes Viral mRNA

In hr-1 infected Hela cells, Katze et al (1981) report wild type levels of E4 transcription but reduced accumulation of E4 mRNA in the cytoplasm of the infected cells. On this basis a model was proposed which claimed that the E1A regulatory protein enhances the accumulation of E4 mRNA by inactivating a cellular factor which destabilizes the viral mRNA before translation. This model implicitly assumes that the destabilizing protein has a relatively short half life, is turned over quickly, and would be continually synthesized during the early stages of viral infection. It should be noted that such a cellular protein has, as yet, not been identified (Katze et al, 1983). The key prediction of this model is that E4 mRNA, because it has been shown to be most sensitive to E1A control (Persson et al, 1981a,b; Katze et al, 1981) would accumulate in the cytoplams of wt or hr-1 infected cells if protein synthesis was inhibited during early stages of infection.

Results from this research study do not support the proposed model. In wt infections of tsH1 cells, incubated at 34^{o}C, abundant E4 mRNA was discovered. When protein synthesis was inhibited during critical early stages of infection there was very little E4 mRNA detected (figures 12 and 13) when, according to the model, wild type levels should have been observed. Similarly, in tsH1 cells infected with hr-1 and incubated at restrictive temperatures E4 mRNA should have been abundant

but little or none was observed (figure 13). In summary, little or no E4 mRNA was observed in either wt or hr-1 infected tsH1 cells when protein synthesis was stringently inhibited.

The results presented in this study would be consistent with the proposed model if the destabilizing protein had a long half life. In this way destabilizing protein synthesized before the block would remain active and prevent accumulation of E4 mRNA during inhibition of protein synthesis. This would predict that in hr-1 infected tsH1 cells E4 mRNA would not accumulate either at restrictive or non-restrictive temperatures since under each circumstance no functional E1A protein is synthesized. In wt infection at 34^{o}C E4 mRNA would accumulate because E1A protein removes the destabilizer but at restrictive temperatures would not because E1A synthesis is inhibited. The results presented in this study are consistent with these predictions but any model which proposes that the destabilizing protein has a long half life would be in contradiction with the original basis for the model. It therefore seems unlikely that the E1A protein regulates E4 expression by inactivating either a short or long lived destabilizing protein.

III E1A Inactivates a Cellular Factor which Represses Transcription from other Early Viral Promoters

In hr-1 and dl 312 infected Hela cells Nevins (1981) reports, in contradiction with Katze et al (1981), that the rate of transcription of all the early genes was severely reduced compared to wild type. Furthermore transcription of the early genes, particularly E4, could be

detected in mutant infected cells if protein synthesis had been inhibited with cycloheximide prior to infection. On this basis Nevins proposed that the role of the E1A gene product in the activation of early viral transcription is to inactivate a cellular factor that prevents transcription from the other early viral products. This model, as did that of Katze et al (1981), assumed that the cellular repressor was unstable and would be continually synthesized during the early stages of viral infection. It should be noted that such a cellular repressor has not been isolated (Imperiale et al, 1983). The key prediction of this model is that in hr-1 or wt infected cells where protein synthesis has been inhibited, early viral transcription particularly that of E4 should be observed at wild type levels (Nevins, 1981).

In this study total accumulation of cytoplasmic poly(A^+) mRNA was measured. It was assumed that the pattern of mRNA accumulation in the cytoplasm of the infected cells was an accurate reflection of the rates of viral transcription in the nucleus. Nevins measured the actual rate of nuclear mRNA synthesis. This assumption was supported by Shaw and Ziff (1982) and Cross and Darnell (1983) who have demonstrated equivalent patterns of early viral transcription by examining accumulated cytoplasmic mRNA by Northern Blot analysis and by quantifying rates of mRNA synthesis in the nucleus by pulse labelling. On this basis it was concluded that in hr-1 or wt infected tsH1 cells, incubated at the restrictive temperatures during early infection there was no evidence suggestive of wild type levels of E4 transcription. This result is inconsistent with the Nevins model. In support of this finding, as

described in the introduction, there have been at least two reports which indicate that E4 transcription was not enhanced by protein synthesis inhibition with cycloheximide prior to infection (Cross and Darnell, 1983; Gaynor and Berk, 1983).

The results presented in this study would be consistent with the Nevins model if the putative cellular repressor had a long half life. As was the case in the Katze proposal, this would contradict the evidence originally cited by Nevins as support for the repressor model. On this basis it is difficult to conclude that early viral transcription is activated solely by the inactivation of a cellular protein repressor.

IV A Positive Effects Model of Early Viral Gene Regulation

If the regulation of early viral gene expression is not mediated through a host cell repressor, as results in this study suggest, then how does E1A exert its effect? Recently evidence has begun to accumulate which indicates that the E1A regulatory protein acts positively to enhance viral transcription (Gaynor and Berk, 1983; Gaynor et al, 1984; Ferguson et al, 1984; Treisman et al, 1983; Green et al, 1983; Imperiale et al, 1983; Feldman et al, 1982). It has been previously observed that in the absence of functional E1A gene products there remained a slow increase in the rate of transcription from the early viral promoters (Nevins, 1981; Jones and Shenk, 1979b). Furthermore when Hela cells were infected with E1A mutants a reduced titre of mutant progeny virus was obtained if the infected cells were incubated for extended periods (Jones and Shenk, 1979b; Katze et al, 1983). These observations led Gaynor and

Berk (1983) to reinvestigate the phenomenon of E1A independent activation of the early viral transcription units.

Gaynor and Berk (1983) observed that the rate of transcription of each of the early genes in dl 312 infected Hela cells was increased at 36h pi compared to 6h pi. On this basis they asked if the E1A independent activation of early transcription was the result of the slow activation of another factor, cellular or viral that could substitute for the E1A protein. Their approach was to coinfect two E1A mutants; virus 15606 which expresses wt E3 mRNA, and dl 312 which expresses unique E3 mRNA species. When coinfected and harvested at 30h pi relatively equal amounts of E3 mRNA was observed from both viruses. When Hela cells were infected with dl 312 and at 24h pi superinfected with 15606 then harvested 6 hours later, only E3 mRNA from d1312 was observed. These results were interpreted as being inconsistent with a model which proposed that E1A independent early transcription occurs because of the delayed induction of a viral or cellular trans-acting function (Gaynor and Berk, 1983).

On this basis they asked if the function of E1A is to accelerate a process required for efficient early transcription. Hela cells were infected with dl 312 and superinfected with wt Ad 5 at 0, 8, 16 and 24h pi. The cells were harvested 8 hours after the superinfection with wt-Ad 5 and assayed for E3 mRNA. Viral E3 mRNa corresponding to dl 312 was first observed at 8 hours after superinfection suggesting that the transcription inducing activity of the E1A protein was potentiated on the d1312 template. To explain these results Gaynor and Berk (1983) proposed

that the function of the E1A regulatory protein is to catalyse the assembly of stable transcriptional complexes without directly interacting with specific viral sequences in the proximity of transcription start sites.

Consistent evidence in support of the transcriptional complex model has emerged from a number of investigators. Ferguson et al (1984) tested the functional activity of E1A protein synthesized in E. coli. To do this the ability of the E1A protein to activate transcription from the E3 promoter was examined in a Xenopus oocyte microinjection assay. When a plasmid vector (pKCAT23), which carried an E3 promoter fused to an intact chloramphenicol acetyltransferase (CAT) gene, was injected into a Xenopus oocyte there was no expression of CAT mRNA but when functional E1A protein was injected with the plasmid abundant CAT expression was observed. In a control experiment, truncated non-functional E1A protein was unable to induce CAT expression when injected in place of the functional E1A protein. From these results Ferguson et al (1984) concluded that the E1A 13S mRNA protein product acted positively to regulate expression from the E3 promoter.

Imperiale et al (1983) reported that transfection of plasmid (pE2), containing only an intact copy of the E2 gene, into 293 cells yielded high levels of the 72 kd E2 gene product. When transfected into mouse L cells (an untransformed cell line) there was very little expression of the 72 kd protein. Interpretation of these results suggested that the presence of the E1A protein allows for the increased expression of the transfected E2 gene. In similar transfection

experiments using a plasmid containing the immediate early gene product (Pr IE) of pseudorabies virus (a Herpesvirus) it was observed that Pr IE was more efficient than was the E1A plasmid in the initiation of E2 expression. This result was consistent with a previous report (Feldman et al, 1982). The significance of this finding is not yet clear but it suggests that these two regulatory proteins mediate their actions by similar mechanisms.

Treisman et al (1983), to test whether the E1A regulatory protein might be capable of activating globin gene transcription, transfected and B-globin genes into 293 cells. They first transfected 293 cells with plasmids containing both the α- and B-globin genes with or without an intact enhancer sequence. B-globin gene transcription was readily detected in the absence of an enhancer sequence and furthermore transcription was not increased by the enhancer in these cells. Identical results were reported for α-globin in gene transcription. When globin genes are transfected into Hela cells no transcription is detected unless the genes are linked to a SV40 enhancer sequence. As additional evidence plasmids containing the SV40 promoter fused to the α-globin genes were constructed. The SV40 enhancer was deleted to varying degrees in each of the plasmids. In Hela cells plasmid with an intact enhancer produced large amounts of the SV40-α-globin fusion product whereas plasmids with less than intact enhancers exhibited dramatically reduced promoter activity. In 293 cells transcription was initiated efficiently on the normal mRNA cap sites regardless of whether the SV40 promoter contained an intact enhancer sequence. In 293 cells it

was discovered that a promoter sequence from the cap site to -21 bp was required for the correct and efficient initiation of transcription. On the basis of these results Treisman et al (1983) concluded that the E1A regulatory protein was capable of relieving the enhancer requirement of both globin genes and the SV40 early promoter by some interaction in critical regions of the promoters.

Green et al (1983) considered that the inactivity of a cloned B-globin gene in Hela cells, in the absence of a viral enhancer was mechanistically similar to the transcriptional inactivity of adenovirus genes, at early times during infection, in the absence of functional E1A gene products. To prove specifically that a product of E1A was responsible for B-globin transcriptional stimulation Green et al carried out co-transfection experiments in which Hela cells were simultaneously transfected with separate plasmids containing the B-globin gene and the Ad 5 E1A gene. As expected B-globin gene plasmid lacking an enhancer does not produce detectable levels of B-globin mRNA. Co-transfection of the B-globin plasmid with the E1A gene plasmid resulted in readily detectable level of steady state B-globin mRNA. In a similar experiment with plasmid containing the immediate early gene of pseudorabies (Pr IE), instead of E1A, readily detectable levels of B-globin mRNa were observed. Consistent with Imperiale et al (1983) the Pr IE gene product was slightly more efficient than E1A in its ability to induce transcription from the B-globin promoter. In these experiments it was observed that deletions in the 5' leader sequence between the cap site and -36 bp severly affected promoter activity. On the basis of these results Green

et al concluded that viral immediate early gene products circumvent the requirements for a linked enhancer sequence. The fact that both E1A and Pr IE appear to be associated with the promoter regions of heterologous genes would suggested that these regulatory proteins are more likely to be positive effectors of transcription rather than inactivators of non-specific cellular repressors of transcription (Green et al, 1983).

Recently Gaynor et al (1984) constructed a recombinant of Ad 5 to test if the other heterologous genes could be activated by the E1A regulatory protein. The E1A gene in this recombinant had been deleted from nucleotides 454 to 1339 and the rat preproinsulin gene (RI-1) was inserted into the E1A deletion. Hela cells were infected with the recombinant and assayed at 36h pi for RI-1 mRNA. Consistent with the previous findings no RI-1 was detected from the infected Hela cells but when the recombinant was used to infect 293 cells there was abundant RI-1 mRNA at 36h pi. Transfection experiments using plasmid RI-1 without any viral sequences only expressed RI-1 mRNA when transfected into 293 cells and not Hela cells, consistent with previous findings. These results demonstrate that the E1A protein can induce expression of a non-viral gene when it is introduced into mammalian cells by infection or transfection (Gaynor et al, 1984).

What has been clearly demonstrated in the studies described above is that the E1A regulatory protein has a transcription inducing activity. This activity appears to be associated with promoter sequences in the 5' leader regions of a variety of eukaryotic inducible genes (Ferguson et al, 1984; Imperiale et al, 1983; Treisman et al, 1983; Green et al, 1983;

Gaynor et al, 1984). The transcription inducing activity is not unique to E1A as it has been shown that the IE gene from pseudorabies has very similar or identical transcriptional inducing activity. Additional support for the transcriptional complex model is reported by Davison et al (1983) who have demonstrated that transcription from the Ad 2 major late promoter, in a partially purified in vitro transcription complexes consisting of the promoter region and at least two other proteins. These factors are bound tightly to the promoter DNA to form a complex that was the substrate for transcription initiation by RNA polymerase II. Taken together these results argue strongly for a positive effects model.

V Summary and Future Approaches

The purpose of this research project has been to examine the regulation of early adenoviral gene expression. As this study comes to a conclusion it appears clear that E1A regulation is not mediated through either host cell destabilizers (Katze et al, 1983) or repressors (Nevins, 1981). There are at least three lines of evidence supporting this conclusion. First, results presented in this study have shown that inhibition of protein synthesis does not enhance early viral gene expression. This result is supported by other investigators (Gaynor and Berk, 1983; Cross and Darnell, 1983). Second, recent evidence from a number of investigators demonstrating that the E1A regulatory protein activates transcription by associating with promoter regions of adenovirus early genes and other eukaryotic inducible genes (Ferguson et al, 1984; Imperiale et al, 1983; Treisman et al, 1983; Green et al, 1983;

Gaynor et al, 1984). Third, by virtue of the wide variety of cell lines in which E1A can induce transcription aruges against the possibility of a generalized repressor protein (Green et al, 1983). Despite this compelling evidence however the host cell destabilizer/repressor models remain a formal, although unlikely possibility.

The transcriptional complex model of E1A regulation requires further development. First, it remains to be conclusively demonstrated that the E1A regulatory protein actually associates with the RNA polymerase II transcription complex. Second, if the E1A regulatory protein is part of the complex then how does its presence catalyse transcription from the other early genes? A possible approach in answering these questions has been developed by Parker and Topal (1984a and b). These investigators have isolated Drosophila regulatory proteins in tight complex with RNA polymerase II in the promoter regions of two Drosphilia inducible genes. Application of the experimental approach developed by Parker and Topol would probably be of value in the adenovirus system.

The relevance of such future studies is clear. By understanding the mechanisms which govern adenoviral early gene expression valuable insights into eukaryotic gene expression will be gained.

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