HUMAN ADENOVIRUS RECOMBINANTS EXPRESSING THE VESICULAR STOMATITIS VIRUS GLYCOPROTEIN

CONSTRUCTION AND CHARACTERIZATION OF HUMAN ADENOVIRUS RECOMBINANTS EXPRESSING THE VESICULAR STOMATITIS VIRUS GLYCOPROTEIN GENE.

by

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ABSTRACT

The potential of human adenovirus (Ad) to serve as a vector for expression of heterologous genes was evaluated. An experimental gene, consisting of sequences coding for the glycoprotein of vesicular stomatitis virus (VSV) attached to the promoter and polyadenylation signal of the herpes simplex virus type 1 (HSV-1) thymidine kinase (TK) gene, was inserted into early region 3 of adenovirus, in both orientations. The TK promoter was functional in both orientations and responded to trans-activation by HSV infection. Abundant expression of VSV G however depended on the presence of a second transcript. This transcript was present only in the recombinant carrying the insertion in the orientation parallel to the E3 promoter (AdG12) and was initiated upstream of the insertion, within Ad sequences.

The potential of Ad recombinants to serve as vaccine vectors was investigated using the recombinant AdG12. Antibody against VSV G was induced in cows, mice, pigs, and dogs in response to infection with AdG12. Protection of mice, immunized with AdG12, against a lethal challenge with VSV demonstrated the biological effectiveness of this immune response.

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LIST OF ABBREVIATIONS

Ad	adenovirus
ara-C	cytosine arabinoside
BSA	bovine serum albumin
CPE	cytopathic effect
cpm	counts per minute
DNA	deoxyribonucleic acid
EDTA	ethylene-diaminetetra-acetic acid
E1	early region 1
E2	early region 2
E3	early region 3
E4	early region 4
G	glycoprotein
h	hour
HSV	herpes simplex virus
M	molar
mg	milligram
min	minute
ml	millilitre
mM	millimolar
mu	map units
ng	nanogram
nt	nucloetide
^{o}C	degree centigrade
ORF	open reading frame
pfu	plaque forming units
rpm	revolutions per minute
RNA	ribonucleic acid
SDS	sodium dodecyl sulfate

TK	thymidine kinase
U	units of enzyme activity
μCI	micro Curie
μg	microgram
V	volts
VSV	vesicular stomatitis virus

1. INTRODUCTION

1.1 RECOMBINANT VIRAL VECTORS

Introduction of biologically active nucleic acids into cells where their activity can be tested is fundamental to the advancement in understanding of many areas of biology. Intact coding information can be expressed in a novel context to characterize protein function or at higher levels to facilitate protein purification. Expression of mutagenized nucleic acids can also contribute to the characterization of protein function and in addition allows dissection of functional properties of non-coding information: properties involved in DNA rearrangements and various aspects of gene regulation. Many of these applications can be served by recombinant viruses expressing heterologous genetic information which is carried as a part of their own genome.

Nucleic acid delivery via recombinant viruses presents major advantages over methods mediated by chemicals such as calcium salts or DEAE dextran. Nucleic acids are more efficiently introduced and can be propagated as well as expressed. A further application of these features of recombinant mammalian viruses is their use as vaccine vectors. This application was pioneered using vaccinia virus and is being further developed using adenovirus.

SV40, papillomavirus, retroviruses, baculovirus (reviewed by Knipe, 1985) and HSV (Smiley et al., 1987) have also been used as expression vectors for the analysis of eukaryotic functional information.

1.2 VACCINES

An ideal vaccine must stimulate the appropriate pathways of the immune system with the appropriate antigen(s), to induce protection against disease caused by an invading pathogen. Classical approaches to vaccination against viral infections involve manipulating the pathogen to either attenuate its virulence or inactivate it. Viruses are inactivated by treating genetically wild type viruses with a chemical, such as formalin, which results in their death. Attenuation of virulence, by the classical approach, is achieved by passaging the virus in an unnatural host to select for genetic mutations. Viruses with reduced virulence are then selected from these mutants (reviewed by Murphy and Chanock, 1985).

Although both of these approaches have produced some effective vaccines, they have not been without their drawbacks. A few of these drawbacks are shared by both inactivated and live attenuated vaccines. Both have, on occasion, induced the diseases against which they were intended to protect, due to incomplete inactivation of the former (Peterson et al., 1955) and incomplete attenuation or reversion (Kew et al., 1981) of the latter. Both

processes can also alter the spectrum of immunogenicity of viral proteins, an effect which can have two undesirable consequences: the immunogenicity of epitopes which have a major protective effect can be reduced and, by priming an improperly balanced immune response, the pathogenicity of subsequent infection with the wild type virus can be enhanced (reviewed by Murphy and Chanock, 1985). Some of these problems may be avoided, in live vaccines, by attenuation of virulence through directed mutagenesis (Buller and Moss, 1985; McDermott et al., 1984).

The risk of exposure to an intact pathogen can be eliminated through the use of isolated antigens: chemically synthesized peptides, proteins manufactured from cloned DNA or polysaccharides. However, isolated antigens are generally less immunogenic than an intact pathogen: both humoral and cellular immunity are stimulated inefficiently (Reiss et al., 1980). Although humoral immunity to isolated antigens can be enhanced by adjuvants which are approved for use in humans (alum) and animals (saponin and mineral oil), these agents are not effective stimulants of cellular immunity. Novel adjuvants, which are under development, will require extensive safety evaluation before their use can be approved (reviewed by Chedid, 1987).

The problems and insufficiencies associated with both classical vaccines and vaccines composed of isolated antigens may be overcome with the use of recombinant viral vaccines. Protective antigens, in their native form, can be delivered by an innocuous viral vector without the risk of exposure to the intact pathogen, to

stimulate both humoral and cellular pathways of the immune response.

1.3 VIRAL VACCINE VECTORS

To serve as an expression vector a virus should display the following basic properties.

(a) It must be able to accept insertions of DNA large enough to direct the synthesis of a typical protein.

(b) Sites on its genome must be identified which can accept these insertions without disrupting essential viral functions.

(c) The control of expression of endogenous functions must be sufficiently well understood to engineer efficient expression of heterologous sequences.

(d) To be versatile, it should have an unrestricted host range so that a variety of recombinants for use in a variety of host species can be easily produced.

To serve as a vaccine vector, a virus should, in addition, be innocuous, physically stable and relatively easy to administer.

Vaccinia virus, which was used to pioneer the development of recombinant viral vaccines, displays many characteristics of an ideal vaccine vector. Its genome can accommodate a large amount of additional genetic information (insertion of 24,700 bp of the phage lambda genome has been reported; Smith and Moss, 1983), which can be inserted without disrupting essential viral functions.

Heterologous genes, which are transcribed from endogenous promoters, can be efficiently expressed (Kieny et al., 1984). Its broad host range has enabled the successful production of numerous model recombinant vaccines. Several species of rodents, foxes, cows, chimpanzees, and humans are among the animals that have shown immune responses to infection with recombinant vaccinia viruses, which have resulted in protection against challenge with the corresponding pathogen (reviewed by Moss and Flexner, 1988). Its use as a human smallpox vaccine has proven it to be physically stable and easy to administer in humans (Fenner et al., 1988). Successful oral vaccination of foxes has demonstrated the ease of administration to animals as well (Blancou et al., 1986).

Vaccinia virus recombinants show excellent potential as vaccines against two pathogens of significant current notoriety: a recombinant expressing the rabies virus glycoprotein has protected foxes against challenge with rabies virus (Blancou et al., 1986); a recombinant expressing the surface antigen of hepatitis B virus has protected chimpanzees against challenge with hepatitis B virus (Moss et al., 1984). Despite these promising practical applications, recombinants of vaccinia virus alone are unlikely to fulfill the needs for all immunizations. Repeated infection by the same vector could induce sufficient immunity to the vector itself to cause elimination of the vaccine before an adequate immune response to the heterologous antigen could be mounted. A major constraint on the further application of vaccinia virus in humans is the concern over safety since complications such as abnormal skin eruptions, severe systemic

infections and encephalitis occasionally result from vaccination with vaccinia virus (reviewed by Fenner et al., 1988).

A second candidate for development as a vaccine vector is the human adenovirus. The basic properties for use as an expression vector have been established for the serotype 5: it can accommodate enough additional genetic information to code for a protein of average size, at sites where essential viral functions are not disrupted (see section 1.5, Structural design and construction). As a vaccine vector, adenovirus displays some advantages over vaccinia virus. Its most important advantage lies in its safety as a vector in humans. Although the safety of serotype 5 has not been evaluated, serotypes 4 and 7 have served as safe and effective vaccines against adenovirus-induced respiratory infections in military recruits (Edmonston et al., 1966; Top et al., 1971). Adenovirus preparations, administered orally in coated capsules, bypassed the respiratory tract and produced inapparent enteric infections. An advantage of secondary importance is the likelihood that adenovirus displays a greater physical stability than vaccinia virus, due to the absence of an envelope.

Several aspects in the application of adenovirus as a vaccine vector require further investigation. The host range of adenovirus and control of expression of heterologous sequences in the adenovirus genome are discussed in section section 1.5, ADENOVIRUS VECTOR DESIGN AND TESTING. Also, since oral administration of coated capsules of adenovirus is not feasible in animals and small children,

alternate routes of administration for these hosts need to be determined.

1.4 ADENOVIRUS OVERVIEW

History, taxonomy and pathogenicity

A large number of adenovirus serotypes have been isolated from a broad range of avian and mammalian species, including humans. Encephalitis, ocular, respiratory and enteric infections are common among the diseases associated with infected organisms. Mammalian as well as avian serotypes have also been shown to induce tumours in hamsters and transform rodent cells in vitro (reviewed by Straus, 1984; Ishibashi, 1984). The discovery of human adenovirus type 12 as the first human virus to have oncogenic potential (Trentin, 1962) prompted extensive studies of several human adenovirus serotypes. Since then adenoviruses have not been shown to cause tumours in their natural hosts (reviewed by Fraenkel-Conrat and Kimball, 1982) and a role for human adenoviruses in human tumours has not been established (reviewed by Graham, 1984a). The intense interest in adenovirus as a possible human tumour virus has however not only provided laboratory models of relevance to the understanding of malignancy but extensive studies of human adenoviruses have unveiled several important aspects of normal cellular and viral physiology (reviewed by Horwitz, 1985). The non-contiguous nature of genetic information

which becomes linked when primary transcripts are spliced was first discovered in adenovirus (Berget et al., 1977; Chow et al., 1977). The DNA of adenovirus was the first non-prokaryotic DNA for which initiation of replication was demonstrated in vitro (Challberg and Kelly, 1979). The strategy for priming DNA replication using protein was discovered in adenovirus (Rekosh et al., 1977).

The family Adenoviridae is divided into two genera; Mastadenovirus, which include human and other animal viruses; and Aviadenovirus, the adenoviruses of birds (reviewed by Horwitz, 1985). The 41 known human serotypes were initially classified into subgroups on the basis of several, largely independent criteria, such as hemagglutination of rat or rhesus monkey red blood cells, oncogenicity, GC content of DNA, electrophoretic mobility of virion proteins, genome homology, and restriction endonuclease profiles of DNA. Classification according to these criteria has turned out to be highly concordant (reviewed by Straus, 1984).

Due to the interest in human adenoviruses, fundamental information about virion structure, genome organization and viral replication has been derived from the studies of several human serotypes, especially serotypes 2, 5, and 12.

Virion structure

The adenovirus virion consists of a non-enveloped icosahedral nucleocapsid whose major structural components are 240 hexons and 12 pentons (reviewed by Horwitz, 1985). The genome is

a linear double-stranded DNA molecule of approximately 36,000 base pairs, with inverted repeats at the termini. The 5' end of each strand is covalently linked to a terminal protein (reviewed by Sussenbach, 1984).

Genome organization

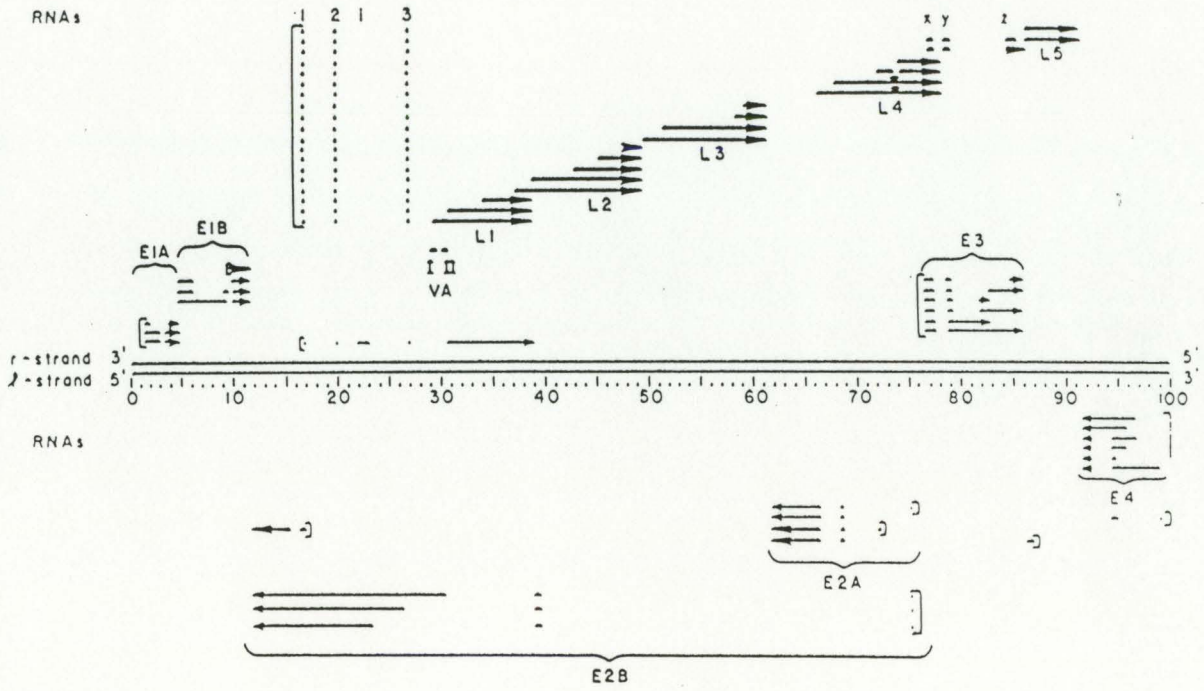
Genetic information is distributed over both strands of the adenovirus genome and is organized into 5 major transcription regions (Fig. 1). Each transcription region coordinates the synthesis of a family of differentially spliced and/or polyadenylated transcripts which code for groups of proteins with functions that are usually related, in the same sense that proteins in a prokaryotic operon are related (reviewed by Cladaras and Wold, 1985). Transcription of the early regions (E1, E2, E3 and E4) begins before DNA replication while transcription of the major late region occurs mainly following DNA replication. Two single transcripts, initiated from their own promoters, are expressed at an intermediate time following infection and code for the virion components protein IX and protein IVa2 (reviewed by Horwitz, 1985).

Early region 1

The products encoded by this region function primarily to control gene expression. Their transcription is initiated by two promoters which divide the region into two non-overlapping subregions E1a and E1b (reviewed by Graham, 1984a).

FIG. 1. TRANSCRIPTION MAP OF ADENOVIRUS TYPE 2.

Arrows show the direction of transcription. Promoters are indicated by vertical brackets. Early mRNAs are designated E; late mRNAs are designated L. All late mRNAs start at 16.3 mu and contain the tripartite leader, designated 1, 2 and 3. Additional leader segments (i, x, y, and z) are found on some late mRNAs. (Modified from Horwitz, 1985.)


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

The major products of the E1a region are two closely related proteins, 289 and 243 amino acids long which differ by 46 amino acids that are unique to the 289 amino acid protein. These products function in trans to modify gene expression at the level of transcription. Two opposing effects on transcription have been described, stimulation and repression, both of which can influence endogenous and exogenous genes (reviewed by Kingston et al., 1985). E1a products initiate the cascade of viral gene expression during the lytic cycle by stimulating transcription of all endogenous promoters which function at early times (Nevins, 1981; Jones and Shenk, 1979; Berk et al., 1979). Heterologous genes, whose expression is stimulated by E1a products, include the human β-globin gene (Green et al., 1983), the rat preproinsulin gene (Gaynor et al., 1984), and several cell cycle dependent genes (Liu et al., 1985). Repression of transcription by E1a products is imposed on genes which are linked to cis-acting transcriptional enhancers. Enhancers of the immunoglobulin heavy chain gene (Hen, 1985), the SV40 early promoter (Velcich and Ziff, 1985), polyoma virus and E1a's own enhancer (Borrelli et al., 1984) have been shown to respond to this effect.

The two E1a functions described above are carried out by independently functioning protein domains. Both domains are present in the 289 amino acid product but only the enhancer repressing domain is present in the 243 amino acid protein (Montell et al., 1982; Velcich and Ziff, 1985; Lillie et al., 1986; Lillie et al., 1987). In addition to their effects on transcription, E1a products

participate in inducing cellular transformation and cellular DNA synthesis. Both of these effects are associated primarily with the transcriptional repression function (Spindler et al., 1985; Lillie et al., 1987; Lillie et al., 1987).

The major products of the E1b region, the 58K and the 19K protein, are encoded in two different reading frames. Although the direct function of the 19K product is not known, an enhanced cytopathic effect which is characterized by the formation of larger than normal plaques (Takemori et al., 1968) and extensive degradation of both host and viral DNA (Ezoe et al., 1981) is a consequence of its absence during infection (Lai Fatt and Mak, 1982; Barker and Berk, 1987). The 58K product functions in a complex with the 34K product of the E4 region (Sarnow et al., 1984). This complex functions at the post-transcriptional level to simultaneously facilitate the accumulation of late viral mRNA and inhibit the accumulation of host mRNA (Babiss et al., 1985; Pilder et al., 1986).

Induction of cellular transformation and maintenance of the transformed state have long been known to depend on what is now known to be the E1 region of adenovirus (Graham et al., 1974; Gallimore et al., 1974). As the products of this region are being identified their roles in cellular transformation and oncogenesis are being evaluated (reviewed by Graham, 1984a). It is currently understood that all four of the E1 products described above contribute to cellular transformation (Montell et al., 1984; Barker and Berk, 1987). Since all four products are modulators of gene expression it is likely that transformation is a consequence of

expression of these functions in the absence of lytic cellular destruction.

Early region 2

The E2 region codes for proteins which function in adenovirus DNA replication. This region is divided into two subregions, E2a and E2b, based on the presence of two polyadenylation signals. The E2a region codes for a 72K protein which is capable of binding both single and double-stranded DNA. It is translated from a coding region which at early times is carried on a transcript initiated by a promoter at 75 mu and at late times on a transcript initiated by a second promoter at 72 mu. The E2b region codes for the viral DNA polymerase and the terminal protein which are expressed from differentially spliced transcripts initiated by the promoter at 75 mu. (reviewed by Peterson 1985; Horwitz 1985).

Early region 3

The E3 region is transcribed from a single promoter into 10 partially overlapping mRNAs which are formed by alternate splicing and polyadenylation at either of two sites. Analysis of the DNA sequence of this region and its mRNA structures suggests that seven to nine proteins are encoded (Cladaras and Wold, 1985). Although it has long been known that this region is not required for virus replication, since deletion of most of it does not prevent replication in cultured cells (reviewed by Klessig, 1985; Berkner and Sharp, 1983),

only recently have the functions of a few E3 products and their possible roles in survival of the virus *in vivo* been determined.

The first E3 product to be characterized was the 19K transmembrane glycoprotein. It is localized in the endoplasmic reticulum where it forms a complex with class I major histocompatibility antigens and specifically blocks their transport to the cell surface. Since recognition of foreign antigens by the cellular immune system occurs in the context of the class I MHC molecules, expression of the 19K protein is thought to help the virus to evade the host cellular immune response (Burgert and Kvist, 1985).

Subsequently Gooding et al., (1988) found that infection of cells with an adenovirus lacking most of the E3 region induced susceptibility to lysis by tumour necrosis factor. This susceptibility is blocked during infections with wild type virus. Resistance to this host antiviral activity, mediated by tumour necrosis factor, was correlated with the presence of an E3-coded 14.7K protein.

Most recently adenovirus infection has been shown to down-regulate the cellular receptor for epidermal growth factor. This function has been correlated with the presence of a 10.4K protein encoded by the E3 region. Sequence analysis of this protein shows it to contain 2 hydrophobic domains: a putative signal sequence and a transmembrane domain. It also contains an 18 amino acid stretch with similarity to the cytoplasmic face of the transmembrane region of the EGF-R. Although the mechanism of EGF-R down-regulation is unknown, it has been hypothesized that the 18 amino acid long stretch mimics the signal on the EGF-R by which ligand binding

induces oligomerization of the receptor prior to its internalization and degradation. The 10.4K protein would thus associate with the EGF-R and induce oligomerization by forming hetero-oligomers. It is unknown whether the observed EGF-R down-regulation results in inhibition or in constitutive stimulation of one of its functions. The consequences of EGF-R down-regulation which might be advantageous to virus survival are also unknown (Carlin et al., 1989).

Both the 19K glycoprotein (Burgert and Kvist, 1985) and the 14.7K protein (Gooding et al., 1988) are believed to enhance virus survival by inhibiting host antiviral mechanisms. The possible benefit to virus survival of interference with normal EGF-R function by the 10.4K protein is less obvious. The common function of products of the E3 region may therefore be more general than the inhibition of host antiviral mechanisms, as the discovery of the first two E3 products suggested, and may be to mediate interactions between specific cell types in the infected organism (Carlin et al., 1989).

Early region 4

Like the E3 region the functions encoded by the E4 region are not entirely understood but are rapidly being unravelled. This region is transcribed from a single promoter into a number of alternately spliced mRNAs. Analysis of the DNA sequence as well as the structure of the mRNAs suggests a coding capacity for at least seven different proteins but only three have been mapped to their appropriate reading frames (reviewed by Bridge and Ketner, 1989).

The 34K protein, which has been mapped to ORF 6, is the one which was described previously to function in a complex with the EIb 58K product to alter the accumulation of both host and late viral mRNA (Babiss et al., 1985; Pilder et al., 1986). The 11K product, which was mapped to ORF 3, is known to associate with the nuclear matrix and ORF 6/7 codes for a 19.5 K protein of which nothing else is known.

Characterization of a mutant which lacks most of the E4 region revealed a complex phenotype: it was impaired in the synthesis viral DNA and viral late proteins, and in the shut-off of host protein synthesis (Halbert et al., 1985). Recently a novel E4 mutant phenotype was characterized in viruses lacking sequences from all but the first ORF. Mutants of this phenotype have a reduced quantity of late viral mRNA, which is thought to result from reduced mRNA stability in the nucleus (Sandler and Ketner, 1989). A higher resolution analysis of a series of E4 deletion mutants showed that deletion of ORF 3 and ORF 6 had a synergistic effect on disruption of late protein synthesis. This study also mutationally separated the impairment in DNA synthesis from the impairment in protein synthesis (Bridge and Ketner, 1989).

The common function of the products of the E4 region, according to current knowledge, appears to resemble that of the E1b region i.e. the post-transcriptional regulation of gene expression. Effects on DNA stability or replication by products of both of these regions may be secondary manifestations of this primary function.

The major late transcription region

Products of this region which have been characterized are virion structural components and proteins involved in virion assembly. Transcription of this region is controlled by a single promoter, located at 16.5 m.u., which is most active at late times following infection but is also the first promoter to be expressed during the lytic cycle (reviewed by Horwitz, 1985). At early times the transcript is terminated at 39 m.u. but at late times transcription continues to 99 m.u. The larger late transcript is processed into five families of mRNAs: L1, L2, L3, L4 and L5, which are defined by their respective polyadenylation signals (Fig. 1). Each family of 3' coterminal mRNAs consists of several alternately spliced members. The earlier transcript is polyadenylated at the L1 site and is alternately spliced into 3 mRNAs coding for 3 proteins. The functions of 2 of the proteins, which are structurally related, are unknown. The other is a virion component associated with hexon (reviewed by Sussenbach, 1984).

Most transcripts carry upstream of their coding sequences a non-translated leader which is formed by the joining of 3 exons and therefore called the tripartite leader. This leader is known to have three effects, which are probably related, on mRNAs to which it is appended: it can enhance their translation (Logan and Shenk, 1984), and has recently been shown by Moore and Shenk (1988), to shorten their nuclear half life and increase their cytoplasmic half life. Less

abundant mRNAs, containing additional exons upstream of the coding sequences, have also been detected (reviewed by Sharp, 1984).

1.5 ADENOVIRUS VECTOR DESIGN AND TESTING

Adenovirus host range

A frequently cited disadvantage of the use of adenovirus as a vaccine vector is its limited host range. A limited range of susceptible hosts would not only limit the application of adenovirus as a vaccine vector but also hinder its development, due to the lack of a small animal model.

The host range of adenovirus was first explored in 1955 by Rowe et al., who inoculated rabbits, mice, guinea pigs, hamsters, white rats, rhesus monkeys, a chimpanzee, kittens, ferrets, and cotton rats with adenovirus, by a variety of routes. After finding no clinical symptoms of disease they concluded that it was apathogenic in these animals. Since then closer examination of some of the same species of animals has revealed evidence of pathogenesis or virus replication. In 1984 Pacini et al inoculated one week old cotton rats with Ad 5 by intranasal instillation and, despite their inability to detect clinical symptoms of disease, recovered virus from the lungs of infected animals. Also, Pereira et al., (1962) reported fatal disease and recovery of virus from several organs of newborn hamsters after subcutaneous inoculation and recently Morin et al., (1987) showed

that Ad 5 replicates in the lungs of 3 to 4 week old hamsters following intranasal inoculation. There is also evidence for the persistence of Ad 5 in the spleen of rabbits (Pereira et al., 1957). The host rage of human adenoviruses may not in fact be as limited as it initially appeared and clearly requires further investigation.

Structural design and construction

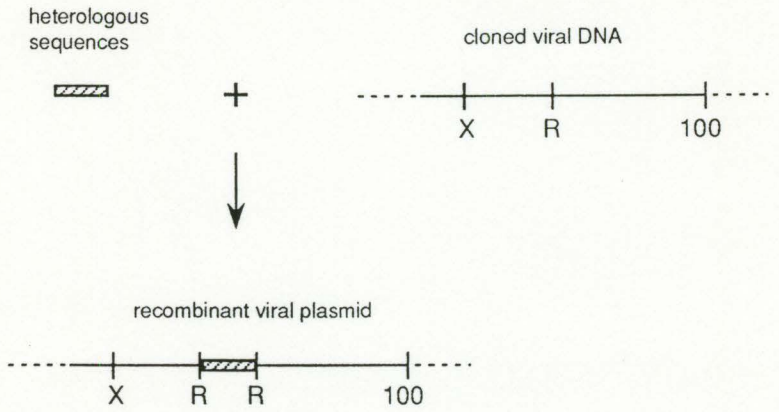
Wild type adenovirus type 5 can package approximately 2 kb of additional genetic information (Jones and Shenk, 1979b; Ghosh-Choudhury et al., 1987), but this limit can be expanded by deleting non-essential sequences. An additional 1880 bp can easily be removed from the E3 region, which is non-essential for virus replication, by deleting the XbaI D fragment from 78.5 mu to 84.7 mu. Non-defective recombinants can be constructed by inserting heterologous sequences into this region. Additional deletions can be made in the E1 region resulting in defective viruses which can however be propagated on 293 cells (Haj-Ahmad and Graham, 1986). Such defective viruses with a limited ability to replicate in normal human cells may have useful applications, such as biocontainment of recombinants expressing pathogenic proteins.

Recombinant adenoviruses are usually constructed by first assembling a portion of the recombinant viral genome from DNA cloned in bacteria. Cloned heterologous sequences are inserted into viral sequences to produce a recombinant viral plasmid as shown in Fig. 2A. The corresponding region of a parental viral genome is

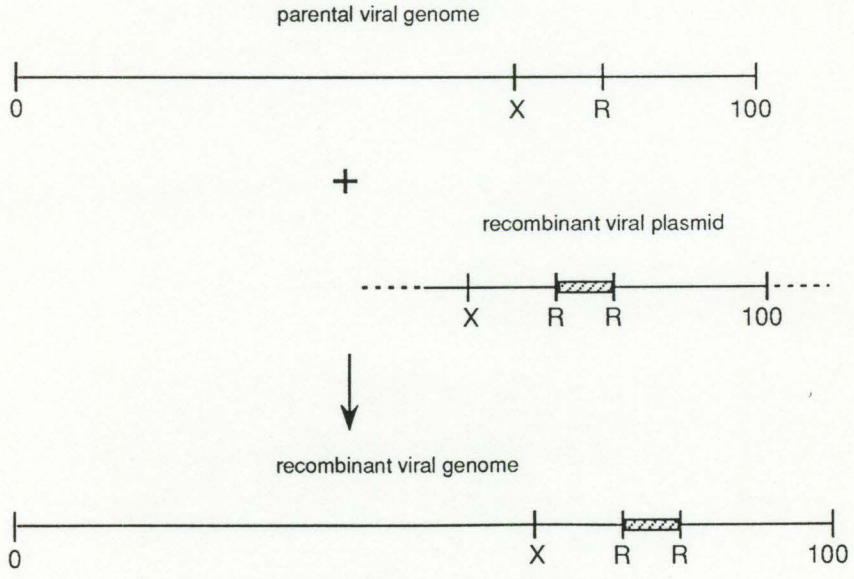
FIG. 2. GENERAL SCHEME FOR CONSTRUCTING RECOMBINANT ADENOVIRUS GENOMES.

Hatched box represents heterologous sequences; dashed line represents bacterial plasmid sequences; solid line represents adenovirus sequences. Positions on the viral genome are: R, restriction enzyme site; 0 and 100 mu, the ends of the genome; X, an arbitrary reference position on the genome. (A) Heterologous sequences are inserted into viral sequences at a resriction enzyme site, to produce a recombinant viral plasmid. (B) The recombinant viral plasmid is used to replace the corresponding sequences on the parental viral genome to produce a recombinat viral genome.

A


heterologous
sequences
cloned viral DNA
X
R
100
recombinant viral plasmid
X
R
R
100

B


parental viral genome
0
X
R
100
recombinant viral plasmid
X
R
R
100
recombinant viral genome
0
X
R
R
100

replaced with the cloned recombinant sequences (Fig. 2B) and infectious virus is produced by introducing DNA into susceptible cells by the calcium technique of Graham and van der Eb (1973); these latter two steps do not necessarily occur in the order mentioned. Several variations of the replacement step are described below; I have not observed a consensus regarding the overall superiority of any one of them.

By the method of Stow (1981), the recombinant viral genome is assembled in vitro by ligating cloned recombinant sequences with viral DNA, after both had been digested with appropriate restriction enzymes. The recombinant viral genome can also be generated by cotransfecting the cloned recombinant sequences with viral DNA and relying on the cellular recombination machinery to replace the parental region by cloned recombinant sequences (Frost and Williams, 1978).

In both of the above approaches, DNA derived entirely from cloned viral sequences can be used. Berkner and Sharp, (1983) recovered infectious virus from several plasmids containing overlapping regions of the viral genome. Subsequently, Graham, (1984b) propagated the entire viral genome as a single bacterial plasmid which was infectious in susceptible mammalian cells and then devised a strategy for selecting recombinant viral genomes, based on this plasmid. The infectivity of the cloned viral genome was first destroyed by an insertion into an essential region: infectivity could then be restored by recombination with viral sequences cloned in a second plasmid, which carried the desired heterologous

sequences inserted in a non-essential region and lacked the inactivating insertion (Ghosh-Choudhury et al., 1987; McGrory et al., 1988).

Expression of heterologous genes

As previously described, adenovirus produces factors which interact with and modify the host transcription apparatus (E1a) as well as factors which regulate gene expression at a post-transcriptional level (E1b, E4). Because the mechanisms by which these factors function are not fully understood their effect on inserted sequences, carrying foreign promoters of transcription and signals controlling transcript processing and stability, cannot be predicted. Expression of heterologous genes therefore requires testing in prototype adenovirus recombinants.

The TK-G experimental gene

To test the function of heterologous regulatory sequences in adenovirus, recombinants which carried an experimental hybrid gene were constructed. The hybrid gene consisted of the promoter and polyadenylation sequences of the thymidine kinase gene of herpes simplex virus type 1, linked to the coding sequences for the glycoprotein of the Indiana serotype of vesicular stomatitis virus. This TK-G hybrid gene was inserted into the E3 region of Ad 5 in both orientations, substituting for most of the E3 coding sequences. A detailed map of the structure of the TK-G hybrid gene is illustrated in Fig. 6.

The HSV TK gene is optimally expressed during the delayed-early period of HSV infection. The accumulation of high levels of TK mRNA during this period depends on the prior synthesis of immediate early trans-acting regulators of gene expression: low basal levels of TK mRNA can however be detected in the absence of these immediate-early regulators (McKnight, 1986).

VSV: the experimental pathogen

VSV is a member of the Rhabdoviridae family. Virions of this family have an enveloped, helical nucleocapsid with a characteristic bullet shape. The single negative-stranded RNA genome codes for five proteins: N, the structural component of the nucleocapsid; G, the single membrane glycoprotein; M, the matix protein which is located between the nucleocapsid and the envelope; and L and NS, both components of the virion-associated transcriptase (reviewed by Emerson, 1985).

Because of its usually mild and often inapparent pathogenicity in humans, in contrast to the severely pathogenic rabies virus, VSV has been the more extensively studied prototype of the Rhabdoviridae family. Its broad host range also makes it amenable to the study of viral pathogenesis and immune responses to infection. It is a pathogen of cattle, swine and horses, of some economic concern, causing vesicular lesions around the mouth and feet. Although complete recovery from disease in these animals usually occurs within two weeks, it can cause a delay in availability of animals for work or slaughter (reviewed by Hanson, 1970).

Since protective immunization of mice and cattle has been achieved with a vaccinia virus recombinant expressing the VSV glycoprotein (Mackett et al., 1985), an adenovirus recombinant expressing the VSV glycoprotein would serve as good model for a novel vaccine vector.

2. MATERIALS AND METHODS

2.1 CELL LINES

Cell lines used in these studies were HeLa (human cervical carcinoma), 293 (human embryonic kidney transformed with adenovirus; Graham et al., 1977), L (mouse fibroblast), Vero (African green monkey kidney), MDCK (Maden Darby canine kidney) and MDBK (Maden Darby bovine kidney)

All cell lines were maintained as monolayers in plastic tissue culture dishes containing approximately 1 ml per 5 cm of growth surface of minimal essential medium F-11 (MEM F-11) supplemented with 10% (v/v) newborn calf serum, 100 U/ml of penicillin and 100 µg of streptomycin. With the exception of 293 cells, monolayers were dispersed by treatment with a solution of 0.5% (w/v) trypsin and 0.2% (w/v) EDTA (GIBCO). 293 cell monolayers were dispersed using a solution of 1% (w/v) potassium chloride, 0.44% (w/v) sodium citrate.

HeLa and L cells were also grown in suspension cultures. Cells were maintained at densities of $2x10^5$ to $1x10^6$ cells/ml of Joklik's modified minimal essential medium (MEM) containing the same supplements.

2.2 VIRUSES

Growth of viruses

All adenovirus stocks, Ad5 strain dl309 (Jones and Shenk 1979b), AdG4 and AdG12, were grown on HeLa cells. Cells were infected with 1 pfu/cell. Monolayers were harvested by scraping with a rubber policeman when cells were beginning to detach from their substrate. Suspension cultures were harvested when inclusion bodies could be visualized in the nuclei of orcein stained cells. Infected cells were centrifuged and resuspended at a density of 1 ml per $2x10^7$ cells in Joklik's modified medium containing 10% (v/v) glycerol. Virus was released from infected cells by 3 cycles of freezing and thawing. Stocks could then be frozen and thawed several more times without a significant decrease in titre.

HSV-1 strain KOS paar5 was grown on Vero cells. Cells were infected with no more than 0.1 pfu/cell and harvested when cells were beginning to detach from their substrate. Cells were scraped with a rubber policeman and resuspended in fresh Joklik's modified medium at a density of approximately $2x10^7$ cells per ml. The cell suspension was then sonicated to break open cells and frozen in small aliquots. Each aliquot was discarded after thawing.

HR-LT Toronto and San Juan strains of the Indiana serotype of VSV were used in these studies. Virus stocks were prepared by infecting L cells at no more than 0.1 pfu/cell and collecting the culture medium when extreme cytopathic effect was apparent.

Stocks were frozen in small aliquots and each aliquot was discarded after thawing.

All infections were carried out by adsorbing virus in 1/10 of the required volume of growth medium without serum during the first hour of infection. Infected cells were maintained in the same media as uninfected cells with the exception of the serum concentration which was reduced to 5% (v/v).

Inclusion body staining

A sample of approximately 10^6 infected cells was pelleted and resuspended in 0.5 ml of a hypotonic solution of 1% (w/v) sodium citrate and allowed to swell for 10 min. Cells were then fixed in Carnoy's fixative (methanol:acetic acid, 3:1) in two steps: first 0.5 ml of fixative was added and then 2 ml of fixative was added 10 min later. Fixed cells were pelleted and resuspended in a few drops of fresh fixative. Individual drops of this suspension were placed on glass microscope slides and allowed to dry for 1 h. Cells were stained with a solution of 2% (w/v) orcein, 50% (v/v) glacial acetic acid for 5 min. After placing a coverslip over the staining solution, cells were examined under the microscope for the presence of inclusion bodies (large densely stained bodies) in the nuclei.

Titration

Virus stocks were titrated on the same cell lines that they were propagated on. 35 mm tissue culture dishes of the appropriate cell line were infected with tenfold serial dilutions of virus stock.

After adsorbing for 1 h infected cells were overlaid with the appropriate solution and incubated until plaques could be visualized as described below.

HSV-infected Vero cells were overlaid with culture medium containing 0.05% (v/v) of human immune gammaglobulin (Connaught). Plaques were identified after about 3 days by direct observation of infected cells.

VSV-infected L cells were overlaid with culture medium containing 0.5% (w/v) melted agarose. Plaques were visualized by observing the fixed, dried monolayer. 1 to 2 days following infection the monolayer was fixed by pouring Carnoy's fixative onto the agarose overlay. After 1 h at room temperature the agarose was lifted off and the monolayer was left to dry.

Adenovirus-infected HeLa cells were overlaid in two steps: first with culture medium containing 0.5% (w/v) agarose immediately after adsorption and then after six days with an equal volume of culture medium with 0.5% (w/v) agarose and 1% (v/v) neutral red (GIBCO 0.033% (w/v) neutral red sodium salt solution). Plaques were visible after 7 to 10 days as white spots on a red background of live cells.

2.3 CONSTRUCTION OF RECOMBINANT ADENOVIRUSES

Purification of adenovirus DNA

Virions were purified from a suspension of approximately $5x10^8$ infected cells. Cells were harvested, resuspended in 10 ml of 0.1M Tris pH 8 and lysed by adding sodium deoxycholate to 0.5% (w/v) and stirring occasionally for 30 min on ice. The viscosity of the solution was reduced using a Tekmar tissumizer until it was slightly higher than water but could easily form drops. For every 3.2 ml of infected cell suspension 1.8 ml of a solution of 0.1 M Tris pH 7 saturated at room temperature with CsCl was added. The suspension was then centrifuged in a Beckman SW 50.1 rotor at 35,000 rpm for 18 h. An opalescent band of virions approximately in the centre of the gradient was collected and dialyzed against a solution of 10 mM Tris pH 8, 1 mM EDTA.

DNA was released from virions by digesting them with pronase. The virion suspension was added dropwise to an equal volume of solution containing 20 mM Tris pH 7.4, 20 mM EDTA, 1% (w/v) SDS and 1 mg/ml pronase and incubated at 37^0C for 3 h. The solution was then phenol extracted, ethanol precipitated and dissolved in 1 ml of water.

Transfection of viral DNA

The calcium technique of Graham and van der Eb, (1973) was used to introduce viral DNA into cells, to recover infectious virus. Viral DNA was precipitated together with calcium phosphate in the

following way; 25 µl of 2.5 M $CaCl_2$ and a total of 10 µg of DNA were added to 0.5 ml of HEPES buffered saline at pH 7.1 (0.8% (w/v) NaCl, 0.037% (w/v) KCl, 0.0125% (w/v) $Na_2HPO_4.2H_2O$, 0.5% (w/v) HEPES, 0.1% (w/v) dextrose). For rescue of the input viral strain the 10 µg total of DNA consisted of 5 µg of viral DNA plus 5 µg of carrier DNA (1 to 2 mg/ml herring sperm DNA vortexed for 5 min). For rescue of a recombinant virus, the carrier DNA was substituted with 5 µg of DNA of the desired recombinant derivative of the plasmid pFGdx1. The precipitate, which formed after 20 min at room temperature was added into the medium of a 60 mm dish 293 cells, approximately 80% confluent. After 4 h the medium was removed and cells were overlaid with 10 ml of MEM F-11 containing 0.5% (w/v) agarose and supplemented as for infected cells. Plaques were visible by direct examination of the monolayer after approximately 7 days.

Identification of recombinants

Recombinant viruses were identified by analysis ethidium bromide stained restriction endonuclease fragments of viral DNA on agarose gels. Viral DNA was prepared from a random selection of plaques, as described below.

Virus was harvested from a plaque by mashing the agar overlay directly over the plaque with a Pasteur pipette and transfering it to a tube containing 1 ml of Joklik's modified MEM containing 10% (v/v) glycerol. To expand the quantity of virus recovered from a plaque, 35 mm dishes of HeLa cells were infected with 0.1 ml of this suspension. When the entire monolayer exhibited

CPE the medium was collected, supplemented with glycerol to 10% (v/v) and frozen for future use as virus stock. The infected cells were solubilized in 0.25 ml of lysing buffer with pronase (10 mM Tris pH 7.4, 10 mM EDTA, 0.5% (w/v) SDS, 0.5 mg/ml pronase) and incubated for 2 to 5 h at 37°C. The cell lysate was then extracted with phenol. If excessive viscosity of the solution interfered with phase separation during phenol extraction, it was reduced by removing cellular high molecular weight DNA by precipitation with salt, according to the method of Hirt (1967): NaCl was added to a final concentration of 1 M and the solution was incubated at 4°C overnight; the precipitated DNA was then removed by centrifugation for 5 min in an Eppendorf microfuge. After extracting with phenol the DNA was precipitated with ethanol and redissolved in 50 µl of water. 5 µl of this solution was sufficient for a single restriction enzyme fragment analysis.

2.4 ANALYSIS OF VIRAL PROTEINS

Metabolic labelling

60 mm dishes of cells were infected with 10 pfu/cell of the virus of interest. The cells were washed with labelling medium (Medium 199 lacking methionine) and incubated at 37°C for the desired length of time in 1 ml of labelling medium containing 10 to 40 µCi of L-[^{35}S]methionine for every hour of incubation. Cells were harvested at the end of the labelling period by scraping into the medium with a rubber policeman.

Immunoprecipitation

Cells from one 60 mm dish were pelleted and solubilized in 1 ml of RIPA buffer (0.1 M Tris pH 7.5, 0.15 M NaCl, 0.1 % (w/v) SDS, 1% (w/v) sodium deoxycholate, 1% (v/v) Triton X-100). After incubating for 10 min on ice, the cell lysate was centrifuged for 15 min at 4°C in an Eppendorf microfuge. The supernatant was collected and could be stored at this stage at -20°C prior to immunoprecipitation. For each immunoprecipitation 0.5 ml of supernatant was incubated with 50 µl of a suspension of protein A sepharose beads (50% (v/v) dry protein A sepharose beads, 50% (v/v) RIPA buffer) and the appropriate quantity of antibody, for at least 4 h at 4°C with continuous agitation. Protein A sepharose beads were then pelleted and washed 4 times in RIPA buffer. Proteins were eluted by resuspending the protein A sepharose beads in 20 µl of 2x sample buffer (50 mM Tris pH 7.2, 2% (w/v) SDS, 5% (v/v) β-mercaptoethanol, 10% (v/v) glycerol, 0.1% (w/v) bromophenol blue) and boiling for 3 min. After pelleting the beads, proteins from 10 µl of supernatant were resolved on a 10% (w/v) SDS polyacrylamide gel.

Several antibody solutions of different specificities and concentrations were used. The Ad5 72K E2a product was immunoprecipitated with the mouse monoclonal antibody H2-19 (Rowe et al., 1984; 10µl of ascitic fluid per reaction). VSV proteins were immunoprecipitated with a rabbit antiserum (10 µl per reaction) and the VSV glycoprotein was immunoprecipitated with a monospecific rabbit antiserum (Bell et al., 1984; 10 µl per reaction).

HSV TK was immunoprecipitated with a rabbit antiserum raised against crystallized HSV TK (2 µl per reaction) prepared by W.C. Summers (unpublished).

SDS polyacrylamide gel electrophoresis

Acrylamide solutions were diluted to the appropriate concentration, from a stock solution containing 30% (w/v) acrylamide and 0.4% (w/v) bisacrylamide. Resolving gels consisting of 10% (w/v) acrylamide in 0.375 M Tris pH 8.9 and 0.1% SDS were polymerized by adding ammonium persulfate to 0.06% and 1 µl/ml of TEMED (Bio Rad). Stacking gels consisting of 3.75% (w/v) acrylamide in 0.0625 M Tris pH 6.8 and 0.1% (w/v) SDS were polymerized by adding ammonium persulfate to 0.1% (w/v) and 2 µl/ml of TEMED. Gels were run in electrophoresis tank buffer (0.05 M Tris pH 8.9, 0.084 M glycine, 0.1% (w/v) SDS) until the bromophenol blue dye migrated to the bottom of the gel.

Autoradiography and fluorography

For autoradiography, gels were fixed for 1 h in a solution of 43% (v/v) methanol, 43% water (v/v), 14% (v/v) glacial acetic acid, dried and exposed to Kodak XAR5 or
XRP1 film at -60^{o}C.

For fluorography, gels were dehydrated by soaking in 2 changes of DMSO (dimethyl sulfoxide), impregnated with PPO (2,5-diphenyloxazole) by soaking in a solution of 20% (w/v) PPO in DMSO,

washed in running water for 1 h to remove DMSO and exposed to X-ray film as for autoradiography.

2.5 INHIBITION OF VIRAL DNA SYNTHESIS

Ara-C was added to a concentration of 20 μg/ml to culture medium after virus adsorption and every 8 h during infection (Gaynor et al., 1982).

2.6 INDUCTION OF VSV GLYCOPROTEIN-MEDIATED CELL FUSION

Approximately 18 h following infection with 10 pfu/cell of AdG12, HeLa cells were exposed to pH 5.25 fusion buffer (1.85 mM $NaH_2PO_4 \cdot H_2O$, 8.39 mM $Na_2HPO_4.7H_2O$, 2.5 mM NaCl, 10 mM HEPES and 10 mM 2-(N-morpholino)ethane-sulfonic acid) for 1 to 2 min (Florkiewicz and Rose 1984). Culture medium was then replaced and incubation at 37°C was continued. The maximum number of fused cells was apparent approximately 1 h later.

2.7 MAPPING THE 5' ENDS OF TRANSCRIPTS

RNA extraction

One or two 150 mm dishes of cells were harvested and washed in cold PBS (phosphate buffered saline). Cytoplasmic membranes were disrupted by resuspending cells in 1 ml of isotonic buffer (0.15 M NaCl, 10 mM Tris pH 7.8, 1.5 mM $MgCl_2$) containing 1% (v/v) NP40, incubating for 5 min at 0^oC and vortexing for 5 seconds. Nuclei were separated from the cytoplasmic extract by centrifuging for 5 min in a Sorval SS34 rotor at 5,000 rpm and 4^oC. RNA could then be extracted from either fraction.

Cytoplasmic RNA was purified by phenol/chloroform extraction modified as follows. An equal volume of buffer consisting of 7 M urea, 0.35 M NaCl, 0.01 M Tris pH 7.8, 0.01 M EDTA, and 1% (w/v) SDS was added to the cytoplasmic extract. After incubating at room temperature for 5 min an equal volume of phenol/chloroform was added and the mixture was shaken for 5 min. Phases were separated by centrifuging for 5 min in Sorval SS34 rotor at 12,000 rpm and 20^oC. The aqueous phase was extracted with phenol/chloroform at least 2 more times or until no interphase was visible and then precipitated with 3 volumes of 95% ethanol at -20^oC overnight. The precipitate was collected by centrifuging for 15 min at 12,000 rpm and 4^oC and washed 4 times with a solution of 95% ethanol:0.4 M potassium acetate pH 5.2 (2.5:1). After drying under vacuum the RNA pellet was dissolved in 0.6 ml of water.

Nuclear RNA was prepared from two 150 cm dishes of cells. Nuclei were dissolved in 2.5 ml of a solution of 4 M guanidinium isothiocyanate, 0.1 M β-mercaptoethanol, and 25 mM sodium citrate at pH 7.0. The solution was then layered on top of 1.5 ml of a 5.7 M CsCl solution in a Beckman Ultraclear ultracentrifuge tube and centrifuged in a Beckman SW 50.1 rotor at 30,000 rpm for a minimum of 8 h. The supernatant was pipetted out of the tube and tube walls were wiped dry with Kimwipes to remove traces of ribonucleases. The pellet was resuspended in 100 µl of 0.5% (w/v) SDS and heated at 55°C for 5 min to dissolve RNA. Undissolved material was removed by centrifuging for 5 min in an Eppendorf microfuge. RNA was precipitated out of the supernatant by adding potassium acetate to 0.3 M and 3 volumes of ethanol. After drying under vacuum the RNA pellet was dissolved on 0.6 ml of water.

Purified RNA preparations were quantitated by measuring the optical density at 260 nm of a 1:10 dilution of the final solution.

SI nuclease mapping:

i. Preparation of a single-stranded 5' end-labelled probe

100 µg of plasmid DNA was digested with restriction enzyme(s) to produce a fragment, (usually 100 to 500 base pairs long) containing the desired sequences. After raising the pH of the solution by adding Tris pH 8 to 50 mM, the digested DNA was dephosphorylated by incubating with 20 U of CIP (calf intestinal alkaline phosphatase) first for 30 min at 37°C and then for 30 min at 50°C. The DNA fragment of interest was purified by resolving it on a

5% (w/v) acrylamide gel, cutting out the appropriate band and eluting it from the acrylamide slice. The purified fragment was dissolved in 50 µl of water.

5µl of purified fragment was 5' end-labelled by incubating at 37°C for 30 min in linker-kinase buffer (see section 2.9, Ligation) with 50 µCi of [γ-^{32}P]ATP and 20 U of T4 polynucleotide kinase. After 2 ethanol precipitations the incorporation of ^{32}P was quantitated by measuring Cerenkov radiation emmitted by the dried pellet . Incorporation of $5x10^5$ to $1x10^6$ Cerenkov counts indicated a sufficiently high specific activity.

To separate the strands of the labelled DNA fragment the dried pellet was dissolved in a solution of 30% (v/v) dimethyl sulfoxide, 1 mM EDTA, 0.05% (w/v) xylene cyanol, and 0.05% (w/v) bromophenol blue, boiled for 2 min and quick-chilled on ice. The solution was then loaded on an approximately 26 cm long strand separating gel consisting of 5% (w/v) acrylamide, 0.1% (w/v) bisacrylamide dissolved in 0.5x TBE. The gel was run at no more than 200 V, to avoid gel heating. The electrophoresis time required to separate the strands was empirically determined for each DNA fragment. For probe B (Fig.6) the gels were run until the xylene cyanol dye migrated to the bottom of the gel and for probe A (Fig.6) gels were run for an additional 45 min at 200V. The resolved strands were located by autoradiography, the correct band was cut out (fast strand for probe A; slow strand for probe B) and the DNA was eluted from the acrylamide slice.

ii. Hybridization and S1 nuclease digestion

For each hybridization 30,000 Cerenkov counts of a single stranded probe and 20 µg of RNA were precipitated out of 0.25 ml of 0.3 M potassium acetate pH 5.2. After washing 2 times with 70% ethanol and drying under vacuum the pellet was dissolved in 30 µl of hybridization buffer at pH 6.4 (0.4 M NaCl, 40 mM PIPES, 10 mM EDTA, and 50% (v/v) recrystallized and deionized formamide). The solution was centrifuged for 5 min in an Eppendorf microfuge to remove debris, transferred to a 0.5 ml microfuge tube and incubated at 42°C for 16 h.

After hybridization the solution was transferred to a 1.5 ml microfuge tube containing 67 U of S1 nuclease (Boehringer Manheim) in 0.15 M NaCl, 50 mM sodium acetate, 5 mM $ZnSO_4$, at pH 4.6 and incubated at 37°C for 1 h. The digestion was terminated by first cooling the solution for 10 min on ice and then adding 50 µl of 4 M ammonium acetate and 30 µl of 0.2 M EDTA. The solution was extracted with phenol/chloroform once and ethanol precipitated together with 10 µg of transfer RNA. The precipitate was centrifuged, dissolved in 0.25 ml of 0.3 M potassium acetate and ethanol precipitated again. After washing with 70% ethanol the pellet was dried and dissolved in 10 µl of sequencing sample buffer (80% (v/v) formamide, 10 mM NaOH, 1 mM EDTA, 0.1% (w/v) bromophenol blue, 0.1% (w/v) xylene cyanol).

The length of the protected probe was determined by running 3 µl of the sample on an 8.5% (w/v) sequencing gel along with appropriate size markers (see below). The gel was exposed to X-

ray film, wet and wrapped in Saran Wrap, after being peeled off of the glass plate by adhering to scrap X-ray film.

Preparation of end-labelled DNA size markers

pBR322 HpaII fragments were 3' end labelled by partly extending recessed 3' ends with Klenow. 5 μCi of [α-^{32}P]dCTP, and 1 U of the Klenow fragment of E. coli DNA polymerase were added to the digested DNA in 11x restriction enzyme buffer (see section 2.9, Restriction enzyme digestion) and incubated for 10 min at room temperature.

Primer extension

The oligonucleotide AB494 (synthesized by the Central Facility of the Institute of Molecular Biology and Biotechnology at McMaster University) was designed by Spessot et al., (1989). It encompasses the 30th to the 54th nucleotide downstream of the 3' splice site of the transcript encoding the adenovirus E4 11K product.

30 to 50 ng of oligonucleotide primer was 5' end-labelled by incubating with 50 μCi of [γ-^{32}P]ATP, 20 U of T4 polynucleotide kinase in linker-kinase buffer (see section 2.9, Ligation) at 37^{o}C for 30 min. 2 to 4x10^{7} cpm of Cerenkov radiation, measured from the dried pellet after 2 ethanol precipitations, indicated a sufficiently high specific activity.

For each primer extension reaction 2x10^{5} cpm of labelled oligonucleotide and 20 μg of RNA were precipitated with ethanol out of 0.25 ml of 0.3 M potassium acetate and dissolved in 8 μl of 10 mM

Tris pH 7.9 and 1 mM EDTA. After adding 2µl of a solution of 10 mM Tris pH 7.9, 1 mM EDTA and 1.25 M KCl the nucleic acids were hybridized at 60°C for 1 h. After hybridization the tube was removed from the water bath and allowed to cool to room temperature. 25 µl of a solution of 20 mM Tris pH 8.7, 10 mM $MgCl_2$, 0.33 mM of each deoxyribonucleotide triphosphate and 10 µg/ml of actinomycin D, containing 10 U of avian myeloblastosis virus reverse transcriptase (Joseph Beard, Life Sciences) was added and the solution was incubated for 1 h at 37°C.

The length of extended products was determined by precipitating the final solution with ethanol, dissolving the pellet in 10 µl of sequencing sample buffer and running 3 µl of the sample on an 8.5% (w/v) sequencing gel as described for SI nuclease mapping.

2.8 CLONING PROCEDURES

Preparation of plasmid DNA

Frozen bacteria were revived by inoculating 5 ml of Luria Broth (1% (w/v) bactotryptone, 1% (w/v) NaCl, 0.5% (w/v) yeast extract) containing 50 µg/ml ampicillin and incubating overnight at 37°C with continuous agitation. The overnight culture was added to 500 ml of Luria broth containing ampicillin and incubated at 37°C with continuous agitation until the optical density of the culture at 660 nm was between 0.5 and 0.7. Plasmid amplification was then

induced by adding Chloramphenicol to 0.18 mg/ml and incubating for an additional 16 to 20 h.

Bacteria were harvested by centrifuging at 5,000 rpm in a Sorval GS3 rotor at 4°C. Cell walls were digested by resuspending the cells in 8 ml of lysozyme digestion buffer (4 mg/ml lysozyme, 50 mM glucose, 25mM Tris pH 8.0, and 10 mM EDTA) and incubating at room temperature for 5 min. Cells were lysed by adding 16 ml of alkaline SDS (0.2 M NaOH, 1% (w/v) SDS) and incubating on ice for 5 min. After adding 12 ml of 3 M potassium acetate to the lysate it was incubated for 15 min on ice and centrifuged for 30 min at 10,000 rpm in a Sorval SS34 rotor. The nucleic acids in the supernatant were precipitated at room temperature with 0.6 volumes of isopropanol for 15 min and collected by centrifugation at 10,000 rpm in a Sorval SS34 rotor for 30 min at 20°C. The pellet was washed with 70% ethanol, dried and dissolved in 3 ml of TE (10 mM Tris pH 8, 1 mM EDTA).

Plasmid DNA was further purified by precipitating RNA out of the solution by adding 6 ml of 5 M LiCl and incubating on ice for 30 min. The precipitate was removed by centrifuging for 15 min at 10,000 rpm in an SS34 Sorval rotor at 4°C. The supernatant was precipitated with 2 volumes of ethanol, centrifuged at 10,000 rpm for 30 min at 4°C, dried and dissolved in 0.5 ml of T.E. Residual RNA was digested with 40 µg/ ml of ribonuclease for 15 min at 37°C. Plasmid DNA was precipitated by adding 0.5 ml of 13% (w/v) polyethylene glycol, 1.6 M NaCl and incubating on ice for 30 min. The precipitate was centrifuged for 5 min in a microfuge and dissolved in

0.5 ml of TE. The solution was phenol extracted and precipitated with ethanol. The purified plasmid DNA was then dissolved in 1 ml of water and stored frozen at -20°C. The DNA concentration was determined by measuring the optical density at 260 nm and confirmed by comparison of ethidium bromide fluorescence on an agarose gel to a known standard.

Transformation of bacteria

This procedure was adapted from Maniatis et al (1982). 100 ml of Luria Broth was inoculated with 1 ml of an overnight culture of the appropriate bacteria: LE392 cells for transformation with pBR322 derivatives and DH5α cells for transformation with pUC 119. The culture was incubated with continuous shaking at 37°C until an optical density at 660 nm of 0.4 to 0.6 was reached. The culture was then centrifuged at 4°C and cells were resuspended in 50 ml of a solution of 50 mM $CaCl_2$, and 10 mM Tris pH 8.0. After incubating on ice for 15 min the cells were again centrifuged and resuspended in 6.7 ml of 50 mM $CaCl_2$ and 10 mM Tris pH 8.0. To attain maximum competence cells were stored at this stage for 24 h at 4°C.

0.2 ml aliquots of competent cells were used for each transformation. DNA was added in ligation buffer (approximately 40 ng of DNA in no more than 100 µl of buffer) and stored on ice for 30 min. Cells were then heat shocked in a 42°C water bath for 2 min. Luria broth without antibiotic was added and cells were incubated for 1/2 h at 37°C. Cells were then spread with a bent glass rod onto Luria agar plates containing 50 µg/ml of ampicillin. Varying amounts

of cells were spread onto several plates to insure a suitable density of plating. Plates were incubated for 16 to 24 h at 37°C.

Screening transformed colonies

The presence of the desired plasmid structure was determined by analyzing restriction enzyme fragments from small scale plasmid DNA preparations from a random selection of transformed bacterial colonies. If a low frequency of the desired recombinant plasmid was expected, the initial screening was performed by hybridizing a radioactive oligonucleotide probe to permeabilized bacterial colonies (see below, Colony Hybridization).

Small scale plasmid DNA preparations.

This procedure is a scaled down and simplified version of the previously described procedure for plasmid DNA preparation. The same reagents were used to prepare a crude nucleic acid solution without further purification of plasmid DNA.

A number of 2 ml overnight cultures (usually 12) from a single transformed colonies were prepared. 1 ml of each culture was centrifuged for 1 min in an Eppendorf microfuge. The pellet was resuspended in 100 µl of lysozyme digestion buffer and incubated for 5 min at room temperature. The cells were lysed with 200 µl of alkaline SDS on ice for 5 min. 150 µl of 3 M potassium acetate pH 5.2 was added to the lysate, incubated on ice for 15 min and centrifuged for 5 min in an Eppendorf microfuge. The supernatant was precipitated with ethanol and collected by centrifuging for 5 min in

an Eppendorf microfuge. The pellet was redissolved in 100 µl of 0.3 M potassium acetate pH 5.2 precipitated again with ethanol. The precipitate was centrifuged, washed with 70% ethanol and dried. The pellet was dissolved in 50 µl of water. 5 µl was sufficient for visualization of ethidium bromide stained restriction enzyme fragments on a gel.

Colony hybridization

Transformed bacterial colonies were transferred from the surface of an agar plate onto a piece of nitrocellulose. Dry nitrocellulose was applied to the surface of the plate and after becoming wet was lifted off with most of the bacteria adhering to it. The bacteria were permeabilized by placing the nitrocellulose on top of 3 layers of Whatman 3MM paper saturated with 0.5 M NaOH (with the bacteria facing away from the paper) for 1 h at room temperature. The nitrocellulose was then transferred onto a pad of 3 layers of Whatman 3MM paper saturated with 20x SSC (3 M NaCl, 0.3M Na citrate pH 7.0) and left for 15 min at room temperature to neutralize the NaOH. After two more changes of 20x SSC saturated pads, each 15 min apart, the nitrocellulose was air dried at room temperature and baked at 80^{o}C for 3 h. The nitrocellulose was then prehybridized in hybridization solution (4x SSC, 0.1% (w/v) BSA, 0.1% (w/v) Ficoll, 0.1% (w/v) polyvinylpyrrolidone, 0.5% (w/v) SDS and 100 µg/ml denatured salmon sperm DNA) for 4 to 6 h at room temperature. The hybridization solution was then replaced with

fresh hybridization solution containing 100 ng of 5' end-labelled oligonucleotide (see section 2.7, Primer extension) and incubated for 16 h a room temperature. After hybridization the nitrocellulose was washed in 4x SSC for 1 h, 2x SSC for 4 h and wrapped in Saran Wrap. Colonies which specifically bound the labelled probe were detected after exposure to X-ray film for approximately 1 h.

2.9 DNA MANIPULATIONS

Elution of DNA from Acrylamide

The band of interest, localized by ethidium bromide fluorescence was cut out of a gel. Approximately 1 cm^2 pieces of acrylamide were placed in 1.5 ml microfuge tubes. After mashing each piece of acrylamide into a paste with a tapered teflon rod it was suspended in 0.6 ml of elution buffer (500 mM ammonium acetate, 10 mM Mg acetate, 1 mM EDTA, and 0.1% (w/v) SDS) and incubated at 37^{o}C overnight. The acrylamide was removed from the suspension first by centrifuging for 5 min in an Eppendorf microfuge and then by passing the supernatant over a column of siliconized glass wool. The DNA was precipitated with ethanol and dissolved in 50 µl of water.

Elution of DNA from agarose

This is an adaptation of the procedure of Maniatis et al (1982) for electroelution of DNA from agarose gels. A slice of agarose containing the band of interest, localized by ethidium bromide fluorescence, was cut out of a gel and placed in a dialysis bag. The minimum volume of 0.5x TBE required to completely immerse the gel slice was added, the bag was tied and placed in a shallow layer of 0.5x TBE in an electrophoresis tank. Application of 100 V overnight through the bag allowed the DNA to migrate out of the gel slice into the surrounding buffer. The buffer was removed from the dialysis bag and the bag was rinsed with a small amount of fresh 0.5x TBE. The DNA solution was then phenol extracted, ethanol precipitated and dissolved in a small volume of water.

Ligation

A total of no more than 5 µg of DNA was incubated with 1 U of ligase in 10 to 20 µl of linker-kinase buffer (50 mM Tris pH 7.4, 1 mM spermidine, 10 mM MgCl, 15 mM dithiothreitol, 200 µg/ml BSA; Maniatis et al., 1982) overnight at 12 to 15^{o}C. To insert fragments into a vector a 2 to 5 fold molar excess of insert DNA was used. To recircularize plasmids the DNA concentration was reduced to approximately 0.3 µg per 100 µl.

Restriction enzyme digestion

Digestions were carried out either in 11X restriction enzyme buffer (1 µl of 0.1 M Tris pH 7.5, 0.5 M KCl, 0.08 M $MgCl_2$ for every 11 µl of digestion solution) or in buffers supplied by restriction enzyme manufacturers. Purified DNA was digested with 1 U of enzyme per ug of DNA at 37°C for 3 to 5 h. Several fold higher enzyme concentrations were used to digest crude DNA preparations.

Ethanol precipitation

After adding potassium acetate pH 5.2 to a concentration of 0.3 M (if the salt concentration of the solution low) and 3 volumes of 95% ethanol to the solution to be precipitated, the solution was cooled at -20°C for approximately 1/2 h or in dry ice until frozen. The precipitate was collected by centrifugation, washed with 70% ethanol and dried under vacuum.

Phenol/chloroform extraction

A solution of phenol was prepared by saturating melted, distilled phenol with 1 M Tris pH 8 and extracting twice with a solution of 0.1 M Tris pH 8 and 0.2% (v/v) β-mercaptoethanol. A chloroform solution was prepared by adding 1/24 volume of isoamyl alcohol

Nucleic acids solutions were purified by shaking or vortexing with an equal volume of either phenol or a 1.1 mixture of phenol:chloroform until an emulsion was formed. The emulsion was centrifuged until the phases were well separated. If the interphase

i. Detection of metabolically labelled VSV G.

To determine the approximate time of expression of VSV G during the lytic cycle, infected Hela cells were labelled with [^{35}S]methionine over broad time intervals. Detectable levels of VSV G could be immunoprecipitated from AdG12-infected cells beginning with the 8 to 16 h interval and continued through the last 24 to 36 h interval (Fig. 4). VSV G could not be detected in AdG4-infected cells.

The slight discrepancy in the mobility of VSV glycoproteins synthesized by AdG12 and the VSV marker, in this figure, is due to a strain difference between them. The gene in AdG12 was cloned from the San Juan strain while the marker proteins are of the HR-LT Toronto strain. Fig. 13 shows the mobility of VSV G expressed by AdG12 and the San Juan strain of VSV to be indistinguishable.

ii. Expression of VSV G at the cell surface: detection of cell fusion activity.

The low pH-induced cell membrane fusion activity associated with VSV-infected cells, was shown by Florkiewicz and Rose (1984) to occur in cells expressing the viral glycoprotein at their surface, in the absence of other viral proteins. This observation was used to test for the presence of VSV G on the surface of cells infected with the adenovirus recombinants. Cells were briefly exposed to pH 5.25 buffer 18 h following infection with AdG4 or AdG12. As shown in Fig. 5 the majority of cells infected with AdG12 were fused following this treatment. This result demonstrates the functional

was large the extraction was repeated. Traces of phenol were removed by one extraction with chloroform followed by precipitation with ethanol.

2.10 DNA SEQUENCING

Sequencing reactions

DNA was sequenced by the method of Sanger et al (1977) using the modified T7 DNA polymerase Sequenase^{TM} Reactions were carried out using reagents supplied in the Sequenase kit according to details outlined in the accompanying manual with the following modifications of Zhang et al., (1988)

(a) The template was prepared by denaturing 2 to 3 μg of plasmid DNA in the presence of 0.2 M NaOH and 0.2 mM EDTA in a total volume of 20 μl at room temperature for 5 min. The solution was then neutralized with 2 μl of 2M ammonium acetate, precipitated with ethanol and dried.

(b) 5 ng of oligonucleotide were hybridized to the template at 65^{o}C for 3 to 5 min and then cooled at room temperature for 30 min.

Primers used for sequencing were the pUC forward sequencing primer (BRL) and the oligonucleotide AB 212 (Fig. 8). AB 212 (5' ACA AAA GGC ACT TCA TAG 3'); synthesized by the Central Facility of the Institute of Molecular Biology and Biotechnology at McMaster University) encompasses the region of the VSV G gene

from 2 nt upstream of the translation initiator codon to the middle of the first RsaI site in the coding sequences.

Sequencing gels

100 ml of acrylamide solution consisting of 8.075% (w/v) acrylamide, 0.425% (w/v) bisacrylamide and 7 M urea were dissolved in freshly prepared TBE (0.1 M Tris, 0.1 M boric acid, 2 mM EDTA pH 8.3) were prepared for each gel. The gel was polymerized with 1 ml of 10% (w/v) ammonium persulfate and 40 µl of TEMED.

Plates were treated to induce adherance of the gel to the short plate and inhibit adherance to the long plate when plates were separated following electrophoresis. The short plate was treated with a solution of 10 ml absolute ethanol, 30 µl glacial acetic acid and 30 µl bindsilane (3-(Trimethoxysilyl)propylmethacrylate). The long plate was treated with a solution of 2% (v/v) repelsilane (dimethyldichlorosilane) in 1,1,1-trichloroethane. Each plate was treated with two applications of the appropriate solution. Each application consisted of spreading 5 ml of the appropriate solution onto the plate, allowing it to dry and then washing with ethanol.

3. RESULTS

3.1 CONSTRUCTION OF RECOMBINANT VIRUSES

Recombinants of human Ad 5, carrying coding sequences for the single glycoprotein of the Indiana serotype of VSV, linked to control sequences of the HSV-1 TK gene were constructed as shown in Fig. 3 The coding sequences for VSV G, contained within the indicated BamHI fragment of the plasmid pSVGL (Rose and Bergman, 1982), were substituted for the sequences between BglII and BamHI sites in the plasmid pTK3B (Varmuza, 1985), which include the translation initiation site and most of the coding sequences of the HSV-1 TK gene.[1] The resulting plasmid, pTK-G, carries a hybrid gene composed of VSV G coding sequences placed between TK promoter and polyadenylation sequences The TK-G hybrid gene was subcloned as an Xbal fragment into the single XbaI site of pMX2 (Graham, 1984), after ligating XbaI linkers to the PvuII fragment isolated from pTK-G The BamHI site at the 3' end of VSV G sequences was destroyed by digesting pTK-G with BamHI, filling in the recessed 3' ends with Klenow and religating. The TK-G gene was then linked to

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[1]This step was carried out by L. Prevec

cloned Ad 5 sequences by inserting the isolated XbaI fragment from the plasmid pMX2 TK-G into the single XbaI site of the plasmid pFGdx1 pFGdX1 contains the BamHI B fragment of Ad 5 (59.5 to 100 mu) with a deletion of the XbaI D fragment (Haj-Ahmad and Graham, 1986) which removes part of the E3 region. Resulting pdxI TK-G plasmids carried TK-G inserts in both orientations (Fig. 3A).

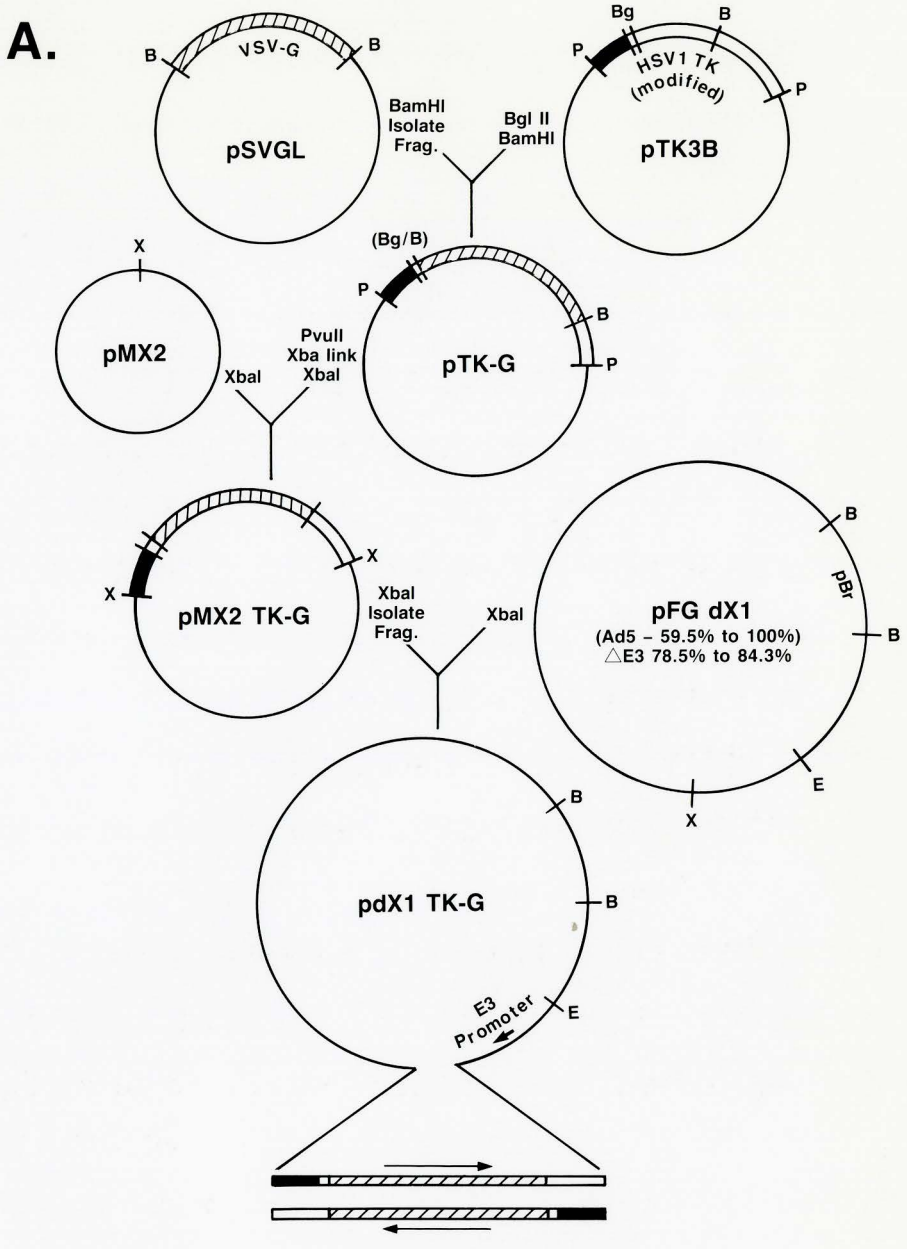
Cloned recombinant adenovirus sequences were rescued into virus by cotransfecting 293 cells with viral DNA from Ad 5 strain dl309 (Jones and Shenk, 1979b) that had been digested with EcoRI and pdxI TK-G plasmid DNA that had been digested with BamHI (Fig. 3B). Viral DNA from resulting plaques was analyzed for the presence of TK-G insertions. Viruses bearing TK-G insertions in both orientations were identified. A recombinant containing the TK-G gene in the orientation parallel to the E3 promoter was designated AdG12; a recombinant containing the TK-G gene in the opposite orientation was designated AdG4.

3.2 EXPRESSION OF VSV G BY A RECOMBINANT ADENOVIRUS IN CULTURED CELLS

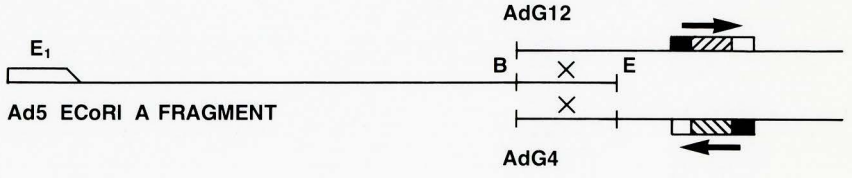
Since the E3 sequences that AdG4 and AdG12 lack are not essential for virus replication (Berkner and Sharp, 1983), the two methods described below allowed detection of VSV G in cultured cells during the course of a lytic infection.

FIG. 3. CONSTRUCTION OF VSV G-EXPRESSING ADENOVIRUSES.

Restriction enzyme sites B, BamHI, Bg, BglII, E, EcoRI, P, PvuII, X, XbaI. Hatched regions represent VSV G coding sequences, solid regions represent HSV-1 TK promotor sequences and open regions represent the remainder of the TK gene, including coding sequences and poly adenylation signal. (A) The TK-G hybrid gene was constructed by removing from the plasmid pTK3B, the BamHI to BglII fragment which contains the translation initiation site and most of the TK-coding sequences, and substituting VSV G coding sequences contained within the BamHI fragment of pSVGL. The resulting plasmid, pTK-G, contains VSV G sequences in the proper orientation relative to TK control sequences The TK-G hybrid gene was subcloned from pTK-G into pMX2 by excising with PvuII, ligating XbaI linkers and inserting into the single XbaI site of pMX2. The BamHI site at the 3' TK-G junction was destroyed by digesting pMX2 TK-G with BamHI, filling in recessed 3' ends with Klenow and religating. The XbaI fragment from pMX2 TK-G, containing the TK-G gene, was inserted into the single XbaI site of pFGdx1. Inserts of the TK-G gene were obtained in both possible orientations relative to the E3 promoter· G4, opposite, G12, parallel. (B) Cloned recombinant Ad 5 sequences in pdx1 TK-G plasmids were rescued into virus by digesting with BamHI, co-transfecting with EcoRI-digested Ad 5 dl309 DNA and screening resulting plaques for recombinants.


A.
VSV-G
B
B
pSVGL
BamHI
Isolate
Frag.
Bgl II
BamHI
Bg
B
P
HSV1 TK
(modified)
P
pTK3B
X
pMX2
Xbal
PvuII
Xba link
Xbal
(Bg/B)
P
B
P
pTK-G
X
X
pMX2 TK-G
Xbal
Isolate
Frag.
Xbal
B
pBr
B
pFG dX1
(Ad5 – 59.5% to 100%)
△E3 78.5% to 84.3%
E
X
B
B
pdX1 TK-G
E3
Promoter
E

B.


AdG12
E1
B
E
Ad5 ECoRI A FRAGMENT
AdG4

i. Detection of metabolically labelled VSV G.

To determine the approximate time of expression of VSV G during the lytic cycle, infected Hela cells were labelled with [^{35}S]methionine over broad time intervals. Detectable levels of VSV G could be immunoprecipitated from AdG12-infected cells beginning with the 8 to 16 h interval and continued through the last 24 to 36 h interval (Fig. 4) VSV G could not be detected in AdG4-infected cells.

The slight discrepancy in the mobility of VSV glycoproteins synthesized by AdG12 and the VSV marker, in this figure, is due to a strain difference between them. The gene in AdG12 was cloned from the San Juan strain while the marker proteins are of the HR-LT Toronto strain. Fig. 13 shows the mobility of VSV G expressed by AdG12 and the San Juan strain of VSV to be indistinguishable.

ii. Expression of VSV G at the cell surface: detection of cell fusion activity.

The low pH-induced cell membrane fusion activity associated with VSV-infected cells, was shown by Florkiewicz and Rose (1984) to occur in cells expressing the viral glycoprotein at their surface, in the absence of other viral proteins. This observation was used to test for the presence of VSV G on the surface of cells infected with the adenovirus recombinants Cells were briefly exposed to pH 5.25 buffer 18 h following infection with AdG4 or AdG12. As shown in Fig. 5 the majority of cells infected with AdG12 were fused following this treatment. This result demonstrates the functional

FIG. 4. EXPRESSION OF THE VSV GLYCOPROTEIN BY RECOMBINANT ADENOVIRUSES.

HeLa cells were labelled with [^{35}S]methionine (80 μCI/dish) during the indicated time intervals following infection with AdG4 or AdG12. Cell extracts were immunoprecipitated with monospecific anti-VSV G rabbit serum and proteins were resolved on 10% SDS polyacrylamide gels. VSV marker proteins were prepared from extracts of [^{35}S]methionine-labelled HeLa cells infected with VSV Indiana strain HR-LT and immunoprecipitated with rabbit anti-VSV serum. The upper band in the VSV marker lane is the mature glycoprotein, two immature forms are directly below it.

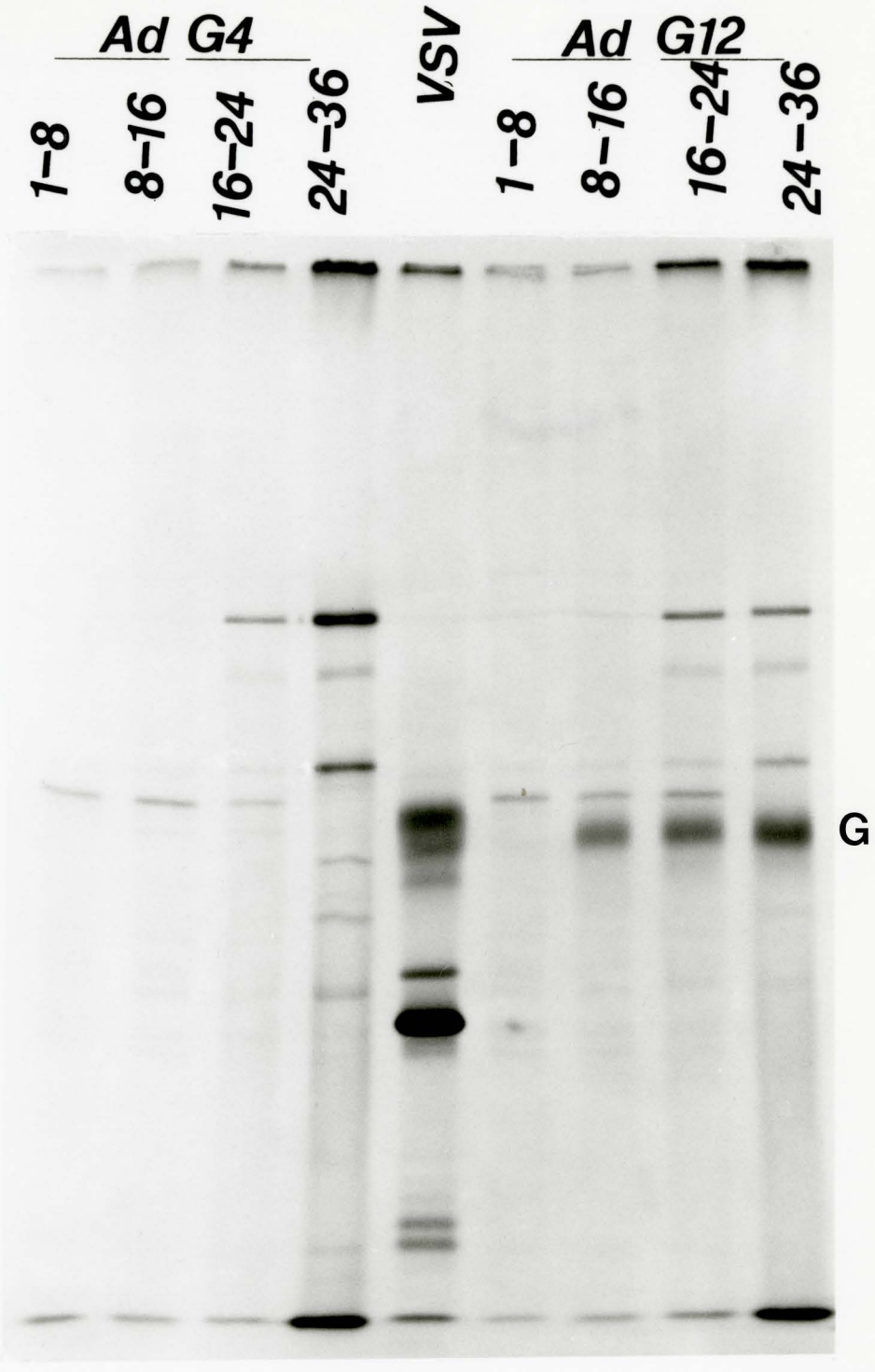
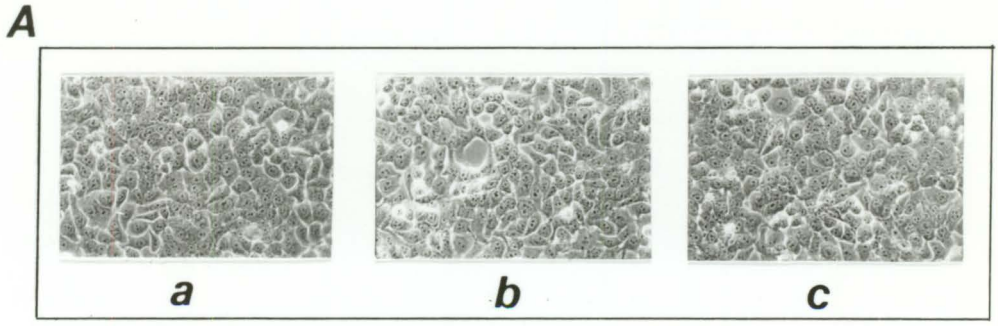
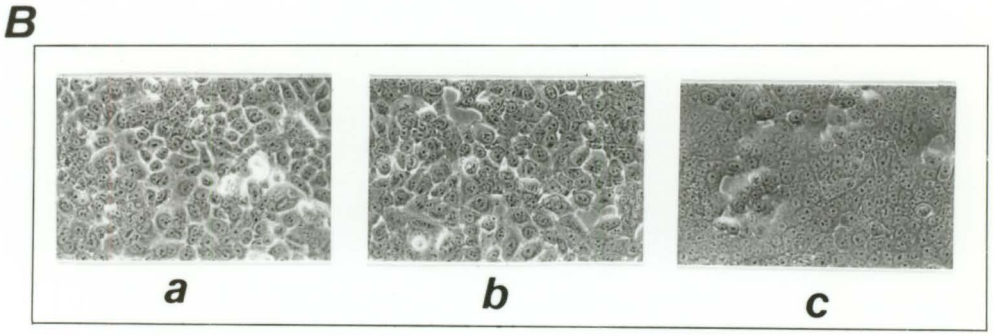

Ad G4
1-8
8-16
16-24
24-36
VSV
Ad G12
1-8
8-16
16-24
24-36
G

FIG. 5. LOW PH-INDUCED FUSION OF ADG12-INFECTED CELLS.

HeLa cells were infected with 10 pfu/cell of Ad 5 dl309 (a), AdG4 (b), or AdG12 (c). (A) Cells were photographed 18 h post infection. (B) The same cells were then exposed to pH 5.25 fusion buffer for 1-2 min as described in Materials and Methods, incubated for an additional 2 h in growth medium and rephotographed. Magnification is 250X.


A
a
b
c


B
a
b
c

integrity of VSV G at the cell surface and together with the previously shown correct electophoretic mobility of the metabolically labelled product, suggests correct post-translational processing and transport.

Cells infected with AdG4 remained unfused following the low pH treatment and resembled cells infected with dl309. This is consistent with the absence of detectable metabolically labelled protein in AdG4-infected cells.

3.3 CONTROL OF VSV G EXPRESSION FROM ADG4 AND ADG12 GENOMES

By metabolic labelling and the cell fusion assay, VSV G was easily detected in cells infected with AdG12 but not AdG4. The difference in expression of the VSV glycoprotein in recombinants with opposite orientations of TK-G gene insertion suggested that transcription of the glycoprotein coding sequences was controlled by flanking adenovirus sequences, rather than the adjacent HSV-1 TK promoter. This idea was tested by looking for the initiation sites of transcripts of VSV G coding sequences.

Analysis of 5' ends of VSV G transcripts.

The 5' ends of transcripts containing VSV G coding sequences were analysed by SI nuclease mapping. Cytoplasmic RNA, extracted from cells 18 h following infection with AdG4 and AdG12,

was hybridized to the single-stranded PvuII to RsaI fragment designated as probe A in Fig. 6. This probe contains 16 nt of VSV G coding sequences as well as all upstream TK sequences of the TK-G hybrid gene. Transcripts initiated by the TK promoter should hybridize with, and protect from SI nuclease digestion, 92 nt of this probe. Because of uncertainty regarding the ability of the TK promoter to function, the actual transcription initiation site and length of homology with the probe were unknown. To allow detection of a wide range of lengths of hybrid molecules, with a wide range of melting temperatures, hybridizations were carried out at two different stringencies: with [+F] and without [-F] formamide.

Although transcripts initiated by the TK promoter were not detected in this experiment, the entire probe was protected by RNA from AdG12-infected cells, under both hybridization stringencies (Fig. 7). This result demonstrates that transcription of VSV G coding sequences in AdG12 is initiated upstream of TK sequences, by adenovirus promoter(s).

The weak signal at approximately 25 nucleotides, observed when RNA from AdG12-infected cells was hybridized to the probe in the absence of formamide [-F], maps to the position of a series of three restriction enzyme sites, which are also present in an inverted order at the 3' end of VSV G sequences. These sites originated from the sequential addition of linkers to the ends of VSV G cDNA (EcoRI, XhoI and BamHI in proximal to distal order from ends of VSV G cDNA: see Fig. 6) during subcloning of these sequences by Rose and

FIG. 6. DETAILED MAP OF THE TK-G HYBRID GENE AND PROBES USED IN S1 NUCLEASE MAPPING.

Shown are. restiction enzyme cleavage sites (bracketed sites were destroyed prior to rescue into virus but were used in the construction of the TK-G gene (see Fig. 3) and in preparation of S1 probes), boundaries between sequences derived from the HSV-1 thymidine kinase gene (TK) and the VSV glycoprotein gene (G); the cap site and polyadenylation signal of the TK gene; VSV glycoprotein coding sequences (stippled) The scale is in nucleotides and numbering begins at the EcoRI site within pBR322 sequences of the plasmid pTK3BG (Fig. 3). Probe A and probe B are single-stranded DNA probes used in S1 nuclease mapping experiments. The PvuII site at 2061 and the RsaI site at 2351 are the boundaries of probe A. The EcoRI site at 2181 and the BglII site at 2312 are the boundaries of probe B

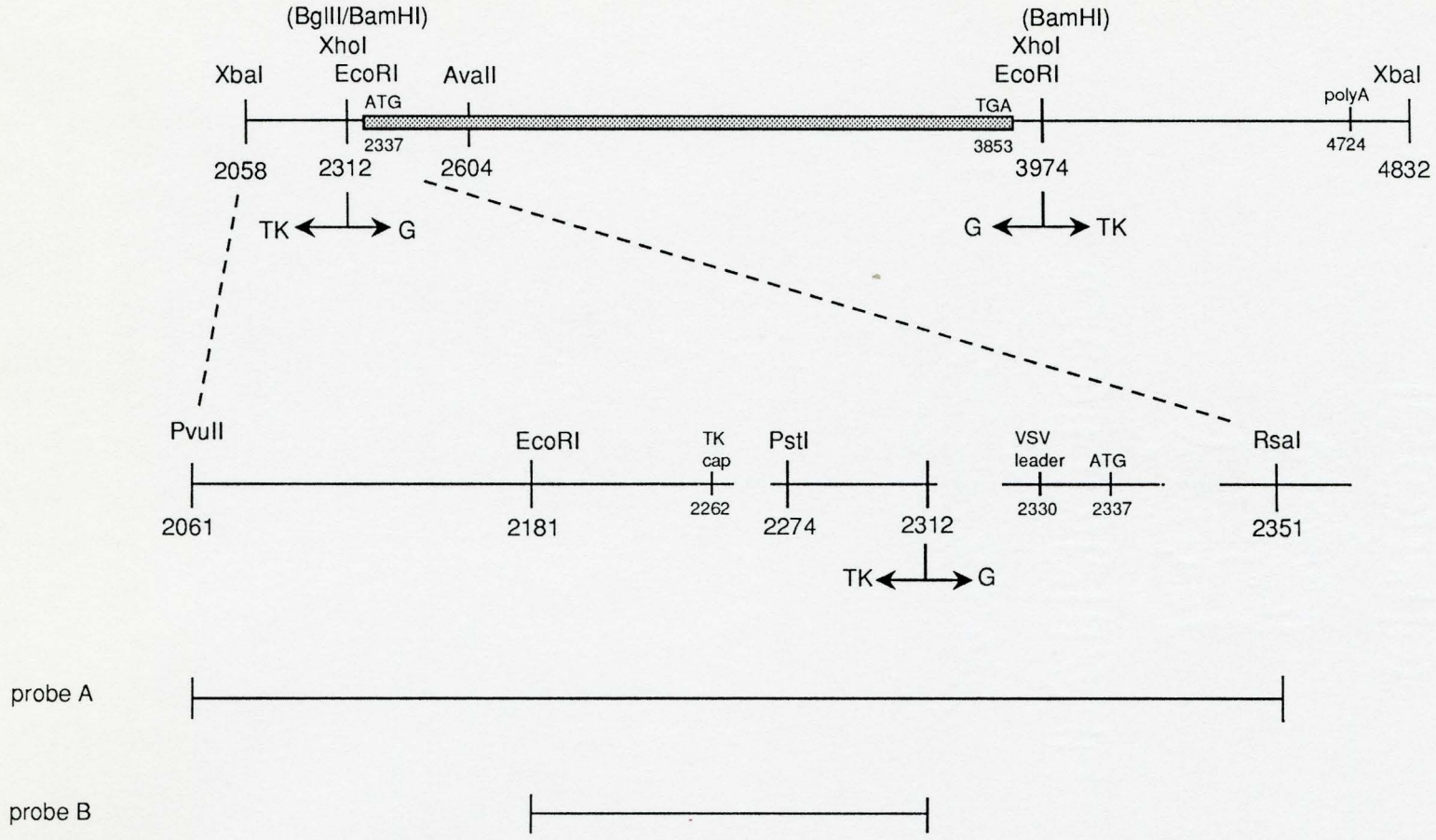
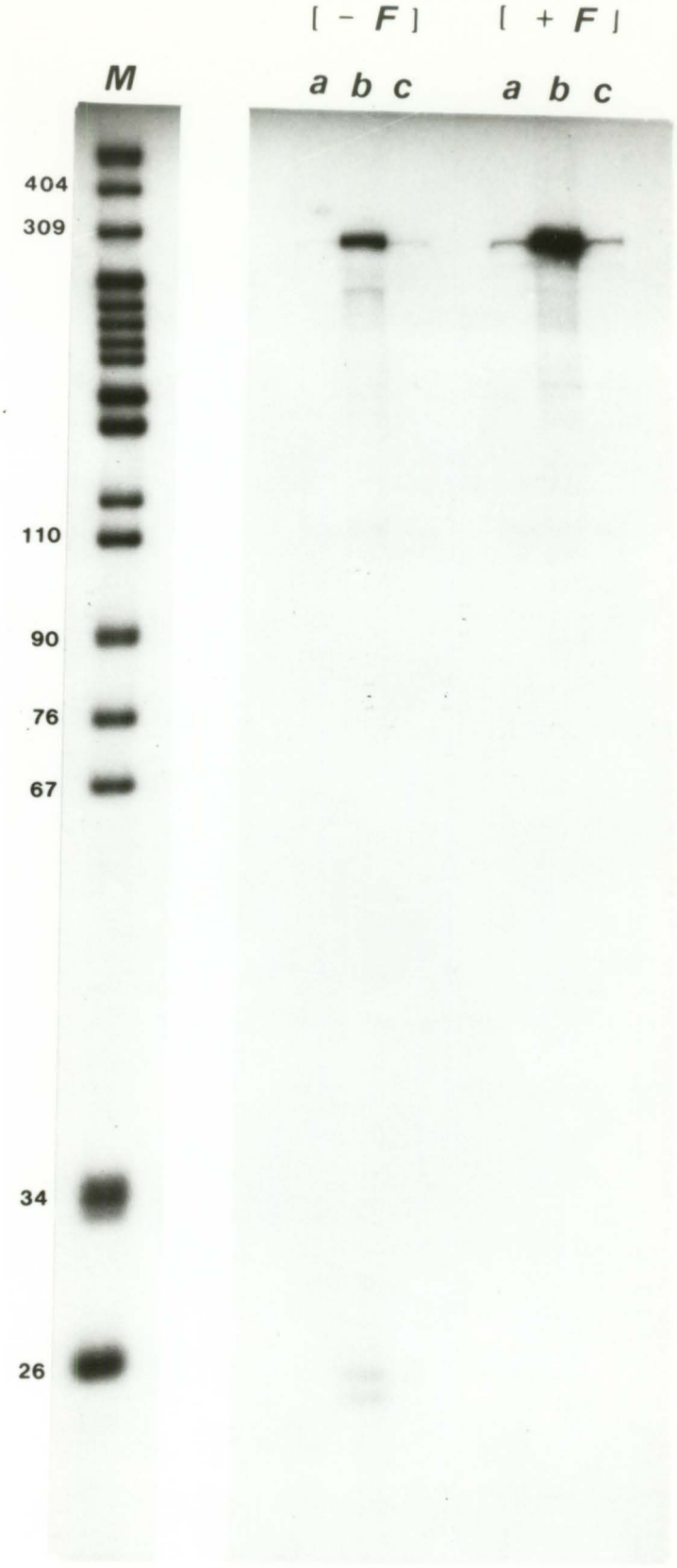

(BglII/BamHI)
XhoI
XbaI
EcoRI
AvaII
ATG
2337
2058
2312
2604
TK
G
(BamHI)
XhoI
EcoRI
TGA
3853
3974
G
TK
XbaI
polyA
4724
4832
PvuII
2061
EcoRI
2181
TK
cap
2262
PstI
2274
2312
TK
G
VSV
leader
2330
ATG
2337
RsaI
2351
probe A
probe B

FIG. 7. SI NUCLEASE ANALYSIS OF CYTOPLASMIC VSV G TRANSCRIPTS FROM ADG4- AND ADG12-INFECTED CELLS.

Cytoplasmic RNA (20 μg) extracted from HeLa cells 18 h following infection with 10 pfu/cell of AdG4 (lanes a), AdG12 (lanes b) or dl309 (lanes c). RNA was hybridized to probe A (see Fig. 6) at 42 ^{o}C using two hybridization stringencies, with ([+F], as described in Materials and Methods) or without [-F] formamide. Following SI nuclease digestion products were resolved on 8% sequencing gels. Lane M contains size marker fragments prepared by 3' end-labelling HpaII-digested pBR322 DNA. The numbers adjacent to the marker fragments represent their length in nucleotides.


[- F]
[+ F]
M
a b c
a b c
404
309
110
90
76
67
34
26

Bergman, (1982). Despite the alteration of both BamHI recognition sequences in the linker series, prior to their rescue into Ad5, there remain 18 consecutive nucleotides which could pair to form a loop in the mRNA molecule. It is therefore likely that this signal results from secondary structure in the mRNA formed by base pairing between these sequences.

Specific protection of lengths of probe both shorter and longer than expected by transcripts initiated from the TK promoter, indicates that the absence of a signal of the appropriate length was not due to unfavourable hybridization conditions.

Sequence determination of TK nucleotides upstream of VSV G coding sequences

To eliminate the possibility that a structural defect in the TK promoter prevented initiation of transcripts at the expected position, the TK-G gene was cloned out of AdG4 and AdG12 and the nucleotide sequence of the TK region upstream of VSV G coding sequences was determined.

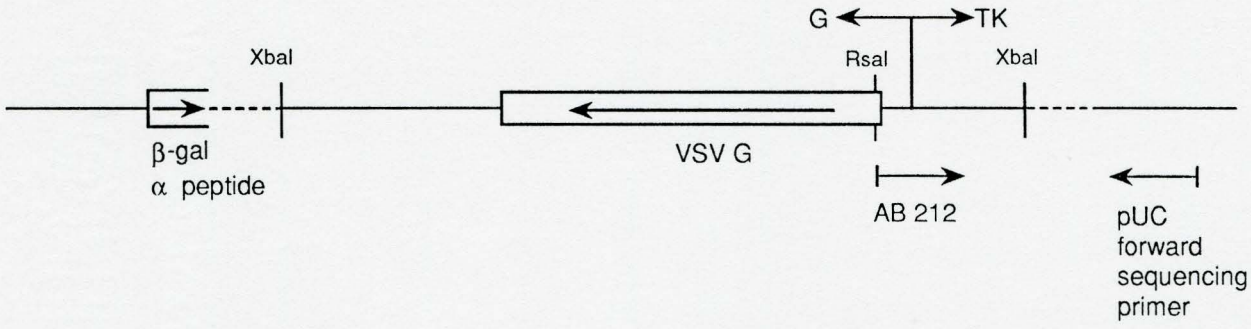
For cloning the TK-G gene from AdG4 and AdG12, small scale viral DNA preparations were used, prepared as for identification of recombinant adenoviruses, except that high molecular weight DNA was removed by high salt precipitation, according to the method of Hirt, (1967). After ligating XbaI fragments of viral DNA to XbaI-digested pUC 119, DH5α cells were transformed to ampicillin resistance. Bacterial colonies containing VSV G coding sequences were identified by detecting radioactive colonies following

hybridization to the ^{32}P-labelled oligonucleotide AB212. Only plasmids carrying inserts with the TK promoter oriented adjacent to the region homologous to the pUC 119 forward sequencing primer were rescued (Fig. 8). Insertion of the TK-G gene in the other orientation would result in expression of VSV G as a β-galactosidase fusion protein, which is likely to be toxic and preclude the rescue of these constructs. Rose and Schafferman, (1981) showed that the VSV G signal sequence, preceded by foreign amino acids, is toxic when expressed in bacteria.

Plasmids containing TK-G insertions derived from both recombinant viruses, pG4-1A1 from AdG4 and pG12-2.5 from AdG12, were sequenced using either the oligonucleotide AB212 or the pUC forward sequencing primer to prime synthesis in the opposite directions, as shown in Fig. 8 The sequence of most of the TK region upstream of VSV G coding sequences was determined in both directions. Short stretches at both ends, 12 nt from the TK-G junction and 15 nt from the former PvuII site at the adeno-TK boundary were sequenced in one direction only Several ambiguities in the sequence ladder were observed, however when the same sequence was read from the other strand it corresponded to the published sequence of the TK gene of HSV-1 strain 101 (Wagner et al., 1981). Since the TK sequences present in AdG4 and AdG12 were derived from HSV-1 strain KOS, these results demonstrate the identity of sequences between the two viral strains in this region of the TK gene and the absence of mutations which could depress the activity of the TK promoter

FIG. 8. MAP OF RECOMBINANT PUC 119 PLASMIDS CONTAINING INSERTS OF THE TK-G GENE DERIVED FROM ADG4 AND ADG12.

The XbaI sites mark the pUC119 and TK-G gene boundaries. Arrows joined at their ends indicate the boundary between sequences derived from the HSV-1 thymidine kinase gene (TK) and VSV glycoprotein gene. Open boxes indicate protein coding regions, arrows within them show directionl sense of the coding information. The oligonucleotide AB212 (see section 2.10, Sequencing reactions) and the pUC forward sequencing primer were used for sequencing in the directions shown by arrows, from the positions of the start of the arrows. The dashed line indicates a short pUC polylinker region, not drawn to scale.


G
TK
XbaI
RsaI
XbaI
β-gal
α peptide
VSV G
AB 212
pUC
forward
sequencing
primer

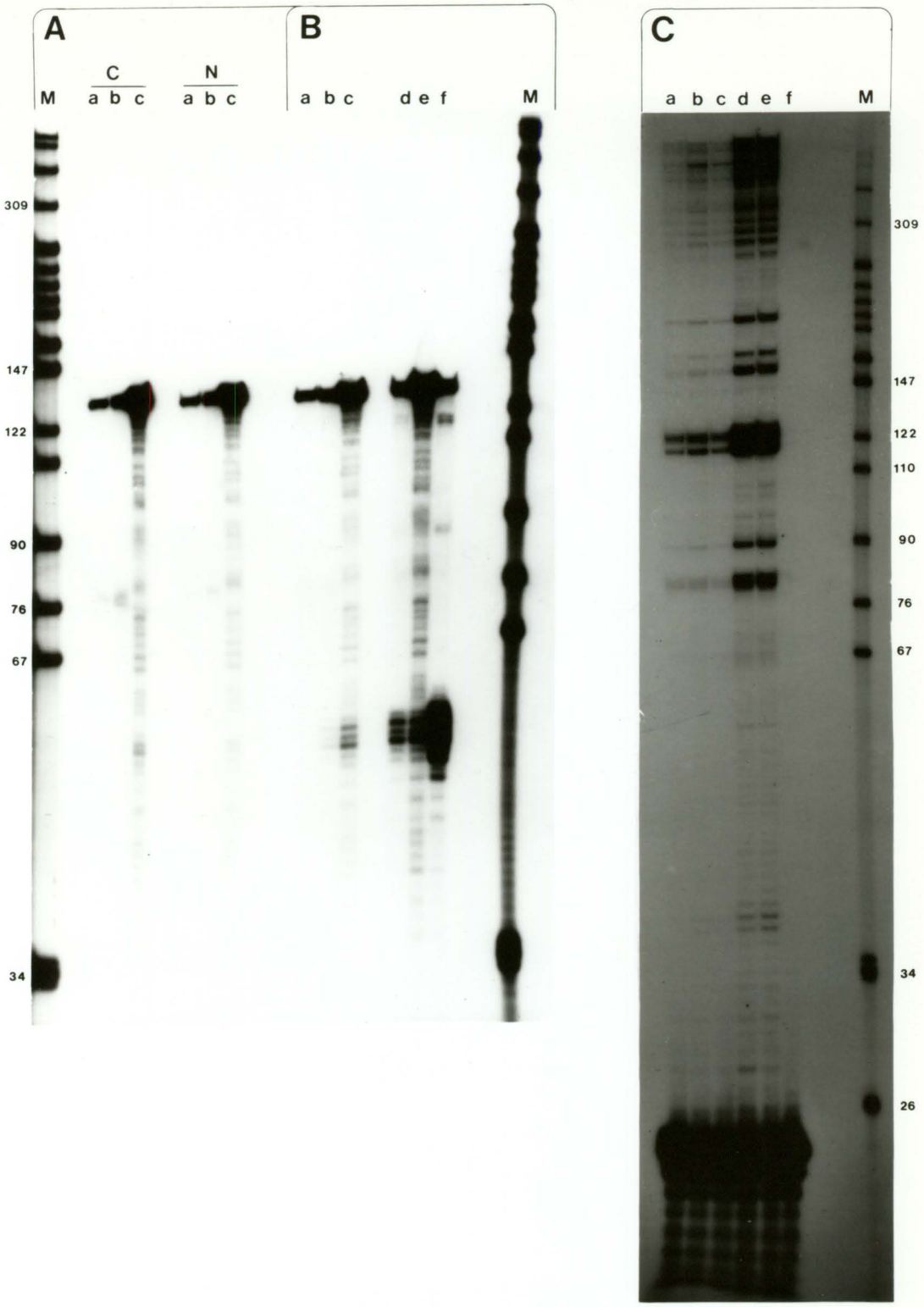
VSV G sequences in the TK-G gene were derived from one of a family of clones containing varying and unspecifed lengths of 5' non-translated leader (Rose and Bergman, 1982). This sequence information also established the length of the leader (7 nt) which was needed for accurate S1 nuclease analysis.

Test for accumulation of TK promoter-initiated transcripts in the nucleus.

Since nucleotide sequence analysis demonstrated that the absence of TK promoter initiated transcripts in the cytoplasm of cells infected with adenovirus recombinants was not due to a structural defect in the TK promoter, the possibility that a post-transcriptional effect could account for this result, was considered. A complex, consisting of the adenovirus 58K E1b product and the 34K E4 product, influences the accumulation of both cellular and viral mRNAs, at the post-transcriptional level, by regulating their transport from the nucleus to the cytoplasm (see section 1.4, Early region 1). The possibility that a selective block in transport, possibly via this 58K/34K complex, was operating on TK promoter-initiated transcripts and causing them to accumulate in the nucleus, was tested. RNA from nuclei as well as the cytoplasm was extracted separately 18 h following infection of HeLa cells. SI nuclease mapping, using the single-stranded EcoRI to BglII fragment designated as probe B in Fig. 6, shows no obvious accumulation of TK promoter-initiated transcripts in the nuclei of either AdG4 or AdG12 infected cells (Fig. 9A). The entire probe however, was protected by

FIG. 9. COMPARISON OF TK LEADER-BEARING TRANSCRIPTS: NUCLEAR AND CYTOPLASMIC TRANSCRIPTS OF ADG4 AND ADG12; CYTOPLASMIC TRANSCRIPTS OF ADTK2 AND ADTK4.

Cytoplasmic (C) or nuclear (N) RNA (panel A), or cytoplasmic RNA only (panels B and C), was extracted 18 h after infecting HeLa cells (panel A), or 293 cells (panels B and C) with adenoviruses: dl309 (lanes a), AdG4 (lanes b), AdG12 (lanes c), AdTK4 (lanes d), AdTK2 (lanes e) or 6 h after infecting 293 cells with HSV-1 strain KOS (lane f). 20 ug of RNA was hybridized to probe B (see Fig. 5) and digested with S1 nuclease (panels A and B) or to the oligonucleotide AB494, which was then extended with reverse transcriptase (panel C). The products were resolved on 8% sequencing gels. Lane M contains size marker fragments prepared by 3' end-labelling HpaII-digested pBR322 fragments. The numbers adjacent to the marker fragments represent their length in nucleotides.


A
B
C
M
C
N
a b c
a b c
a b c d e f
M
a b c d e f
M
309
147
122
90
76
67
34
309
147
122
110
90
76
67
34
26

both nuclear and cytoplamic RNA from AdG12-infected cells, indicating the presence of transcripts initiated upstream of the region encompassed by the probe. These transcripts should include the ones which were found to protect the entire probe used in Fig. 7, which contains the entire upstream TK region as well as VSV G coding sequences

Comparison between transcription of the HSV-1 TK gene and transcription of the TK-G hybrid gene

The lack of detectable VSV G synthesis in AdG4 was inconsistent with the results of Haj-Amahd and Graham, (1986), who detected TK enzyme activity in an adenovirus recombinant bearing the entire HSV-1 TK gene inserted in the same E3 antiparallel orientation. To see if this apparent inconsistency in detection of the two proteins could be accounted for by a difference in the accumulation of cytoplasmic transcripts, the quantities of these trancripts were compared. The 5' ends of transcripts bearing TK leader sequences common to both genes were analysed by SI nuclease mapping.

Cytoplasmic RNA was harvested from 293 cells 18 h following infection with AdG4, AdG12 and the two adenovirus recombinants bearing the entire TK gene in opposite orientations. AdTK2, E3 parallel and AdTK4, E3 antiparallel. RNA samples were hybridized to the same single-stranded probe as in Fig. 9A. This analysis revealed, in all four adenovirus recombinants, the presence of transcripts initiated at the position expected for the TK promoter

and comigrating with a signal generated by HSV RNA (Fig. 9B). In addition, transcripts initiated upstream of the TK promoter were detected in AdTK2 as in the analogous AdG12 recombinant.

The overall patterns of transcription in recombinants bearing the entire TK gene were the same as in recombinants bearing the TK-G hybrid gene with one exception: a small excess in the quantity of TK promoter initiated transcripts was observed in AdG12 compared to AdG4, but no comparable difference was apparent in the AdTK recombinants.

To control for relative quantitative differences in amounts of viral RNA in this experiment, the same four RNA samples were analyzed for the presence of E4 transcripts. Primer extensions were performed using the oligonucleotide AB494, which was designed to detect transcripts encoding the E4 11K product (Spessot et al., 1989). AB494 primes the synthesis of a family of extension products 120 to 126 nt long. These products appear in Fig. 9C as a pair of bands, one about 122 nt long and the other slightly shorter than 120 nt. The slight deviation of the smaller band from the expected length can be explained by the fact that the sequence of the region of Ad 5 immediately upstream of the primer has not yet been determined but was assumed to be the same length as the Ad 2 genome (GenBank accession JO1969 JO1973 JO1975). The weaker bands below are probably due to pauses in reverse transcription while the weaker bands above could represent some of less abundant, alternately spliced E4 mRNAs (Tigges and Raskas, 1984). This analysis revealed no quantitative differences in E4 transcripts,

between samples containing the same insert in different orientations. The small quantitative difference in TK initiated transcripts between the two VSVG-bearing recombinants is therefore not due to a procedural error A difference in the quantities of E4 transcripts, between recombinants bearing different inserted genes, was observed. the viruses bearing the TK gene produced more E4 transcripts. This is an indication either of a greater accumulation of E4 transcripts in the AdTK viruses than in TK-G bearing viruses, from a similar number of genomes, or transcription from a larger number of AdTK genomes. If the latter alternative were true, it suggests a larger particle to pfu ratio for the AdTK viruses, since infections were carried out using the same number of pfu of each virus.

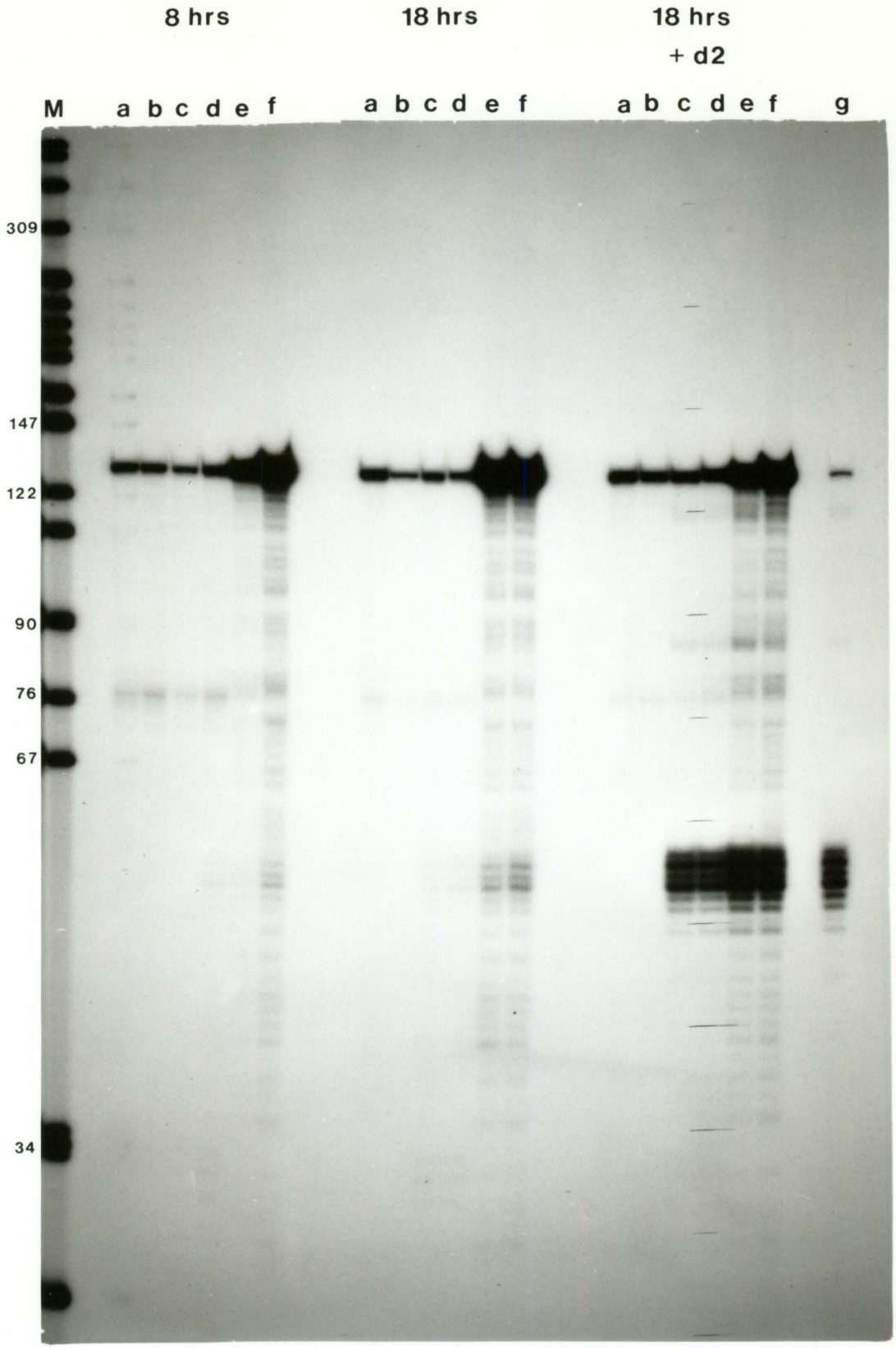
Response of the TK promoter to superinfection by HSV-1

The functional integrity of the TK promoter, in TK-G bearing adenovirus recombinants, was verified by testing its responsiveness to stimulation by HSV transactivating proteins For this purpose cells infected with adenovirus recombinants were superinfected with an HSV-1 mutant, HSVTKd2, having a deletion in the TK gene (Smiley, 1980) SI nuclease analysis of cytoplasmic RNA harvested 18 h following infection with recombinant adenoviruses showed a significant increase in the quantity of TK promoter-initiated transcripts in response to superinfection with HSVTKd2 (Fig. 10).

Because of a small apparent difference in the quantity of TK promoter initiated transcripts observed in HeLa cells compared to 293 cells (compare Fig. 9A with Fig. 9B), each infection in this

FIG. 10. RESPONSE TO SUPERINFECTION WITH HSV-1 OF TK LEADER-BEARING TRANSCRIPTS.

HeLa cells (lanes a, c and e) or 293 cells (lanes b, d and f) were infected with 10 pfu/cell of Ad5 dl309 (lanes a and b) AdG4 (lanes c and d) or AdG12 (lanes e and f). Cytoplasmic RNA was extracted 8 or 18 h after infecting with adenoviruses and 6 h after superinfecting with 2 pfu/cell of the HSV-1 TKd2 mutant. 20 μg of RNA was hybridized to probe B (see Fig. 6) and following digestion with S1 nuclease products were resolved on 8% sequencing gels. Lane g contains an S1 analysis of RNA extracted from HSV-1-infected cells to indicate the normal start site for TK-initiated transcripts (not for quantitative comparison). Lane M contains size marker fragments prepared by 3' end-labelling HpaII-digested pBR322 fragments. The numbers adjacent to the marker fragments represent their length in nucleotides.


8 hrs
18 hrs
18 hrs
+ d2
M
a b c d e f
a b c d e f
a b c d e f g
309
147
122
90
76
67
34

experiment was carried out in both cell types, to investigate the possibility of a host mediated effect on the efficiency of TK promoter function. The indistinguishable quantities of TK promoter initiated transcripts in each cell type, seen at 18 h following infection, does not support the idea of such an effect.

The 8 h infections were included to test for differences in the kinetics of expression of VSV G coding transcripts between the two cell types At this time point there are more TK promoter-initiated transcripts and more transcripts initiated upstream of the probe in adenovirus sequences in AdG12-infected 293 cells than in HeLa cells. These results suggest that adenovirus infection proceeds more rapidly in 293 cells than in HeLa cells.

Translation of transcripts of TK and TK-G genes

In the previous section, adenovirus recombinants were shown to produce at least two classes of transcripts of both the HSV-1 TK gene and the TK-G hybrid gene when inserted in the E3 parallel orientation one initiated by the TK promoter and one initiated in adenovirus sequences. Only one class of transcript, that initiated by the TK promoter, was detected in recombinants carrying the heterologous gene in the E3 antiparallel orientation. The ability of each class of transcript to be translated can therefore be determined by comparing the synthesis of the heterologous protein, in recombinants carrying the heterologous gene inserted in opposite orientations

i. VSV glycoprotein synthesis by AdG4

Because only one class of transcript of VSV G sequences was detected in AdG4-infected cells, that initiated by the TK promoter, detection of VSV glycoprotein synthesis would demonstrate translation of this class of transcript. Since previous attempts to detect VSV G in AdG4-infected cells by immunoprecipitation of metabolically labelled protein were unsuccessful, the sensitivity of the assay was increased by labelling cells with a higher concentration of [^{35}S]methionine and by enhancing detection of radioactivity with fluorography Fig 11A shows the results of immunoprecipitating, with anti-G antiserum, extracts of 293 cells labelled for 2 h, beginning 17 h following infection with adenoviruses. Synthesis of a protein of the correct mobility, in AdG4-infected cells, demonstrates that TK promoter initiated transcripts are translated into glycoprotein

ii. TK protein synthesis by AdTK2 and AdTK4

In TK-G-bearing recombinants, the synthesis of a greater quantity of VSV G in the E3 parallel orientation is correlated with the presence of an abundant transcript, which is initiated in upstream adenovirus sequences. To see if this correlation is maintained in TK bearing recombinants, synthesis of TK protein was examined in AdTK2 and AdTK4.

293 cells were metabolically labelled with [^{35}S]methionine 17 to 19 h following infection with AdTK2 and AdTK4 and extracts were immunoprecipitated with a monospecific anti-TK antiserum. As

FIG. 11. COMPARISON OF SYNTHESIS OF HETEROLOGOUS PROTEINS IN FOUR ADENOVIRUS RECOMBINANTS: ADG4, ADG12, ADTK4 AND ADTK2.

293 cells were labelled with 160 μCi/dish (panel A) or 40 μCi/dish (panel B) of [^{35}S]methionine 17 to 19 h after infecting with 10 pfu/cell of the indicated adenoviruses. Cell lysates were immunoprecipitated with anti-VSV G serum (A) or anti-TK serum (B). Marker proteins from VSV- or HSV-infected cells were labelled with [^{35}S]methionine 5 to 7 h after infection and immunoprecipitated from cell extracts with anti-VSV serum (panel A) or anti-TK serum (panel B).

A
VSV
dl 309
Ad G4
Ad G12
G
B
VSV
mock
dl 309
AdTK4
AdTK2
HSV
TK

seen in Fig. 11B, a greater quantity of TK protein is synthesized by AdTK2 which has the TK gene inserted in the E3 parallel orientation.

In this experiment TK protein from AdG4- and AdG12-infected cells is detected against a background of a co-migrating protein which is not likely to be TK-related, since it is also detected in dl309-infected cells. The two proteins directly below TK, which are not seen in dl309-infected cells, are probably products of the TK gene initiated at the two downstream, in-phase, translation initiator codons (Harr et al., 1985). The larger proportion of these proteins in Ad-infected cells than in HSV-infected cells may reflect a difference in initiator codon usage during the course of infection by the two viruses

TK-bearing recombinants exhibit an orientation dependent difference in synthesis of TK protein which is analogous to VSV G-bearing recombinants for both heterologous genes the quantity of protein is greater when inserted in the E3 parallel orientation. The correlation of a greater quantity of protein with the presence of abundant transcripts initiated in adenovirus sequences, indicates that these transcripts are translated.

Kinetics of Expression of VSV G from AdG12

Since the majority of VSV G detected in AdG12 is translated from transcripts initiated in adenovirus sequences, definition of the kinetics of VSV G expression would assist in identifying these sequences. To distinguish between early and late kinetics, dependence of VSV G expression on DNA synthesis was determined.

Fig. 12 shows that inhibition of DNA synthesis with ara-C, in cells labelled 14 to 18 h following infection, had a similar effect on the detection of VSV G as on an adenovirus early gene product: the E2a 72K protein, synthesis of both proteins was slightly reduced but not eliminated

Additional evidence for early kinetics of VSV G expression was obtained by more precisely defining the time of onset of VSV G synthesis, relative to the adenovirus 72K protein. By labelling for 1 h intervals, the earliest time during which VSV G synthesis could be detected was 6 to 7 h following infection. This corresponds to the earliest time interval for detection of the E2a 72K protein (Fig. 13).

The expression of VSV G in AdG12 prior to DNA synthesis implies that its expression is controlled by an adenovirus early promoter

3.4 THE POTENTIAL OF ADENOVIRUS AS A VACCINE VECTOR

The practical utility of recombinant viral genomes as vaccine vectors depends on their ability to deliver antigens into a wide range of animal hosts and to express the antigens in sufficient quantities to stimulate an immune response. Both of these factors were tested with the VSV G-expressing recombinant AdG12.

AdG12 has the potential of serving not only as a vaccine for the disease VSV produces in cattle but also as a safe experimental model for more serious pathogens, such as the related rabies virus.

FIG. 12. VSV GLYCOPROTEIN SYNTHESIS IN ADG12-INFECTED CELLS IN THE PRESENCE OF ARA-C.

HeLa cells were labelled with [^{35}S]methionine during the indicated number of hours after infection with Ad5 dl309 (lanes a), AdG4 (lanes b) or AdG12 (lanes c) in the absence or presence (+ara-C) of ara-C. One half of each cell extract was immunoprecipitated with anti-VSV G serum (A) and the other half of the same extract was immunoprecipitated with the monoclonal antibody H2-19 specific for the adenovirus E2a 72K protein (B). VSV marker proteins of the Indiana San Juan strain are shown.

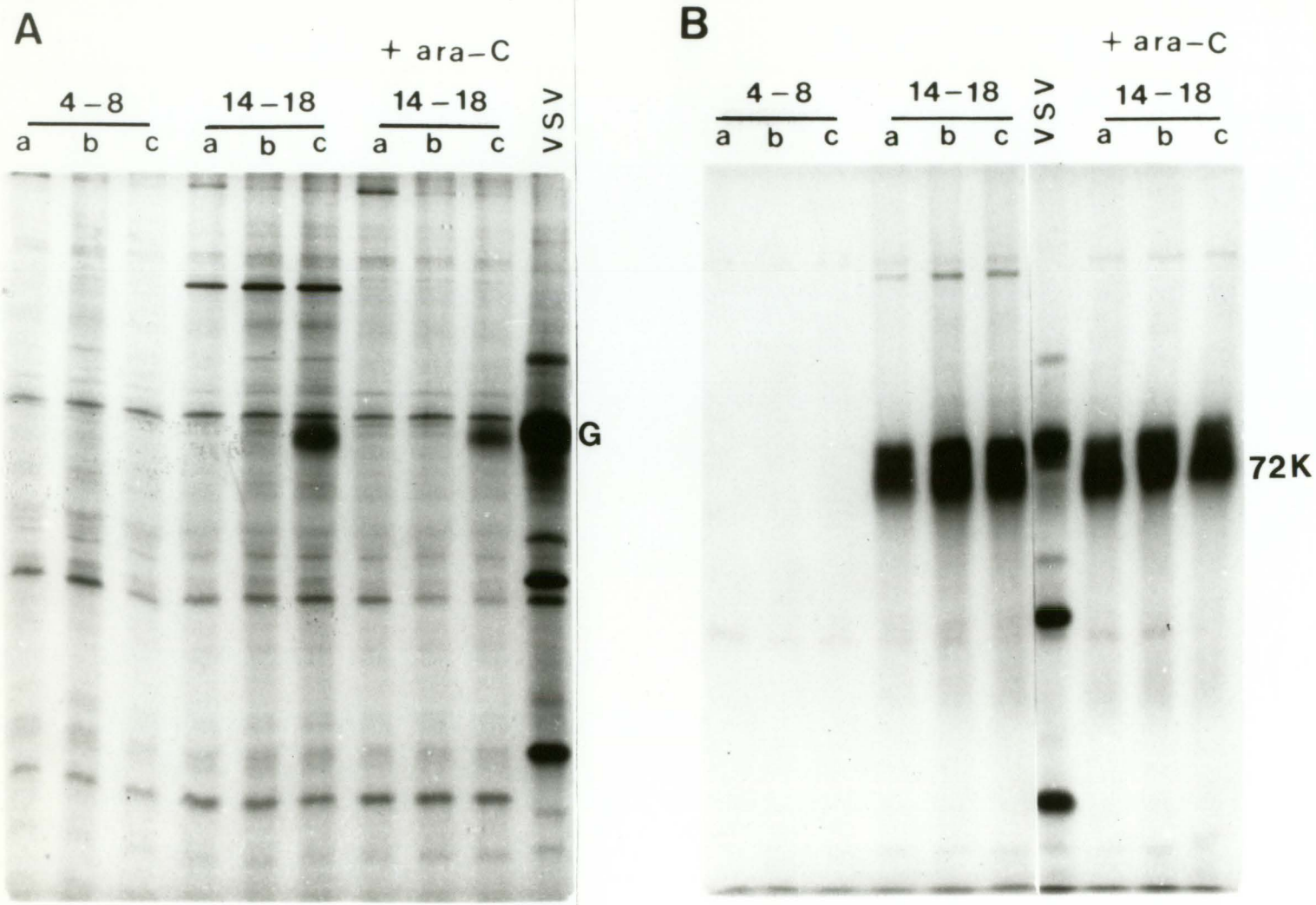
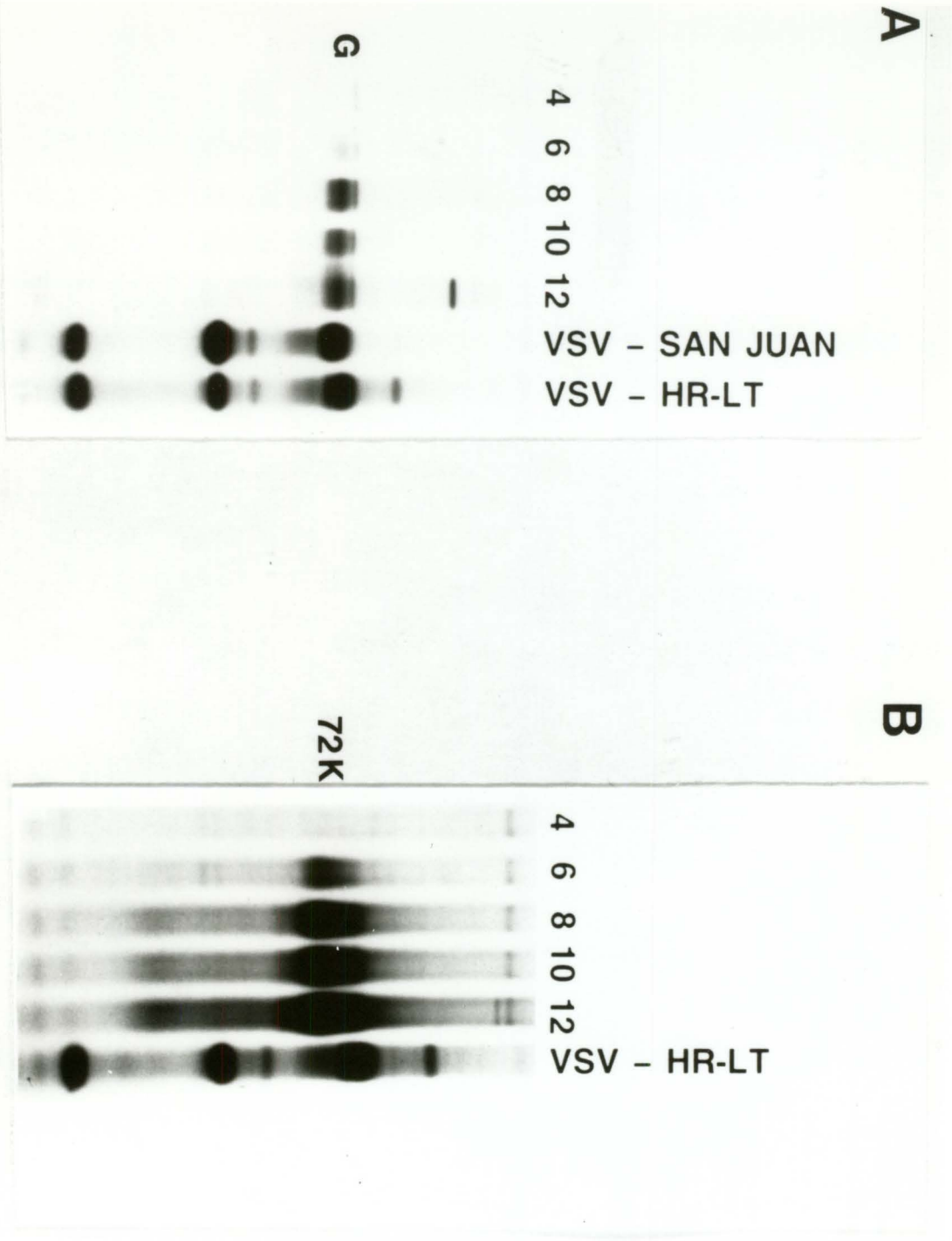

A
+ ara–C
4–8
14–18
14–18
a b c a b c a b c
VSV
G
B
+ ara–C
4–8
14–18
14–18
a b c a b c VSV a b c
72K

FIG. 13. TIME OF ONSET OF VSV GLYCOPROTEIN EXPRESSION IN ADG12-INFECTED CELLS.

HeLa cells were infected with AdG12 and labelled with [^{35}S]methionine for 1 h beginning at the indicated times post infection. One half of each cell extract was immunoprecipitated with anti-VSV G serum (A) and the other half of the same extract was immunoprecipitated with the monoclonal antibody H2-19 specific for the adenovirus E2a 72K protein (B). Proteins were resolved on 10% SDS polyacrylamide gels. VSV marker proteins from two strains of the Indiana serotype, San Juan and HR-LT, are shown. The glycoprotein expressed in AdG12 was cloned from the San Juan strain and comigrates with the mature form of the glycoprotein of the corresponding VSV strain.


A
G
4 6 8 10 12
VSV - SAN JUAN
VSV - HR-LT
B
72K
4 6 8 10 12
VSV - HR-LT

Host systems for testing both of these applications were chosen: bovine for VSV and canine for rabies. The mouse was also tested as a host, to investigate its potential to serve as a small experimental animal.

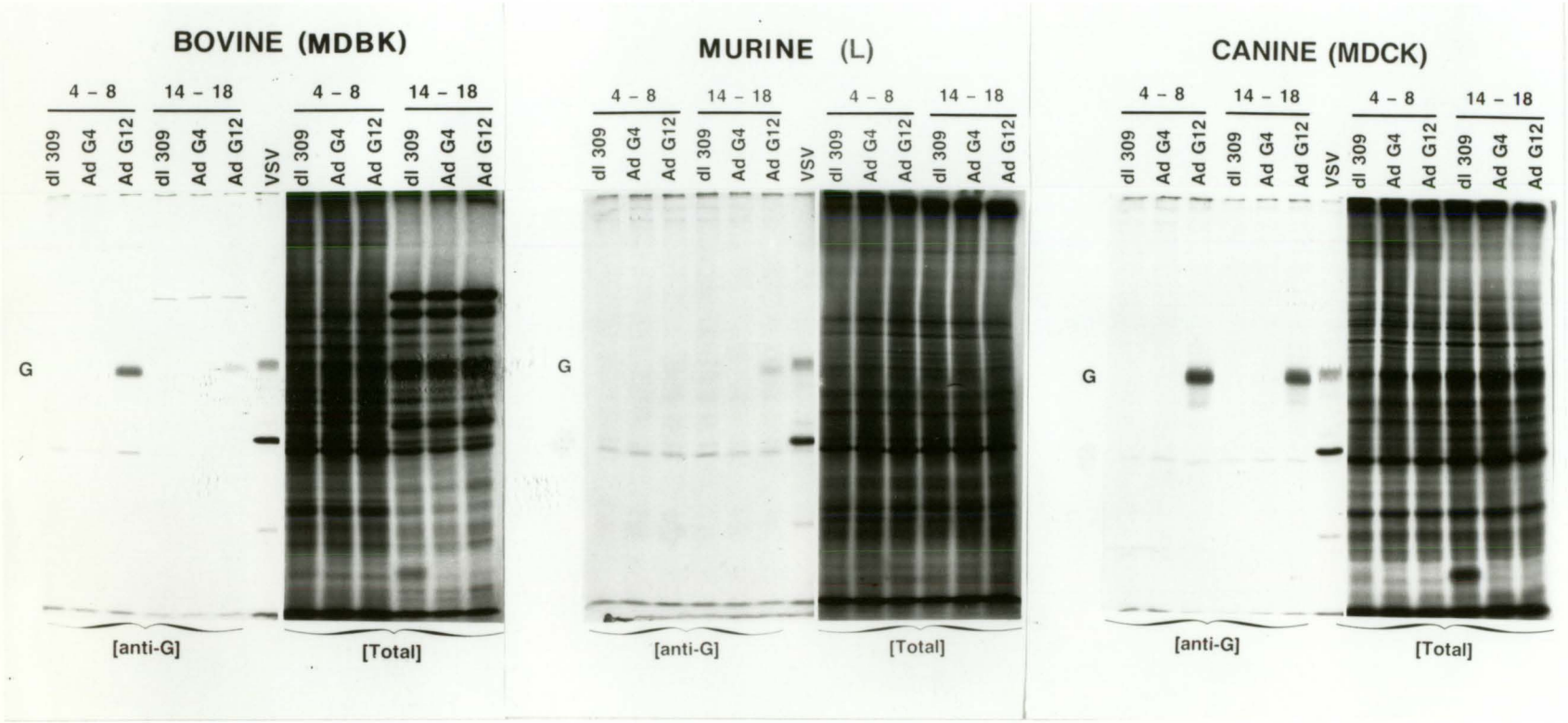
VSV G expression and virus replication in cultured bovine, canine and murine cells.

The host range of adenovirus recombinants was explored by infecting cultured murine fibroblast (L), bovine epithelial (MDBK), and canine epithelial (MDCK) cells. Cells infected with AdG4, AdG12 and Ad5 strain dl309 were labelled with [^{35}S]methionine for 4 h beginning at 4 or 14 h following infection. Infected cell extracts were tested for the expression of VSV G and for replication of the virus. To test for VSV G expression one portion of the each extract was immunoprecipitated with anti-G antiserum and analyzed by electrophoresis. To test for virus replication another portion of the total extract was analyzed by electrophoresis: a change in the total protein profile between the two labelling periods, resulting from the appearance of late viral proteins and inhibition of host protein synthesis, indicated virus replication (Fig. 14).

In bovine (MDBK) cells infected with AdG12, VSV G synthesis was easily detected during both labelling intervals. Changes in the total protein profiles indicated replication of all three viruses. MDBK cells therefore resemble human cells as adenovirus hosts: they are permissive for virus replication and VSV G is expressed with early kinetics.

FIG. 14. EXPRESSION OF THE VSV GLYCOPROTEIN IN BOVINE, MURINE AND CANINE CELLS.

MDBK (bovine), L (murine) or MDCK (canine) cells were labelled 4 to 8 or 14 to 18 h after infecting with 10 pfu/cell of the indicated adenoviruses. One portion of each cell extract was immunoprecipitated with anti-VSV glycoprotein serum [anti G] and another portion of the same extract was left untreated [total]. Proteins were resolved on 10% SDS polyacrylamide gels. VSV marker proteins were prepared from cells infected with the VSV Indiana HR-LT strain.


BOVINE (MDBK)
MURINE (L)
CANINE (MDCK)
4 – 8
14 – 18
dl 309
Ad G4
Ad G12
VSV
G
[anti-G]
[Total]

Canine cells infected with AdG12, also produced easily detectable levels of VSV G at both labelling intervals. No change in the total protein profile was observed following infection with any of the three adenoviruses. The latter observation, together with the fact that MDCK cells continue to grow and can be passaged after being infected with 10 pfu/cell of dl309, show that they are non-permissive for adenovirus replication. Synthesis of VSV G in these cells shows that adenovirus can enter these cells and express some of its products.

In murine cells infected with AdG12, VSV G synthesis was detected only during the 14 to 18 h interval No change in the total protein profile was observed with any of the three viruses. Since Tremblay et al., (1985) showed that the kinetics of adenovirus gene expression is retarded in mouse cells, the 14 to 18 h interval in this experiment, probably represents the early phase of infection and corresponds to results seen 4 to 8 h following infection of human or bovine cells

Immunization of animal hosts with AdG12[2]

The expression of VSV G in AdG12-infected bovine, canine, and murine cultured cells encouraged investigation of the ability of AdG12 to induce an immune response in the corresponding animals. AdG12, purified twice by isopycnic centrifugation was administered to mice, calves, pigs and dogs by intraperitoneal injection (ip),

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[2]L. Prevec, K.L. Rosenthal, L. Belbeck and J.B. Derbyshire carried out much of the experimental work in this section.

subcutaneous injection (sc) or intranasal spray (in). Blood samples were taken from the animals prior to and following administration of AdG12 and sera were analyzed for VSV neutralizing activity. As shown in Table 1, 100 fold dilutions of sera taken from all four species of animals following administration of AdG12, showed significant VSV neutralizing activity

To test the effectiveness of immunization with AdG12, mice were challenged with a lethal dose of VSV Mice infected with a single ip injection of 10^8 pfu of AdG12 were challenged one month later with $5x10^8$ pfu of VSV (B particle stocks of the Indiana ST strain, Prevec and Kang, 1970), administered by tail vein injection. Fig.15 shows that immunization with AdG12 provided a significant protective effect on survival of a lethal dose of VSV The neutralizing antibody titres of mouse sera shown in Table 1 are representative of this effect, since in both cases mice were immunized in the same way

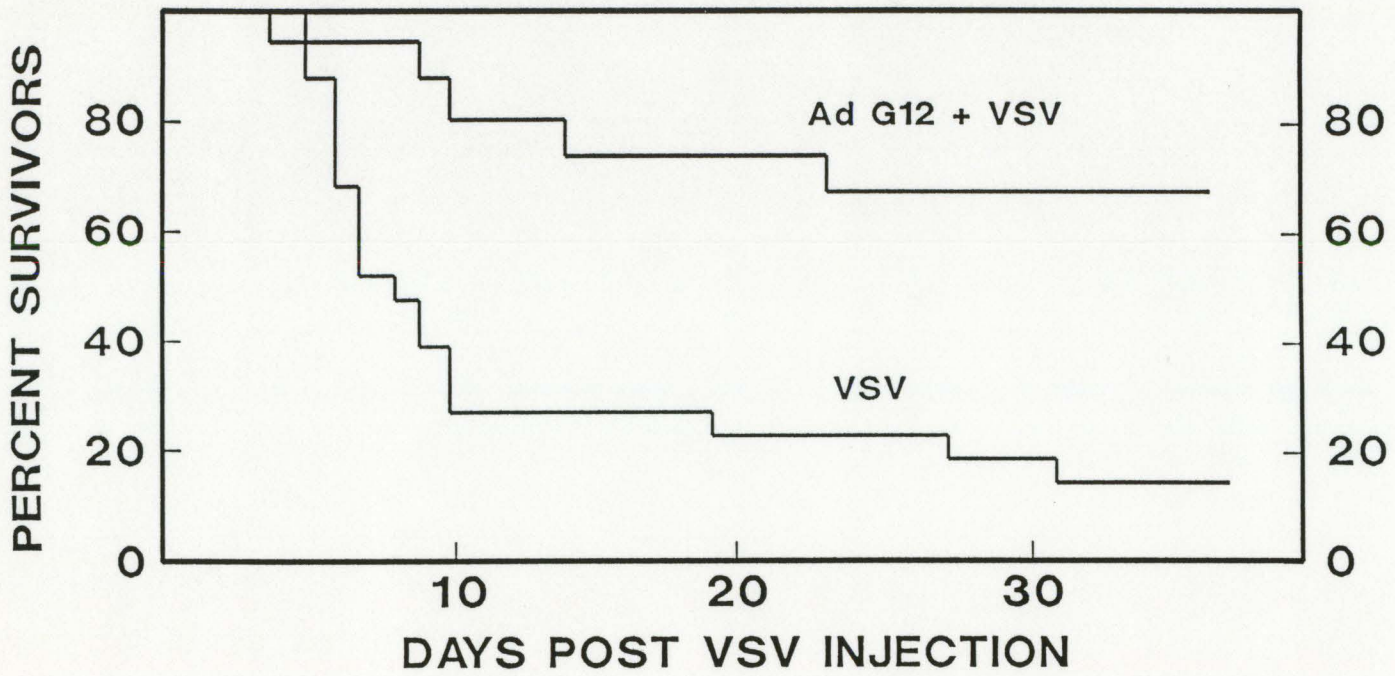
TABLE 1. INDUCTION OF VSV-NEUTRALIZING ANTIBODY BY ADG12.

Mice (5 to 6 weeks old) were given the indicated single doses of virus. Calves (4 months old) and pigs (2 months old) were given a first dose of 10^7 pfu of AdG12, and after 10 days a second dose of 10^8 pfu of AdG12. Dogs (over 6 months old) were given a first dose of $5x10^7$ pfu and after 14 days a second dose of $6x10^8$ pfu. Virus was administered by intraperitoneal injection (ip), subcutaneous injection (sc) or by an intranasal spray (in). Blood samples were taken from animals at the indicated times following administration of virus. One µl of each serum sample was diluted 100 fold and tested by plaque assay for VSV neutralizing actitvity. Log plaque reduction = log N_0/N where N_0 is the starting number of plaques and N is the number of plaques surviving serum neutralization. Superscripts indicate the N_0 values in separate assays: [a]$2.0x10^6$; [b]$2.0x10^5$; [c]$4.4x10^5$.

Animal	Virus	Route	Dose (pfu)	Time (days)	Log plaque reduction
Mouse	[a]none				-.15
	[a]VSV	ip	10^3	42	2.9
	[a]VSV	ip	10^5	42	3.3
	[a]VSV	ip	10^7	42	>3.3
	[a]AdG12	ip	10^8	42	>3.3
Calf 1	[b]none				-.08
	[a]AdG12	in	10^7	10	-.32
	[b]AdG12	in	10^7+10^8	24	1.4
Calf 2	[b]none				0
	[a]AdG12	sc	10^7	10	0.90
	[b]AdG12	sc	10^7+10^8	24	2.5
Pig 1	[b]none				-.10
	[a]AdG12	sc	10^7	10	1.1
	[b]AdG12	sc	10^7+10^8	24	2.6
Pig 2	[b]none				-.06
	[a]AdG12	in	10^7	10	1.0
	[b]AdG12	in	10^7+10^8	24	1.3
Pig 3	[b]none				.12
	[a]AdG12	sc	10^7	10	1.3
	[b]AdG12	sc	10^7+10^8	24	3.3
Dog 1	[c]AdG12	in	5×10^7	14	0.72
	[c]AdG12	in	$5\times10^7+6\times10^8$	30	2.3
Dog 2	[c]AdG12	sc	5×10^7	14	0.77
	[c]AdG12	sc	$5\times10^7+6\times10^8$	30	>3.6

FIG. 15. PROTECTION OF MICE FROM VSV BY IMMUNIZATION WITH ADG12.

A group of 15 mice were immunized by an ip injection of 10^8 pfu of AdG12 followed 4 weeks later by a challenge with 10^8 pfu of VSV administered by tail vein injection (upper line). A second group of 25 mice were challenged with VSV in the same way without previous immunization with AdG12 (lower line). A third group of 20 mice were given the immunizing dose of AdG12 alone and only one of these died over the following 68 days of monitoring. Mouse cages were monitored daily and deaths were recorded.


PERCENT SURVIVORS
80
60
40
20
0
Ad G12 + VSV
VSV
10
20
30
DAYS POST VSV INJECTION

4. DISCUSSION

4.1 ADENOVIRUS AS AN EXPRESSION VECTOR

To asses the utility of human Ad5 as a vector for expression of heterologous genes, non-defective recombinants expressing the glycoprotein of VSV were constructed. VSV G coding sequences, attached to promoter and polyadenylation sequences of the HSV-1 TK gene, were inserted into the partly deleted E3 region of Ad5, in both orientations. One recombinant, AdG12, carrying the inserted sequences in the orientation colinear with the E3 transcription unit, produced easily detectable quantities of VSV G during the course of lytic infection of HeLa cells. The AdG12-synthesized glycoprotein was indistinguishable from the VSV-synthesized glycoprotein on the basis of low pH-induced cell fusion activity and electrophoretic mobility. This implies correct post-translational processing and transport to the cell surface.

The utility of AdG12 as an expression vector, in species other than human, was demonstrated by infecting cultured bovine (MDBK), murine (L), and canine (MDCK) cells. The permissiveness for adenovirus replication differed among these cell lines: MDBK cells were fully permissive, L cells were semi-permissive (Tremblay et al., 1985) and MDCK cells were non-permissive. VSV G synthesis was

however easily detected in cells of all three species. Two basic conclusions can be drawn from these results:

(1) Adenovirus can efficiently penetrate cells of all three species.

(2) A heterologous gene can be expressed, without complete virus replication.

These conclusions suggested the possibility of inducing an immune response to antigens expressed by AdG12, in the corresponding species of animals and therefore the potential applicability of Ad 5 as an immunization vector. Another feature born out of these results is the possibility of efficiently delivering foreign sequences, without lytic destruction of the recipient cells by the vector: this was achieved by infecting MDCK cells with AdG12.

4.2 ADENOVIRUS AS AN IMMUNIZATION VECTOR

To assess the utility of human Ad5 as an immunization vector several mammalian animal species were infected with AdG12. VSV neutralizing activity was detected in the sera of dogs, mice, cows and pigs infected with AdG12. The biological effectiveness of this immune response was demonstrated by its protective effect on mice which were challenged with a lethal dose of VSV.

Infection of dogs, pigs and mice with AdG12 is consistent with reports of infection of these animal species with wild type Ad 5. Ad 5 specific antibodies were detected in dogs, infected most likely

by human contact (rather than by deliberate inoculation; Shortridge and Hu 1976; Winters 1979). Respiratory tract lesions were observed in piglets following inoculation with human Ad 5, which had been previously passaged on porcine kidney cells (Betts et al., 1962). Necrotic lesions in livers and spleens were induced by inoculation of mice with human Ad 5 (Postlethwaite, 1973).

The results presented in this study on induction of immunity to an adenovirus recombinant in several mammalian species, together with the reports mentioned above and previously (see section 1.5, Adenovirus host range), on induction of immunity to non-recombinant Ad 5, indicate that the host range of Ad 5 is sufficiently broad to allow both its development and application as a vaccine vector.

The outcome of infection with AdG12 and other recombinant adenoviruses, which lack coding sequences for most products of the E3 region, may differ from infection with wild type Ad 5. Interference with the host's antiviral mechanisms and the host's response to a growth factor are functions of individual E3 products which have been described to date (see section 1.4, Early region 3). One consequence of a large deletion, affecting most E3 coding sequences, was described by Morin et al (1987): viruses lacking E3 sequences similar to those of AdG12, replicated in hamster lungs to the same maximum titre as wild type Ad 5 but persisted for a shorter time than wild type adenovirus. The impact of this alteration in persistence, as well as other consequences of deleting E3 functions,

on the overall pathogenicity of Ad 5 is currently unknown and in need of further investigation.

Another form of adenovirus-induced pathogenesis, which is known to occur only in unnatural hosts, is the induction of tumours. Human adenoviruses have induced tumours in rodents and baboons, under experimental conditions (reviewed by Graham, 1984a). Some non-human species could therefore be at risk of developing tumours as a result of immunization with recombinants of a human adenovirus.

The safety of a recombinant viral vaccine depends not only on the vector but also on the heterologous immunogen that it expresses. Individual proteins can have intrinsic toxic effects, such as the cell fusion activity of viral glycoproteins (reviewed by Chany et al., 1987) or produce toxic effects through the immune responses that they induce, such as induction of autoimmunity (Onodera et al., 1982).

In addition to influencing the safety of a vaccine, the choice of immunogen also determines its effectiveness. Choosing an effective immunogen requires an understanding of the pathogenesis of the disease. Stimulating an immune response to viral surface antigens provides adequate protection against diseases whose pathogenesis depends on acute infection. The importance of immunity to viral surface antigens in these pathogens is well illustrated by the association of influenza virus pandemics with major changes in viral hemagglutinin or neuraminidase (reviewed by Murphy and Chanock, 1985). This type of immunity however does

not appear to be effective in eliminating persistent or latent pathogens such as HSV (reviewed by Wildy, 1987) or HIV (reviewed by Gallo et al., 1989).

4.3 CONTROL OF EXPRESSION OF HETEROLOGOUS GENES INSERTED IN THE E3 REGION OF ADENOVIRUS.

The substantially reduced synthesis of VSV G by the recombinant AdG4 carrying a TK-G hybrid gene identical to AdG12 but inserted in the opposite E3 antiparallel orientation was surprising and suggested an unexpected mechanism for control of its expression. Transcriptional control of the TK-G gene by adenovirus sequences, when it was inserted in the E3 parallel orientation, was demonstrated by analyzing the 5' ends of transcripts. This finding is consistent with analyses of the control of expression of adenovirus recombinants carrying other heterologous genes in the E3 region, which were reported during the course of this study. Morin et al., (1987) demonstrated expression of sequences coding for the major surface antigen of hepatitis B virus, which were inserted without a promoter. Johnson et al., (1988) showed expression of sequences coding for the HSV-1 glycoprotein B (gB) linked to the SV40 early promoter, regardless of the orientation of insertion. Surprisingly, analysis of transcripts of the SV40-gB gene, when inserted in the E3 parallel orientation, revealed that the SV40 sequences did not initiate transcription but rather functioned as a splice acceptor for

transcripts initiated upstream. A similar analysis of transcripts of this gene, when inserted in the opposite orientation, has not yet been performed and the promoter(s) initiating its transcription in this orientation remain unidentified.

Transcripts of the TK-G gene initiated by the TK promoter were also detected. Their presence was independent of the orientation of insertion. This result supports the work of Moore and Shenk (1988) who demonstrated correct initiation of transcription of the HSV-1 TK gene by its own promoter from a human adenovirus genome. The number of transcripts of VSV G sequences initiated by the TK promoter was much lower than the number initiated by adenovirus sequences and in initial experiments they were not detected. Their detection in subsequent experiments was probably due to the use of preparations of probes with higher specific activities which improved the sensitivity of these experiments.

Because of the initial difficulty in detecting transcripts initiated by the TK promoter, the structural integrity of the TK promoter and its capacity to function in the context of an adenovirus infection, required verification. The selective transport of mRNA out of the nucleus of adenovirus infected cells, possibly by the complex consisting of the E1b 58K product and E4 34K product (see section 1.4, Early region 1), was investigated as a mechanism which could obscure detection of TK promoter-initiated transcripts in the cytoplasm. The failure to detect an accumulation of TK promoter-initiated transcripts in the nuclei of AdG4- or AdG12-infected cells,

indicated that the difficulty in detecting them in the cytoplasm was not exclusively due to a block in their transport.

The structural integrity of the TK promoter was demonstrated by nucleotide sequence analysis of the upstream TK region of the TK-G hybrid gene, cloned out of both recombinants. This analysis showed the correspondence of every nucleotide of this region of the TK gene to the published sequence of the TK gene of HSV-1 strain 101 (Wagner et al., 1981). Since TK sequences present in the TK-G gene were cloned from the HSV-1 KOS strain, this result also demonstrates the absence of sequence differences between the two viral strains, in this region of the gene.

The functional integrity of the TK promoter was demonstrated by its susceptibility to stimulation by HSV immediate-early products, provided in trans. Superinfection with HSV-1 resulted in a substantial increase in the quantity of TK promoter-initiated transcripts in both AdG4- and AdG12-infected cells.

The ability of both classes of VSV G transcripts to be translated, was deduced by comparing the synthesis of the glycoprotein in cells infected with each recombinant. Translation of TK promoter-initiated transcripts was demonstrated by detecting VSV G synthesis in AdG4-infected cells, since AdG4 directs the synthesis of only one class of VSV G transcript. The correlation of a much greater quantity of VSV G synthesis with the presence of the abundant adenovirus-initiated transcripts in AdG12-infected cells suggests translation of this transcript.

An identical pattern of expression of the entire HSV-1 TK gene was demonstrated in a pair of adenovirus recombinants bearing insertions of this gene analogous to the TK-G gene insertions in AdG4 and AdG12. A small number of TK promoter-initiated transcripts was observed regardless of the orientation of insertion and an abundant class of transcript, initiated upstream of TK sequences, was detected when the gene was inserted in the E3 parallel orientation. A larger quantity of TK protein synthesis was also correlated with the synthesis of the more abundant adenovirus-initiated transcript.

The kinetic class of the adenovirus promoter controlling synthesis of the abundant VSV G transcript, in AdG12-infected cells, was identified by defining the kinetics of VSV glycoprotein synthesis. Early kinetics of expression was demonstrated by the early time of onset of VSV G synthesis: VSV G synthesis was first detected at the same time as synthesis of the 72K product of the E2 region. This result implicates the E3 promoter in the control of VSV G expression. The E3 promoter, which was not removed as part of the E3 deletion (see Fig.6), normally transcribes endogenous sequences that are in the position of TK-G gene in AdG12.

Definition of the kinetics of VSV G synthesis as early was supported by the persistence of VSV G synthesis when DNA synthesis was abolished with ara-C, however, a reduction in VSV G synthesis was also observed under these conditions. This could be partly accounted for by a reduction in the number of viral templates, however a similar reduction in the synthesis of the E2a 72K product was also observed, even though its transcriptional control is more

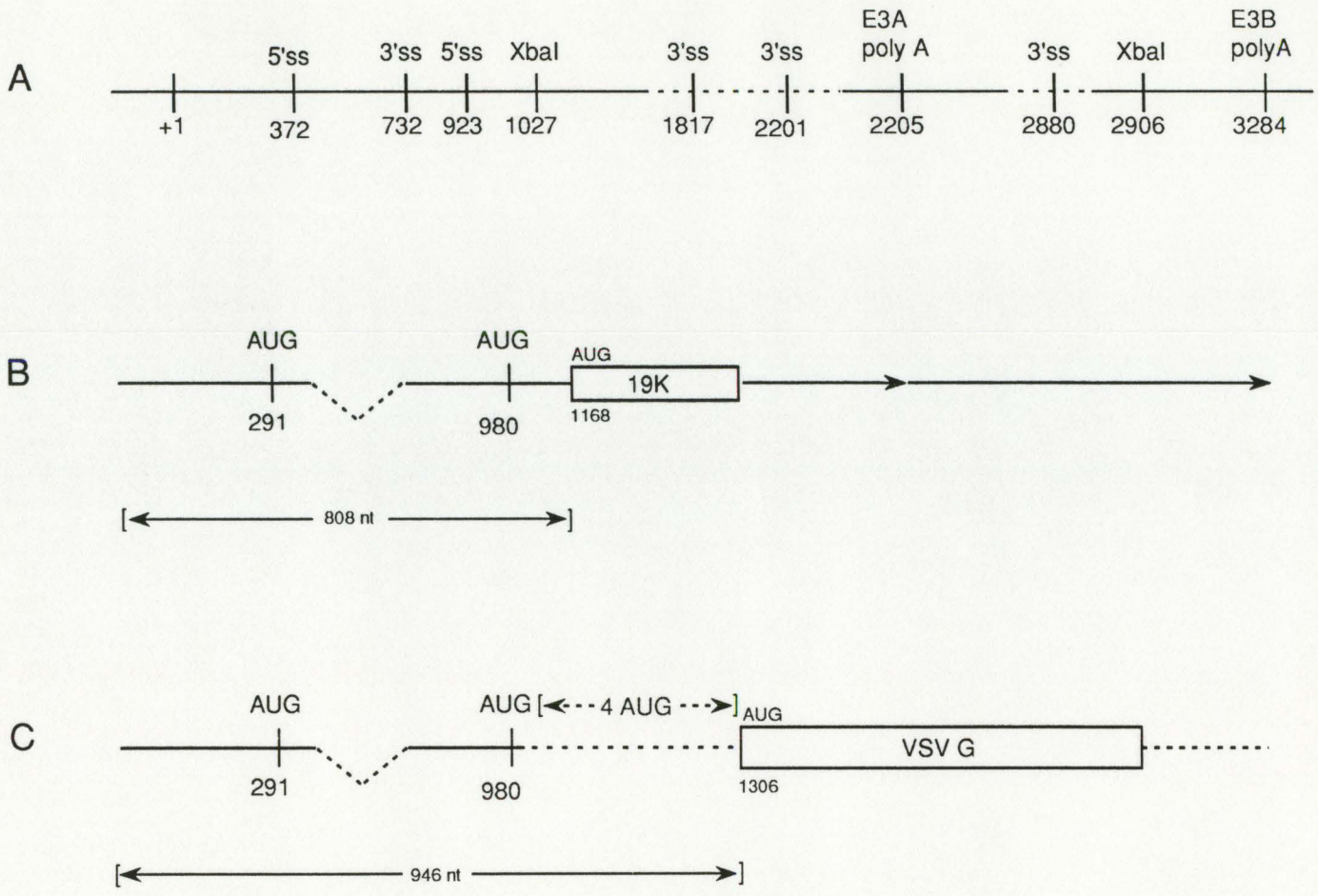
complex. Synthesis of the 72K product is controlled by two promoters: one at 75 mu which functions before DNA synthesis and a second at 72 mu which functions after DNA synthesis (see section 1.4, Early region 2). The promoter at 72 mu would be unable to function in the presence of ara-C. This feature would contribute to the reduction in synthesis of the 72K protein perceived under these conditions. Participation of this second component in the reduction of 72K synthesis, in the presence of ara-C, brings to mind the possibility that a second promoter may also contribute to the expression of VSV G, at late times during infection. The putative second promoter would likely be the major late promoter, since it normally transcribes endogenous sequences that are in the position of the TK-G gene, at late times during infection.

4.4 POSSIBLE STRUCTURE OF THE VSV G TRANSCRIPT INITIATED BY THE ADENOVIRUS E3 PROMOTER

Although its actual structure was not experimentally determined, a palausible structure for a VSV G transcript initiated by the E3 promoter can be deduced by examining the structures of normal E3 transcripts. Fig. 16A illustrates the positions of the ends of these transcripts and major splice sites. The 5' regions of most E3 transcripts contain the first exon and lack the first intron

FIG. 16. STRUCTURES OF NORMAL AD 5 E3 mRNAs AND POSSIBLE STRUCTURE OF THE E3 PROMOTER-INITIATED VSV G RNA.

Numbers below the lines indicate the number of nucleotides from the transcription initiation site; dashed lines indicate regions not drawn to scale; open boxes indicate protein coding regions. (A) Transcript processing signals of the E3 region: splice sites, 5'ss and 3'ss; polyadenylation signals, E3A poly A and E3B poly A. Sequences between the XbaI sites are deleted in AdG12. (B) Structures of transcripts coding for the 19K glycoprotein. The 808 nt long leader upstream of 19K coding sequences contains 2 AUGs. (C).Possible structure of the VSV G coding transcript initiated from the E3 promoter. The 946 nt long leader upstream of VSV G coding sequences contains a total of 6 AUGs.


A
5'ss
3'ss
5'ss
XbaI
3'ss
3'ss
E3A
poly A
3'ss
XbaI
E3B
polyA
+1
372
732
923
1027
1817
2201
2205
2880
2906
3284
B
AUG
AUG
AUG
19K
291
980
1168
808 nt
C
AUG
AUG
4 AUG
AUG
VSV G
291
980
1306
946 nt

(Cladaras et al., 1985). The resulting leader usually carries translation initiator codons upstream of the one which initiates the most abundant product (Cladaras and Wold, 1985). These features are illustrated in the structure of the transcript coding for the 19K glycoprotein (Fig 16B). The 5' region of the VSV G transcript (Fig. 16C) would differ from the 19K transcript in having a slightly longer leader (946 nt instead of 808 nt) which contains an additional 4 upstream AUGs

The usage of an AUG for initiation of translation, according to the model of Kozak (1984), depends both on its proximity to the 5' end of a transcript and on the similarity of its flanking nucleotides to a preferred sequence. The most conserved feature of this preferred sequence is a purine three nucleotides upstream of the AUG. Cladaras et al., (1985) reported that the usage of translation initiator codons on adenovirus E3 transcripts, correlates well with the occurrence of Kozak's preferred sequence.

The degree of interference in translation of VSV G by upstream initiator codons could be predicted by applying Kozak's model. By examining each AUG that lies upstream of VSV G coding sequences for the presence of a purine three nucleotides upstream, preferential initiation of translation can be predicted to occur at the normal VSV G initiator codon and at one other AUG, which is located upstream in TK-derived sequences. Removal of the strong upstream initiator codon alone should provide a substantial improvement in the efficiency of VSV G translation. The efficiency of translation may be further improved by removing the remaining upstream AUGs.

The AUG at position 291 (Fig. 16C) is in phase with VSV G coding sequences and could therefore give rise to an adenovirus E3/VSV G fusion product. The expected size of this fusion product is approximately 25K longer than VSV G (a total of 85K) however, no AdG12-specific product of this approximate size was detected (see Figs. 4, 11A, 12A). The absence of detectable levels of this fusion product is consistent with evidence for the weak potential of this AUG as a translation initiator codon: its flanking sequences do not correspond to the preferred sequence for a strong initiator codon, according to Kozak's model; its presence close to the 5' end of most E3 transcripts, some of which are known to code for a major product that is initiated downstream (Cladaras and Wold, 1985) suggests its inability to interfere with downstream initiation of translation.

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