AN ADENOVIRUS TYPE 5 RECOMBINANT VECTOR ENCODING THE HSV-1 PROTEIN, ICP4

STUDIES ON THE ROLE OF THE HERPES SIMPLEX VIRUS ICP4 PROTEIN IN ADENOVIRUS GENE EXPRESSION

By

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ABSTRACT

Many viral transcriptional activators have been shown to activate genes of heterologous systems. To assess the ability of the herpes simplex virus ICP4 trans-activating protein to complement an adenovirus mutant lacking its own trans-activator, the E1a protein, I constructed an adenovirus type 5 vector containing a temperature sensitive ICP4 gene, under control of its own promoter, within the E1 region of the genome. The recombinant virus expresses ICP4 in human cells which are permissive (293) or nonpermissive (KB and R970-5) for E1a$^-$ viral replication, and at levels which approximate those obtained in herpes simplex infection. The adenovirus encoded protein is functional in that it complements an ICP4 deletion mutant of herpes simplex virus, however it is incapable of complementing adenovirus E1a$^-$ mutants for viral growth or DNA replication. At the level of activation of gene expression, ICP4 stimulates the expression of the adenovirus E2a gene but not that of other early genes. My results indicate that ICP4 does not possess all of the functions of the E1a proteins and, furthermore, that adenovirus early genes differ in their susceptibility to heterologous trans-activators.

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LIST OF ABBREVIATIONS

Ad	adenovirus
bp	base pairs
Ci	Curie
Da	Dalton
dATP	deoxy Adenosine-triphosphate
dCTP	deoxy Cytidine-triphosphate
dNTP	deoxy Nucleotide-triphosphate
DNA	deoxyribonucleic acid
E	early
EDTA	ethylene diamine tetraacetic acid
Hepes	N-2-hydroxyethylpiperazine-N'-2-ethane sulfonic acid
HIV	human immunodeficiency virus
HSV	herpes simplex virus
ICP	infected cell polypeptide
IE	immediate early
k	kilo (thousand)
L	litre
m	milli (thousanth)
M	molar
mRNA	messenger ribonucleic acid
moi	multiplicity of infection
MW	molecular weight
PBS	phosphate buffered saline
pfu	plaque forming unit
rpm	revolutions per minute
RIPA	radioimmunoprecipitation assay (buffer)
RT	room temperature
SDS	sodium duodecyl sulfate
TK	thymidine kinase
ts	temperature sensitive
Tris	Tris(hydroxymethyl)aminomethane
u	micro (millionth)
V_{MW}	viral molecular weight
w/v	weight/volume
wt	wild type
x g	centripetal force relative to gravity

I would like to dedicate this thesis to all of my family for offering support and strength throughout this and many other endeavors.

INTRODUCTION

Regulation of Cell Function

Unicellular and multicellular organisms have a requirement for a coordinated regulation of differentially expressed genes. Some gene products are needed on a continuous basis and therefore are expressed throughout the life cycle of the cell, while expression of others is tightly regulated to match cellular needs at specific times. The level of induction of cellular genes may be a response to external stimuli, such as hormones, or to internal controls dependent on the physiological state of the cell. The mechanism whereby protein levels are adjusted may involve processes affecting the state of the protein itself, e.g., by changing the activity or half-life of the protein. Alternatively, the regulation may be through mechanisms affecting the transcript encoding the protein, e.g., by altering the half-life or the rate of transcription of the message. Other levels of control may involve compartmentalization of specific components including messages, activators/inhibitors of protein synthesis, or the proteins themselves.

Mechanisms of regulation of gene expression may be studied using viral systems. Viral life cycles offer

relatively simple models of gene regulation which are usually dependant on machinery present in the host cell. Therefore clues to the mechanisms involved in the regulation of cellular gene expression may be derived from studies of viral transcriptional regulation within infected cells. In addition, genes may be easily altered by introducing mutations into specific regions of the promoter or coding region. Also, variations in the multiplicity of infection allow the copy number of templates per cell to be controlled, and allow the introduction of the virus into the cells at specific times during the cell cycle.

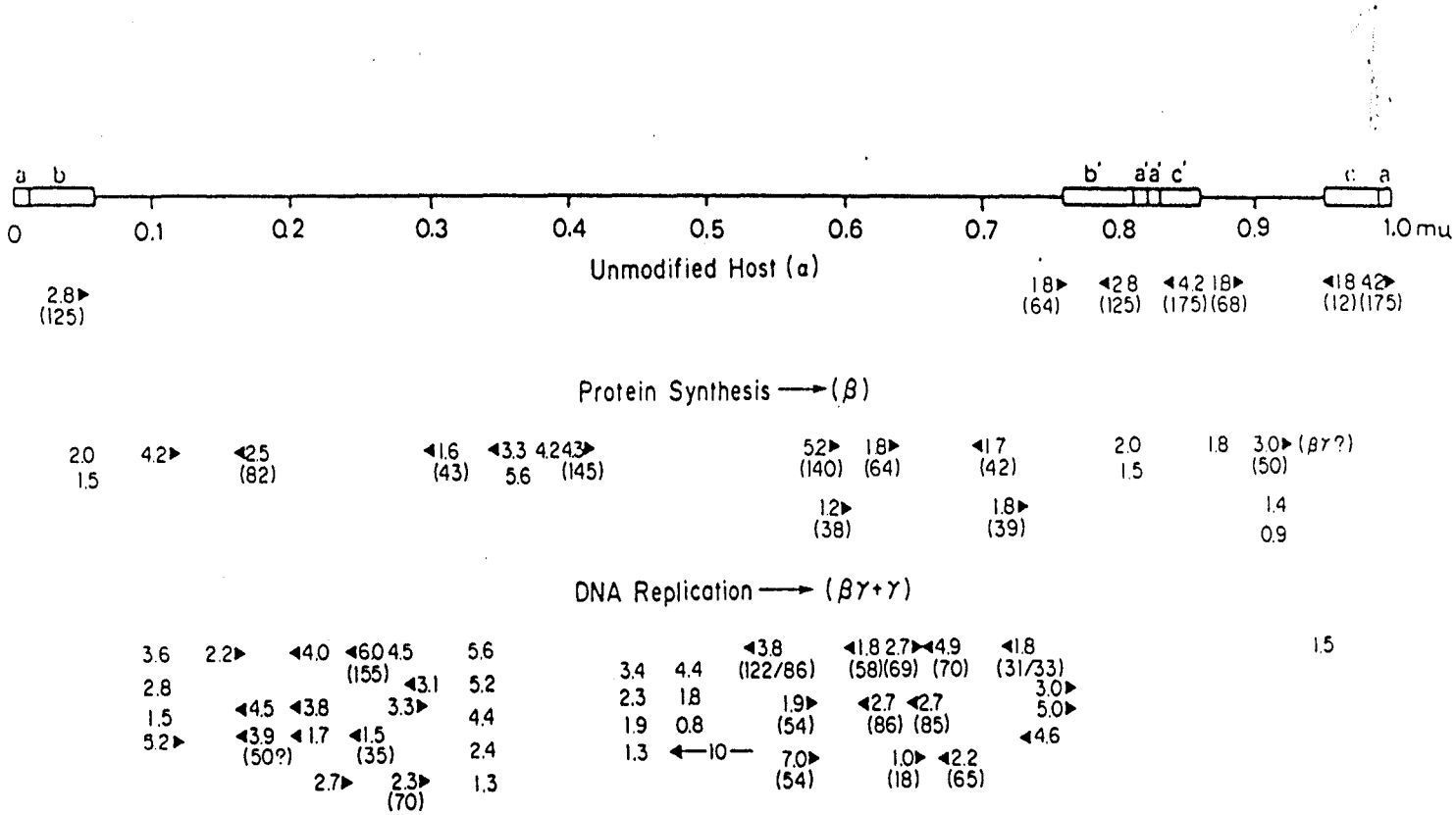
Herpes Simplex Virus

Six herpes viruses have been isolated from humans to date: herpes simplex virus type 1 and type 2, Varicella-zoster virus, Epstein-Barr virus, cytomegalovirus and human B lymphotropic virus (Spear and Roizman, 1981; Roizman and Batterson, 1985; Salahvddin et al., 1986). Herpes simplex virus type 1 (HSV-1), belonging to the family alphaherpesviridae, has a wide host range in both animals and cultured cells. Infection with this virus usually manifests as cutaneous lesions; however more rarely a systemic infection may lead to encephalitis (Nahmias and Roizman, 1973). HSV-1 primarily infects epithelial cells; infection of

superficial nerve endings may result in the virus remaining in a latent state within nerve ganglia. Virus may be periodically induced to reactivate from the dormant state, possibly as a result of immunoincompetence of the host.

The HSV-1 virion consists of a double stranded linear DNA genome enclosed in an icosahedrally symmetric capsid and is enveloped in a trilaminar membrane which appears to be derived from the nuclear membrane of the infected cell (see Roizman and Furlong, 1974; Roizman and Batterson, 1985 for review). The capsid consists of 162 pentameric and hexameric protein capsomeres. Between the capsid and the outer envelope is an asymmetrically distributed electron-dense material referred to as the tegument (see Roizman and Furlong, 1974; Roizman and Batterson, 1985 for review). The genome has a molecular weight of approximately 100 x 10^6 Daltons and consists of two covalently linked segments, each containing primarily unique sequences flanked by inverted repeats (Fig. 1)(Sheldrick and Berthelot, 1974; Wadsworth et al., 1975). The longer (L) segment comprises 82% of the genome and consists of unique (U_L) sequences bracketed by inverted repeats (ab-b'a'). The shorter (S) segment comprises the remaining 18% and also consists of unique (U_S) sequences flanked by inverted repeat sequences (a'c'-ca). Thus overall the genome is

Fig. 1. HSV-1. A genomic map of HSV-1 including a diagramatic representation of the temporal relationship of immediate early, early and late transcriptional phases with respect to the onset of replication of the viral DNA. (Reprinted from; The Herpesviruses, vol. 3. Ed. Bernard Roizman. Plenum Press, New York, 1985).


a b
b' a' a c'
c a
0
0.1
0.2
0.3
0.4
0.5
0.6
0.7
0.8
0.9
1.0 mu
Unmodified Host (a)
2.8▶ (125)
1.8▶ (64)
◀2.8 (125)
◀4.2 1.8▶ (175) (68)
◀1.8 4.2▶ (12) (175)
Protein Synthesis ⟶ (β)
2.0 1.5
4.2▶
◀2.5 (82)
◀1.6 (43)
◀3.3 4.2 4.3▶ 5.6 (145)
5.2▶ (140)
1.8▶ (64)
◀1.7 (42)
2.0 1.5
1.8
3.0▶ (50)
(βγ?)
1.2▶ (38)
1.8▶ (39)
1.4
0.9
DNA Replication ⟶ (βγ+γ)
3.6
2.8
1.5
5.2▶
2.2▶
◀4.0
◀4.5
◀3.8
◀3.9 (50?)
◀1.7
◀6.0 4.5 (155)
◀3.1
3.3▶
◀1.5 (35)
2.7▶
2.3▶ (70)
5.6
5.2
4.4
2.4
1.3
3.4
2.3
1.9
1.3
4.4
1.8
0.8
←10—
◀3.8 (122/86)
1.9▶ (54)
7.0▶ (54)
◀1.8 2.7▶ (58) (69)
◀4.9 (70)
◀2.7 (86)
◀2.7 (85)
1.0▶ (18)
◀2.2 (65)
◀1.8 (31/33)
3.0▶
5.0▶
◀4.6
1.5

itself flanked by direct repeat sequences referred to as the "a" sequences. This sequence is also located at the L/S junction in an orientation that is inverted with respect to the terminal "a" sequences (see Roizman and Batterson, 1985 for review). During replication of the virus, U_L and U_S may become inverted with respect to each other and therefore viral replication results in the production of four isomeric forms of the genome (Hayward et al., 1975).

Lytic Infection

The lytic cycle of the virus (see Spear, 1985; Roizman and Batterson, 1985 for review) begins with the attachment of the virions to the cell by interaction with receptors on the cell surface. The viral membrane then appears to fuse with the cell membrane, thus releasing the nucleocapsid into the cytoplasm. The nucleocapsid is transported to a pore on the nuclear membrane, through which the viral DNA enters the nucleus. After viral DNA replication has occurred, the viral genomes are packaged into preformed capsids in the nucleus. This is followed by attachment of the nucleocapsid to patches of modified nuclear membrane, followed by envelopment. Egress of the virus particle may be similar to a reverse phagocytosis process involving a pathway through the Golgi apparatus, similar

to egress of glycosylated proteins (see Roizman and Batterson, 1985 for review).

Viral Macromolecular Synthesis and Regulation of Gene Expression

Within HSV, at least three classes of sequentially synthesised transcripts are produced in a tightly regulated cascade (see Honess and Roizman, 1974; Spear and Roizman, 1981; Harris-Hamilton and Bachenheimer, 1985; Roizman and Batterson, 1985; Godowski and Knipe, 1986 for review). All transcripts are produced in the nucleus of the infected cell by utilizing the host-cell RNA Polymerase II (Constanzo et al., 1977), are capped and methylated and have a poly A tail. Infection of cells results in a rapid shut-off of host macromolecular synthesis (see Roizman and Batterson, 1985 for review). The first viral genes to be transcribed, the immediate early (IE) or α genes, most of which code for proteins involved in regulating subsequent viral genes at the transcriptional level. The second class of genes, early (E) or β, encodes proteins whose primary functions deal with the replication of the viral genome. The final late (L) or τ phase of transcription results in the production of proteins involved in the assembly and egress of the viral particles (see Roizman and Batterson, 1985).

HSV genes each contain their own promoters and upstream regulatory regions, minimally composed of a TATA box and a CAP site (Everett, 1984b; Johnson and Everett, 1986). The temporal appearance of many of the viral transcripts is so tightly regulated that β genes are not transcribed until α products are present (Honess and Roizman, 1974), and some τ genes are not transcribed in the absence of viral DNA synthesis (Honess and Roizman, 1974; Holland et al., 1980; Harris-Hamilton and Bachenheimer, 1985). The mechanism involved in activation of viral genes is not yet understood, but studies to date have indicated that the upstream promoter-regulatory regions of the genes define their kinetic class. Point mutation and deletion studies have confirmed that IE, E and L viral genes each have distinct promoter regions (Machem and Roizman, 1982a; Machem and Roizman, 1982b; Machem and Roizman, 1982c).

The first viral transcripts are expressed in the absence of de novo protein synthesis, peak at 2 to 4 hours post-infection, and are referred to as immediate early (IE) or α transcripts. Immediate early upstream regulatory sequences may be divided into three regions. A TATA box motif exists most proximal to the CAP site, usually around the -30 bp range. Upstream from this region, somewhere between the TATA box and -110 bp exist CAAT boxes as well as GGGCGG regions referred to as GC

boxes (Machem and Roizman, 1982a, 1982c). These multiple GC regions are bound by the cellular transcription factor, Sp1 which increases the transcriptional rate about 25 fold (James and Tjian, 1985). A third region, having the consensus sequence TAATGARAT (with R representing a purine) is located at about -115 bp relative to the CAP site (O'Hare and Hayward, 1987). This region has been found to respond to a virion protein referred to as $V_{MW}65$, which is carried into the cell upon infection, and stimulates expression from IE promoters via the TAATGARAT sequences (Batterson and Roizman, 1983; Campbell et al., 1984; O'Hare and Hayward, 1984; Lang et al., 1984;; O'Hare and Hayward, 1985).

Five proteins produced from the IE transcripts have been identified and are named α or ICP 0, 4, 22, 27 and 47. All of these are modified by phosphorylation, and all are localized in the nucleus with the exception of ICP47 which is localized in the cytoplasm of infected cells (Pereira et al., 1977; Ackermann et al., 1984). Some of the IE proteins appear to be involved in regulating the synthesis of the subsequent class of polypeptides at the transcriptional level.

The immediate early product of importance to this study is the one whose transcript is produced from the α4 gene and is a modulator of HSV gene expression.

This 175,000 Dalton protein, referred to as ICP4 or $V_{MW}175$, has been found essential throughout the infective cycle for productive infection (Watson and Clements, 1978; Dixon and Schaffer, 1980; Frink et al., 1981). Studies have indicated that ICP4 is required for both the transition from immediate early to early protein synthesis (Clements et al., 1977; Dixon and Schaffer, 1980; Everett, 1984a; Quinlan and Knipe, 1985) and for the activation of some late genes (DeLuca et al., 1984). ICP4 is also capable of regulating the expression of its own transcript as well as of other α transcripts. This has been shown primarily in infections with the temperature sensitive mutant, tsK, at the non-permissive temperature (NPT) which results in the overproduction of immediate early proteins (Preston, 1979; O'Hare and Hayward, 1985b; Pizer et al., 1986; Zhang et al., 1987) and secondly using protein synthesis inhibitors during the initial stages of HSV infection, which results in a higher expression of IE transcripts in comparison to a non-inhibited infection (Harris-Hamilton and Bachenhiemer, 1985; Zhang et al., 1987). In addition, several studies have indicated that ICP4 is capable of activating transcription from promoters of several heterologous genes of both viral and cellular origin (Feldman et al., 1982; Everett, 1984b; Trembley et al., 1985). The large size of the ICP4 protein may

allow for several functional domains, thus accounting for the complex observations made in relation to its transactivating role. One study indicated that two temperature sensitive mutants of ICP4 demonstrated different abilities to transactivate certain genes at the non-permissive temperature (DeLuca and Schaffer, 1985), adding to the complex nature of the transactivating function of the protein. Whether or not ICP4 acts by binding directly to DNA is not clear, but strong evidence indicates that ICP4 associates with regulatory regions of viral genes only in the presence of cellular lysate, indicating the involvememt of cellular factors in the binding (Fenwick et al., 1978; Freeman and Powell, 1982; Faber and Wilcox, 1986; Beard et al., 1986; Kristie and Roizman, 1986a and b; Muller, 1987). Which cellular proteins are influenced by ICP4 has not yet been elucidated, however, McKnight and Tjian (1986) have suggested that ICP4 may interact with a TATA-binding factor.

The α0 gene product has a molecular weight of approximately 110,000 Daltons, and is also referred to as $V_{MW}110$ or ICP0. It has the ability to activate several promoters in trans including those of early and late HSV genes (Quinlan and Knipe, 1985; O'Hare and Hayward,1985a; Mauromara-Nazos et al., 1986), several cellular genes, the adenovirus type 5 E3 gene, and the

simian virus 40 early gene (Gelman and Silverstein, 1985; O'Hare and Hayward, 1985a and b, Sacks and Schaffer, 1987; Gelman and Silverstein, 1987a). However, the role of $V_{MW}110$ in the life cycle of HSV is still unclear since one study indicated that reduction of the ICP0 product below 10% of wild-type infection levels had no effect on virus production (Sandri-Goldin et al., 1987).

The α22 gene produces a 68,000 Dalton protein referred to as $V_{MW}68$ or ICP22. Transient transfection assays have indicated that ICP22 is not capable of activating plasmid-borne E or L viral gene regulatory regions (Mauromara-Nazos et al., 1986). However, ICP22 may be required by the virus for the activation of some late genes (Sears et al., 1985).

Experiments using viruses carrying a temperature sensitive mutant of the ICP27 ($V_{MW}63$) protein have demonstrated that it is essential for the full expression of several late genes (Sacks et al., 1985; Rice and Knipe, 1988). In fact, a temperature sensitive ICP27 mutant demonstrated increased levels of IE transcription, and reduced levels of L transcription at the non-permissive temperature (Sacks et al., 1985). In spite of these results, it has not been demonstrated that ICP27 is capable of activating plasmid-borne copies of early or late genes (Mauromara-Nazos et al., 1986).

Deletion mutants of the α47 gene encoding the ICP47 ($V_{MW}12$) protein are still capable of replication in cultured cells. Therefore ICP47 appears non-essential in infection of cultured cells (Longnecker and Roizman, 1986; Umene, 1986; Brown and Hardland, 1987). However, transient transcription assays have demonstrated that this protein is capable of augmenting the activation of viral genes by ICP4 and ICP0 despite not having any stimulatory effect on its own (O'Hare and Hayward, 1985a).

The second class of transcripts, the early (E) or β, appears before the onset of viral DNA replication, reaches a maximum at 5 to 7 hours post-infection and requires α proteins for their expression. Viral DNA replication occurs in the nucleus of infected cells and is first detected at about 3 hours and continues to 12-15 hours post-infection (Roizman et al., 1965). The β class has been further subdivided into β_1, expression of which subsides prior to DNA replication, and β_2 which continues to be expressed into late times of infection (Honess and Roizman, 1974). The upstream sequences required for regulation of early genes extends to about -70 bp from the CAP site (Costa et al., 1985; Smiley et al., 1983) and thus far, the only regulatory regions that have been identified are a TATA box, at about -30 bp from the CAP site, and CAAT motifs and GC boxes as

distal transcription signals upstream from the TATA box (Frink et al., 1981; Eisenberg et al., 1985). The early promoters are constitutively active in expression systems when introduced in a plasmid form, and deletions or mutations which alter the response of these promoters to transactivators also alter the constitutive expression of plasmid-borne copies of the mutated regulatory regions (Frink et al., 1981; Everett, 1983; Eisenberg et al., 1985).

The final, late (L) or τ phase, occurs after the onset of viral DNA replication and is further subdivided into τ_1 genes, which appear at early times but increase in expression after the onset of viral DNA replication, and τ_2 genes which are true late transcripts, appearing only after genomic replication has commenced. The synthesis of IE and E proteins, as well as ongoing replication of the viral genome are required to obtain maximum expression of late genes (Honess and Roizman, 1974; Holland et al., 1980; Harris-Hamilton and Bachenheimer, 1985). Late promoters appear to contain only a TATA box and CAP site (Frink et al., 1981; Johnson and Everett, 1986); in fact, one study indicated that when all of the regions upstream of the TATA box of a plasmid-borne copy of the gD promoter (an early gene) were deleted, and an HSV-1 origin of replication was included in the plasmid construct, the altered promoter

was regulated as a late gene (Johnson and Everett, 1986).

The mechanism of transactivating proteins in differentially regulating HSV genes has not yet been elucidated. Differences in response to transactivators appear even within the same class of genes. An example of this was demonstrated with two early genes, gB and gD, which can be differentially regulated by ICP4 (Arsenakis et al., 1988), with gD being susceptible to down-regulation and gB to stimulation by ICP4 (Everett, 1984; Arsenakis et al., 1988). ICP4 may bind to a region just downstream of the gD CAP site to confer this suppression (Everett, 1987). The evidence thus far indicates that cellular transcription factors are responsible for the activation of viral genes. Viral regulators, such as ICP4, appear to interact with these factors resulting in an alteration of the response level of the specific promoters to the cell transcriptional machinery. Other theories have ICP4 involved in the assembly, accumulation or compartmentalization of the transcriptional complexes. ICP4 has been found diffusely distributed in the nucleus of the infected cell at early times, and concentrated in globular replication compartments after the onset of viral DNA replication (Ackermann et al., 1984; Knipe et al., 1987). The ICP4 protein may also interact with more

than one pathway, however many more studies would have to be conducted before a more stringent hypothesis may be put forward.

Adenoviruses

Adenoviruses (see Horwitz, 1985 for review) from the family adenoviridae, were discovered in 1953. A transmissible agent with the ability to cause degeneration of epithelial tonsil tissue was isolated during attempts to establish tissue culture lines from tonsils and adenoidal tissue (see Sussenbach, 1984 for review). This agent was subsequently identified as the causative factor in a large number of upper respiratory ailments, was defined as a virus, and henceforth was referred to as adenovirus. Adenoviruses have been subclassified on the basis of hemagglutination patterns with rat or rhesus monkey red blood cells, DNA base composition (GC content), DNA homology and oncogenic potential. Adenovirus of the serotype 12 (Ad12) was the first description of a human virus causing tumours in animals (Trentin et al., 1962), however, association of adenovirus in malignancies of humans has not been demonstrated.

The structure of the virus consists of double stranded, linear DNA encapsidated in non-enveloped, regular icosahedrons of approximately 65 to 80 nm in

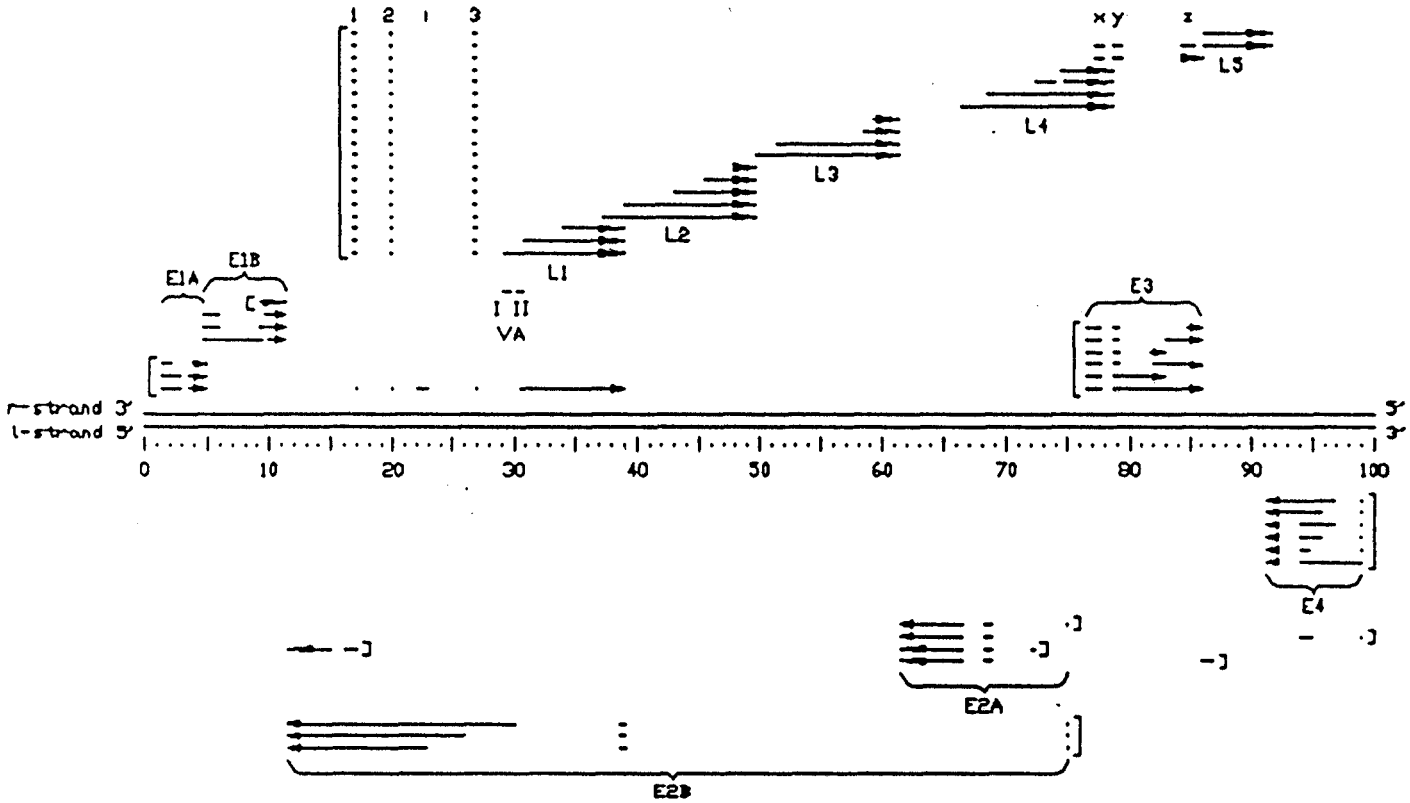
diameter. The capsids consist of 20 triangular surfaces and 12 vertices, with a fiber protein extending outward from each vertex (see Horwitz, 1985 for review).

The genome has a molecular weight of approximately 24 x 10^6 Daltons and includes inverted terminal repeats of 100 to 140 base pairs. Covalently linked to a dCMP at each 5' end is a 55,000 molecular weight polypeptide, referred to as the terminal protein (Rekosh et al., 1977), which plays an important role as primer in the replication of viral DNA (see Horwitz, 1985 for review).

Lytic Infection

The virus particle attaches to specific receptors on the cell surface via its fiber protein. The particle is then introduced into the cell by a mechanism that is not yet fully understood, but involves the loss of fiber protein from the virion (see Horwitz, 1985 for review). The nucleoprotein is then transported across the cytoplasm to pores on the nuclear membrane and the DNA subsequently enters the nucleus by an energy dependent mechanism (see Horwitz, 1985 for review). Transcription, viral DNA replication and virion assembly occur within the nucleus. The assembly of virions begins by the formation of capsomeres in the cytoplasm. An intermediate capsid, referred to as a "light

Fig. 2. Adenovirus type 5. A representative diagram of the Ad5 genome indicating transcripts and coding regions.


1
2
3
x y
z
L5
L4
L3
L2
L1
E1A
E1B
I II
VA
E3
r-strand 3'
l-strand 5'
5'
3'
0
10
20
30
40
50
60
70
80
90
100
E4
E2A
E2B

intermediate" is subsequently formed and is finally located in the nucleus. The entry of the viral genome into the intermediate capsid appears to occur before the association of core proteins with the DNA (see Horwitz, 1985 for review). After packaging and core protein formation, all of the precursor structural proteins are cleaved to their mature states. This results in the tightening of the capsid into a mature form, which then egresses through the nuclear membrane and out of the cell in a process which does not involve the rupturing of the cell surface, probably involving the microtubules of the cell (see Sharp, 1984; Horwitz, 1985 for review).

Viral Macromolecular Synthesis and Regulation of Gene Expression

The cascade of transcription has been conveniently subdivided into four phases as follows; pre-early peaking from 0 to 2 hours post-infection, early from 2 to 6 hours, intermediate from 6 to 12 hours and late from 12 to 36 hours post infection with viral DNA replication being the standard division between intermediate and late. Transcripts are capped, methylated and have a poly A tail (see Horwitz, 1985 for review). Infection of cells results in a shut-off of host macromolecular synthesis (Hodge and Scharff, 1969; Flint, 1982).

The E1a major transcripts are referred to as pre-early because they may be transcribed in the absence of de novo protein synthesis. It is an E1a function that is responsible for the subsequent transcription of the early classes of genes, E1b, E2, E3 and E4. Studies on the need for and function of E1a have been conducted with E1a deletion mutants such as Ad5 dl312 (Jones and Shenk, 1979a and b). It has been demonstrated that dl312 grows as well as wild type Ad5 on 293 cells, which constitutively express the E1 functions, whereas it is severely restricted on other human cell lines (Jones and Shenk, 1979a). Transcriptional regulation by E1a products has been most often monitored by comparing the levels of transcription of an early gene, the 72,000 Dalton DNA binding protein (72K), in wild type virus or dl312 infections. These types of studies demonstrated that at 6 to 8 hours post infection, 72K transcripts were plentiful in wild type infections but were approximately 300 fold lower in dl312 infected cells (Berk et al., 1979; Nevins, 1981). However, many studies indicated that there was not an absolute requirement for the E1a products, since the effects observed in their presence could be mimicked by using high multiplicity of infections, or using protein synthesis inhibitors prior to infection (Nevins, 1981).

The E1a region codes for two major and a number

of minor transcripts. The two major transcripts are also the two largest, and are designated 13S and 12S. The only difference between the 12S and 13S transcripts is that the 12S transcript has the region coding from amino acids 140 to 185 of the 289 residue 13S product spliced out. The larger product appears to be the one involved in transactivation (Moran et al., 1986) since mutants with a deletion in 12S are capable of replication [at least in exponentially growing cells (Spindler et al., 1985)]. However, it appears that the 12S product does play a role in lytic infection in G_o arrested cells and may drive these cells from G_o to S-phase, thus allowing maximal replication of the virus (Spindler et al., 1985). The 12S product appears to direct the G_o to S-phase transition via a different route than that normally used in the absence of virus (Cheetham and Bellett, 1982). The 12S product may also act as a repressor of certain viral genes as indicated by a study in which it was determined in vitro that 13S stimulated transcription from the 72K early promoter while the 12S product inhibits expression from the 72K late promoter (Guilfoyle et al., 1985; Guilfoyle et al., 1986; Leff and Chambon, 1986). The E1 products also play a role in the transformation of primary cells (Graham et al., 1974; Shiroki et al., 1979; Ruley, 1983; Moran et al., 1986b). Both the 12S and 13S products are

required to immortalize primary cells (Shiroki et al., 1979; Ruley, 1983). The E1b products confer a complete transformed state on the cells, therefore both E1a and E1b functions are required to obtained a fully transformed cell line.

Several investigators have identified cellular proteins which are involved in E1a mediated activation of some of the adenovirus early genes. It has been determined that the activity of the TATA-box transcription factor (TFIID) is increased due to the presence of E1a (Spangler et al., 1987; Leong et al., 1988). The increased activity of TFIID has also been observed in the presence of the pseudorabies immediate early trans-activating protein (Abmayr et al., 1985; Abmayr et al., 1988). On the other hand, the induction of the Ad5 E2A promoter by E1a involves the increased activity of the cellular factor, E2F (Reichel et al., 1988). Further analysis is required to see what factors are involved in the activation of other adenovirus genes and how the activity of these factors is increased.

Under stringent inhibition of protein synthesis, it was found that the E1a transcripts are not the only ones formed, and are not even the first (Lewis and Mathews, 1980). Two late transcripts, one coding for a 13.5 K protein and the L1 transcript are produced first. These transcripts are presently referred to as

immediate-early, although they were originally classified as late because of their accumulation at late times in infection. All of the pre-early and early genes are regulated via their own promoters. Most of the late genes are maximally transcribed after the onset of DNA replication which occurs in the nucleus of the infected cell via a semiconservative mechanism starting at about 7 to 9 hours post infection and results in the continuous elongation of both strands (Kelly, 1985). The mechanism of the early to late switch is not well understood but probably occurs by a cis acting function (Thomas and Mathews, 1980). The late genes of adenovirus have contributed greatly to the understanding of RNA processing since it was the study of transcription in adenoviruses that led to the discovery of the process of mRNA splicing. A single late promoter, referred to as the major late promoter, is responsible for the expression of late genes via the production of full-length transcripts (Sharp, 1984) followed by the splicing out of appropriate sequences to give the final transcripts. There are also some transcripts which peak at intermediate times but do not require viral DNA synthesis for their production and therefore are referred to as intermediate transcripts.

The promoters and upstream regulatory regions of several of the early genes of Ad5 are quite different in

terms of sequences important for activation. The E1b promoter contains a TATA box, GC box (Sp1 binding), CAAT motifs and several upstream enhancer-like elements which appear in vitro to be of importance only when the GC box is deleted (see Berk, 1986 for review; Parks et al., 1988). The promoter/regulatory region of the E2A 72 kDa protein is quite complicated (see Berk, 1986 for review). First of all, there are two promoters. One is used both at early and late times of infection and is referred to as the early promoter or E2A-E. The other, located approximately 3 map units further upstream is referred to as the E2A-L promoter, since it is used only at late times. The early promoter is stimulated by the 13S product of E1a (Guilfoyle at al., 1985; Guilfoyle et al., 1986; Bhat et al., 1987) and actually consists of two overlapping promoters (Zajchowski et al., 1985). One promoter, which transcribes about 80% of the transcripts, has a poor-fit TATA box as well as several upstream elements which are imperfect inverted repeats, quite similar to repeats found in the upstream regulatory regions of HTLV-I and HTLV-II promoter regions (Chen et al., 1985). The second promoter has a better-fit TATA box and is located about 25 bp upstream from the poor-fit TATA of the major promoter. It also has a region at about -50 to -60 bp upstream from the TATA sequences which, when deleted, results in the

suppression of the minor start site. The E2A-L promoter has a TATA box, Sp1 binding sites and CAAT boxes (Bhat et al., 1987). E1a products are not required for expression from this promoter, but the 12S product is capable of suppressing transcription from this site (Guilfoyle et al., 1985; Guilfoyle et al., 1986; Leff and Chambon, 1986). The E3 promoter and upstream control elements are comprised of a TATA box as well as several Sp1 binding sites (Leff et al., 1985). The E4 regulatory regions also has a TATA box and multiple Sp1 binding sites, however it appears to also be under the influence of an enhancer region that is quite homologous to the enhancer elements located upstream of E1a (Gilardi and Perricaudet, 1984).

The modes of action of the E1a products in transactivation are not yet fully understood, however enough information has accumulated to implicate an interaction with cellular factors. In fact, a cellular E1a-like factor has been postulated. If appropriate conditions are met, the absence of E1a has no effect on the replication of the virus, therefore the requirement of E1a for replication is not as stringent as the requirement for transactivators in other viral systems, such as the absolute requirement for ICP4 by HSV. The requirement for E1a can be bypassed by inhibiting cellular protein synthesis (Parsons and Green, 1971;

Craig and Raskas, 1974), by high multiplicities of infection (Gaynor and Berk, 1983), or by allowing the infection with an E1a deletion mutant to proceed for an appropriate amount of time (33 hours)(Imperiale et. al., 1984). In addition, early transcription in dl312 infected murine embryonal carcinoma cells is not detected until the cells are induced to differentiate (LaThangue and Rigby, 1987). These types of results indicate that cellular proteins are capable of stimulating expression from viral genes in the absence of E1a products. Specific regions of early viral promoters are protected from exonuclease III digestion after adenovirus infection. It has been determined that these sites are required for both basal constitutive expression and for response to E1a products (Murthy et al., 1985; Kovesdi et al., 1986). When cells are infected by an E1a deletion mutant, and sufficient time is allowed to pass (33 hours), protection from exonuclease III is observed on the same regions of the early promoters as observed with wild type infections (Kovesdi et al., 1986). This information implies that E1a interacts in some manner with a transcription complex which will eventually bind the promoter, even in the absence of E1a proteins. E1a perhaps accelerates the assembly or targeting of the complex to the DNA. Further analysis of E1a targets and associated cellular

factors will have to be done in order to understand the complete effects of E1a upon infection.

Models of Gene Regulation

The mechanism of action of viral transactivators is still unclear. Studies have demonstrated that viral transactivators fully or partially complement transactivator defects in heterologous viral systems (Feldman et al., 1982; Imperiale et al., 1983; Tevethia and Spector, 1984; Trembley et al., 1985; Leiberman et al., 1986; Wong and Levine, 1986; Tevethia et al., 1987; Felser et al., 1987; Felsner et al., 1988), or activate various cellular genes (Kao and Nevins, 1983; Green et al., 1983; Svensson and Akusijarvi, 1984; Stein and Ziff, 1984; Imperiale et al., 1984; Gaynor et al., 1985; Smiley et al., 1987). The transactivators from various viral systems are usually quite diverse in size and do not display any great degree of homology in terms of coding sequence. Some viral activators do associate with the promoter regions of target genes (Kovesdi et al., 1986; Siva Raman et al., 1986; Kovesdi et al., 1986; Muller, 1987), however, whether this association is direct or involves interaction with cellular factors is still being investigated, although most evidence indicates an association to DNA via cellular factors (Kristie and Roizman, 1986; Muller, 1987).

Adenovirus as a Vector

Viruses which are defective in terms of their capacity to replicate, yet retain infectivity offer an attractive mode of delivering a foreign gene into cells without having to worry about the effects of a lytic infection. If the wild type form of the virus chosen is also not a health threat to the host, then its choice as a vector is more inviting. Such is the case for adenovirus type 5. Studies on the packaging constraints of the virus have indicated that up to 2 Kbp of foreign DNA may be introduced into the Ad5 genome without impeding packaging (Ghosh-Choudhury et al, 1987). In addition, 5.5 kbp may be deleted from the viral chromosome by removing the E3 region (found to be non-essential for viral replication in cultured cells, Berkner and Sharp, 1983; Saito et al., 1985) and the E1 region. The latter is essential in the life cycle of the virus, however, its absence can be circumvented by replicating the virus in 293 cells, which constitutively express the E1 functions. Vectors based on the Ad5 virus have been developed (Haj-Ahmad and Graham, 1986), and a similar approach was used in constructing the virus used in this study.

Purpose of Study

Several lines of evidence suggest that viral transactivating proteins may operate through common cellular regulatory pathways. Many studies have indicated that transcriptional regulators from one viral system are capable of regulating heterologous viral systems, some to a greater degree than others.

Indications of cellular involvement in the regulation of viral gene expression come from studies which demonstrate that transactivators, such as the HSV ICP4 and Ad5 E1a products, do interact with cellular proteins, and some of these cellular plus viral protein complexes do interact with viral promoter/regulatory regions (Yee and Branton, 1985; Harlow et al., 1986; Kovesdi et al., 1987; Muller, 1987). In addition, transactivation by the ICP4 and E1a products is not restricted to viral genes, since cellular genes introduced into cells by transfection, or integrated into viral genomes, are themselves subject to regulation by these transactivators (Green et al., 1983; Allan et al., 1984; Gaynor et al., 1984; Everett, 1985; Gaynor et al., 1985; Smiley et al., 1987). Interestingly, these same genes are not activated in their endogenous location (Gaynor et al., 1985). Several cellular genes are activated when in their natural chromosomal position, but these appear to be unique genes in that

their requirement is tied to special circumstances, such as the 70,000 Dalton heat shock gene (Imperiale et al., 1984). It was found that these genes also had different degrees of basal transcriptional activity, with the level of transcription being dependant on the cell line. Cell lines which exhibit a relatively high degree of basal transcription of the heat shock gene also display a similar high level of E1a-like activity. In these cell lines, the requirement for E1a products for viral replication are overcome if sufficiently high levels of E1a deletion mutant are used for infection, or a sufficiently long infection is allowed to proceed (Imperiale et al., 1984).

Testing of individual transactivators on individual genes has indicated that Pseudorabies virus, Human cytomegalovirus, Epstein-Barr virus and Herpes Simplex virus immediate-early transactivators were capable of activating at least one adenovirus early gene (Feldman et al., 1982; Imperiale et al., 1983; Tevethia and Spector, 1984; Lieberman et al., 1986; Wong and Levine, 1986; Tevethia et al, 1987). In addition, the HSV-1 early genes could be activated by the immediate early products of Human cytomegalovirus, VZV and pseudorabies virus (Everett, 1984a; Everett and Dunlop, 1984). The ICP4 and ICP0 products are also capable of inducing the Human Immunodeficiency virus LTR promoter

in a manner dependant on cell-type (Ostrove et al., 1987). These types of studies have also indicated that some transactivators are capable of substituting completely for endogenous transactivators in some viral systems (Spector and Tevethia, 1986; Felser et al., 1987). These heterologous interactions lend a tool which may make it possible to elucidate the mechanisms governing gene regulation in cell systems. Tracing the path of viral transactivators, and their interactions with host cellular proteins will help in the understanding of which cellular proteins are involved in the viral gene regulation, and ultimately lead to an understanding of the normal function of these cellular proteins. The ICP4 product has already demonstrated the capability of transactivating at least one early gene of adenovirus, the E2A gene encoding the 72 kDa protein (Trembley et al., 1985). These experiments involved the infection of mouse cells (Z4) which have been transformed with and constitutively express the ICP4 gene, with an E1a deletion mutant of Ad5. The infection resulted in the production of the 72 kDa protein while infection of untransformed mouse cells with the same virus did not. However, mouse cells are not permissive for Ad5 replication, therefore this experimental design did not allow for a complete assessment of the extent of substitution of E1a by ICP4. Therefore, an adenoviral

construct having the E1 region replaced by the gene of the HSV-1 ICP4 gene was made. This would offer a method of infecting cells and assessing the full extent of substituting of ICP4 for the E1a transactivation function. Also, an important consequence of this project was the development of a tool for efficiently introducing the ICP4 gene into a wide range of cell types; the introduction of ICP4 being dependant only on the susceptibility of the cells to adenovirus attachment and penetration.

MATERIALS AND METHODS

Cells

The human 293 cell line (Graham et al., 1977) was obtained from F. L. Graham and grown in monolayers at 37° C in MEM F11 medium (GIBCO) supplemented with 10% newborn calf serum. The 293N3S variant (Graham, 1987) was grown in suspension at 37° C in Joklik's medium (GIBCO) supplemented with 10% horse serum. Both types of 293 cells were split at a ratio of 1:2 to 1:3 when monolayers reached confluency or suspensions reached a cell count of 5×10^5 cells/mL, respectively. Human R970-5 (Rhim et al., 1975) and KB cells, and monkey Vero and E5 (DeLuca et al., 1985) cells were grown in monolayers at 37° C in α-minimal essential medium (α-MEM) plus 10% newborn calf (R970-5 and Vero) or fetal calf serum (E5). The E5 cells, obtained from P. A. Schaffer, support replication of the ICP4 deletion mutant, HSV-1 d120 (DeLuca et al., 1985). All of these cells were split at a ratio of 1:5 to 1:10 once monolayers reached confluency, and were maintained at 37° C. All media were further supplemented with 1% penicillin/streptomycin (GIBCO) and 1% L-glutamine (Sigma).

Viruses

Wild type Ad5, Ad5 mutants dlE3dX2 (Haj-Ahmad and Graham, 1986), dl312 (Jones and Shenk, 1979b; Shenk et al., 1979), dlE1,3 (Haj-Ahmad and Graham, 1986), and the AdICP4 recombinant virus were propagated in 293 or 293N3S cells and titred on 293 cells. HSV-1 KOS and the d120 mutant (DeLuca et al., 1985) were propagated in Vero and E5 cells respectively, and titred on E5 cells. Purification of viral DNA was as follows: 293N3S cells were grown in suspension to a concentration of approximately 2-4 x 10^5 cells/mL. (2-3 litres), pelleted by spinning at 1000 r.p.m. for 30 minutes, and resuspended in approximately 100 mL. phosphate buffered saline (PBS). Adenovirus (wild type or mutant) was added at an m.o.i. of 5 to 10 p.f.u./cell for 1 hour at 37° C with constant, slow stirring. The virus infected cells were then returned to the original volume of medium, and growth in suspension was continued for 3 more days. At this time, cells were again harvested by centrifugation, and resuspended in 18.0 mL. of 100 mM Tris pH 8. 2.0 mL. of 5% sodium deoxycholate (Sigma) was added, and the cells were disrupted with an omnimixer until the viscosity decreased to approximately that of water. 1.8 mL of cesium chloride-saturated 100 mM Tris pH 8 was added for every 3.2 mL lysate. This was transferred into Beckman quick-seal tubes and

centrifuged for 20 hours at 35,000 r.p.m. in the SW50 rotor. Virus bands (clearly evident in the CsCl gradient) were extracted by syringe, and were lysed and protein digested by mixing 1:1 with a mixture containing 0.5 mg./mL. pronase, 0.01 M Tris pH 7.4, 0.01 M EDTA disodium salt, 0.4 % SDS, and incubating at 37° C overnight. The protein were then extracted several times with equal volumes of 100mM Tris, pH 8 saturated phenol. Viral DNA was finally recovered by precipitation with ethanol. Stock solutions of virus were prepared as follows: monolayers of 293 cells (in the case of wild type or mutant adenoviruses) E5 cells (d120) or Vero cells (HSV-1 KOS) were infected with virus at approximately 0.01 pfu/cell (HSV) or 1 pfu/cell (Adenovirus). When full cytopathic effect was attained, cells were harvested, rinsed three times with PBS, resuspended in approximately 1 mL. PBS/10^7 cells, sonicated, and virus titred.

Plasmid Preparations

Plasmid pGX164, containing the ts ICP4 gene from HSV-1 17 tsK (Watson and Clements, 1978; Preston, 1979a + b) was obtained from C. M. Preston. pXC3 and pTR00, used in the construction of pXCX2, were provided by Y. Haj-Ahmad (1986). Purification of plasmid DNA was according to Maniatis et al. (1982). Briefly, for

small scale isolation, approximately 1 mL. of overnight bacteria cultures were centrifuged for 15 seconds in the microfuge. The pellets were resuspended in 100 uL lysing solution containing lysozyme (50 mM Glucose, 25 mM Tris-Cl pH 8, 10 mM EDTA, 5 mg/mL. lysozyme) and left on ice for 15 minutes. 200 uL alkaline SDS (0.2 N NaOH, 1% SDS) was added, followed by gentle inversion to mix, and the samples were left on ice for an additional 10 minutes. At this point, 150 uL of 5 M potassium acetate (pH 4.8) was added to each sample, inverting gently to mix, and the samples were left on ice for one hour. Samples were then centrifuged in the microfuge for 10 minutes. The supernatants were removed, mixed with 1.0 mL cold 95% ethanol, placed at -20° C for 15 minutes, and centrifuged for 10 minutes in the microfuge. The pellets were then resuspended in 100 uL H_2O, and reprecipitated with the addition of 100 uL isopropanol. Samples were pelleted in the microfuge by centrifugation for 10 minutes. The final pellets were dried under vacuum and resuspended in 100 uL H_2O. Large scale preparations of plasmid were similar with the following exceptions. Bacterial pellets from a 500 mL overnight culture were resuspended in 40 mL of 5 mg/mL lysozyme solution and left at room temperature for 10 minutes. 80 mL of alkaline SDS was then added, and the solution was gently mixed and placed on ice for 5

minutes. 40 mL 5 M potassium acetate was added, the solution gently mixed and placed on ice for 15 minutes. 10 mL H_2O was added, the samples mixed, spun at 6000 rpm for 15 minutes in the Sorvall at 4° C. The supernatant was filtered through cheesecloth to remove debris, and was added to 0.6 volumes of isopropanol, and was then centrifuged at 6000 rpm for 10 minutes in the Sorvall. The pellets were resuspended to a final volume of 5.0 mL in 0.1 X SSC (15 mM NaCl, 1.5 mM sodium citrate, pH 7.0), transferred to 50 mL Corning tubes and mixed well on the vortex. 2.0 mL of a 1 mg/mL pronase, 0.8% SDS solution was added, followed by mixing and incubation for 30 minutes at 37° C. 8.4 grams of CsCl was then dissolved in the solution, and this was left on ice for 15 minutes prior to centrifugation at 3000 rpm for 10 minutes. The supernatant was distributed into 2 VTi 65 tubes and balanced by weight. 200 uL of 6 mg/mL ethidium bromide was layered on top, followed by mineral oil to fill the tubes. Tubes were then sealed, and spun at 55,000 rpm overnight at 15° C. DNA bands were retrieved by syringe, followed by repeated extraction with isoamyl alcohol to remove the ethidium bromide. The DNA was recovered by diluting the final DNA/CsCl solution with TE buffer (10 mM Tris-Cl pH 8, 1 mM EDTA) 1:4, and precipitating with 2.5 volumes of cold ethanol.

DNA Restriction and Modification

All restriction enzymes and DNA modifying enzymes, such as T4 DNA ligase, calf intestinal alkaline phosphatase, mung bean nuclease, were purchased from Bethesda Research Laboratories, Pharmacia or Boehringer Manheim Corporation, and were used according to the specifications set out by the manufacturers.

DNA Transfection

For rescuing of the AdICP4 recombinant virus, 2 ug of AddlE3dX2 DNA, cleaved with ClaI and XbaI, and 4 ug of pRYICP4 DNA were cotransfected into semiconfluent 293 cells grown in 60 mm dishes in MEM-F11 (GIBCO) using the calcium technique (Graham and van der Eb, 1973) as follows. Viral, plasmid and carrier DNA were diluted in 0.5 mL Hepes buffered saline (5g/L Hepes, 8g/L NaCl, 0.37g/L KCl, 0.125g/L Na_2HPO_4-$2H_2O$, 1g/L dextrose, final pH 7.1). $CaCl_2$ was added (2.5 M stock) to a final concentration of 125 mM. The mixture was then incubated for 30 minutes at room temperature to allow DNA-calcium phosphate precipitates to form. Once the precipitate formed, the solution was added to the cells. After four to five hours at 37° C, the medium was removed, and the monolayers were treated for two minutes with 20% glycerol in MEM-F11. Monolayers were rinsed three times with MEM F-11, then overlayed with a 1:1 mixture of 1%

agarose and 2X MEM F-11 supplemented with 10% horse serum, 0.2% yeast extract, 2% penicillin/streptomycin, and 2% L-glutamine. Prior to addition onto the cells, the overlay was maintained at 44° C to prevent agarose solidification. Once the overlay had solidified, samples were placed at 39.5° C until plaques appeared. Subsequent plaques were picked, expanded and titred on 293 cells.

Separation of DNA

DNA digests were separated and identified by electrophoresing through 1 % agarose gels, cast and run in Tris-acetate (TAE) buffer (40 mM Tris-acetate, 2 mM EDTA, pH 8.0). DNA bands were made visible through the addition of 0.1-0.5 ug/mL ethidium bromide to the TAE buffer, and observed under ultra-violet light.

Labelling of DNA with ^{32}P-orthophosphate

For several of the experiments, replicating viral DNA was labelled with 50-100 uCi/mL ^{32}P-orthophosphate in 199 phosphate-free medium (GIBCO), followed by restriction digestion and agarose gel electrophoresis. The gels were subsequently dried under vacuum onto Whatmann 3mm filter paper, and exposed to Kodak X-Omat K diagnostic film. DNA produced from the primer extension reactions were separated on a urea-acrylamide gel consisting of 8% acrylamide [42 g urea, BioRad, 20 mL 40% acrylamide-bisacrylamide (20:1), 3.8

mL 1.6% ammonium persulphate, 10 mL Tris-borate buffer/100 mL final volume, 80 uL TEMED (Bio-Rad)] and was electrophoresed in an IBI thermoplate sequencing apparatus.

Southern Transfer and Hybridization

The Southern transfer was by the method of Wahl et. al (1979) as follows: agarose gels containing separated DNA fragments were first washed twice, for 5 minutes, then for 10 minutes in 250 mL of 0.25 M HCl. This was followed by 2 washes of 10 minutes and 15 minutes respectively in 0.5 M NaOH, 1 M NaCl. The final two washes, for 15 and 30 minutes respectively, were with 0.5 M Tris-Cl, pH 7.4, 3 M NaCl. All of the washes were done with gentle shaking. The gel and a sheet of nitrocellulose were each soaked in 20X SSC (3 M sodium acetate, 0.3 M sodium citrate, pH 7). The blotting materials were stacked from the bottom up as follows: 4 to 5 sheets of 20X SSC soaked 3mm Whatman paper, agarose gel, nitrocellulose, 2 sheets of 20X SSC soaked 3mm Whatman paper, 4 sheets of dry 3mm Whatman paper and finally a stack of 5 to 8 cm of paper towels. The transfer was allowed to proceed overnight.

Once the transfer was complete, the nitrocellulose was washed twice, 30 seconds each, in 2X SSC, blot dried between sheets of 3mm Whatman paper, and finally dried for 2 to 4 hours at 80° C.

Probe DNA was nick-translated with α^{32}P-dCTP using the nick-translation kit of Bethesda Research Laboratories. The nitrocellulose was pre-hybridized in a pre-hybridization buffer for 4 hours at 42° C (50% $v/_v$ formamide, 5X SSC, 10% of 50X Denhardt's solution containing 20 mg/mL sonicated, denatured salmon sperm DNA, 0.05 M Tris-Cl pH 7.5)(50 x Denhardt's consists of 1% ficoll, 1% polyvinylpyrolidine, 1% bovine serum albumin dissolved in H_20). The hybridization was overnight at 42° C in a hybridization buffer (60% formamide, 6X SSC, 1X Denhardt's plus salmon sperm DNA, 1% dextran sulphate, 20 mM Tris-Cl pH 7.5) containing 1-2 x 10^6 cpm/mL labelled probe which was first boiled for 5 minutes. Once the hybridization was complete, the nitrocellulose was washed several times as follows: three times 5 minutes with 2X SSC, 0.2% SDS at room temperature; three times 60 minutes with 2X SSC, 0.2% SDS at 65° C. Finally, the nitrocellulose was exposed to Kodak X-ray film.

Immunoprecipitation and Immunoblotting of Proteins

For detection of the ICP4 protein produced in infected human cells, the rapid lysis method of Metzler and Wilcox (1985) was used. Monolayers of cells in 100 mm dishes were rinsed three times with PBS, and harvested in ice cold PBS with a rubber policeman.

Cells were pelleted by centrifugation at 3000 rpm for 5 minutes at 4° C, then resuspended in 400 uL ice cold disruption buffer [50 mM Tris-HCl pH 7, 8.5% (w/v) sucrose, 5% (w/v) 2-mercaptoethanol, 2% (w/v) sodium-dodecyl-sulphate] containing 10 mM Na-p-tosyl-L-lysine-chlorometylketone (TLCK, Sigma) and 10 mM N-tosyl-L-phenyl-alanine-chloromethylketone (TPCK, Sigma) protease inhibitors. Following sonication (2 x 30 seconds on ice), 125 uL of 4x sample buffer (Laemmli, 1970) was added and the samples were boiled for 3 minutes. Aliquots of 100 uL were electrophoresed on 6% polyacrylamide SDS gels. Proteins were then electroblotted onto nitrocellulose for 8 hours at 225 mA at 4° C using a Bio-Rad trans-blotting apparatus (25 mM Tris-HCl pH 8.3, 192 mM glycine, 20% methanol). Immunostaining of the protein was according to Johnson et al. (1984) using the anti-ICP4 P2 rabbit serum (W.R. Clements, unpublished) as a first antibody, followed by goat anti-rabbit serum conjugated to alkaline phosphatase (Jackson Immunoresearch). The procedure was as follows: the nitrocellulose bound protein was pre-blocked with BLOTTO (5% (w/v) skim milk powder, 0.01% Antifoam A emulsion, Sigma, 0.05% polyoxyethylene-sorbitan momolaurate (Tween 20, Sigma), 0.0001% thimerosal, Sigma), then was exposed to the first antiserum, (diluted 1:5000 in BLOTTO) for 90 minutes,

followed by four five minute washes with BLOTTO. Exposure to the second antiserum, (also diluted 1:5000 in BLOTTO) was for 60 minutes, followed again by four five minute washes in BLOTTO, and finally by two two minute washes in 15 mM borate buffer, pH 9.5. Colour developement was by the method of Blake et al. (1984) as modified by Persson and Bacchetti (1987). This involved a 30-120 minute exposure to the development solution (0.1 M Tris-HCl pH 9.2, 0.01% (w/v) nitro blue tetrazolium (Sigma), 0.05 mg/mL 5-bromo-4-chloro-3-indolyl phosphate (Sigma), 4 mM $MgCl_2$).

Preparing proteins for immunoprecipitations involved labelling of infected cells with ^{35}S-methionine (Amersham or ICI, 40 uCi/60 mm plate in medium containing 1/10th the normal amount of methionine), washing the monolayers three times with PBS, then pelleting the cells in PBS. Final cell pellets were resuspended and sonicated (4 x 15 seconds) in RIPA buffer (50 mM Tris-HCl pH 7.2, 150 mM NaCl, 0.1% (w/v) sodium dodecyl sulphate, 1% (v/v) Triton-X-100) plus 1 mM phenylmethylsulfonyl fluoride (Sigma) and 0.11 trypsin inhibitor units of aproteinin (Sigma) per mL. Aliquots of 500 uL of supernatants were mixed with 100 uL of formalin-treated Staphylococcus aureus (Cullen and Schwartz, 1976) suspension and 10 uL of the appropriate antibody and placed on a slow rotating wheel for 2 hours

at 4° C. Immunocomplexed products were washed four times with RIPA buffer by centrifugation and resuspension (in fresh RIPA) of the Staphylococcus aureus bacteria. The final pellets were resuspended in 50 uL of 2X sample buffer (Laemmli, 1970) and loaded on 9% (for the 72kD antigen) or 12% (for the 19kD antigen) SDS-polyacrylamide gels. Gels were fluorographed by the method of Bonner and Laskey (1974) by pre-soaking the gels in dimethyl sulfoxide (DMSO) twice for 30 minutes each time, followed by a 3 hour soak in DMSO containing 20% (w/v) PPO toluene based scintillation fluid. Finally, the gels were rehydrated by soaking in H_2O for 30 minutes prior to drying onto 3mm Whatman filter paper under vacuum.

Separation of Proteins

Proteins were separated by sodium dodecyl sulphate polyacrylamide gel electrophoresis. The gels were prepared as follows: the resolving gel consisted of 6%, 9% or 12% acrylamide-bisacrylamide (30:0.8)(Bio-Rad), 0.375 M Tris-HCl pH 8.8 (Bio-Rad electrophoresis grade), 0.1% SDS (Bio-Rad electrophoresis grade), 0.1% glycerol (BDH chemicals), 0.1% ammonium persulphate (BDH chemicals) and 15-20 uL N,N,N',N'-tetramethylethylenediamine (TEMED, Bio-Rad) per 42 mL resolving gel. A 1 to 2 cm stacking gel was layered onto the resolving gel and consisted of 12% acrylamide-

bisacrylamide (30:0.8), 0.125 M Tris-HCl pH 6.8, 0.1% SDS, 0.05% glycerol, 0.0625% ammonium persulphate and 15 uL TEMED/20 mL stacking gel. Gels were electrophoresed at 4-5 V/cm for 12 to 18 hours with a reservoir buffer consisting of 0.025 M Tris-HCl and 0.192 M glycine (Bio-Rad electrophoresis grade) at a pH of 8.3. Proteins were identified by autoradiography of immunoprecipitates or immunostaining.

Primer Extension

Oligonucleotides corresponding to sequences of 20 or 25 nucleotides downstream from the appropriate message cap site were synthesised (Central Facility of the Institute of Molecular Biology and Biotechnology, McMaster University) to serve as the primers. The oligomers were end labelled by the method of Jones et al. (1985) with ^{32}P-ATP (NEN) in the following manner. 50 ng of the oligomer was suspended in a kinasing buffer (20 mM Tris-HCl pH 9.5, 1mM spermidine-HCl, Sigma, 0.1 mM EDTA) to a final volume of 40 uL and incubated at 90 C for two minutes. To this, 5 uL of a second kinasing buffer (0.5 M Tris-HCl pH 9.5, 0.1 M $MgCl_2$, 50 mM dithiothreitol, Sigma and 50% (v/v) glycerol plus the ^{32}P-ATP (50 uCi) and 10 units of T4 DNA kinase) was added, followed by a 60 minute incubation at 37° C. The DNA was finally precipitated by adding 200 uL of 2.5 M

ammonium acetate, 10 ug tRNA (BMC) and 750 uL of 95% ethanol.

RNA was extracted from infected cells by the method of Chirgwin et al. (1979) or Berk and Sharp (1977).

The first method involved the harvesting of infected cells at the appropriate time (indicated in results) by rinsing the monolayers three times with PBS followed by pelleting of the cells in Corex tubes. Two to three mL of an extraction solution [4 M guanidinium isothiocyanate (BRL), 0.5% N-laurylsarcosine (Sigma), 25 mM sodium citrate, 0.1 M 2-mercaptoethanol (BDH chemicals), 0.1% antifoam A (Sigma), pH 7.0] was added to each cell pellet, which were then vortexed for at least two minutes. The cell suspension was then layered onto 1.8 mL of 5.7 M CsCl in 25 mM sodium acetate pH 5.0 in SW50.1 centrifuge tubes (Beckman). Centrifugation was for at least 12 hours at 35,000 rpm at 20° C. The RNA pellet was resuspended in 300 uL H_2O. NaCl was added to a final concentration of 0.1 M, followed by precipitation with 2.5 volumes of 95% ethanol. The RNA was pelleted by a 30 minute centrifugation at 10,000 rpm (Sorvall) followed by drying. The dried pellets were resuspended in 160 uL H_2O, and the A260/A280 optical density readings were measured and recorded.

The method of Berk and Sharp required that the harvested cells be first lysed with phosphate buffered saline containing 0.65% Nonidet P40 (Sigma) for 10 minutes at 0° C. Cell nuclei were then sedimented by centrifuging at 5000 rpm for 5 minutes in the Beckman table-top centrifuge. The cytoplasmic fraction was then removed and mixed with an equal volume of an extraction buffer (7 M urea, 0.35 M NaCl, 0.01 M Tris-Cl pH 7.4, 0.01 M EDTA, 1.0% SDS). This mixture was extracted twice with equal volumes of phenol-chloroform [equal volumes of phenol and chloroform:isoamyl alcohol, 24:1 (v/v)]. The aqueous phase was precipitated by adding $NaCH_3COOH$ (0.4 M final, pH 5.2) and 2.5 volumes of 95% ethanol followed by centrifugation. The pellets were washed two times by redissolving in 0.4 M sodium acetate, adding 2.5 volumes of 95% ethanol, followed by centrifugation.

Primer extensions were carried out by the method of Jones et al. (1979). Briefly, 10-30 ug of dried RNA was resuspended in 8 uL hybridization buffer (10mM Tris-HCl pH 7.9, 1 mM EDTA) containing approximately 2 x 10^5 dpm of labelled oligonucleotide. To this, 2 uL of a second salt solution (1 mM EDTA, 1.25 mM KCl) was added followed by a 60 minute incubation at 65° C. After cooling to room temperature, 25 uL of a nucleotide tri-phosphate solution [20 mM Tris-HCl pH 8.7, 10 mM $MgCl_2$,

5 mM dithiothreitol, 0.33 mM of each dNTP (Pharmacia), 10 ug/mL actinomycin D, (Sigma) and 10 units of AMV reverse transcriptase (Life Sciences Inc.)] were added, followed by incubation for 60 minutes at 37° C. Finally, the DNA was ethanol precipitated and resuspended in loading buffer. The samples were electrophoresed on an 8% urea-acrylamide gel using an IBI thermoplate sequencing apparatus. The molecular weight marker included in the gel consisted of 3' ^{32}P end-labelled, Hpa II digested pBR322 DNA.

RESULTS

The purpose of this study was to assess potential similarities in the functioning of two viral transactivators, the ICP4 protein of HSV-1 and the E1a product(s) of Ad5. In particular, it was of interest to determine the extent of complementation of E1a defects by ICP4 in the lytic cycle of Ad5. Several studies have indicated that the transactivating proteins of many viruses interact with cellular proteins in order to carry out their gene activation function. Also, some viral transactivators are capable of activating genes in heterologous viral systems. Further investigation in this area may result in the elucidation of the cellular pathways being used by viruses for transcription, and their role in the normal cell cycle. Previous studies with HSV ICP4 and Ad5 E1a by Trembley et al. (1985) had demonstrated that an ICP4 protein constitutively expressed in mouse cells was capable of increasing the levels of the 72,000 Dalton DNA binding protein (72K) from an infecting Ad5 virus. This same study also indicated that the presence of both functional ICP4 and E1a products resulted in a decrease in the level of 72K. A full assessment of the extent of substitution of E1a by ICP4 functions was not possible in the system used by

Trembley et al., (1985) since mouse cells are not permissive for adenoviral replication.

The method chosen to assess the full extent of substitution of E1a by ICP4 was to construct a recombinant Ad5 virus which lacked the E1a gene, and in its place carried the HSV ICP4 gene. The extent of substitution for E1a function by ICP4 was assessed by infecting cells with this virus and evaluating its levels of transcription, translation, viral DNA replication and viral replication relative to wild type Ad5 and E1a mutant levels.

Construction of AdICP4 : an Ad5 Recombinant Virus Coding For HSV ICP4

Studies by Ghosh-Choudhury et al., (1987) have indicated that the maximum Ad5 genome size that could be properly encapsidated was approximately 38 kilobase pairs (kbp), only 2 kbp larger than the wild type genome. Also, any recombinant required that both long terminal repeat regions of the genome (see figure 4) be intact in order for genomic replication and encapsidation to take place.

The method chosen to meet these considerations was modeled after a system developed by Haj-Ahmad and Graham (1986). In their system, an Ad5 vector having the E1 and E3 regions deleted was produced. The E3

Fig. 3. HSV-1. A genomic map of HSV-1 including a detailed diagram of one of the two short repeat sequences (c and c') which contain the ICP4 gene. The upstream regulatory region of the ICP4 gene is also presented, indicating the CAP site, TATA box, distal upstream elements, far upstream elements and pertinent restriction endonuclease cleavage sites.

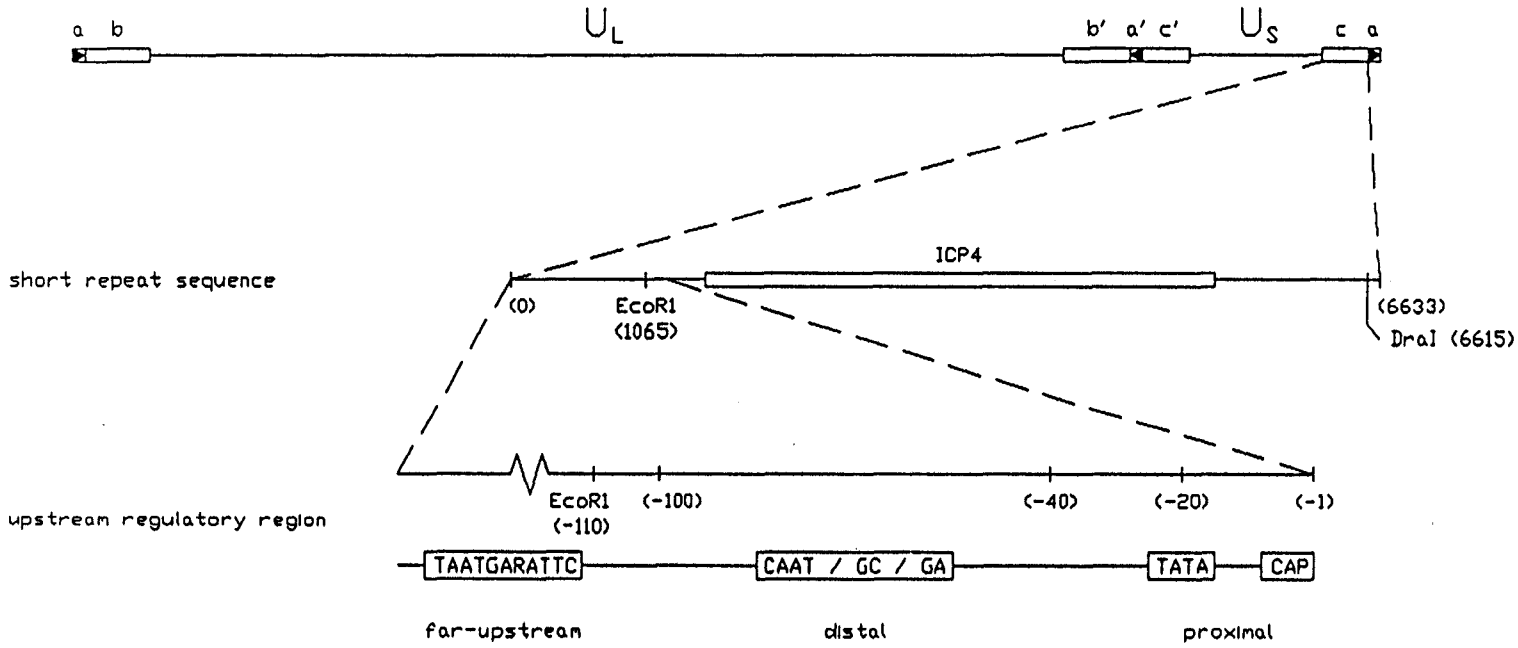

a b
U_L
b' a' c'
U_S
c a
short repeat sequence
ICP4
(0)
EcoR1
(1065)
(6633)
DraI (6615)
upstream regulatory region
EcoR1
(-110)
(-100)
(-40)
(-20)
(-1)
TAATGARATTC
CAAT / GC / GA
TATA
CAP
far-upstream
distal
proximal

Fig. 4. Representative diagrams of A) the Ad5 genome indicating transcripts and early and late coding regions: B) a HindIII restriction map of the Ad5 genome: C) the dlE3dX2 genome indicating the E3 deletion and the unique ClaI and XbaI restriction endonuclease sites. Also shown is the pXCX2 plasmid indicating the E1 deletion from its adenovirus sequences.

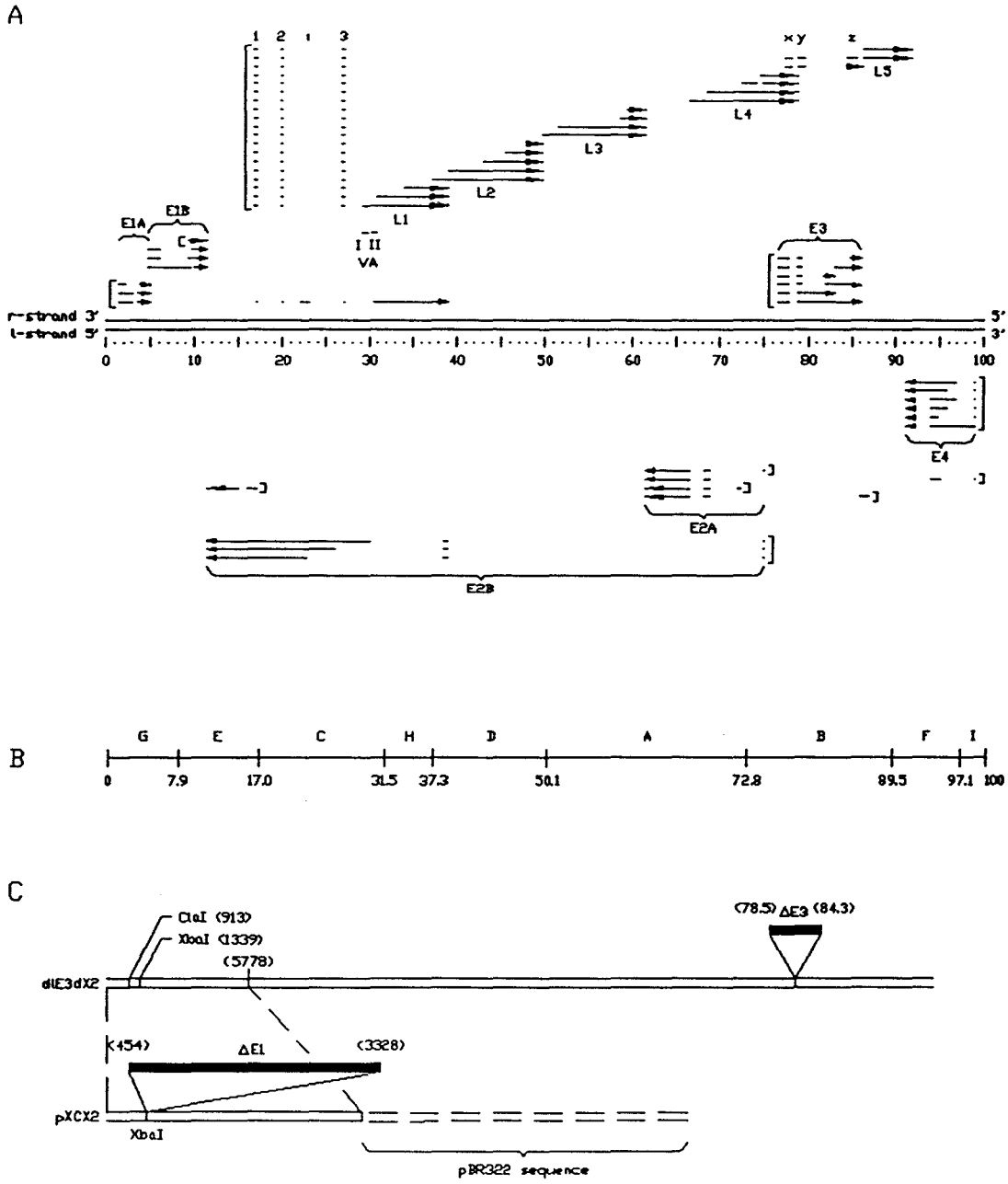

A
1 2 3
x y z
L1
L2
L3
L4
L5
E1A
E1B
I II
VA
E3
r-strand 3'
t-strand 5'
5'
3'
0 10 20 30 40 50 60 70 80 90 100
E4
E2A
E2B
B
G E C H D A B F I
0 7.9 17.0 31.5 37.3 50.1 72.8 89.5 97.1 100
C
ClaI (913)
XbaI (1339)
(5778)
(78.5) ΔE3 (84.3)
dlE3dX2
(454)
ΔE1
(3328)
pXCX2
XbaI
pBR322 sequence

Fig. 5. Construction of Ad5 recombinant virus encoding the HSV-1 ts ICP4 gene. A). The ts ICP4 gene was supplied from pGX164 and was sub-cloned into a portion of pBR322 via the EcoR1 and DraI restriction sites. XbaI linkers were subsequently added within these sites resulting in pRS4, which carries the ICP4 gene flanked by XbaI restriction sites.

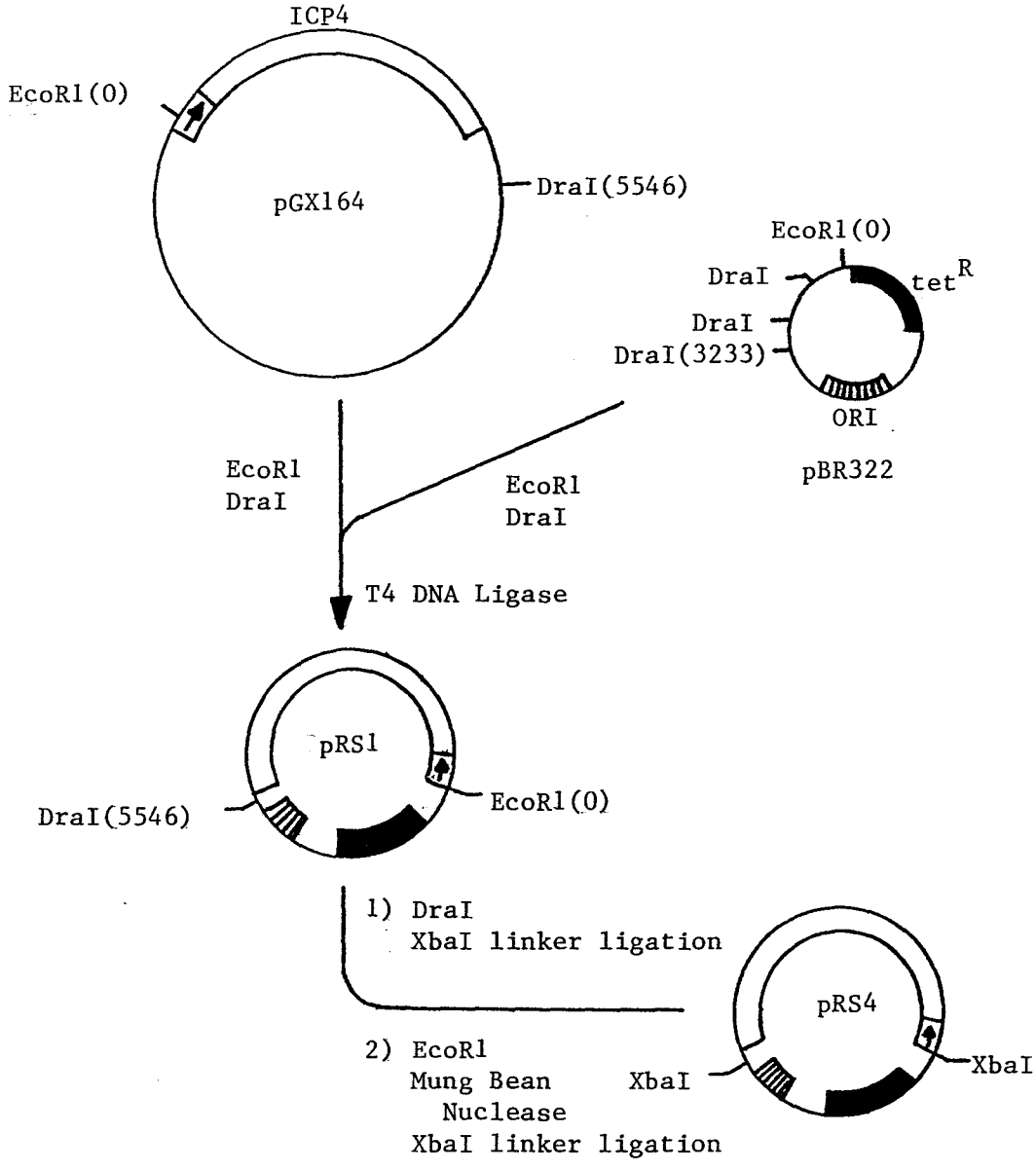

ICP4
EcoRl(0)
pGX164
DraI(5546)
EcoRl(0)
DraI
tet^R
DraI
DraI(3233)
ORI
pBR322
EcoRl
DraI
EcoRl
DraI
T4 DNA Ligase
pRS1
DraI(5546)
EcoRl(0)
1) DraI
XbaI linker ligation
pRS4
2) EcoRl
Mung Bean
Nuclease
XbaI linker ligation
XbaI
XbaI

Fig. 5. B). A second plasmid, pXCX2, carrying the extreme left end of the Ad5 genome, containing the segments from 0 to 452 nt (segment 1), and 3328 to 5778 nt (segment 2) separated by an XbaI site was constructed as follows: segment 1 was supplied by the EcoR1 to XbaI fragment of pTR00, and was sub-cloned into pXCX, supplying segment 2. This plasmid was derived from pXC3 by substituting an XbaI site for the Bgl II sites.

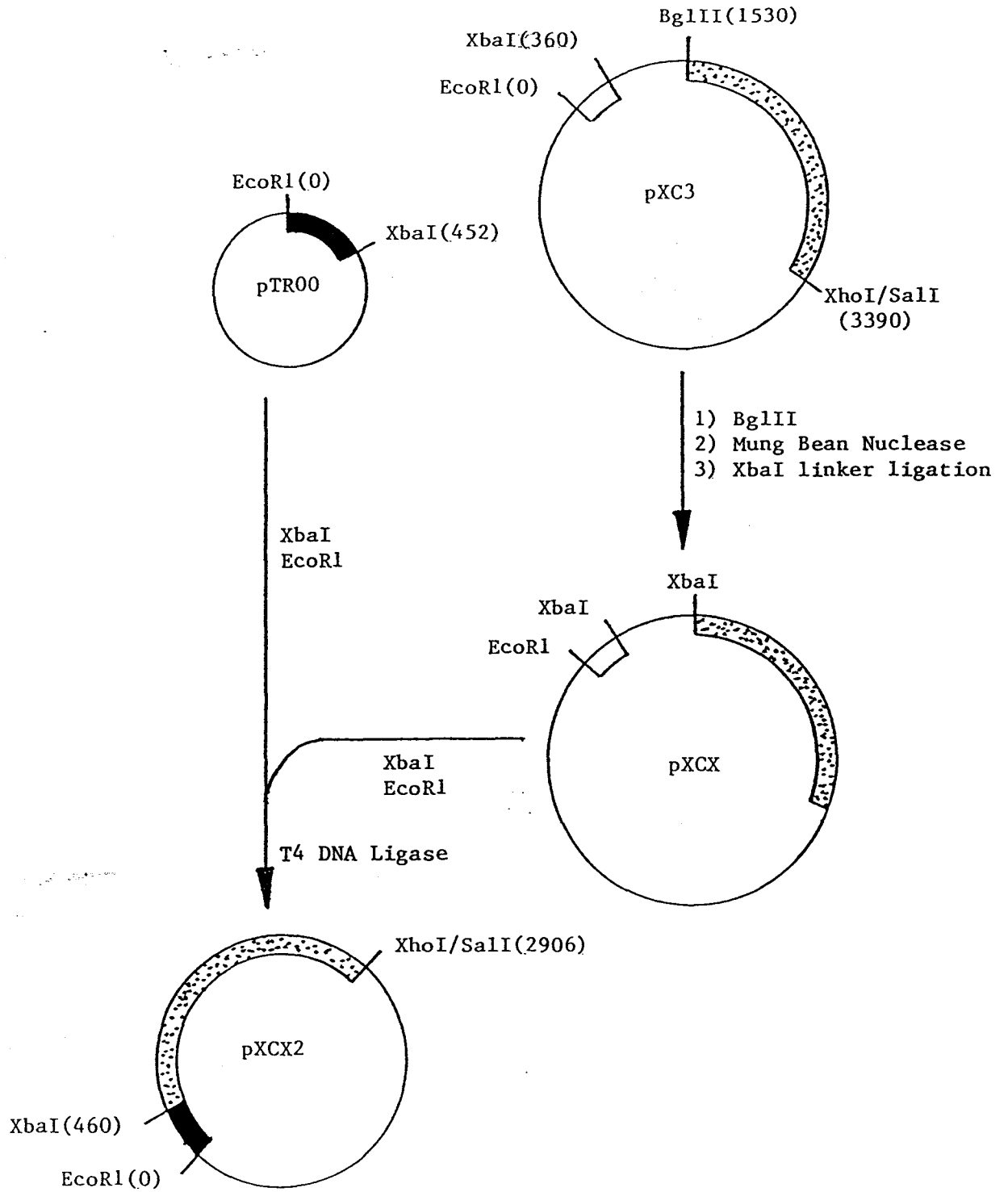
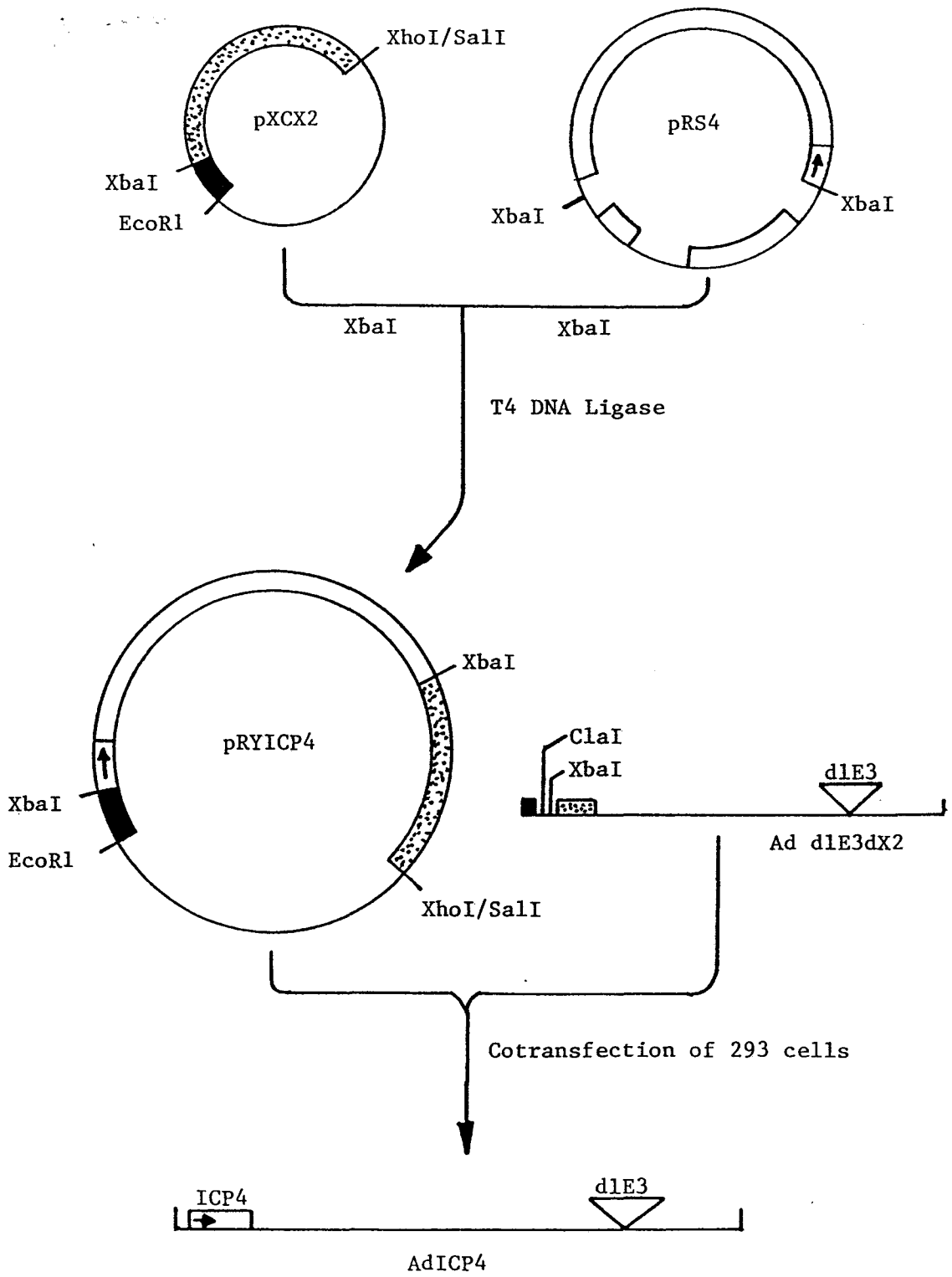

BglII(1530)
XbaI(360)
EcoRI(0)
pXC3
XhoI/SalI
(3390)
EcoRI(0)
XbaI(452)
pTR00
1) BglII
2) Mung Bean Nuclease
3) XbaI linker ligation
XbaI
EcoRI
XbaI
XbaI
EcoRI
pXCX
XbaI
EcoRI
T4 DNA Ligase
XhoI/SalI(2906)
pXCX2
XbaI(460)
EcoRI(0)

Fig. 5. C). The ICP4 gene, now flanked by XbaI sites, was cloned into the XbaI site of pXCX2 in a rightward orientation, producing pRYICP4. pRYICP4 was rescued into virus by cotransfection with AddlE3dX2 DNA (cleaved at the unique ClaI and XbaI sites) in 293 cells at 39.5° C.


XhoI/SalI
pXCX2
XbaI
EcoRl
pRS4
XbaI
XbaI
XbaI
XbaI
T4 DNA Ligase
pRYICP4
XbaI
XbaI
EcoRl
XhoI/SalI
ClaI
XbaI
dlE3
Ad dlE3dX2
Cotransfection of 293 cells
ICP4
dlE3
AdICP4

region was found to be non-essential for viral replication in cultured cells and provided an ideal location for insertion of foreign genes. The E1 defect was circumvented by replicating the vector, with or without an insert, in 293 cells, which constitutively produce E1 proteins (Graham et al., 1977). Similarly, my study included the production of an Ad5 in which the deleted E1 region is replaced with the ICP4 gene, and also has the E3 region deleted. The ICP4 gene was introduced into the vector through an in vivo recombination event between homologous regions of two co-transfected DNA fragments: an Ad5 vector DNA and a plasmid containing Ad5 sequences and the ICP4 gene. The total amount of deleted Ad5 DNA was approximately 5.0 kbp (2.9 kbp from E1 and 1.9 kbp from E3). The inserted ICP4 gene was approximately 5.6 kbp in length, therefore the recombinant has a genome of 36.6 kbp. The cloning is outlined in figure 5 and described in the following text.

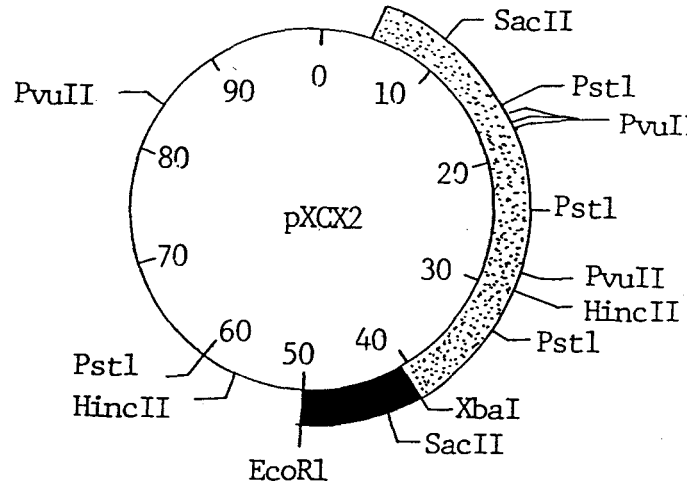
The ICP4 coding region was obtained from HSV-1 strain 17tsK (Preston, 1979a + b; Watson and Clements, 1980). The rationale for using a temperature sensitive mutant of ICP4 was two-fold. First of all, the availability of the ts gene would allow modulation of ICP4 function by temperature shifts, thus providing a direct control for the effects of the protein.

Secondly, Trembley et al. (1985) have observed inhibition of Ad5 transcription when both functional ICP4 and E1a are expressed in mouse cells. Were this phenomenon to occur also in human cells, the use of ts ICP4 might allow for the isolation and propagation of the recombinant virus in 293 cells grown at the non-permissive temperature.

The original ICP4 gene which was contained in an EcoR1-DraI fragment in the plasmid pGX164 (obtained from C.M. Preston), was subcloned into pBR322 through these two restriction sites. Further cloning (see below) required that these sites be converted to XbaI restriction sites, resulting in the plasmid pRS4. The cloning preserved most of the upstream regulatory sequences of the ICP4 gene [omitting only the most upstream region responsible for response to the virion transactivator, $V_{mw}65$ (Lang et al., 1985; Machem and Roizman, 1982a + b + c)], as well as the poly-A signal at the 3' end. The XbaI flanked ICP4 gene was subsequently cloned into the unique XbaI site of the plasmid pXCX2, resulting in the plasmid pRYICP4. The plasmid pXCX2 contains two regions of Ad5 sequences: the extreme left 452 nucleotides and the region corresponding to nucleotides 3328-5778, joined by a unique XbaI site. This results in the deletion of the E1 coding region, while preserving the left end inverted

terminal repeat and provides a region of 2450 base pairs of Ad5 sequences for homologous recombination with the viral vector DNA (see Figures 4 and 5). The ICP4 gene was cloned in a rightward orientation in the pRYICP4 plasmid. This construct was confirmed by restriction endonuclease patterns of pRYICP4 and pXCX2 (Fig. 7). An example of this analysis is as follows: the map of pRYICP4 in figure 6 indicates that digestion of the DNA with the restriction endonuclease Pst 1 will produce fragments of 7.21, 3.78, 0.73, 0.43 and 0.03 kbp in length. Lane 9 of figure 7 is the pattern of such a digest electrophoresed on an agarose gel. Bands representing the two largest fragments are clearly visible, however the 0.73, 0.43 and 0.03 kbp fragments were too small to be retained on this gel. Lane 10 is the pattern of the same plasmid digested with the restriction endonuclease XbaI. In this case, the bands representing the 6.62 and 5.55 kbp fragments are clearly visible. Digestion with both of these enzymes results in the 7.21 kbp Pst 1 fragment being reduced to 5.55, 1.20 and 0.48 kbp by Pst 1. The 6.62 kbp XbaI fragment was also reduced to 3.78, 1.20, 0.73. 0.48, 0.43 and 0.03 kbp by Pst 1. Lane 14 is the result of such a digest. The 5.55, 3.78 and 1.20 kbp bands are evident in this pattern. Use of other restriction endonucleases confirmed the structures of pXCX2 and pRYICP4.

Fig. 6. A restriction endonuclease map of the plasmids pXCX2 and pRYICP4 signifying pertinent sites.


SacII
PstI
PvuII
PvuII
0
10
20
PstI
pXCX2
30
PvuII
HincII
PstI
40
50
60
70
80
90
PvuII
PstI
HincII
XbaI
SacII
EcoRI

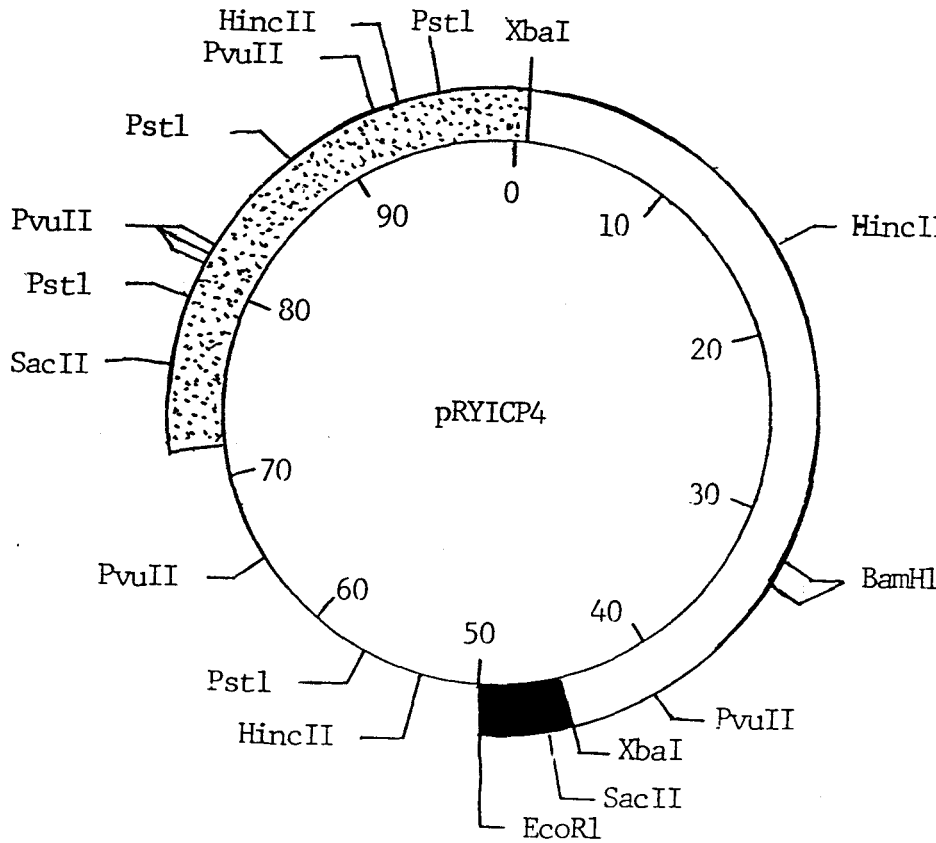
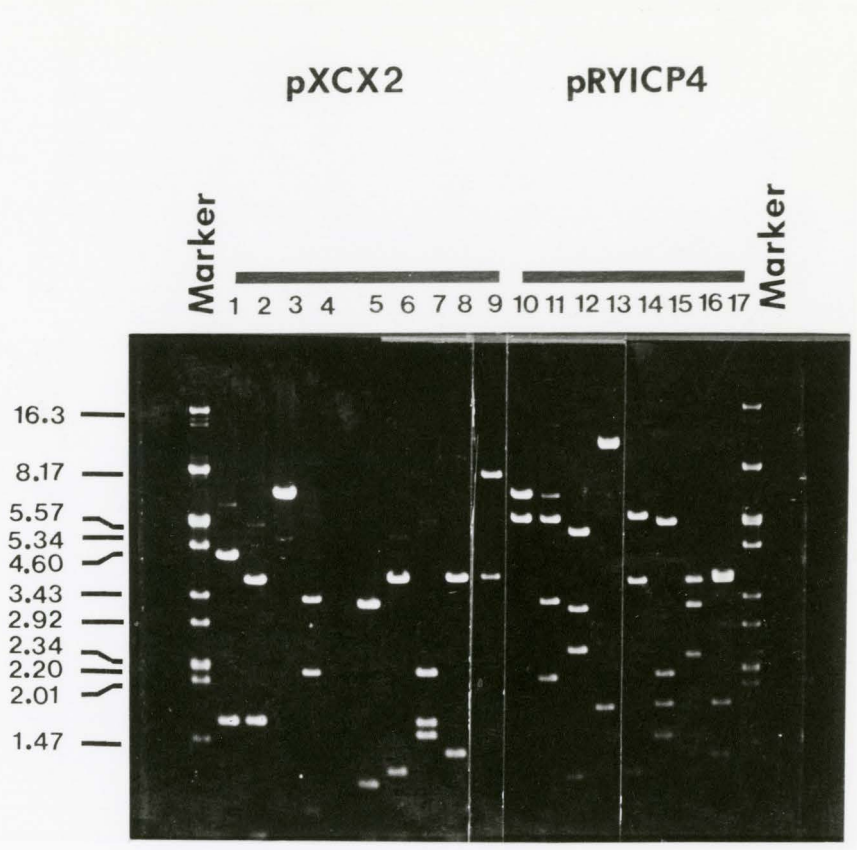

HincII
PstI
XbaI
PvuII
PstI
0
10
HincII
90
PvuII
PstI
80
20
SacII
pRYICP4
70
30
PvuII
BamHI
60
40
50
PstI
PvuII
HincII
XbaI
SacII
EcoRI

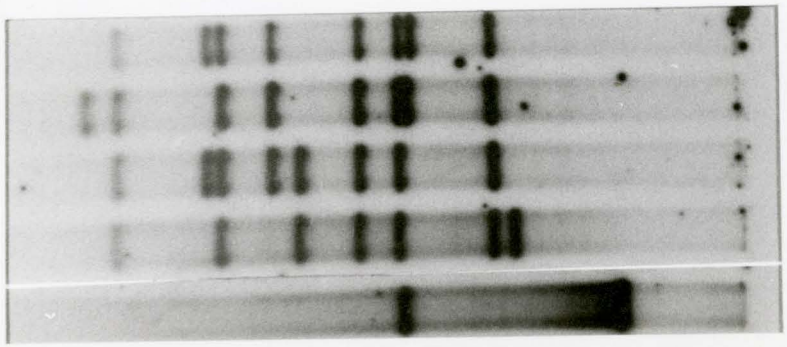
Fig. 7. A restriction digest pattern confirming pRYICP4. The fragment patterns of pRYICP4 are compared to those of the parental plasmid, pXCX2. The digests are as follows: SacII: lane 1; Pst1: lanes 2 and 9; XbaI: lanes 3 and 10; PvuII: lanes 4 and 11; HindIII: lane 12; Bam H1: lane 13; SacII + Pst1: lane 5; XbaI + Pst1: lanes 6 and 14; PvuII + Pst1: lanes 7 and 15; HincII + Pst1: lanes 8 and 16; BamHI + Pst1: lane 17. The sizes of the marker fragments are noted in the figure.


pXCX2
pRYICP4
Marker
1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16 17
Marker
16.3
8.17
5.57
5.34
4.60
3.43
2.92
2.34
2.20
2.01
1.47

The viral recombinant was produced by cotransfecting pRYICP4 and the DNA of Ad5 dlE3dX2 into 293 cells at the non-permissive temperature. The reason for using Ad5 dlE3dX2 was two fold. First of all, dlE3dX2 has a deletion of the E3 region (between 78.5 and 84.7 map units). Secondly, Ad5 dlE3dX2 has unique ClaI and XbaI sites at nucleotides 917 and 1339 respectively. Prior to transfection, the vector DNA was rendered non-infectious by cleaving at these sites, thus removing the extreme left end of the genome which is essential for its replication. In vivo homologous recombination with pRYICP4 regenerated the extreme left end of the recombinant, thus allowing replication. The extreme left end was repaired during the infective process removing the pBR322 portion of pRYICP4 (Stow, 1982). All of the transfections resulted in a total of 89 plaques, of which only one corresponded to the desired viral recombinant. Viruses were identified by extracting DNA from plaques and digesting the DNA with the restriction endonuclease HindIII. Digests were then electrophoresed and migration patterns were compared to a HindIII digest of wild type Ad5. Digestion of the Ad5 genome with HindIII produces 9 fragments, A through I, of sizes 8.2, 5.8, 5.34, 4.6, 3.43, 2.92, 2.8, 2.0 and 1.0 kbp respectively. All of these fragments except for the I band, which is too small to be retained on the

gel, appear in lane 1 of figure 8. The Ad5 mutant, dl312, which has a deletion in E1a, has a digestion pattern similar to that of Ad5 except for an alteration in the migration of the G fragment, which migrates below the H band (lane 2). Similarly, the digestion pattern of dlE3dX2 has only one alteration relative to the Ad5 pattern. In this case, the B fragment shifts from 5.8 kbp to 3.9 kbp due to the deletion of the E3 region (lane 3). This new band is referred to as B'. This same deletion accounts for the B to B' shift observed in the HindIII digestion pattern of AdICP4 (lane 4). In addition, the E and G bands no longer exist, and a new fragment of 8.95 kbp has been created. The deletion of the E1 region of AdICP4 has coincidently removed the HindIII restriction site dividing the E and G fragments. Since the ICP4 gene, which now resides between the undeleted portions of the E and G fragments, contains no HindIII restriction sites, the new larger fragment emerges. Digestion of AdICP4 with XbaI (lane 5) excised the 5.55 kbp insert containing the ICP4 gene. Also apparent is a large band representing the 28 kbp fragment produced by the XbaI digest. An 0.46 kbp fragment was also excised in the digestion but a corresponding band does not appear in lane 5 due to the small size of the fragment. Most of the 89 isolates appeared to be mutants with a deletion in the G

Fig. 8. A autoradiogram of HindIII restriction digest patterns of Ad5 as well as Ad5 deletion mutants and includes the recombinant, AdICP4. Lane 1, wild type Ad5 displaying the characteristic HindIII digest pattern; lane 2, Ad5dl312; lane 3, Ad5dlE3dX2 with the characteristic shift from the 5700 bp B fragment to the 3800 bp B' fragment; lane 4, AdICP4 with the same B to B' shift as well as the disappearance of the G and E fragments due to their fusion to the ICP4 gene, and the appearance of this new fusion product at 8950 bp. Included in lane 5 is an XbaI digest of AdICP4 confirming that the 5550 bp ICP4 XbaI fragment does reside in the viral construct. The sizes of the Ad5 fragments A through I are as follows: 8.2, 5.8, 5.34, 4.6, 3.43, 2.92, 2.8, 2.0 and 1.0 kbp respectively.



1 wt (Hind III)
2 dl312 (Hind III)
3 dlE3dX2 (Hind III)
4 AdICP4 (Hind III)
5 AdICP4 (Xba I)

fragment. This deletion is probably a result of nuclease activity on the viral fragments before or during the transfection process, and the rejoining of the terminal portion of the G fragment to the remainder of the dlE3dX2 genome.

Expression of ICP4

It was important to determine if the ICP4 gene contained in the vector would produce protein. This was assessed by immunostaining protein from infected cells with the ICP4-specific P2 rabbit antiserum (W.R. McClements, unpublished) in a Western blot. Infections with AdICP4 or HSV-1 were carried out at 34° C (permissive temperature for ICP4) in 293 (permissive for E1 mutants), Kb and R970-5 cells (non permissive for E1 mutants), as well as at 37° C in 293 cells for comparison. In addition, infections with ICP4 of R970-5 and Kb cells were done using different multiplicities of infection. The AdICP4 infected cells were harvested at several times post-infection in order to assess the kinetics of protein accumulation. The cells were processed by a quick-lysis method (see Methods) to avoid the degradation of ICP4 protein observed in human cells by Metzler and Wilcox (1985). Aliquots of lysates representing equal numbers of cells were separated on acrylamide gels, and subsequently transferred to

nitrocellulose and immunostained (Figures 9 and 10). In all cases results were compared to a standard lysate prepared from cells harvested at 6 hours after infection with 20 pfu/cell of wild type HSV-1 (Figures 9 and 10).

Figure 9 shows the results obtained from a Western blot of AdICP4 infected 293 cells, incubated at 34° C or 37° C and harvested as a function of time post infection. The 37° C infection showed slightly faster kinetics, as would be expected at higher temperatures. The levels of ICP4 approached that of the control HSV-1 infected cells at 16 to 20 hours post infection, then plateaued.

Figures 10A and 10B are results obtained from Western blots of KB and R970-5 cells infected with AdICP4 respectively. As in figure 9, the first lane contains the ICP4 protein produced from HSV-1 infection of the respective cells. The Western blots indicate that ICP4 is produced in both cell types, is first detected at about 12 hours post infection and continue to accumulate at least until 48 hours post infection. At late times, ICP4 has accumulated to levels comparable to those of HSV-1 infections. The effect of m.o.i. was simply to elevate the levels of ICP4 at each time point due to an increase in the template number.

Therefore, ICP4 is produced in all three cell types, and adequate levels are produced if the

Fig. 9. Expression of ICP4 in human 293 cells infected with AdICP4. 293 cells were infected with 25 pfu/cell at two different incubation temperatures, 34° C and 37° C. Lysates were prepared by the rapid lysis method of Metzler and Wilcox (1985), and amounts representing equivalent cell numbers were electrophoresed and immunoblotted. Results are compared to a standard immunoblot of cells infected with wild type HSV-1 (KOS) at an m.o.i. of 20 and harvested at 6 hours post infection.

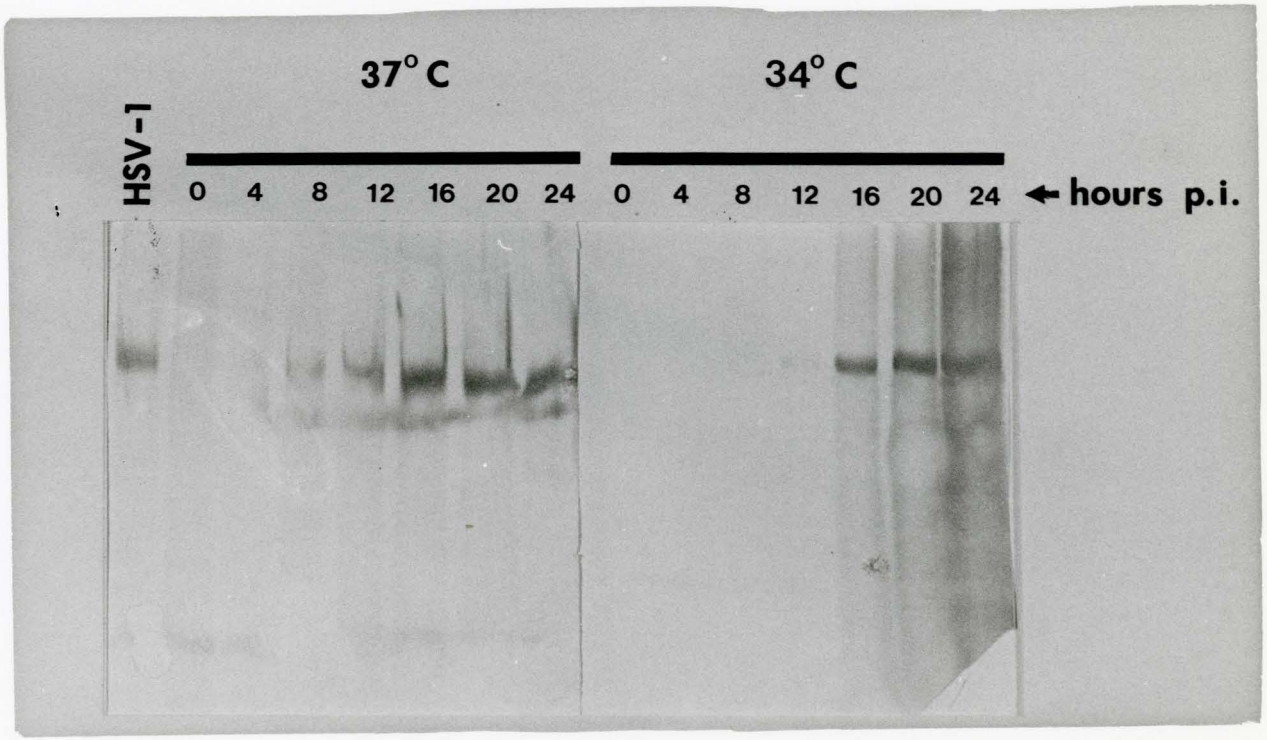
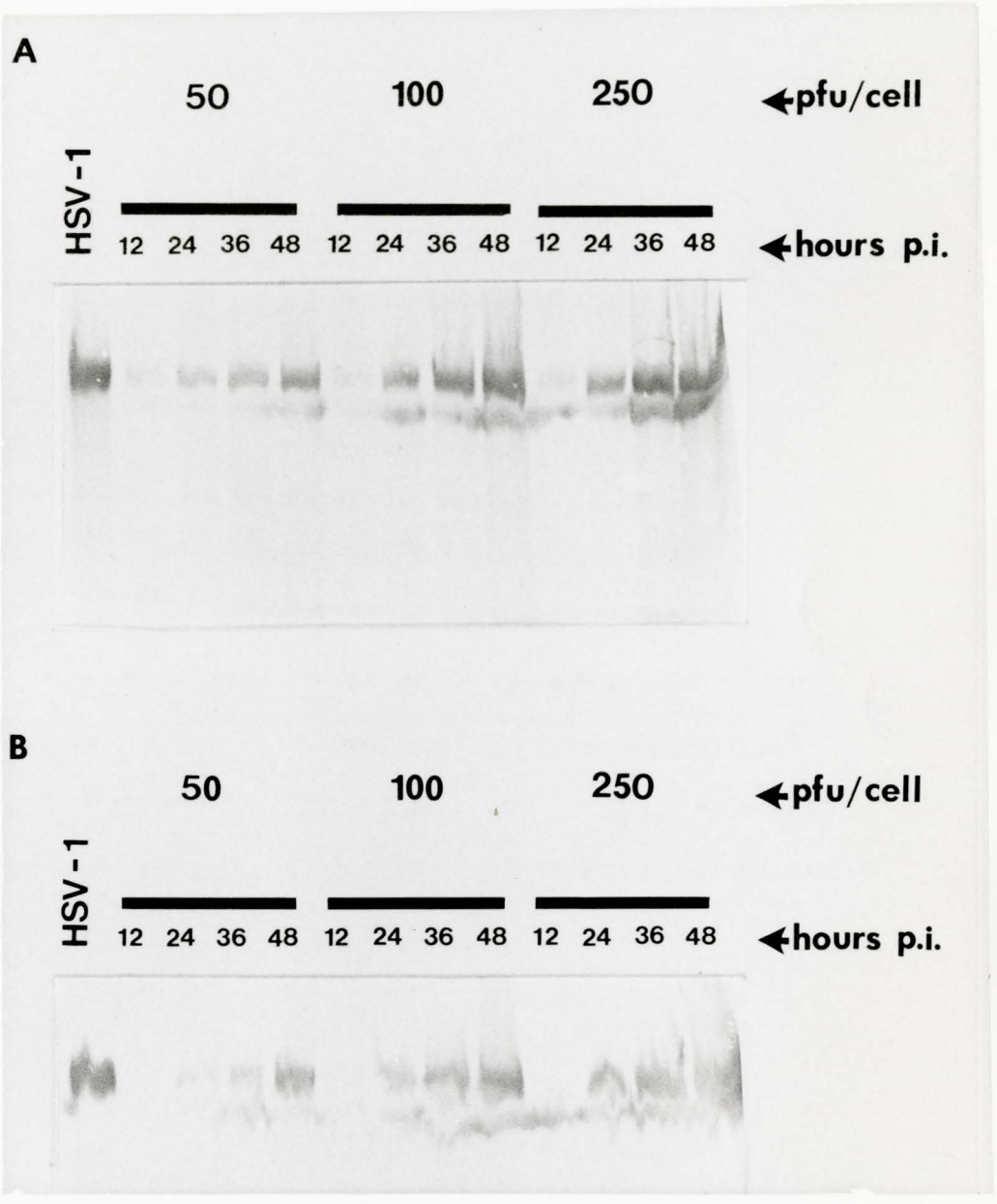

37° C
34° C
HSV-1
0 4 8 12 16 20 24
0 4 8 12 16 20 24
← hours p.i.

Fig. 10. Expression of ICP4 in KB and R970-5 cells.
Expression of ICP4 in human KB (panel A) and R970-5 (panel B) was assessed by infection of cells with AdICP4 at 50 pfu/cell, 100 pfu/cell and 250 pfu/cell as indicated; cells were harvested at 12, 24, 36 and 48 hours post infection as indicated. Lysates were prepared by the rapid lysis method of Metzler and Wilcox (1985), and amounts representing equivalent cell numbers were electrophoresed and immunoblotted. Results are compared to a standard immunoblot of cells infected with wild type HSV-1 (KOS) at an m.o.i. of 20 and harvested at 6 hours post infection.

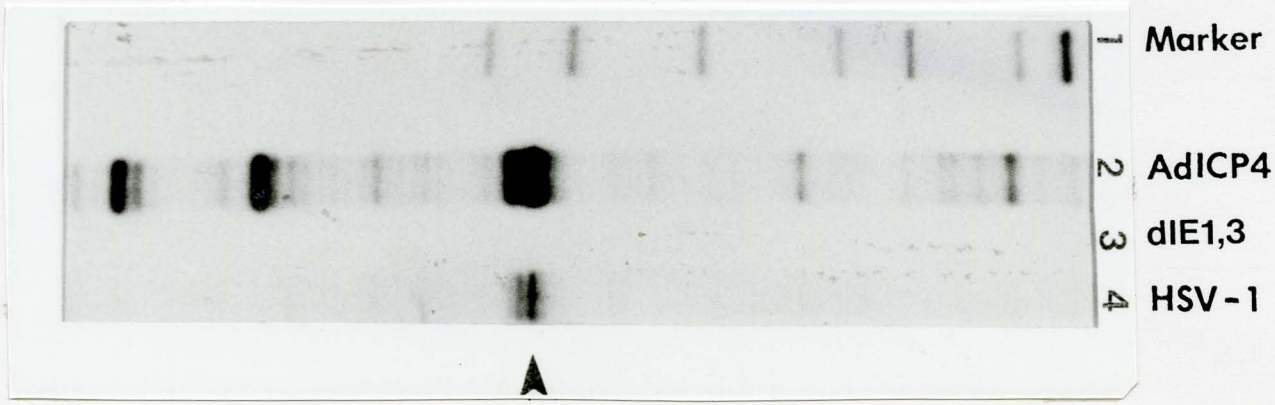

A
50
100
250
pfu/cell
HSV-1
12 24 36 48 12 24 36 48 12 24 36 48
hours p.i.
B
50
100
250
pfu/cell
HSV-1
12 24 36 48 12 24 36 48 12 24 36 48
hours p.i.

appropriate time post infection is allowed for accumulation.

Mapping The Start Site of The ICP4 Transcript

The construction of AdICP4 resulted in all of the E1a promoter, less the TATA box, remaining upstream of the ICP4 promoter. Therefore it was of interest to see which promoter was being used to drive the transcription of ICP4. This was assessed by extending a primer complementary to a region stretching from +52 to +77 relative to the ICP4 mRNA CAP site with reverse transcriptase, followed by electrophoresis of the resultant DNA fragments on a denaturing urea-acrylamide gel (Figure 11, lane 2). The result is compared to similar primer extensions using mRNA extracted from HSV-1 infected cells (lane 4) as well as from cells infected with Ad5 dlE1,3, which has the E1 and E3 deletions, but does not carry the ICP4 gene (lane 3). Bands corresponding to extension products 74 to 75 nucleotides in length were apparent from both HSV and AdICP4 infected cell extracts. The ICP4 transcript is made from multiple start sites therefore extension products should range from 73 to 77 nucleotides (Murchie and McGeoch, 1982). The majority of the transcriptional start sites in the AdICP4 derived messages are identical to those of HSV-1 infection (lanes 2 and 4, see arrow).

Fig. 11. Localization of 5' CAP site of ICP4 message. The location of the CAP site produced on the mRNA of ICP4 transcribed from AdICP4 was assessed by conducting a primer extention in the 3' direction. The primer was 25 nt long representing the complement to a region located +53 to +77 relative to the wild type CAP site from HSV-1 infections. The primer was end labelled with $^{32}P\gamma ATP$, hybridized to whole RNA isolates from AdICP4 infected (lane 2) and Ad5 dlE1,3 infected 293 cells (lane 3), and HSV-1 infected VERO cells (lane 4) as marker. Lane 1 represents DNA fragments obtained from a HpaII restriction endonuclease digest of the plasmid pBR322. The marker fragments from largest to smallest are as follows: 182, 162, 124, 112, 92, 78 and 69 nucleotides respectively.


1
Marker
2
AdICP4
3
dIE1,3
4
HSV-1

However, several DNA fragments of shorter length than the one representing the major transcriptional start site appear in lane 2, probably representing pause sites where the reverse transcriptase may have fallen off of the template. There is no indication that the E1a promoter is being used since the expected fragment size would be approximately 160 nucleotides in length, and no such product is evident in lane 2.

Complementation of HSV-1 ICP4 Mutant Virus

In order to ensure that the ICP4 produced from the viral recombinant was functional, the ability of AdICP4 to complement an ICP4 deletion mutant strain of HSV-1, d120 (DeLuca et al., 1985), was assessed. Infections were carried out in human R970-5 cells by first infecting with AdICP4 and incubating at 34° C or 39.5° C, followed by superinfection with d120 or wild type HSV-1 (Table 1). Control infections involved using Ad5 dlE1,3 followed by d120 or wt HSV-1, or simply infecting with d120 alone. Cells were harvested when cytopathic effect seemed complete (36 to 48 hours post-superinfection for HSV, 48 to 72 hours for AdICP4 plus d120) or at 48 to 72 hours for those cells not exhibiting any cytopathic effect. The progenies were titred by plaque assay on E5 cells, which are monkey cells constitutively expressing ICP4 (DeLuca et al.,

1985). Since these cells are not permissive for Ad5, progeny can only result from d120 complemented by ICP4 from AdICP4 or HSV-1. In all cases, the AdICP4/d120 infected cells required a longer infection period to achieve full cytopathic effect; also the cytopathic effect eventually observed was different from that of HSV infected cells. Characteristic HSV infection results in well rounded cells, looking like balloons attached to the bottom of the dish. The AdICP4/d120 infected cells appeared unhealthy but exhibited a different morphology than HSV-1 infected cells. The results in table 1 indicate that virus yield from AdICP4/d120 infections were much lower than from HSV-1 infections, ranging from circa 0.5% (from the coinfection of d120 and AdICP4 at 20 pfu/cell) to between 5 and 10% (100 pfu/cell of AdICP4 followed by d120 after a 24 hour interval) of HSV-1 infected cell yield. Also, the yields from AdICP4 plus d120 infected cells did not greatly exceed the initial d120 virus input; the increase ranged from 1.65 to 45.7 fold. However, the yield resulting from dlE1,3 plus d120 was substantially less than the initial viral input, being 100 or more fold lower (virus yield being less than 0.01 pfu/cell). In addition, the cells infected with AdICP4 plus d120, and incubated at 39.5° C did not produce a higher virus yield than the negative controls, dlE1,3

plus d120 infected cells. However, cells infected with HSV-1 under any conditions were capable of producing a high virus yield, even at 39.5° C (286 pfu/cell). Therefore, the results indicate that prior infection by AdICP4 results in complementation of d120 in a temperature dependant manner. The presence of an adenoviral vector (Ad5 dlE1,3) did not interfere with HSV-1 (KOS) replication, and temperature also had little effect on final titre. The trend observed in table I suggests that the yields of d120 were dependant on the level of ICP4 as indicated by the dependance on the amount of AdICP4 used in the initial infection or the time interval before superinfecting with d120. These results indicate that ICP4 levels may be rate limiting for the replication of d120.

Replication in 293 Cells at Permissive Temperature

The observations of Trembley et al. (1985) indicated that inhibition of expression of the 72K gene occurs in the presence of both functional ICP4 and E1a products. We assessed if this inhibition was extended to viral replication in human cells. This was accomplished by titering AdICP4 on the E1 expressor cells, 293, at the permissive and non permissive temperatures, and comparing the results to those of an infection by the Ad5 E1 deletion mutant, dlE1,3 which is known to replicate in these cells (Table 2). The

Table I. Complementation of the HSV-1 d120 mutant by AdICP4.

V_1 (m.o.i.)		V_2 (hr p.i.)		Virus yield	
				34° C	39.5° C
AdICP4	(20)	d120	(0)	1.65	0.02
AdICP4	(20)	KOS	(0)	360.0	ND
AdICP4	(20)	d120	(16)	3.9	0.03
AdICP4	(20)	KOS	(16)	645.0	ND
AdICP4	(20)	d120	(24)	17.6	0.03
AdICP4	(20)	KOS	(24)	675.0	ND
AdICP4	(100)	d120	(24)	45.7	ND
dlE1,3	(50)	d120	(20)	0.01	0.006
dlE1,3	(50)	KOS	(20)	284.0	286.0
mock	--	d120	--	0.02	ND

TABLE I. Complementation of the HSV-1 d120 mutant by AdICP4. R970-5 cells were first infected with an Ad5 virus at various m.o.i.'s, incubated at 34° C, and were subsequently superinfected with 1 pfu/cell of a second virus, V_2, at the times indicated. Following infection, the incubation temperatures were maintained at 34° C or shifted to 39.5° C, as indicated under virus yield. Resultant progeny were titred on E5 cells and are expressed as the amount of infectious HSV-1 (in pfu) recovered per infected cells. Note: ND = not done.

results clearly indicate that AdICP4 titers as well as dlE1,3 at all indicated temperatures. The only significant observation is that both viruses grow at a more rapid rate at higher temperatures. These experiments reveal that the AdICP4 recombinant could be efficiently propagated at 34° C in 293 cells, indicating that the presence of functional ICP4 and E1a was not detrimental to virus viability.

Complementation of Ad5 E1a Functions

The ability of ICP4 to complement E1a functions was assessed at several different levels: production of infectious virus, viral DNA replication, and the activation of adenoviral genes usually under the control of E1a functions.

i) In order to test for the production of infectious virus, the AdICP4 recombinant had to be supplemented with an E1b coding region, which was lacking in the recombinant. This was accomplished by coinfecting AdICP4 with the Ad5 deletion mutant dl312, which has a deletion in the E1a gene but carries the entire E1b gene. Infections with the wild type Ad5, dl312 alone and AdICP4 alone were carried out as controls. Another viral recombinant, AdICP0, with the HSV-1 ICP0 gene substituting for the E1a region was provided by Saul Silverstein and was also included in

Table II. Replication of AdICP4 in 293 Cells.

Viruses	Incubation Temperature	Virus yield
dlE1,3	34° C	4.38×10^1
AdICP4	34° C	1.25×10^2
dlE1,3	37° C	3.13×10^2
AdICP4	37° C	3.38×10^2
dlE1,3	39.5° C	3.13×10^2
AdICP4	39.5° C	2.75×10^2

TABLE II. Replication of AdICP4 in 293 cells at different temperatures. 293 cells were infected with either AdICP4 or Ad5dlE1,3 at 25 pfu/cell, incubated at 34° C, 37° C or 39.5° C and harvested at 48 hours p.i. Resultant progeny were titred on 293 cells and expressed as the amount of infectious virus recovered per cell.

this study. Cells infected by these various viruses were harvested and titred on 293 cells (Table 3). In the case of wild type infections, cytopathic effect was fully developed after two (KB) or four (R970-5) days, at which time these cultures were harvested. The AdICP4, AdICP0, dl312, AdICP4/dl312 and AdICP4/AdICP0/dl312 infected samples did not show a typical cytopathic effect at these times and therefore infections were allowed to proceed for one more day. Cells were then harvested and virus was titred on 293 cells. The results in table 3 indicate that infections with any of the E1a deleted viruses yielded a similar quantity of progeny, and this yield was substantially less than that of wt Ad5 infected cells. Therefore, neither ICP0, ICP4 nor a combination of both was capable of complementing the E1a deletion in terms of viral replication. Of interest is the observation that some progeny of AdICP4 and dl312 were produced in the KB cells, albeit at very low levels, an observation that was not made with the R970-5 cells. This observation supports those of several other investigators regarding the difference in permissiveness for E1a mutants in different cell lines (Nevins, 1981; Imperiale et al., 1984; LaThangue and Rigby, 1987).

ii) AdICP4 viral DNA replication was assessed by following the procedures of the previous experiment

Table III. Replication of AdICP4, AdICP0 and dl312

Cells	Viruses	hr p.i.	Virus yield
R970-5	Ad5	96	2.3×10^4
R970-5	AdICP4	120	≤0.1
R970-5	dl312	120	≤0.1
R970-5	AdICP4 + dl312	120	≤0.1
R970-5	AdICP0	120	≤0.1
R970-5	AdICP0 + AdICP4 + dl312	120	≤0.1
KB	Ad5	48	2.1×10^4
KB	AdICP4	72	2.5×10^1
KB	dl312	72	9.3×10^1
KB	AdICP4 + dl312	72	2.1×10^2

TABLE 3. Replication of AdICP4, AdICP0 and dl312 in non-expressor human cells. R970-5 or KB cells were infected at 34° C with 20 pfu/cell of wild type Ad5, dl312 or AdICP4, 30 pfu/cell of AdICP0, and 10 pfu/cell each of AdICP4 and dl312 or AdICP4, AdICP0 and dl312 in the cells indicated. Infected cells were harvested at the times indicated representing either complete cytopathic effects (wild type infections) or a further 24 hour incubation (for viral mutants). The resultant viruses were plaque assayed on 293 cells and yields are expressed as infectious virus recovered per infected cell.

up to the point of harvest of infected cells. Instead of harvesting the cells immediately, they were cultured for an additional four hours in medium containing ^{32}P-orthophosphate, which would result in the labelled phosphate being incorporated into replicating DNA. The DNA from infected cells was subsequently isolated, digested with HindIII and electrophoresed. An autoradiogram of the results is presented in figure 12. Lanes 1 and 5, containing DNA from infected KB and R970-5 cells respectively, show the typical pattern of HindIII digested wild-type Ad5 DNA. Lanes 2, 3 and 4, containing DNA from AdICP4, dl312 and AdICP4 plus dl312 infected KB cells, indicate the presence of a small amount of viral DNA. In contrast to this, lanes 6, 7 and 8 containing DNA extracted from R970-5 cells infected in the same manner as the KB cells do not contain viral DNA. These results indicate that the ICP4 function is not capable of substituting for E1a even to the point of DNA synthesis. The results paralleled those of infectious virus production in that no DNA replication beyond that of control virus was observed for the experimental samples. Only R970-5 cells were used from this point onwards due to the detected permissiveness of KB cells to infection by E1a defective mutants. Kevin Inchley of our lab confirmed that viral DNA replication is not activated by ICP4, using Southern

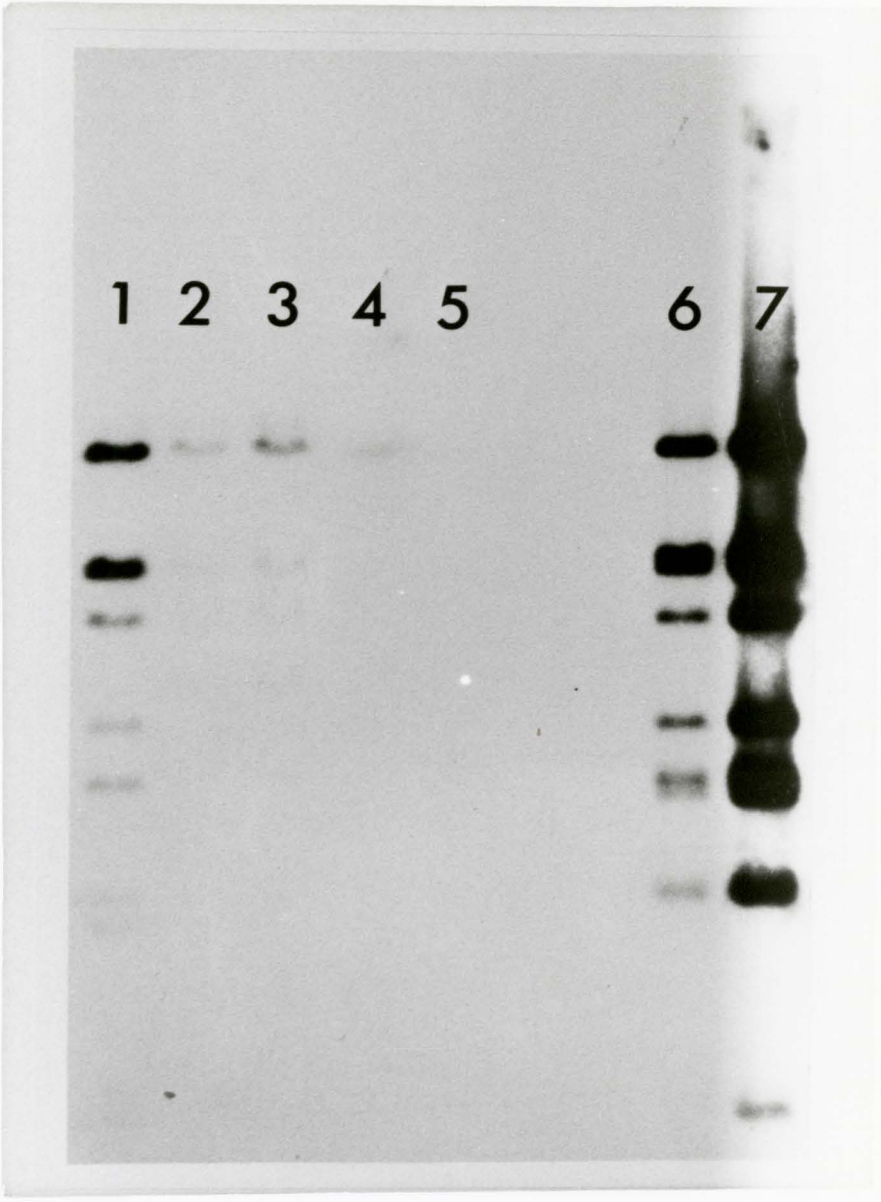
blotting for the detection of viral DNA. R970-5 cells were infected with Ad5 (100 pfu/cell), AdICP4 (100 or 200 pfu/cell), dl312 (100 or 200 pfu/cell) or AdICP4 plus dl312 (100 pfu/cell each). All cells were harvested at 48 hours post-infection, DNA was extracted, digested with HindIII, then electrophoresed. 5 ug of total DNA was loaded for all samples except from Ad5 infected cells which had only 0.1 ug. The DNA was then transferred from the gel to nitrocellulose, and probed with Ad5 DNA nick-translated with α^{32}PdCTP. An autoradiogram of the Southern blot is presented in figure 13. The bands were scanned with a densitometer in order to establish the relative quantities of DNA from sample to sample. The results of these scans are as follows: relative to the quantity of DNA from the Ad5 infected cell sample, the DNA from cells infected with AdICP4 plus dl312 was 0.55%, with AdICP4 was 0.29% and with dl312 was only 0.06%. The banding pattern of the HindIII digested DNA from AdICP4 plus dl312 infected cells indicates that DNA from both viruses is present. According to these results, Ad5 produces several hundred fold more viral DNA that AdICP4, dl312 or a combination of the two. Therefore ICP4 does not appear to aid in the replication of adenovirus lacking E1a.

iii) The final set of experiments were aimed at assessing to what extent ICP4 could substitute for E1a

Fig. 12. Viral DNA replication. R970-5 or KB cells were infected at 34° C with 20 pfu/cell of wild type Ad5, AdICP4 or dl312, or 10 pfu/cell each of AdICP4 and dl312. The cells were labelled with ^{32}P-orthophosphate for the final 4 hours of infection. Cells infected with wild type Ad5 showed fully developed cytopathic effect after two (KB) or four (R970-5) days, at which time these cultures were harvested. The AdICP4, dl312, and AdICP4/dl312 infected samples did not show a typical cytopathic effect at these times and therefore infections were allowed to proceed for one more day. DNA was extracted from infected cells and analysed by digesting with the restriction endonuclease HindIII and separating the resultant fragments on an agarose gel. The gel was then dried by vacuum and exposed to X-ray film.

Kb
R970-5
wt
AdICP4
dl312
AdICP4+dl312
wt
AdICP4
dl312
AdICP4 + dl312
1
2
3
4
5
6
7
8

Figure 13. Viral DNA Replication. R970-5 cells were infected as follows: 100 pfu/cell each of AdICP4 and dl312 (lane 1); 100 or 200 pfu/cell of AdICP4 (lanes 2 and 3); 100 or 200 pfu/cell of dl312 (lanes 4 and 5); 100 pfu/cell of wild type Ad5 (lanes 6 and 7). The cells were incubated at 34° C for 48 hours, at which time cells were harvested. DNA was extracted from cell lysates as indicated in Methods, digested with the restriction endonuclease HindIII, and separated by electrophoresis on an agarose gel. The amounts of DNA electrophoresed were as follows: 5 ug in lanes 1 to 5; 0.1 ug in lane 6 and 1.0 ug in lane 7. DNA was then transferred to nitrocellulose and hybridized with ^{32}P *in vitro* labelled Ad5 genomic DNA. Autoradiography was for 5 hours. (The experiment was conducted by Kevin Inchley).


1 2 3 4 5 6 7

Mw x10−3
34°
39.5°
Ad ICP4
d1312
Ad ICP4
d1312
wt
93
69
46

in the activation of Ad5 genes. Two target genes were selected for immunoprecipitation experiments, the one coding for the 72kDa DNA binding protein and that coding for the E1b, 19kDa protein. For detection of the 72 kDa protein, cells were first infected with 10 pfu/cell of AdICP4 or 30 pfu/cell of dl312, and incubated at 34° C for a period of 16 hours to allow for the accumulation of the ICP4 protein. At this time, the infected cultures were maintained at 34° C or shifted to 39.5° C for an additional 20 hours. Wild type Ad5 infections were for only a 20 hour period, since this was sufficient to accumulate the 72K protein. Cells were labelled with ^{35}S-methionine for the final four hours of infection, then lysed in RIPA buffer and imunoprecipitated with the H2-19 monoclonal antibody specific for 72K protein. The samples were then electrophoresed on an SDS-acrylamide gel, and the resultant gel was autoradiographed (Figure 14). The 72K product is readily detected by immunoprecipitation of lysates from cells infected with Ad5. Cells infected with AdICP4 and incubated at 34° C also produced a strong band corresponding to the 72K protein, and the intensity of this band is comparable to that obtained from wild type Ad5 infected cells. Neither of the controls, cells infected with dl312 and incubated at 34° C or 39.5° C, nor the AdICP4 infected cells incubated at

39.5° C displayed a significant amount of the 72K protein. These results indicate, in agreement with the studies by Trembley et al. (1985), that tsICP4 is capable of activating the production of the 72K protein, and does so in a temperature dependant and efficient manner.

A similar protocol was followed to assess the effect of ICP4 on the production of the E1b 19kDa protein. Since the E1b region was deleted from the AdICP4 construct, dl312 was included in the infections with AdICP4 in order to supply the E1b gene. R970-5 cells were first infected with 10 pfu/cell of AdICP4 and incubated at 34° C for 16 hours. At this time, cells were superinfected with 10 pfu/cell of dl312 and maintained at 34° C or shifted to 39.5° C for an additional 36 hours. Cells infected with wild-type Ad5 were only incubated for a 36 hour period. In addition, to assess the amount of 19kDa protein produced from a non-replicating template, cells were also infected with the Ad5 mutant, ts125. The genome of this virus replicates only at the permissive temperature, 34° C. Labelling with ^{35}S-methionine was done for the final 6 hours of infection. Cells were then harvested, lysed and the 19kDa protein was immunoprecipitated with the 19-C1 antipeptide serum specific for the E1b 19kDa protein and finally electrophoresed on an SDS-acrylamide

gel. An autoradiogram of the results is given in figure 15. A strong band representing the 19kDa protein is evident from lysates of cells infected with Ad5 at both 34° C and 39.5° C. Corresponding bands in the samples from AdICP4/dl312 infected cells are barely detectable. It is difficult to say with certainty if these bands represent the 19kDa protein, but if they do they appear to be of approximately equal intensity at both temperatures indicating that their production is independant of ICP4. An Ad5 infection for 36 hours results in the replication of Ad5 DNA, therefore increasing the template number of E1b genes. In order to assess the quantity of 19 kDa protein produced from a replicating versus that of a non-replicating viral genome (AdICP4), cells were also infected with the Ad5 mutant ts125. In this case, the band representing the 19kDa protein was evident in lysates from cells incubated at the permissive temperature, 34° C, but not at the non-permissive temperature, 39.5° C. Taken together, these results do not give a clear indication of what effect ICP4 has on the production of the 19kDa protein. What is clear is that an easily detectable band can be obtained from lysates of cells infected with Ad5 if the length of infection permits the increase in template number by genomic replication. If the template number is not increased, as for ts125 infected cells

Fig. 14. Activation of expression of the Ad5 early 72 kDa protein by ICP4. R970-5 cells were mock infected or infected with 10 pfu/cell of AdICP4 or 30 pfu/cell with dl312 and incubated at 34° C. After 16 hours, the mock cells were infected with 10 pfu/cell of wild type Ad5. The various infections were then maintained at 34° C or shifted to 39.5° C for an additional 20 hours, of which the last 4 involved labelling with ^{35}S-methionine.
Immunoprecipitations were carried out with monoclonal antibody H2-19 against the 72K protein (arrow) and the gel was exposed for 3 days.

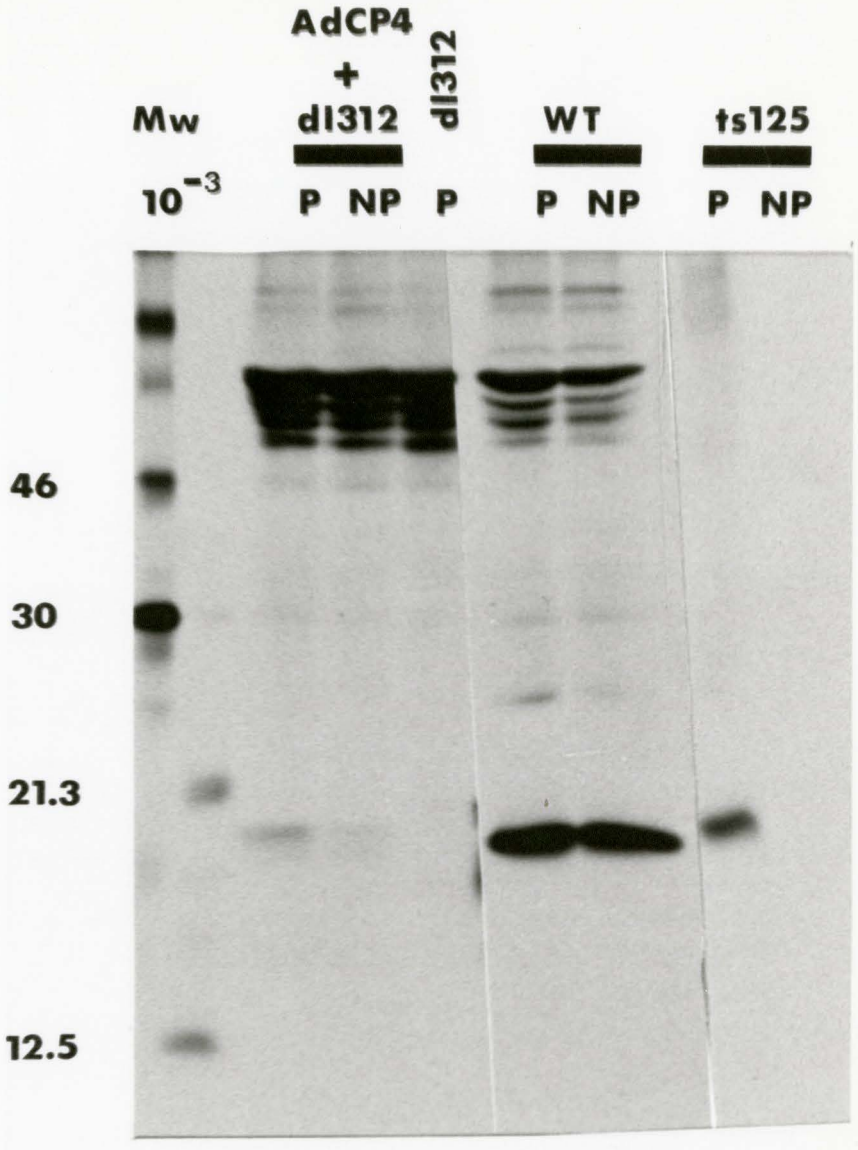
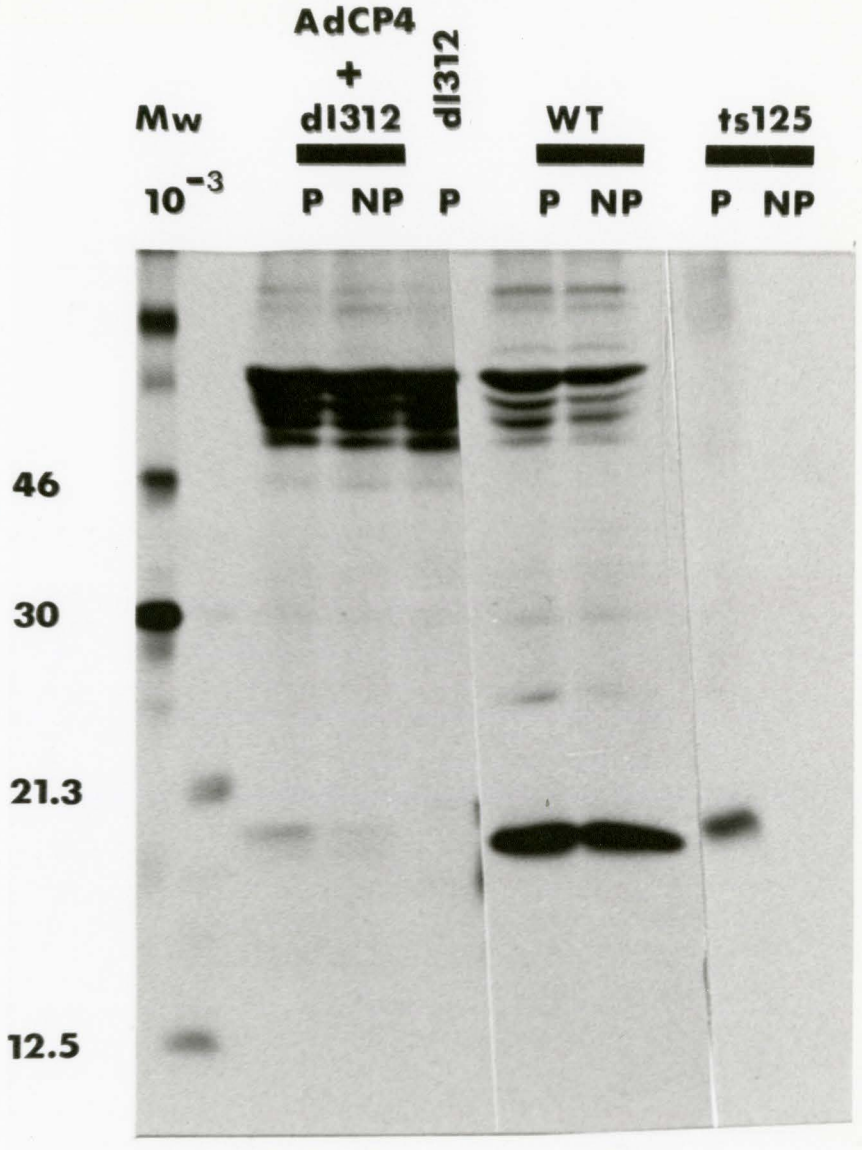

AdCP4
+
dl312
dl312
WT
ts125
Mw
10^-3
P NP P P NP P NP
46
30
21.3
12.5

Fig. 15. Activation of expression of the the Ad5 early E1b 19 kDa protein by ICP4.
R970-5 cells were mock infected or infected with 10 pfu/cell of AdICP4 and incubated at 34° C (permissive termperature, P). After 16 hours, the mock cells were infected with 10 pfu/cell of wild type Ad5, the Ad5 temperature sensitive mutant ts125, or dl312, and the cells previously infected with AdICP4 were superinfected with 10 pfu/cell of dl312. Infections were then continued at 34° C or shifted to 39.5° C (non-permissive temperature, NP) for an additional 36 hours, of which the last 6 involved labelling with ^{35}S-methionine. Immunoprecipitations were carried out with the 19-C1 antipeptide serum specific for the E1b 19K protein and the gel was exposed for 6 days.


AdCP4
+
dl312
dl312
WT
ts125
Mw
10^-3
P NP P P NP P NP
46
30
21.3
12.5

incubated at 39.5° C, then the quantity of 19kDa protein that can be immunoprecipitated is very small. Since the dl312 genome does not replicate in the absence of E1a products, the cells infected with AdICP4 plus dl312 would not produce a quantity of 19kDa protein sufficient for detection by this method, even if ICP4 was affecting the levels of the 19kDa protein.

Subsequent experiments were aimed at determining the level of activation of Ad5 early genes by ICP4 at the level of transcription. E1b was not assessed in these experiments since the gene is not present in AdICP4.

Oligonucleotide primers representing regions just downstream of the CAP sites for E2A-E (+148 to +167), E3 (+47 to +71) and E4 (+102 to +126) messages were synthesized by the Central Facility of the Institute of Molecular Biology and Biotechnology, McMaster University, for use in primer extension analysis of RNA extracted from R970-5 cells infected with Ad5, dl312, or AdICP4. Cells were infected with virus at an m.o.i. of 100 pfu/cell and incubated at either 34° C or 39.5° C for 24 hours in the presence of cytosine arabinoside in order to prevent the viral templates from replicating. Since it has been determined that the AdICP4 genome does not replicate in R970-5 cells, it was desirable to obtain messenger

transcribed from non-replicated wild-type Ad5 DNA as a proper positive control. After the 24 hour incubation, cells were harvested and RNA was extracted as indicated in methods and materials. RNA was then subjected to the primer-extension procedure. The results indicate that extension products of RNA preparations from Ad5 infected cells are produced from all three primers as seen in figures 16 (experiment by Silvia Bacchetti) and 17. The E2A-E primer, which hybridizes to message encoding the 72K protein, produces an extension product approximately 167 nucleotides long, as well as one of approximately 75 nucleotides (Fig. 16). This second product may be due to an incompletion of the extension due to a pause of reverse transcriptase at a site susceptible to this event. The extension products appear for both Ad5 infected sample (lane 1) and the AdICP4 infected sample which was incubated at 34° C (lane 3), but not the one incubated at 39.5° C (lane 4) or dl312 infected sample (lane 2). The intensities of the bands for the wild type and AdICP4 are not too dissimilar indicating that AdICP4 is producing an appreciable quantity of ICP4 message relative to the wild type infection. The E3 and E4 primers produce extensions of approximately 71 nucleotides and 120 to 126 nucleotides respectively. Extension products of proper length were obtained from RNA extracted from Ad5 infected cells (Fig. 17, lanes

1). A small quantity of extension products was also obtained from AdICP4 infected cells, but this is not attributed to the action of ICP4 since the products appear at both the permissive and non-permissive temperatures for ICP4 (lanes 4 and 5).

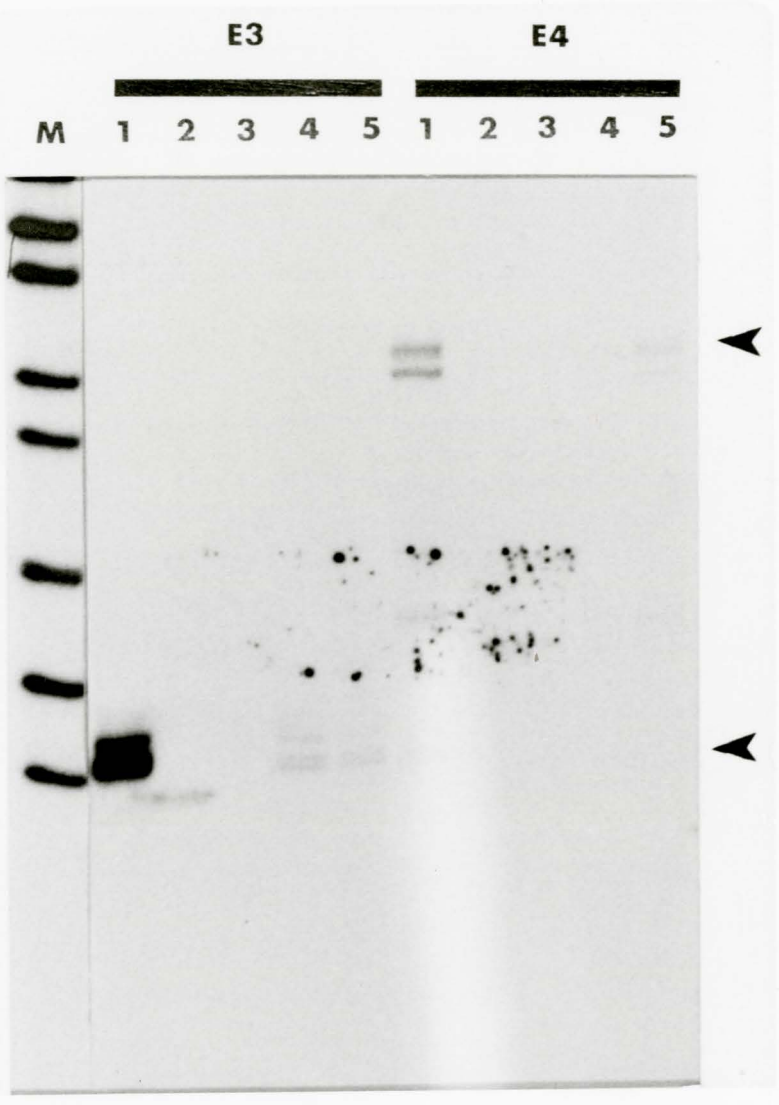
These results indicate that the presence of ICP4 does increase the level of 72K gene expression, and does so by acting at the level of transcription. Even though primer extension analysis may not be very quantitative, the amount of E2A-E message produced from AdICP4 infected cells appears to be only three or four fold less than the amount produced from wild-type infection. The result obtained by immunoprecipitation of 72K from cell lysates infected with AdICP4 and Ad5 (figure 14) indicated that there was not a great deal of difference in the intensities of the bands produced from wt and AdICP4 infected cells. However, ICP4 does not appear capable of activating the E3 or E4 promoters, at least not to the same extent as for the E2A-E promoter.

In conclusion, we have demonstrated that tsICP4 has the ability of stimulating the production of the Ad5 early gene product, 72K, and it does so in a temperature dependant manner. The activation occurs at the level of transcription. This effect seems to be unique to the E2A-E promoter under the conditions tested in this study.

Figure 16. Activation of E2 transcription in response to ICP4. R970-5 cells were infected with 100 pfu/cell each of Ad5 (lane 1), Ad dl312 (lane 2), or AdICP4 at 34° C (lane 3) or 39.5° C (lane 4) in the presence of cytosine arabinoside (20ug/mL). RNA was extracted from all cultures at 24 hours post infection and primer extension analysis was performed as outlined in Methods using an oligonucleotide complementary to the E2 message from nucleotides 5 to 24 downstream from the 3' splice acceptor site (Kruijer et al., 1981). Autoradiography was overnight. (The experiment was conducted by Silvia Bacchetti and Thomas Hupel).


E2a
1 2 3 4

Figure 17. Activation of E3 and E4 transcription in response to ICP4. R970-5 cells were infected with 100 pfu/cell each of Ad5 (lanes 1), Ad dl312 at 34° C (lanes 2) or 39.5° C (lanes 3), or AdICP4 at 34° C (lanes 4) or 39.5° C (lanes 5) in the presence of cytosine arabinoside (20 ug/mL). RNA was extracted from all cultures at 24 hours post infection and primer extension analysis was performed as outlined in Methods using oligonucleotides complementary to nucleotides 47 to 71 downstream of the E3 message cap site (Sussenbach, 1984) and nucleotides 29 to 53 downstream of the 3' splice acceptor site of the E4 message (Tigges and Raskas, 1984). The plasmid pBR322 digested with HpaII and labelled by nick-translation with ^{32}P-dCTP was used as a size marker to determine the size of the primer extension products for E3 (71 nt) and E4 (120-126 nt). The marker fragments from largest to smallest are as follows: 162, 149, 124, 112, 92, 78 and 69 nucleotides respectively. Autoradiography was overnight.


E3
E4
M
1
2
3
4
5
1
2
3
4
5

DISCUSSION

The activation of both cellular and viral genes requires the coordinated activity of many factors, possibly present in different abundancy, in order to achieve efficient production of message. Since all gene products are not required at all times, several different mechanisms might be involved in the differential expression of genes. In particular the activation of transcription of many viral genes requires the coordinated activity of both viral and cellular factors (Harlow et al., 1986; Kovesdi et al., 1987; Muller, 1987), of which the latter are utilized also in the transcriptional activation of the host genes. Therefore, viral transcriptional activation occurs via mechanisms involving the interaction of viral activator proteins with the resident cellular transcriptional machinery.

It has been shown that viral trans-activating proteins are capable of activating genes of heterologous viruses and of cells. Previous work had specifically shown that the HSV-1 ICP4 protein can activate the promoter of the E2a gene of adenovirus type 5 (Trembley et al., 1985). The purpose of the present study was to determine the extent to which the HSV transactivator, ICP4, could substitute for the Ad5 transactivator, E1a,

in the context of the adenovirus lytic cycle. To this end the ICP4 gene was introduced into an adenovirus genome lacking the E1 coding region. Using this recombinant virus, AdICP4, I obtained results indicating that ICP4 is capable of substituting E1a only in the activation of transcription of one adenovirus early gene, the E2a 72 kDa DNA binding protein. The remaining Ad5 early genes were not activated, nor was there any evidence of genomic replication under the conditions tested.

Properties of AdICP4

Substantial quantities of ICP4 protein were produced from infection of human R970-5, KB or 293 cells with the AdICP4 recombinant virus. However, the kinetics of ICP4 accumulation from infected 293 cells, which constitutively express the E1 functions, was significantly different from that obtained in infected R970-5 or KB cells. In AdICP4-infected 293 cells maintained at 34° C, the accumulation of ICP4 protein to levels comparable to those of HSV infected cells (harvested at 6 hours post-infection) required a length of infection of between 12 to 16 hours, at which time the levels of ICP4 plateaued (Figure 9). In contrast, production of ICP4 in R970-5 or KB cells was slower, and levels comparable to HSV infected cells were attained

with a length of infection in excess of 40 hours, with no apparent plateau occurring up to the longest infection time assayed, i.e., 48 hours (Figure 10).

The above differences in the kinetics of ICP4 production can be explained by considering that AdICP4 is capable of replicating only in 293 cells (E1 deletion mutants of Ad5 are capable of replicating with the same kinetics as wild-type Ad5 when they infect 293 cells). Replication of viral DNA during Ad5 infection of 293 cells starts at 7 to 9 hours post infection. As the AdICP4 genome replicates, the template number of the ICP4 gene increases. Assuming that each template produces ICP4 message at a similar rate, overall protein levels would increase with increasing template number. The plateau effect observed in ICP4 production is probably a reflection of the feedback auto-regulating ability of this protein (O'Hare and Hayward, 1985b; Godowski and Knipe, 1986). Infection of R970-5 or KB cells with AdICP4 does not result in a significant amount of viral DNA replication, therefore the template number is relatively stable. A plateau in ICP4 accumulation was not observed in these cells, at least up to 48 hours probably due to the slow rate of production of the ICP4 protein.

The observation that AdICP4 is capable of replicating in 293 cells at 34° C appears to conflict

with a previous study which showed that the presence of both functional ICP4 and E1a products result in an inhibition of expression of the 72 kDa protein (Trembley et al., 1985). However, the experimental conditions used in that study and in the present one differ significantly. Firstly the studies of Trembley et al. were conducted in mouse cells, while my study using AdICP4 was in human cells. Perhaps the observed inhibition of expression of the 72 kDa product is specific for mouse cells and is mediated by cellular factor(s) which are not present in human cells. In addition, 293 cells constitutively express the E1 functions, therefore there is a substantial quantity of E1a products present at the time of infection of these cells with AdICP4. Since the accumulation of ICP4 occurs slowly, expression of the viral genome can proceed utilizing the E1a products long before ICP4 accumulates to levels which might be sufficient for inhibition. In contrast, the work of Trembley et al., 1985, utilized cells (Z4) which constitutively expressed ICP4, thus this protein rather than the E1a products was present early in infection.

The HSV ICP4 deletion mutant d120 was capable of replicating in cells infected with AdICP4 and incubated at the permissive temperature (34° C), but not at the nonpermissive temperature (39.5° C). This demonstrates

that the ICP4 protein produced by AdICP4 is capable of complementing the ICP4 defect of d120. The virus yield however did not approach that obtained in wild-type HSV infected cells; in fact, even using 100 pfu/cell of AdICP4 and superinfecting with d120 after 24 hours, the yield of d120 obtained from AdICP4/d120 infected cells was less than 10% of that from HSV infected cells. A reason for the low level of complementation may have to do with the quantity of ICP4 protein available for transactivation. The Western blot data (figure 10B) indicates that at 24 hours post-infection, even using 100 pfu/cell of AdICP4 the level of ICP4 is only about 25% of that in HSV infected cells. This in itself does not explain the low level of complementation since the level of ICP4 protein in a cell line which constitutively expresses ICP4 (Persson et al., 1985) is about 5% of that produced in wild-type HSV infected cells. The infection of these cells with d120 results in a virus yield comparable to that of HSV infected cells (J.R. Smiley, personal communication). However a major difference between the ICP4 gene in the AdICP4 genome and the Z4 cells does exist. The cloning of the ICP4 gene into the adenovirus vector resulted in the removal of the most-distal regulatory region of the promoter which is responsible for ICP4 response to the HSV virion protein, $V_{MW}65$. The ICP4 gene contained in

Z4 cells possessed all of the upstream regulatory regions found in the wild-type HSV ICP4 gene, and therefore was able to respond to the virion protein $V_{MW}65$. Persson has demonstrated that infection of Z4 cells with d120 (which delivers the virion protein), results in a substantially higher rate of ICP4 protein production (Persson, 1987). Since AdICP4 neither contains the sequence required for response to $V_{MW}65$, nor has the capacity to replicate its genome in R970-5 cells, the level of ICP4 will not be elevated. Therefore, the net result is that the ICP4 protein produced from the AdICP4 virus is functional in terms of its role in the HSV life-cycle, however the amount of this protein appears limiting.

Although the amount of ICP4 expressed by AdICP4 was not sufficient to fully complement the ICP4 defect of d120, it could activate the expression of the Ad5 72 kDa protein. This activation occurred as efficiently as with the E1a products and the effect was temperature sensitive. The results of these experiments indicate that a 24 hour infection of R970-5 cells with AdICP4 at 10 pfu/cell is sufficient to produce a quantity of 72 kDa product similar to that found in wild-type Ad5 infection. This suggests that AdICP4 may be more potent in the transactivation of the E2A promoter than in complementing the ICP4 defect of d120 virus in terms of

viral replication.

Promoter Regions of ICP4 and E1a Target Genes

The results of my study indicate that ICP4 is capable of stimulating expression only from the E2 promoter, and not the E3 or E4 promoters of Ad5. The similarities or differences between promoter regions of different HSV-1 and Ad5 viral early genes may help in the understanding of why ICP4 is limited to transactivation of the E2A gene. The promoter most used as a model system for assessing transactivation by ICP4 in HSV is the thymidine kinase (tk) promoter. Although Persson et al. (1985) have shown that not all HSV early genes are transactivated by ICP4, the tk gene proved to be a target of ICP4 activation. Upstream regulatory sequences found to be important for promoter function for the tk gene are the TATA box, CAAT boxes and GC rich regions. It has been determined that a defect in any of these regulatory sequences results in a decrease in transcription from this gene at both the basal level, and in terms of susceptibility to ICP4 activation (Everett, 1984b). However, defects in the TATA box have a much more pronounced effect on the susceptibility of the promoter to activation by ICP4 (Coen et al., 1986).

The Ad5 early promoter which most closely resembles the tk promoter is that of the E1b gene. It

also has a TATA box, CAAT boxes and CG rich regions. However, it was not possible based upon the results of my study to determine whether or not ICP4 had any effect on the E1b promoter. Conditions were such that the system was not sufficiently sensitive to accurately assess the levels of an E1b product, the 19 kDa protein, in wild type Ad5 infections. Therefore, it was not possible to determine if the negative result for 19 kDa protein production from AdICP4/dl312 infected cells was truly negative, or the induced levels were below the lowest detectable level. Even though the effect of ICP4 on the E1b promoter was not elucidated in this study, it is important to discuss its promoter region. It has been determined that the TATA box is the critical sequence for activation by E1a products (Wu et al., 1987). In addition, the TATA box is also the critical sequence for response of the major late promoter of adenovirus to E1a transactivation (Leong et al., 1988). E1a products activate these promoters by increasing the activity of the TATA-box binding factor (Abmayr et al., 1988) and do so by increasing the activity of this factor and not merely by increasing its abundance (Abmayr et al., 1985; Spangler et al., 1987). This results in an increase in the number of templates being transcribed (Leong and Berk, 1986).

HSV late genes which are activated by ICP4 have

only a TATA box as an element of their promoter (Johnson and Everett, 1986; Homa et al., 1986). However, these genes are not activated until viral replication has occurred (Johnson and Everett, 1986). This implies that ICP4 activation of promoters composed only of a TATA box may not be very efficient, whereas the activation of this same type of promoter by E1a appears very efficient. Therefore, ICP4 may not fully complement the E1a defect due to its inability to efficiently activate promoters which respond mainly to the activity of TATA-box binding factors.

The promoter regions of the E2A gene are quite complex and have been described in the introduction. The unique sequences present in the E2A promoter in comparison to the other early promoters are the upstream imperfect inverted repeats. Similar inverted repeats have been found in the upstream regulatory regions of HTLV-I and HTLV-II promoters (Chen et al., 1985). Studies have indicated that sequences upstream of the TATA box are important for stimulation by E1a products (Kovesdi et al., 1986), and that these sequences respond to the action of the cellular transcription factor, E2F (Kovesdi et al., 1986; SivaRamen et al., 1986; Reichel et al., 1988). The 13S product of E1a induces transcription from the E2A promoter via the E2F factor by increasing the amount of E2F in infected cells

(Reichel et al., 1988). The kinetics of E2F appearance parallel that of the 72 kDa protein in that a rapid increase in E2F production starts at 5 hours post infection and peaks at 7-8 hours post infection (Reichel et al., 1988). The E2F factor is part of a protein-DNA complex which forms on the E2A promoter upon Ad5 infection; the length of time required for the formation of this complex, which can occur in the absence of E1a products, is decreased in the presence of 13S products (Kovesdi et al., 1987). It has also been determined that binding of E2F to Ad5 DNA is unique to the E2A promoter, and that this factor does not associate with the E1b, E3, E4 and the Ad5 major late promoter (Kovesdi et al., 1987). Perhaps the ICP4 protein also interacts with a transcriptional regulatory pathway in which the E2F factor operates. Studies will have to be conducted in order to determine if ICP4 has the same effect on the production of E2F as do the E1a products.

It has been previously demonstrated in a transient transfection assay that ICP4 does not activate the E3 promoter <u>in vitro</u> (O'Hare et al., 1986) which agrees with the findings of this study. The E3 promoter is relatively simple, having only a TATA box and GC rich regions (Leff et al., 1985). Both the TATA box and upstream regions are required for activation by E1a products. Conversely, the E4 promoter, which is also

not activated by ICP4, contains a TATA box, GC rich regions and upstream enhancer elements which involve the cellular transcription factor ATF. These upstream elements are important for activation by E1a products (Gilardi and Perricaudet, 1984) indicating again that E1a transactivates via several diverse mechanisms, two of which appear to involve a TATA binding factor and the E2F factor respectively. In addition, E1a activates promoters via these two factors by using two different mechanisms. The TFIID-mediated activation of promoters is increased by altering the activity of the factor while the E2F-mediated activation is enhanced by increasing the quantity of E2F in the cell.

The fact that other viral activators can substitute for E1a and ICP4 is also of importance to this discussion. Transient transfection assays have demonstrated that the immediate early proteins of pseudorabies virus (PRV), varicella-zoster virus (VZV) and cytomegalovirus (HCMV) are capable of transactivating early genes of HSV (Everett, 1984a). The major immediate early protein of VZV has considerable homology to ICP4 but those of the other two viruses are quite different from ICP4 (McGeoch et al., 1986). The immediate early protein of cytomegalovirus is capable of complementing the defect of E1a$^-$ adenovirus when the two viruses are used in co-

infection, resulting in replication of the defective adenovirus (Tevethia and Spector, 1984; Tevethia et al, 1987). Furthermore, it has been demonstrated that two cytomegalovirus transactivating proteins are responsible for this complementation (Tevethia et al., 1987). The immediate early (IE) proteins of Epstein-Barr virus (EBV) and the pseudorabies virus (PRV) are capable of activating early genes of adenovirus (Feldman et al., 1982; Imperiale et al, 1983; Abmayr et al., 1985; Gaynor et al, 1985; Wong and Levine, 1986). Studies have shown that the PRV IE protein activates the adenovirus E1b promoter by the same mechanism as E1a products do (Wu and Berk, 1988). Both E1a products and PRV IE protein activate the E1b promoter by facilitating the formation of TFIID protein-DNA complexes (Abmayr et al, 1988; Wu and Berk, 1988). In addition, β-globin genes transfected into cells require only the TATA box for activation by Ad E1a and the IE protein of PRV (Green et al., 1983) thus giving further indications that the two proteins interact with the same cellular transcriptional mechanisms. Unfortunately, whether or not PRV-IE protein can fully substitute for E1a was not determined in these studies. Infection with PRV results in a rapid shut-off of host-macromolecular synthesis, therefore co-infection with an adenovirus may result in the same fate for potential adenovirus replication. This problem can

be circumvented by isolation and subcloning of the PRV gene coding for the IE protein, therefore making it available for transient transfection assays rather than co-infection with an E1a$^-$ adenovirus mutant.

The ICP4 protein is a very large protein and may have more than one domain responsible for the activation of HSV genes (DeLuca et al., 1984; DeLuca and Schaffer, 1985). Many studies indicate that ICP4 is not the only immediate early HSV protein that is capable of transactivation of early genes (Everett, 1984a; DeLuca and Schaffer, 1985; O'Hare and Hayward, 1985a and b; Quinlan and Knipe, 1985). In fact, ICP0 and ICP4 transactivate HSV early genes better synergistically than either alone (Everett, 1984a; O'Hare and Hayward, 1985a; Quinlan and Knipe, 1985). ICP4 is involved in localizing other HSV early proteins to the nucleus of infected cells (Knipe and Smith, 1986; Knipe et al., 1987) therefore the presence and proper localization of HSV proteins other than ICP4 may be necessary for the complete activation of all HSV genes even though ICP4 is sufficient to activate many of the HSV early genes (Persson et al., 1985).

Thus, it appears that E1a products and ICP4 operate via different mechanisms, except for an overlap in the activation of the E2A promoter of Ad5. One major difference in the function of these two activators

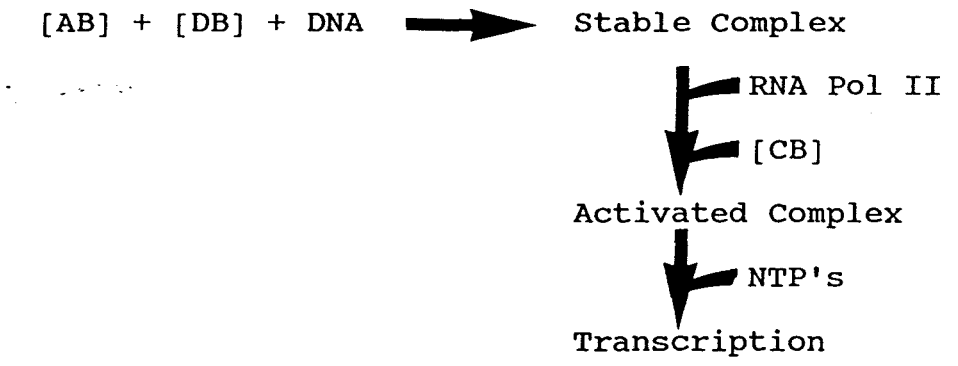
should be pointed out. First of all, the requirement of E1a is not absolute (Imperiale et al., 1984) whereas a herpes simplex virus will not replicate in the absence of ICP4 protein (DeLuca et al., 1985). Secondly, the activation of delayed genes in adenovirus infection is carried out by E1a products alone, while HSV infection requires several other viral factors in addition to ICP4. The method by which ICP4 activates the E2A gene is still a mystery but the mechanism involved may be elucidated by introducing mutations into the promoter region of the E2A gene and observing the effects of these mutations on the response to ICP4 relative to E1a products. Also, further studies on E2F may indicate that ICP4 specifically activates the E2A promoter through this factor, but cannot interact with the factors involved in the activation of the remaining adenovirus early genes.

Models of Gene Regulation

Both ICP4-mediated and E1a-mediated activation ultimately results in the transcription of viral genes by the host cell RNA Polymerase II; the increase in levels of transcripts comes from an increase in the number of actively transcribed templates (Leong and Berk, 1986). The viral activators may be involved in catalyzing the increase in active template used by RNA

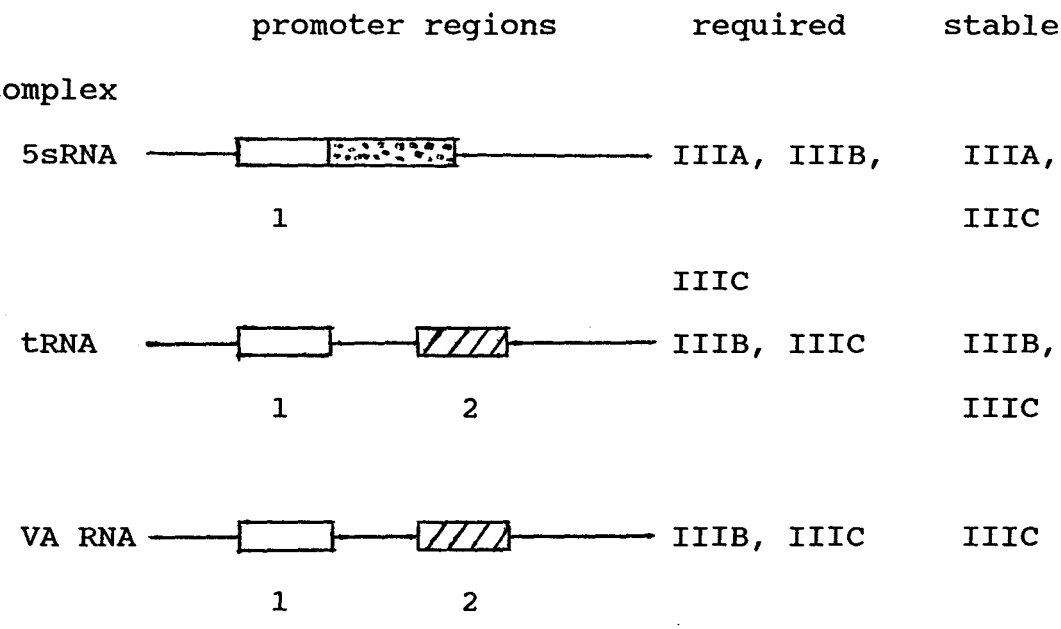
Pol II, however it is unclear if this is accomplished by altering the levels of cellular factors (Yoshinaga et al., 1986; SivaRamen et al., 1986; Kovesdi et al., 1986). Active complexes are formed upon mixing DNA promoter templates, RNA Pol II and cellular lysates (Fire et al., 1984) and are stable for many rounds of replication of the template (Bogenhagen et al., 1982). If the amounts of factors are limited, newly introduced templates are not activated because the factors and polymerase are stably associated with the original templates (Fire et al., 1984) and may continue to be, for as many as 40 rounds of replication (Bogenhagen et al., 1982). Therefore, the control asserted by viral activators may involve a change in the number of inactive and active complexes.

A model describing this activity was deduced from studies on three chromatographically isolated cellular factors, [AB], [CB] and [DB], which are involved in DNA/protein complex formation (Fire et al., 1984). These factors, in cooperation with DNA promoter regions and RNA Pol II form a stable complex which proceeds to transcribe from the templates. A schematic representation of the postulated process is outlined. It is important to realize that others, as yet undetected factors, may be involved in the process:



This scheme does not take into account the ability of the transcriptional machinery to discriminate between diverse promoters. This problem may best be approached by looking at a model system of another transcriptional protein, RNA Polymerase III. This enzyme is responsible for the transcription of the adenoviral VA RNA, as well as of cellular tRNA and 5sRNA genes (Lasser et al., 1983). Several chromatographically separated factors which are involved in RNA Pol III transcription have also been isolated from cell lysates (Lasser et al., 1983). Transcription was studied by introducing RNA Pol III, appropriate DNA templates and these factors, referred to as IIIA, IIIB and IIIC, into Xenopus oocytes. The factors are limiting in that if supplied in limiting quantities, newly introduced templates will not form transcriptional complexes, as the factors are already associated within stable complexes. The requirements of these factors for transcription, as well as the factors remaining stably

associated in the transcriptional complex are as follows:

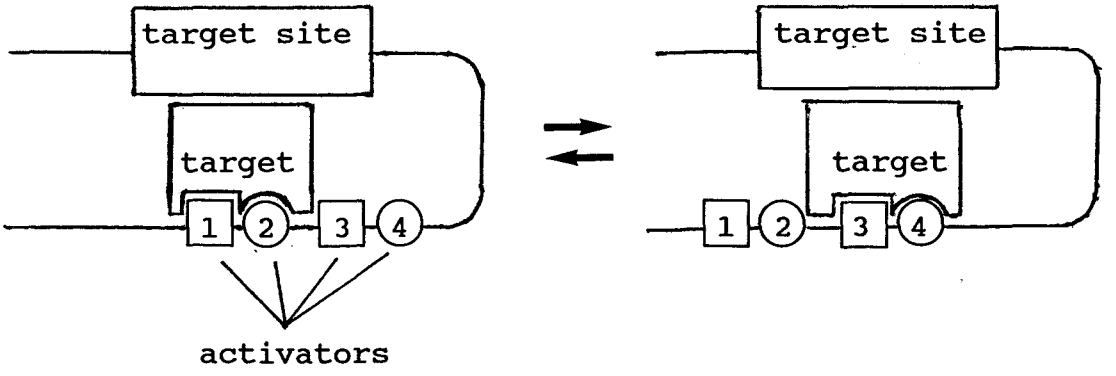


The data used to deduce this model indicate that the same factors may be used differently by the promoters of various genes, thus supporting the hypothesis that similar regulatory pathways may be used by activators of various viruses.

A recent model of gene activation by transactivators has been postulated by Ptashne (1988). He proposes that activators, such as those that bind to enhancer regions, have two important sites, one which is involved in binding (directly or indirectly) to the DNA and another which is responsible for activating the

transcription by interacting with polymerase or its complex (Ptashne, 1988). The DNA binding portion of the activator is quite specific, requiring unique sequences on the DNA. By contrasts the activation site is quite general and can act on a transcriptional complex as long as it is bound to DNA. It has been determined that the activating region of many activators, including the E1a products, are made up of amphipathic α-helices with one face being negatively charged and the other face being hydrophobic (see Ptashne, 1988 for review). Other factors, such as viral transactivators, may act by bringing cellular activators which are bound to DNA closer to the polymerase complex or the viral transactivators may bind to cellular proteins already bound to DNA, therefore the transactivator becomes available to activate the polymerase complex (Ptashne, 1988). If a gene has diverse activator binding regions on its promoter, several different viral activators may act on the same promoter through different cellular activators.

This model is as follows:



ICP4 and E1a products may affect transcription by bringing cellular activators closer to the polymerase, thus allowing the polymerase to be stimulated. Conversely, they may assemble the transcriptional machinery and bring it to the promoter region, or bind to the DNA through some cellular factor and acting as activators of transcription themselves. If ICP4 and E1a are capable of binding cellular factors which are limiting, whether they are bound to DNA or not, then an excess of ICP4 or E1a may "soak up" these factors before they have a chance to bind DNA. If this process prevents DNA binding, transcription will be inhibited. This may explain the inhibition of transcription observed by Trembley et al. (1985) for the E2A promoter in the presence of ICP4 and E1a.

In conclusion, it is evident that much more research into the activities of ICP4 and E1a products must be performed in order to better elucidate how genes, both cellular and viral, are regulated at the level of transcription. A continuation of this study may prove essential to the understanding of how cellular factors are involved in transcriptional regulation, since it has shown that two diverse transactivators act to stimulate the same promoter *in vivo*.

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