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PROTEIN COMPARISONS AMONG SIX ISOLATES OF ENTEROVIRUS 70

A Thesis Submitted to the
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Master of Science
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School of Medicine

By

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ABSTRACT

Enterovirus type 70 (EV70), a causative agent of acute hemorrhagic conjunctivitis emerged suddenly as a novel human pathogen in Ghana, West Africa in 1969. Despite a relatively brief history of passage in humans, the virus has already affected an estimated 80 million people as it has spread in a pandemic fashion to all continents except Australia. While the origin of this highly infectious virus remains obscure, RNase T1 fingerprinting has shown that all isolates of EV70 are derived from a common ancestor. It is possible, therefore, to trace the changes in EV70 from the time of emergence to the most recent isolates. The major objective of this study was to determine if changes in the RNA genome of EV70 have led to detectable differences in structural and nonstructural polypeptides among 6 isolates of the virus collected from different regions of the world between 1971-1981. Protein analyses included one dimensional SDS-PAGE, two dimensional high resolution electrophoresis, peptide mapping and immunoprecipitation and revealed a surprisingly similar protein complement between isolates whose genomes differ by about 4%. These studies are a first step towards localizing regions of the genome which have changed since EV70 appeared as a human pathogen.

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DEDICATION

This thesis is dedicated to my family.

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LIST OF ABBREVIATIONS

AHC	acute hemorrhagic conjunctivitis
CA24	coxsackie virus A24
CDC	Centres for Disease Control
Ci	curie
CPE	cytopathic effect
CSF	cerebrospinal fluid
DEP	diethylpyrocarbonate
EDTA	ethylene diamine tetraacetic acid
EMCV	encephalomyocarditis virus
EV70	enterovirus 70
FMDV	foot-and-mouth disease virus
HAV	hepatitis A virus
HELF	human embryonic lung cells
HRV	human rhinovirus
IEF	isoelectric focusing
K	thousand
mA	milliampere
MAb	monoclonal antibody
MEM	minimal essential medium
mmol	millimole
m.o.i.	multiplicity of infection
MW	molecular weight
NIH	National Institutes of Health

nm	nanometre
PEG	polyethylene glycol
pI	isoelectric point
pfu	plaque forming units
RNase	ribonuclease
SDS	sodium dodecyl sulphate
SEC	Singapore epidemic conjunctivitis
TBS	Tris buffered saline
TMEV	Theiler's murine encephalomyocarditis virus
WHO	World Health Organization

INTRODUCTION

Enterovirus 70: Historical Background

1. Pandemic Spread of the Virus

Enterovirus 70 (EV70) has had a relatively brief history as a human pathogen. The first outbreaks of a new, highly infectious type of hemorrhagic conjunctivitis were reported in Ghana, West Africa in June 1969 (Chatterjee et al., 1970). Referred to as 'epidemic hemorrhagic conjunctivitis' or 'Apollo 11 disease' (because it coincided with the Apollo 11 moon landing (Kono, 1975)), this new clinical entity spread throughout West Africa and reached North Africa by December 1970. During 1970-1971, outbreaks of a disease with identical clinical and epidemiological features occurred in Asia and spread rapidly to involve the whole south east region of this continent, as well as India (Mirkovic et al., 1973). Japan was also affected but to a lesser extent than other Asian countries (Kono et al., 1972). Between 1971-1972, epidemics of hemorrhagic conjunctivitis were reported in the Middle East and small nosocomial outbreaks occurred throughout Europe. Extensive epidemics swept through Thailand, Singapore and India between 1974-1975 (Reeves et al., 1986). Most areas affected by epidemic conjunctivitis during the first pandemic (1969-1971) were subject to sporadic epidemic outbreaks

between 1972-1979. During these years, the disease apparently remained confined to Europe and Asia (Yin-Murphy, 1984; Reeves et al., 1986).

A second pandemic in 1980-1981 involved India, Pakistan, Nigeria and, for the first time, the Americas. In the Americas, the disease occurred initially in Brazil and spread rapidly to the Caribbean, Central America, Florida and North Carolina (Morgan et al., 1981; Malison et al., 1984; Waterman et al., 1984; Asbell et al., 1985; Reeves et al., 1986). As of 1982, the only continent not yet affected by this disease was Australia (Yin-Murphy, 1984).

Laboratory investigations following the first pandemic of hemorrhagic conjunctivitis revealed an unusual etiologic situation. A variant of coxsackie A24 (CA24) isolated from conjunctival scrapings was found to be responsible for the 1970 epidemic of conjunctivitis in Singapore and was referred to as Singapore epidemic conjunctivitis (SEC) virus (Yin-Murphy and Lim, 1972; Mirkovic et al., 1974). However, an antigenically distinct virus known as acute hemorrhagic conjunctivitis (AHC) virus was subsequently recovered from similar outbreaks in Japan in 1971 and identified as a previously unknown human enterovirus (Kono et al., 1972). Serological studies of the 1971 Singapore epidemic revealed that the etiologic agent responsible during that year was identical to the AHC virus, not the SEC virus (Yin-Murphy and Lim, 1972). The AHC virus was subsequently classified by

the World Health Organization (WHO) enterovirus reference centre as enterovirus type 70 (EV70) (Mirkovic et al., 1973).

This highly unusual situation, wherein two antigenically distinct viruses causing clinically indistinguishable syndromes emerged simultaneously, prompted Kono (1978) to suggest that these agents may constitute an acute hemorrhagic conjunctivitis virus complex and have a common origin. Studies by Natori and co-workers (1984) however, have clearly shown that there is no genetic homology between the CA24 variant and EV70 agents of acute hemorrhagic conjunctivitis.

While EV70 has spread to most regions of the world, CA24 has thus far remained restricted to parts of South East Asia and the Indian subcontinent (Kono 1985). In 1983 it was estimated that 70-80 million people around the world have been infected with EV70. This sudden emergence and pandemic spread from an original African focus in 1969 is paralleled only by the appearance and spread of novel influenza virus strains (Kono, 1975; Wadia et al., 1983).

2. Pathogenesis and Epidemiology

The target organ of EV70 is the conjunctiva and the temperature sensitive nature of the virus (optimum temperature of 32-34^{o}C) in all probability reflects adaptation to this region of the body (Miyamura et al., 1974; Stanton et

al., 1977; Hung and Kono 1979). Clinically, the conjunctivitis runs a rapid and normally benign course. Following a short incubation period (approximately 24 hours) predominant ocular signs and symptoms typically include sudden acute onset, lacrimation, pain, edema, seromucous discharge and subconjunctival hemorrhage. Systemic involvement is rare, with fever infrequently reported. Complete recovery usually ensues within 5-10 days. However, in rare cases, neurological complications 2-5 weeks following or, infrequently, accompanying EV70 ocular infection do occur, but such complications are mainly confined to adulthood (Mirkovic et al., 1973; Yin-Murphy, 1984; Asbell et al., 1985).

That EV70 does possess neurovirulent properties has been confirmed by inoculating it into the central nervous system of cynomologus monkeys (Kono et al., 1973). The resultant histopathological changes in the spinal cord were similar to the lesions seen in acute poliomyelitis, and the neurovirulence of the J670/71 prototype strain of EV70 appears to approximate that of live attenuated poliovirus vaccine strains (Kono et al., 1973; Kono, 1978). Generally, manifestations of neurological sequelae are reminiscent of acute paralytic poliomyelitis and include radicular pains in muscles and limbs and asymmetrical flaccid paralysis. Permanent incapacitation occurs in 1:4 cases and rough estimates of the frequency of neurological complications due

to AHC range from 1:10,000 to 1:17,000 cases (Hung and Kono, 1979). The attack rates may well be higher: failure to report neurological sequelae and the association between EV70 and radiculomyelitis going unnoticed by physicians has contributed to the lack of reliable epidemiological data (Hung and Kono, 1979). While the virus has never been isolated from the conjunctiva or cerebro-spinal fluid (CSF) of patients with neurological complications, neutralizing antibodies to EV70 in serum and CSF have been reported and this suggests invasion of the nervous system by the virus (John et al., 1981; Kono et al., 1981c).

3. Transmission

Explosive outbreaks of EV70 related acute hemorrhagic conjunctivitis occur in crowded coastal areas of developing countries. Generally, epidemics are much less severe in developed nations. While it appears that hot rainy periods favour the epidemic spread of EV70 in tropical coastal regions, outbreaks have been reported at all times of the year in countries having a seasonal climate (Hierholzer et al., 1975; Yin-Murphy, 1984).

Transmission of acute hemorrhagic conjunctivitis which may occur through a variety of vehicles, seems to involve bringing the virus directly into the eye. The role played by a fecal-oral route of spread, as in other enterovirus infections, is unclear for there has been little experimen-

tal evidence that the alimentary tract can support massive multiplication of the virus (Hung and Kono, 1979). Both direct person to person contact via hands contaminated with ocular secretions as well as indirect contact via fomites (namely face-cloths, towels, and bedding) have been shown to play a large role in the transmission of this highly infectious agent (Arnow et al., 1977). The virus is stable for up to 21 days when suspended in tap water at room temperature and communal wash basins contaminated with ocular secretions have also been implicated in the spread of the disease (Arnow et al., 1977; Hung and Kono, 1979). One of the distinguishing features of the conjunctivitis is extensive intrafamilial spread. Major factors involved in secondary spread within families have been identified as crowded living quarters with communal bathrooms comprising the single most important risk factor. Several epidemiological studies have demonstrated the importance of personal hygiene in the control of this contagious virus (Arnow et al., 1977; Malison et al., 1984; Reeves et al., 1986).

4. Origin of EV70

The origin of this relatively 'new' and highly infectious human viral pathogen remains obscure. However, T_1 RNase fingerprinting studies have revealed that all isolates of EV70 are derived from a common ancestor and it has been postulated that the original strain emerged in West Africa

in 1966 +/- 15 months, 3 years prior to the first recorded outbreaks (Takeda et al., 1984; Miyamura et al., 1986). EV70 possesses several unique features which distinguish it from other human enteroviruses and Kono and co-workers (1981a) have suggested three possible origins for the virus:

1) a formerly non pathogenic human virus
2) an animal enterovirus
3) an insect picornavirus.

The putative progenitor must have mutated in some way so as to acquire pathogenicity for humans. Seroepidemiologic studies of animal sera in Japan and Africa have demonstrated a high frequency of virus neutralizing titres in several domestic animals even prior to the first pandemic in 1969. One interpretation of such findings is that EV70 may have evolved from an animal enterovirus which commonly infects a number of domestic species (Kono et al., 1981b; Sasagawa et al., 1982). However, attempts to neutralize EV70 with antisera prepared against 4 swine and 8 bovine enteroviruses failed (Sasagawa et al., 1982). Another interesting and unusual feature of the virus is its wide host range in vitro. While most human enteroviruses are restricted in growth to primary or continuous cell cultures of primate origin, EV70 behaves quite differently. In addition to human and simian cell lines, it has also been successfully propagated in a variety of cells of non-primate origin such as bovine, porcine, rat, hamster and rabbit (Yoshii et al.,

1977). This wide host range also suggests an animal origin. Moreover, the interaction of EV70 with receptors on erythrocyte surfaces is similar to that found in cardioviruses (rodent picornaviruses) and not the other human enteroviruses. However, an antigenic relationship between EV70 and cardioviruses could not be demonstrated (Utagawa et al., 1982).

5. Evolution of EV70

Rapid changes in viral RNA over time, a well known phenomenon, appears in part to be a result of the inherent error-prone nature of RNA replication (Holland et al., 1982). A notable example of such genomic evolution occurs in the prototype and best studied enterovirus, polio, where it has been shown that greater than 100 genetic changes can occur in oral vaccine virus genomes during passage through as few as one or two individuals (Kew et al., 1981) as well as during epidemic transmission (Nottay et al., 1981). In contrast, T_1 RNase fingerprinting analyses of EV70 isolates and RNA homology studies have revealed that despite great numbers of intervening infections the earliest (1971) and most recent (1981) strains are closely related (Kew et al., 1983; Natori et al., 1984; Takeda et al., 1984). Thus, genome conservation appears to be much greater in EV70 than in poliovirus, and only one basic genotype of EV70 is found to be in circulation at any one time (Kew et al., 1983). It

has been suggested that the comparatively slow rate of genomic change may be due to an absence of selective antibody pressure on the virus (Kew et al., 1983). EV70 is in most cases transmitted directly to the eye and following a short incubation period, multiplies there for only a brief period of time (Takeda et al., 1984). Therefore it may not be subject to the pressures exerted upon other enteroviruses such as polio which often survive and multiply in the gut for weeks (Kew et al., 1983). In summary, a relatively short passage history in humans and a comparatively stable genome has made it possible to estimate the base changes in the viral RNA genome between EV70 isolated shortly after its emergence in 1969 and the second pandemic in 1981. It has been estimated that approximately 4% of the nucleotides differ between the earliest and most recent isolates (Takeda et al., 1984).

Picornaviruses

1. Description

Enterovirus 70 belongs to one of the largest families of viral pathogens - the PICORNAVIRIDAE. This group of viruses has been subdivided into four genera (see Table 1) which differ from one another in pathogenicity, buoyant density, acid lability, sedimentation coefficients and viral serology. However, all picornaviruses do share similar struc-

TABLE 1

The PICORNAVIRUS Family

Genus Enterovirus	
Polioviruses	types 1-3
Coxsackie viruses, group A	types A1-22,24
Coxsackie viruses, group B	types B1-6
Echoviruses	types 1-9,11-27, 29-34
Human enteroviruses	types 68-72*
enteroviruses of other species	34 types
Genus Rhinovirus	
Human Rhinoviruses	>110 types
Bovine Rhinoviruses	2 types
Genus Cardiovirus	
Encephalomyocarditis (EMC) virus	1 type
Genus Aphthovirus	
Foot-and-mouth disease virus (FMDV)	7 types

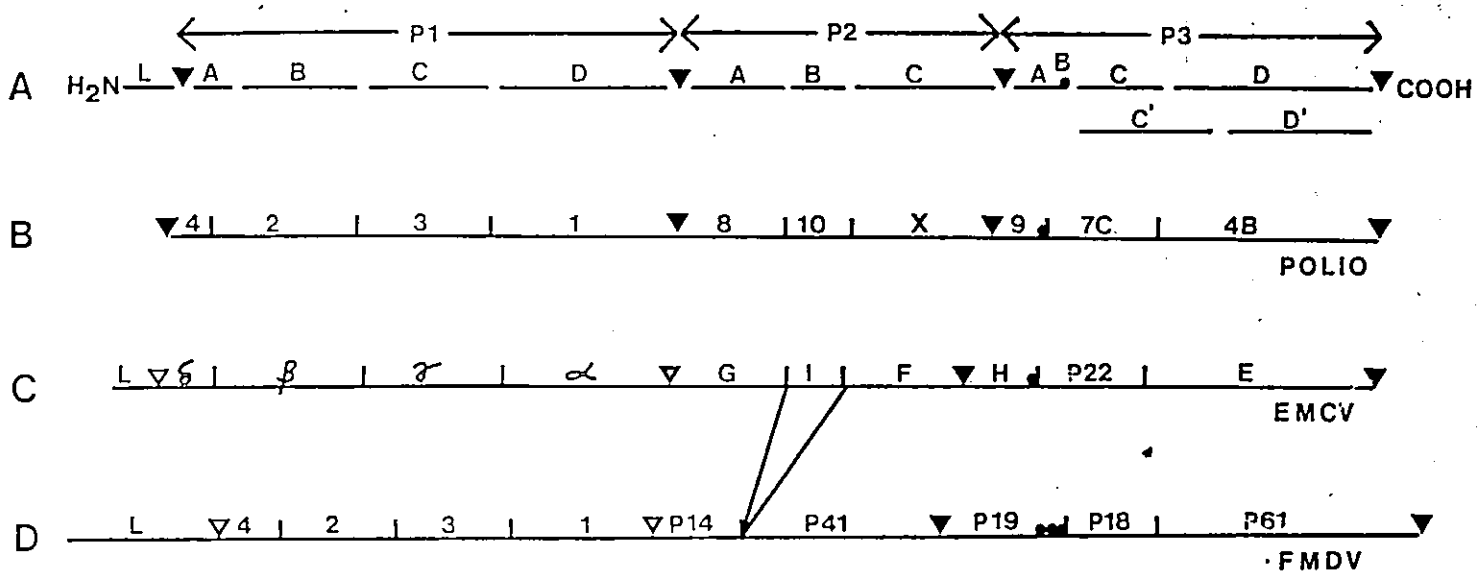
*Enterovirus 72 = Hepatitis A virus
Adapted from Melnick (1983)

tural components and replication strategies (Putnak and Phillips 1981; Rossmann et al., 1985). Mature particles have an external diameter of approximately 30 nm, lack carbohydrate or lipid and are of icosahedral symmetry. The genome consists of one molecule of infectious positive sense RNA with a molecular weight of approximately 2.5×10^6 (Matthews, 1982). A small viral coded protein VPg (MW 2,400 in poliovirus) is covalently linked to the 5'-end of all nascent viral RNA and negative strand template RNA. This protein may serve to prime RNA synthesis and/or regulate encapsidation of RNA (Pettersson et al., 1978; Putnak and Phillips, 1981). Like eucaryotic mRNA, picornaviral RNA molecules possess a polyadenylic acid sequence at the 3'-end which varies in length between 10 and 150 nucleotides (Figure 1) (Yogo and Wimmer, 1972; MacNaughton and Dimmock, 1975).

2. Picornaviral Proteins

Picornavirus protein nomenclature, has in the past, been confusing and cumbersome. This is because similar proteins had been variously designated depending upon the particular virus (Figure 1). To avoid further confusion, a new (L434) systematic nomenclature has been adopted for these viruses (Figure 1) and will be used throughout this thesis (Rueckert and Wimmer, 1984). Traditional poliovirus nomenclature is given in brackets.

Figure 1. Schematic diagram of an idealized picornavirus polyprotein. The L434 protein nomenclature (Rueckert and Wimmer, 1984) and general genome organization are shown (A). Traditional protein nomenclature and genome organization for poliovirus, encephalomyocarditis virus (EMCV) and foot-and-mouth disease virus (FMDV) are shown in B, C and D respectively. Solid triangles (▼) represent junctions between the leader (L), P1, P2 and P3 regions of the genome. Solid black circles (•) represent the viral coded protein 3B (VPg). The deletion in the P2 region of FMDV is indicated by the lines extending from the corresponding region in EMCV to the missing portion of FMDV. C' and D' refer to alternate cleavage proteins which have been identified in poliovirus, human rhinovirus 1-A (HRV-1A) and FMDV. Diagrams adapted from Rueckert and Wimmer, 1984.


A
H_2N
L
A
B
C
D
A
B
C
A
B
C
D
COOH
C'
D'
P1
P2
P3
B
4
2
3
1
8
10
X
9
7C
4B
POLIO
C
L
G
I
F
H
P22
E
EMCV
D
L
4
2
3
1
P14
P41
P19
P18
P61
FMDV

The production of picornaviral proteins involves a complex series of proteolytic cleavages following translation of the RNA (Figure 1). Early pulse-chase experiments using radiolabelled amino acids, amino acid analogs and proteolytic inhibitors indicated that under these conditions, poliovirus RNA is translated into a single large primary polyprotein precursor (NCVPOO) with a molecular weight of 247,000. The size of this precursor protein differs among the various picornaviruses. In the absence of proteolytic inhibitors, this polyprotein is cleaved while in the nascent state into the total complement of viral specific proteins (Holland and Kiehn, 1968; Jacobson and Baltimore, 1968a; Summers et al., 1972; Pallansch et al., 1984). Rueckert and Wimmer (1984) (Figure 1) have divided the polyprotein into three fundamental regions based upon the products of the major primary cleavages:

P1 region - the 5'region which generates coat proteins 1A(VP4), 1B(VP2), 1C(VP3), 1D(VP1).

P2 region - the midpiece which generates proteins 2A, 2B (unknown function) and 2C (a putative viral protease).

P3 region - the 3'region which encodes VPg, another viral protease, 3C(7c), and the viral polymerase, 3D(4b).

Several mechanisms to explain processing of NCVP00 into P1, P2 and P3 have been suggested in the literature. Kitamura and co-workers (1981) and Palmenberg and Rueckert

(1982) have suggested that the early stages of infection with poliovirus include self cleavage of P3 or the trans action of one viral polypeptide upon another to release both the viral protease 3C and replicase 3D. In support of this suggestion is the work of Parks et al. (1986) which has shown that protein 3C in encephalomyocarditis virus is capable of monomolecular autocatalysis. Recent evidence has also indicated that viral protein 2A may be the protease that is responsible for the initial cleavage event between P1 and P2 regions in poliovirus and probably also in coxsackie, rhino and hepatitis viruses (Toyoda et al, 1986; Palmenberg, 1987). The majority of secondary cleavages which generate both the structural and nonstructural viral protein products have been shown to be catalyzed by a viral coded enzyme, 3C (and perhaps also by 2C) (Korant et al., 1979; Palmenberg et al., 1979). The final protein processing event in virion morphogenesis is the maturation cleavage of 1AB(VP0) to 1A(VP4) and 1B(VP2). This step occurs when RNA associates with the maturing particle and may involve an autoproteolytic cleavage wherein both the viral RNA and serine residues on protein 1B interact to cause 1AB scission (Palmenberg, 1987; Rossmann et al.,1987)

3. Genome organization

Many different experimental approaches have been used to locate the viral proteins in the genome. Summers and Maizel

(1971) and Taber et al., (1971) utilized the drug pactamycin, an inhibitor of protein synthesis initiation to map the proteins. Assuming that protein initiation occurs only at the 5'end of the RNA, proteins coded farthest from this site are most efficiently labelled with radioactive amino acids added at different times after initiation is inhibited. These studies, in conjunction with tryptic peptide mapping experiments (Jacobson et al., 1970), which demonstrated precursor/product relationships among proteins common to a certain region of the RNA, helped to establish the location of the proteins in the poliovirus genome. In recent years, RNA and protein sequence analysis have confirmed the earlier results, and in addition, have permitted comparison of the genome organization in all four genera of the family Picornaviridae.

Despite the great diversity amongst the picornaviruses with respect to pathogenicity, host range, serology and various biophysical properties, the overall organization of the genome is quite well conserved between poliovirus, foot-and-mouth disease virus (FMDV), encephalomyocarditis virus (ECMV), human rhinoviruses (HRV) and hepatitis A virus (HAV) (Figure 1). Pertinent features of the RNA genome of picornaviruses include a genomic length of approximately 7500 nucleotides, a 5'noncoding region of approximately 700 bases, a 5'initiation site, a long open reading frame (approximately 6600 nucleotides) coding for a single

translation product as well as a short noncoding region at the 3'end which includes a poly A tail (Kitamura et al., 1981; Racaniello and Baltimore, 1981; Ticehurst et al., 1983; Forss et al., 1984; Palmenberg et al., 1984; Stanway et al., 1984; Najarian et al., 1985; Skern et al., 1985).

There are several differences among the picornaviruses with respect to genome size and protein location which are worth noting (Figure 1). FMDV appears to be distinct from the others in various ways. First, the genome has approximately 1000 more nucleotides than the three types of poliovirus. Most of these are added to the leader area which precedes the P1 region. While EMCV also possesses a leader sequence it is much shorter than that of FMDV. In both viruses the leader sequence is translated into small leader peptides with an unknown function. (Forss et al., 1984; Palmenberg et al., 1984; Palmenberg, 1987). In addition, the midpiece or P2 region of FMDV lacks the 2B protein and the P3 region includes three tandem VPg genes. In other picornaviruses, only one VPg gene is present. Cardio- and aphthoviral genomes are also distinguished by a poly C tract of variable length in the 5'noncoding region (Forss et al., 1984; Palmenberg et al., 1984). In HAV the initial portion of P1 has undergone a deletion and thus 1A (VP4) is coded for at the end of the P1 region in contrast to all other picornaviruses (Najarian et al., 1985). Other differences among this family of viruses occur in cleavage recognition

sites between the proteins. In polio the majority of proteolytic cleavages occur at glutamine-glycine pairs (Kitamura et al., 1981). The cleavage specificity in EMCV, FMDV, HRV 14 and HAV is less stringent and can occur at a number of other amino acid pairs in addition to glutamine-glycine (Forss et al., 1984; Palmenberg et al., 1984; Stanway et al., 1984; Najarian et al., 1985).

The virion structural proteins 1A(VP4), 1B(VP2), 1C(VP3) and 1D(VP1) are each present in 60 copies (Rueckert, 1965; Stoltzfus and Rueckert, 1972; McGregor et al., 1975). Virion morphogenesis begins with the aggregation of 5 coat protein precursors (P1 in Figure 1). This aggregate takes on a pentameric structure and the precursor protein is then cleaved into 1AB(VP0), 1C(VP3) and 1D(VP1). Following the assembly of the cleaved pentamers into the procapsid (or empty capsid), viral RNA is added and this structure is known as the provirion. The final maturation step involves the cleavage of 1AB(VP0) to 1A(VP4) and 1B(VP2) (Jacobson and Baltimore, 1968b; Fernandez-Tomas and Baltimore, 1973; Melnick, 1983; Palmenberg, 1987).

4. Role of Capsid Proteins

A variety of experimental approaches have been used to elucidate the topological relationships among the four picornavirion structural proteins 1A(VP4), 1B(VP2), 1C(VP3) and 1D(VP1) (Figure 1). Iodination of external proteins on

intact bovine enterovirus and mengovirus particles, as well as chemical modification studies using intact rhino-, mengo- and poliovirions have demonstrated that 1D is labelled or modified to a greater extent than are proteins 1B and 1C. The smallest capsid protein, 1A, is inaccessible to these chemical reagents suggesting an internal position in the virion (Talbot et al., 1973; Carthew and Martin, 1974; Lonberg-Holm and Butterworth, 1976; Lund et al., 1977). Chemical cross linking with bifunctional reagents has revealed that proteins 1C and 1D are adjacent proteins in the poliovirus capsid (Wetz and Habermehl, 1979). These proteins are also coded for on contiguous regions of the viral RNA (Figure 1). Wetz and Habermehl (1979) speculated that adjacent arrangement of 1C and 1D may contribute significantly to the topography of the capsid while protein 1B(VP2) may be an elongated interior protein capable also of contributing to the surface of particles.

Tryptic peptide mapping studies of capsid proteins in several different serotypes and in different subtypes of the same serotype of FMDV clearly demonstrated that proteins 1A (VP4) and 1B (VP2) are more conserved than 1C or 1D (VP3 or VP1) in all the FMDV isolates examined (Robson et al., 1980). Similarly, amino acid sequence homology comparison between poliovirus, HRV 2, HRV 14 and coxsackie B virus indicated that proteins 1C and 1D show the least sequence homology. In contrast, 1A and 1B showed much greater

sequence conservation (Stanway et al., 1984; Skern et al., 1985; Tracy et al., 1985). These findings have prompted several researchers to speculate that such sequence conservation indicates a critical structural role for proteins 1A and 1B while 1C and 1D may contribute more to antigenicity and host cell receptor binding properties of the virus (Robson et al., 1980; Skern et al., 1985). No doubt, particle conformation also plays a crucial role in antibody induction and attachment to host cells. Hughes and coworkers (1984) demonstrated that neutralizing monoclonal antibodies and polyclonal antisera to HAV fail to recognize viral proteins when virions are disrupted, pointing to the importance of viral protein conformation in epitope recognition. In addition, empty capsids which lack RNA and so called 'A' particles which contain RNA but lack 1A, do not bind to susceptible cells (Putnak and Phillips, 1981).

A great deal of experimental evidence supports a very important role for capsid protein 1D in terms of virus neutralization as well as host cell attachment. Advances in three dimensional resolution of picornaviral structure have revealed a large cleft or 'canyon' on the surface of the virion which has been suggested as the host cell receptor-binding site (Rossmann et al., 1985; Rossmann et al., 1987). Protein 1D residues line the bottom of this canyon. Residues of both 1D and 1C are found on the walls and these include some of the least well conserved amino acids in picorna-

viruses, perhaps explaining the diversity in host cell receptor specificity among these viruses (Rossmann et al., 1985). In contrast, 1D residues at the bottom of the canyon, which appear to be inaccessible to antibodies and thus, not subject to antibody pressure, could remain constant, thereby allowing a particular virus to retain its own receptor specificity (Rossmann et al., 1985; Rossmann et al., 1987). Many recent studies have revealed that the major antigenic neutralization sites are found in protein 1D and in some viruses these have been localized to protrusions on the surface of the virion (Minor et al., 1983; Hughes et al., 1984; Chow et al., 1985; Linemeyer et al., 1985; Rossmann et al., 1985; Sherry et al., 1986).

5. Changes in EV70 Proteins

While the RNA genomes of several EV70 isolates have been characterized and compared by T_1 RNase fingerprinting (Kew et al., 1983; Takeda et al., 1984; Miyamura et al., 1986), prior to undertaking this project there existed a paucity of information concerning the biochemical nature of the structural and nonstructural proteins of the virus. Therefore, we decided to approach the study of the evolutionary change in EV70 by comparing polypeptides among 6 isolates of the virus collected between 1971 and 1981 from various regions of the world. These isolates represent both major pandemics as well as the interpandemic period (Table 2).

Previous work has demonstrated that these isolates differ in several respects:

1) at the RNA level - T_1 RNase fingerprinting revealed that:

a) J670 and R6 from the first pandemic are closely related

b) V1250, KW97 and 1604 from the 1981 epidemic are quite closely related to each other

c) RU3573, a 1975 isolate is similar to both the 1971 and 1981 strains and therefore may represent an evolutionary intermediate (Kewet al., 1983; Takeda et al., 1984)

2) biologically - isolation rates of EV70 during the 1971 pandemic ranged between 25-47% but were much lower, usually 0-8%, for the 1981 pandemic (Kono et al., 1972; Desmyter et al., 1981; Malison et al., 1984)

3) antigenically - 1981 strains give four fold lower neutralization titres with antiserum to J670 than with homologous antiserum (Kew et al., 1983).

The major objective of this project was to compare the polypeptides among these 6 isolates of EV70 to determine if changes, which have led to antigenic and biologic differences between the isolates, have also been manifested as detectable differences in viral proteins. This study is a first step towards localizing those regions of the EV70

TABLE 2

ORIGIN OF EV70 ISOLATES

ISOLATE	YEAR OF ISOLATION	LOCATION	OUTBREAK
J670*	1971	Japan	Pandemic
R6	1971	Morocco	Pandemic
RU3573	1975	S. Vietnam	Localized
V1250	1981	Honduras	Pandemic
KW97	1981	Key West, FL	Pandemic
1604	1981	Pakistan	Pandemic

*prototype strain

genome which have been subject to change as the virus has evolved during its short history as a human pathogen.

6. Precedent Studies

Various approaches have been used to probe for changes in nucleic acids and proteins which may contribute to differences in the pathological, biological or antigenic properties between viral isolates. T_1 RNase oligonucleotide fingerprinting analysis of poliovirus isolates has been invaluable for tracing the genetic and epidemiological relationships between clinical poliovirus isolates (Minor, 1980; Kew et al., 1981; Nottay et al., 1981). This approach has also been used in evolutionary studies of EV70 (Kew et al., 1983; Takeda et al., 1984). More recently, RNA sequence analysis has proved instrumental in determining genetic relationships among picornaviruses belonging to the same genus, as well as between viruses in different genera (Forss et al., 1984; Toyoda et al., 1984; Skern et al., 1985; Tracy et al., 1985).

Other researchers have chosen to compare viral proteins. One dimensional SDS-PAGE (Laemmli, 1970) is a simple method which has been used extensively in examining the viral protein complement for possible alterations in electrophoretic mobility. High resolution two-dimensional electrophoresis (O'Farrell, 1975) is a powerful tool for determining differences between proteins as it can detect changes in

both molecular weight and isoelectric point (net charge). This technique has been applied to the characterization of closely related isolates of the mouse picornaviral pathogen, Theilers murine encephalomyelitis virus (TMEV) and to the study of the properties of EMCV capsid proteins (Churchill and Radloff, 1981; Lorch and Friedmann,1983; Rozhon et al., 1985). Cleveland peptide mapping is a simple yet stringent test of protein identity and has been used to probe for differences between subtypes and serotypes of FMDV (Robson et al., 1980). Furthermore, monoclonal antibodies, by virtue of their restricted specificity, provide a means by which the fine structure of a protein may be examined at the antigenic level. These antibodies then are extremely useful in the comparison of closely related viruses (Stanley et al., 1985). In the present study these protein analyses were applied to the characterization of 6 different isolates of EV70 in order to detect possible differences in the viral protein complement.

MAIN OBJECTIVES OF THE STUDY

In summary, the main objectives of this study were:

1) to establish growth conditions and a plaque assay procedure for EV70

2) to develop a suitable purification protocol for the virus

3) to study the biophysical properties of EV70 (RNA and protein complement) in terms of molecular weight so as to verify the previous estimates of other investigators

4) to probe for differences in the proteins among 6 isolates of EV70 collected from different geographic locations between the years 1971-1981.

MATERIALS AND METHODS

1. Cells

A continuous line of monkey kidney cells, LLC-MK2, was obtained from Flow Laboratories (Rockville, MD) and used throughout this study for the propagation and quantitation of EV70. Monolayers were grown in 60 or 100 mm polystyrene dishes (Nunc/Gibco Canada, Montreal, Quebec) at 37°C in a 5% CO_2 atmosphere in a Shellab incubator (Johns Scientific, Toronto, Ontario). Cell culture medium (MEM) consisted of Earle's minimum essential medium (autoclavable powder; Gibco Canada) supplemented with 5% sterile fetal bovine serum (FBS; Bocknek Organic Materials, Rexdale, Ontario), 0.2% (wt/vol) sodium bicarbonate ($NaHCO_3$; Cellgro Mediatech, Washington, DC), 2mM L - glutamine (Cellgro Mediatech) and 50 micrograms/ml of gentamycin sulphate (Roussel Laboratories, Wembley, England). To passage cells, monolayers were washed with Tris buffered saline (TBS; 137 mM NaCl, 5 mM KCl, 0.7mM Na_2HPO_4, 5.6 mM glucose and 25 mM Tris - HCl pH 7.2) and trypsinized in 0.05% trypsin -0.02% EDTA (Gibco Canada) for 2-5 minutes at 37°C. Cells were removed from the plates by gently pipetting up and down, centrifuged (Minifuge 2, Heraeus Christ GMBH, Osterode am Harz, W. Germany) in a sealed rotor at 360 x g for 5 minutes at 20°C,

resuspended in fresh MEM and added to new culture dishes. Normally the cells were split in a ratio of 1:5.

2. Virus

Isolates of EV70 (J670, R6, RU3573, KW97, V1250 and 1604 (Table 2)) were kindly provided by Dr. M. Hatch and Dr. M. Pallansch, Centre for Disease Control (CDC), Atlanta, Georgia. The passage histories of these strains were not given. The prototype, J670, was plaque purified twice prior to the production of stock virus. However, for all other isolates, stocks were prepared directly from the inoculum received from CDC. Virus infections were carried out in the absence of FBS.

To prepare stocks of virus, newly confluent monolayers of LLC-MK2 cells were infected at low multiplicities of infection (m.o.i. = 0.001-0.01) in MEM at 33°C. After one hour, the inoculum was removed, replaced with fresh MEM (5 ml) and incubated at 33°C until the cytopathic effect (CPE) was maximal, usually between 24 and 36 hours post infection. Following two cycles of freezing and thawing, cell debris was removed by centrifugation (Minifuge 2) for 10 minutes in a sealed rotor at 3,980 x g at 4°C and the medium was aliquoted and stored frozen at -80°C. All stocks were titred by the plaque assay technique.

3. Plaque Assay

Medium was removed from confluent monolayers of LLC-MK2 cells in 60 mm dishes. Cells were infected with duplicate 0.2 ml samples of serially diluted virus for 1 - 2 hours at 33°C, overlaid with 3.5 ml of a 1:1 mixture of two times concentrated (2x) MEM:1.6% agarose and incubated for a further four days at 33°C. At this time, a second overlay which contained 0.2 mg/ml of neutral red dye was added. Plaques were counted 4-8 hours later and virus titres were expressed in plaque forming units per ml (pfu/ml). Alternatively, instead of a neutral red overlay, cells were fixed on day 4 for 2 hours with a 10% formal saline solution and then stained briefly with 0.1% (w/v) crystal violet dye.

4. Growth Curve

The replication of EV70 in LLC-MK2 cells was examined by performing a time course experiment. Cells were grown to confluency in 100 mm culture dishes and infected with the J670 prototype strain of EV70 at m.o.i.'s of 10, 1, 0.1 and 0.01 for two hours at 33°C. At this point, monolayers were washed twice with MEM to remove nonadsorbed virus. Two ml of fresh MEM were added to each dish and incubation at 33°C was resumed. At various time intervals post infection, 1 ml samples were removed and residual medium was aspirated and replaced with fresh MEM. Sample aliquots were stored at

-80°C for plaque assay. The extent of CPE was also recorded throughout the experiment.

5. Radioactive Labelling of Viral Macromolecules

To prepare EV70 virions containing radioactively labelled RNA, confluent cells were infected at a m.o.i. of 0.1-0.2 in MEM for one hour at 33°C. At this time, the inoculum was removed and replaced with fresh MEM also containing 5 micrograms/ml of actinomycin D (Boehringer Mannheim, Dorval, Quebec). Incubation was continued for one hour and then 50 microcuries/plate of [5,6-3H] uridine (30-50 Ci/mmol; New England Nuclear (NEN), Lachine, Quebec) was added. Infection was allowed to proceed until virtually all cells in the monolayer became rounded due to viral CPE (approximately 11 hours). The plates were then scraped with a rubber policeman and culture supernatant fluid and cells were frozen at -80°C. Virus was subsequently purified from these harvests.

Both EV70 and cellular proteins were labelled with L-[^{35}S] methionine. Basically, the infection procedure was the same as that described for uridine labelling. In all experiments, monolayers were washed twice with warm TBS when approximately 75% of the cells were rounded and incubated in 2 ml of methionine-free medium (Flow Laboratories, Rockville, MD), with or without actinomycin D, for one hour. Eventually, the use of actinomycin D in such protein labelling experiments was discontinued since host

cell protein synthesis was rapidly shut off by the virus. Following starvation for methionine, 50 microcuries/plate of ^{35}S-methionine (1450 Ci/mmol; Amersham Canada, Oakville, Ontario) was added and cells were pulse labelled for one hour, then chased for the same length of time in MEM containing an excess of non-radioactive methionine. Alternatively, cells and supernatant were harvested immediately following the pulse and frozen at -80^{o}C.

6. Virion Purification

Two methods were routinely employed to purify EV70. The first method has been described elsewhere (Natori et al., 1984), however, several modifications were made. EV70 infected cells and culture supernatants labelled with ^{3}H-uridine or ^{35}S-methionine were frozen and thawed twice, subjected to Dounce homogenization and the homogenate was then clarified by centrifuging at 7,740 x g for 10 minutes at 4^{o}C (Beckman J2-21M centrifuge, JA-20 rotor). Supernatants were maintained on ice while pellets were dissolved in a small volume of 1% (wt/vol) Nonidet P-40 (NP40) in TN buffer (100 mM NaCl, 50 mM Tris-HCl pH 7.2) to ensure the release of all cell bound virus. Pellets were placed on ice for 30 minutes and then spun down as described above. Supernatants were pooled and a concentrated solution of polyethylene glycol (PEG, Carbowax 6000; 40% wt/vol) and NaCl (2.5 M) was then added to a final concentration of 8%

PEG and 0.5 M NaCl. Following the addition of protease inhibitors phenylmethylsulfonyl fluoride (PMSF; United States Biochemical Corp., Cleveland, OH) and benzamidine hydrochloride (BHCl; Sigma Chemical Company, St. Louis, MO) to 1 mM concentration, the suspension was mixed well and placed on ice overnight (approximately 18 hours). The next day, the suspension was centrifuged at 12,100 x g for 20 minutes at 4°C (Beckman J2-21M centrifuge, JA-20 rotor). Pellets were suspended in 200-400 microlitres of TN buffer, layered onto a 12 ml 10-40% linear sucrose gradient and centrifuged at 154,000 x g (Beckman L3-50 ultracentrifuge) in an SW41 rotor for 3.5 hours at 4°C. Fractions were collected by bottom puncture and aliquots were added to aqueous scintillation cocktail (Formula 963 Dupont / NEN, Lachine Quebec) and assayed for radioactivity in a Beckman LS-250 liquid scintillation counter. Peak radioactive samples were pooled, diluted in TN buffer and virus was pelleted in an SW41 rotor at 210,000 x g for 2.5 hours. Tubes were well drained and pellets were resuspended in a small volume of an appropriate buffer (depending upon the intended use of the preparation).

The second purification protocol which will be referred to as the 'rapid method' was adapted from the one used for the purification of poliovirus (Pincus et al., 1986). Briefly, subconfluent or newly confluent LLC-MK2 cells in 100 mm culture dishes were infected at a m.o.i. of approximately

0.1 as described above. At approximately 6-8 hours post-infection, the medium was aspirated, cells were washed twice with warm TBS and methionine-free medium containing 15 microcuries/ml of ^{35}S- methionine and 1:20 volume of normal MEM (containing non-radioactive methionine) was added. The infection was continued overnight at 33°C until complete destruction of the monolayer had occurred. Cells and supernatants from infected cultures were frozen and thawed twice. Debris was then pelleted by centrifugation in a Beckman SW28 rotor at 32,000 x g and 20°C for 15 minutes. The supernatants were transferred to new SW28 tubes and 10% (wt/vol) sodium dodecyl sulfate (SDS) was added to a final concentration of 0.5%. After gentle but thorough mixing, the virus was pelleted by centrifugation at 110,000 x g for 4 hours at 24°C (SW28 rotor). Supernatants were discarded, tubes were drained well, and the virus pellets were resuspended in a small volume of an appropriate buffer and stored at -80°C.

7. RNA Extraction and Electrophoresis

Purified, ^{3}H-uridine labelled virions, prepared by the sucrose gradient method, were dissolved in a small volume (500 microlitres) of NENS buffer (50 mM Na acetate pH 5.1, 100 mM NaCl, 10 mM EDTA, 0.5% SDS) containing 200 micrograms/ml of proteinase K (Boehringer Mannheim). After a 30 minute incubation at 37°C, 50 micrograms of phenolized

dextran was added as carrier and RNA was extracted twice with phenol:chloroform:isoamyl alcohol (25:24:1) and twice with chloroform alone. 2.5 volumes of 99% cold ethanol was added to to the extract and this was placed at -20°C for 12-24 hours to permit RNA precipitation. This step was followed by centrifugation in a Fisher model 235B micro-centrifuge (13,000 x g) at 4°C for 15 minutes or in the Beckman J2-21M centrifuge for 30 minutes at 17,000 x g and 4°C depending on the sample volume. The precipitate was redissolved in diethylpyrocarbonate-treated water (DEP-H_2O) and a 5 M solution of DEP- treated NaCl was added to attain a final concentration of 0.1 M salt. This was then re-precipitated by the addition of 2.5 volumes of 99% ethanol and left at -20°C for 12-24 hours. As described above, the RNA was pelleted, the supernatant was drained and the pellet well dried. The RNA (pellet) was then prepared for electro-phoresis by resuspending in RNA sample buffer (20 micro-litres H_2O, 8 microlitres 0.1 M Na_2HPO_4 + 0.5% SDS, 12 microlitres glyoxal and 40 microlitres dimethylsulfoxide (DMSO)) and heating for 15 minutes at 65°C. Electrophoresis of the RNA sample was performed using a mini submarine gel apparatus (Hoefer Scientific/Technical Marketing Associates, Ottawa, Ontario) and a 1% agarose gel containing 10 mM Na_2HPO_4 for several hours at 50V. Gels were prepared for fluorography by soaking in EN^{3}HANCE for one hour. They were then rinsed in cold water for an additional hour and dried

onto Whatman paper under vacuum for 1 hour with heat. Gels prepared in this manner were placed in Kodak cassettes and exposed to X-ray film (Dupont Cronex, E.I. Dupont deNemours, Wilmington, DE) at -80°C.

8. Polyacrylamide Gel Electrophoresis

Electrophoresis of viral and cellular proteins was carried out using the Laemmli (1970) discontinuous SDS- polyacrylamide gel system (SDS-PAGE). Samples to be electrophoresed were suspended in modified 2x Laemmli sample buffer (2x SB) consisting of 4% SDS (wt/vol), 20% glycerol (vol/vol), 10% 2-mercaptoethanol (vol/vol), 60 mM Tris-HCl pH 6.8 and 0.1% (wt/vol) bromophenol blue dye. Slab gels consisting of 12 or 15% polyacrylamide with 0.1% SDS (resolving gel) were overlaid with 3 cm stacking gels containing 4.8% polyacrylamide and 0.1% SDS. The Tris-glycine-SDS running buffer used was that described by Laemmli (1970). All samples in 2x SB were heated for at least 5 minutes at 100°C to ensure complete denaturation of proteins and were then loaded into the wells of the stacking gel. Normally electrophoresis was carried out at 20-25 milliamps (mA) constant current until the tracking dye had reached the end of the resolving gel (approximately 16 hr). The glass plates were removed and the gels fixed for 1 hour in 30% methanol, 10% acetic acid and then prepared for fluorography as described for RNA electrophoresis. Molecular weights of proteins were

estimated by constructing a standard curve from the migration of either high molecular weight prestained protein markers (Bethesda Research Laboratories, Life Technologies Inc., Gaithersburg, MD) or ^{14}C methylated protein markers (Amersham) which were included in each gel.

9. High Resolution Two Dimensional Electrophoresis

Both virion proteins and infected cell lysates were examined by two dimensional high resolution electrophoresis. Details of this method have been described elsewhere (O'Farrell, 1975), however, several modifications were made and these changes will be briefly explained. Virions were labelled overnight with ^{35}S-methionine as has been described, purified by the sucrose gradient method and resuspended in lysis buffer (LB; 9.5 M urea, 2% (wt/vol) NP-40, 2% (wt/vol) Ampholines consisting of 1.6% pH range 5-7 (Biorad Laboratories, Mississauga, Ontario) and 0.4% (wt/vol) pH range 3-10 (Pharmacia Canada, Dorval, Quebec) and 5% (vol/vol) 2-mercaptoethanol).

For the preparation of cellular lysates, EV70 infected cells were labelled with ^{35}S-methionine for 1 hour and then harvested immediately by scraping the plate with a rubber policeman. Cells were pelleted briefly (5 minutes) at 360 x g and 4°C (sealed rotor Minifuge 2 centrifuge) and were then washed once in cold TBS and pelleted once more. The cell pellets were then resuspended in 250 microlitres/plate

of cold TBS, transferred to Eppendorf tubes and were pelleted for 5 seconds in the microcentrifuge. At this point, the cells were resuspended in 100 microlitres/plate of swelling buffer (10 mM Tris-HCl pH 7.0-7.5, 1 mM EDTA, 1% (wt/vol) NP-40, 100 micrograms/ml RNase A (Boehringer Mannheim, Montreal, Quebec), 1 mM PMSF and 1 mM BHCl)) and were placed on ice for 10 minutes. After a brief spin (15 seconds) to remove nuclei, each 100 microlitre aliquot of supernatant was added to Eppendorf tubes containing 0.1 g urea, 8 microlitres of 2-mercaptoethanol (4% vol/vol) and 8 microlitres NP-40 (4% vol/vol). These samples were stored at -80°C. Immediately prior to electrophoresis in the first dimension, the samples were thawed and 2% (wt/vol) pH range 3-10 ampholines (Pharmacia Canada) were added.

First Dimension

Electrophoresis in the first dimension involved isoelectric focusing (IEF) of proteins in polyacrylamide tube gels containing urea. O'Farrell (1975), gives a detailed description of the preparation of tube gels and this procedure was followed, however, tubes were not siliconized prior to use. Slight modifications were also made to the pre-run times and actual electrophoresis conditions. Gels were pre-run for 10 minutes at 200 V, 15 minutes at 300 V and 20 minutes at 400 V, samples were applied and electrophoresis continued at 400 V for 12 hours. At this point, the

gels were immediately extruded into 10 ml of IEF equilibration buffer (10% wt/vol glycerol, 5% vol/vol 2-mercaptoethanol, 2.3% wt/vol SDS, .0625 M Tris-HCl pH 6.8) and then frozen at -80°C. All 6 isolates of EV70 were run simultaneously in the first dimension. The pH gradients were checked by including an isoelectric focusing prestained protein marker (Electran Range 4.7-10.6, BDH Chemicals, Toronto, Ontario) and by directly measuring the gradient using a mini electrode which could be easily moved along the surface of the gel (Orion/Fisher Scientific, Ottawa, Ontario).

Second dimension

Electrophoresis in the second dimension consisted of SDS-PAGE as has been described for protein electrophoresis. However, it was necessary to change the procedure slightly so as to accommodate the first dimension tube gel. Polyacrylamide gels were cast using 2 bevelled plates and instead of using a comb to form the wells, a single well was made at one side of the gel using a spacer. A one dimensional marker such as a sample of radiolabelled infected cell lysate was placed in this well. Following thawing and equilibration for 30 minutes at room temperature, each tube gel was carefully laid between the 2 bevelled plates so that it rested against the surface of the stacking gel. Gels were secured at both ends with a few

drops of 1% agarose in equilibration buffer. Electrophoresis was started at 20 mA and continued until the dye had reached the bottom of the resolving gel, usually 12-16 hours. The slab gels were then processed for fluorography as has been described. It was not possible to carry out second dimensional analysis on all 6 isolates at the same time. Therefore, they were run in pairs and conditions were carefully standardized.

10. Peptide Mapping

This procedure was adapted from Cleveland et al. (1977). Cytoplasmic extracts of EV70-infected cells and mock-infected cells prepared by the RIPA buffer lysis method as described for immunoprecipitation of viral proteins (see below) were first electrophoresed on 12% polyacrylamide-SDS gels, dried without fixing or fluorography and then exposed to X-ray film. The desired protein bands were cut out of the gel and then chopped into small pieces. The gel pieces were equilibrated at room temperature for 30 minutes in equilibration buffer (0.125 M Tris-HCl, pH 6.8, 0.1% SDS) and were either used immediately or stored frozen at -80°C. Rehydrated gel pieces were placed into the bottom of the wells of a 15% SDS-polyacrylamide resolving gel with a extra long stack (4.5 cm) of 3.75% polyacrylamide. Approximately 15-20 microlitres of 2x SB were added to each well to fill in the spaces around the gel pieces. Equal volumes

of 2 mg/ml Staphylococcus aureus V8 protease (Miles Scientific, Naperville IL) in water and 2x SB were well mixed and 10-20 microlitres of this protease solution (1 mg/ml (wt/vol) final concentration) were applied to each well. Electrophoresis was started at 10 mA until the dye front had traversed 3/4 of the stacking gel at which point the power was turned off for 45 minutes to allow protease digestion to occur. Following proteolysis, the electrophoresis was resumed at 25 mA constant current for 16 hours or until tracking dye had reached the end of the gel. Gels were then fixed, prepared for fluorography, dried and exposed to X-ray film.

11. Immunoprecipitation of Viral Proteins

Cellular and viral proteins were labelled with ^{35}S-methionine and cells were harvested after a one hour pulse. The preparation of cellular lysates involved washing the cells in cold TBS several times and lysing them in 200 microlitres/plate of 50 mM Tris-HCl, pH 7.2, 100 mM NaCl, 1.0% (wt/vol) sodium deoxycholate (NaDOC), 1% (vol/vol) Triton X-100 (TX-100), 0.1% SDS and 1 mM each of PMSF and BHCl (this mixture will be referred to as RIPA buffer). Cell debris and nuclei were removed by immediately pelleting in a microcentrifuge for 30 seconds. The supernatants (cytoplasmic extract or lysate) were aliquoted and stored at -80°C.

Normally 20 microlitres of cytoplasmic extract were added to 10 microlitres of murine monoclonal antibody, Balb C mouse, monkey or rabbit antisera (Table 3). To encourage antigen-antibody formation, these mixtures were allowed to sit on ice for 1-2 hours. Then, 50 microlitres of protein A-Sepharose CL-4B beads (200 mg/ml) (Pharmacia Canada) and 400 microlitres of RIPA buffer were added to each sample. Incubation was continued with end-over-end mixing at 4°C for at least 2 hours. The beads were then pelleted in a micro-centrifuge for 20 seconds, washed 3-5 times with cold RIPA buffer and resuspended in a small volume of 2x SB (20-50 microlitres). Samples were heated for 5 minutes at 100°C to elute and denature immune complexes and were applied to 12% polyacrylamide-SDS gels for analysis. Following electro-phoresis, gels were processed for fluorography as has been described.

TABLE 3

ANTIBODY SOURCES

	Type	Source
Monoclonal Antibodies		
74-5G	IgG1 non-neutralizing	L. Anderson, CDC, Atlanta
73-7H	IgG1 non-neutralizing	L. Anderson, CDC, Atlanta
Polyclonal Antisera		
Mouse		B. Brodeur, LCDC, Ottawa
Monkey		R. Kono, NIH, Tokyo
Rabbit		R. Kono, NIH, Tokyo

RESULTS

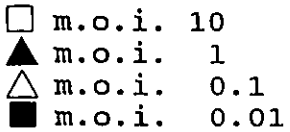
1. Growth of EV70 in LLC-MK2 cells

Prior to undertaking this project, EV70 was not being routinely propagated, titred and purified in our laboratory. Thus it was necessary, as a preliminary step, to establish these procedures.

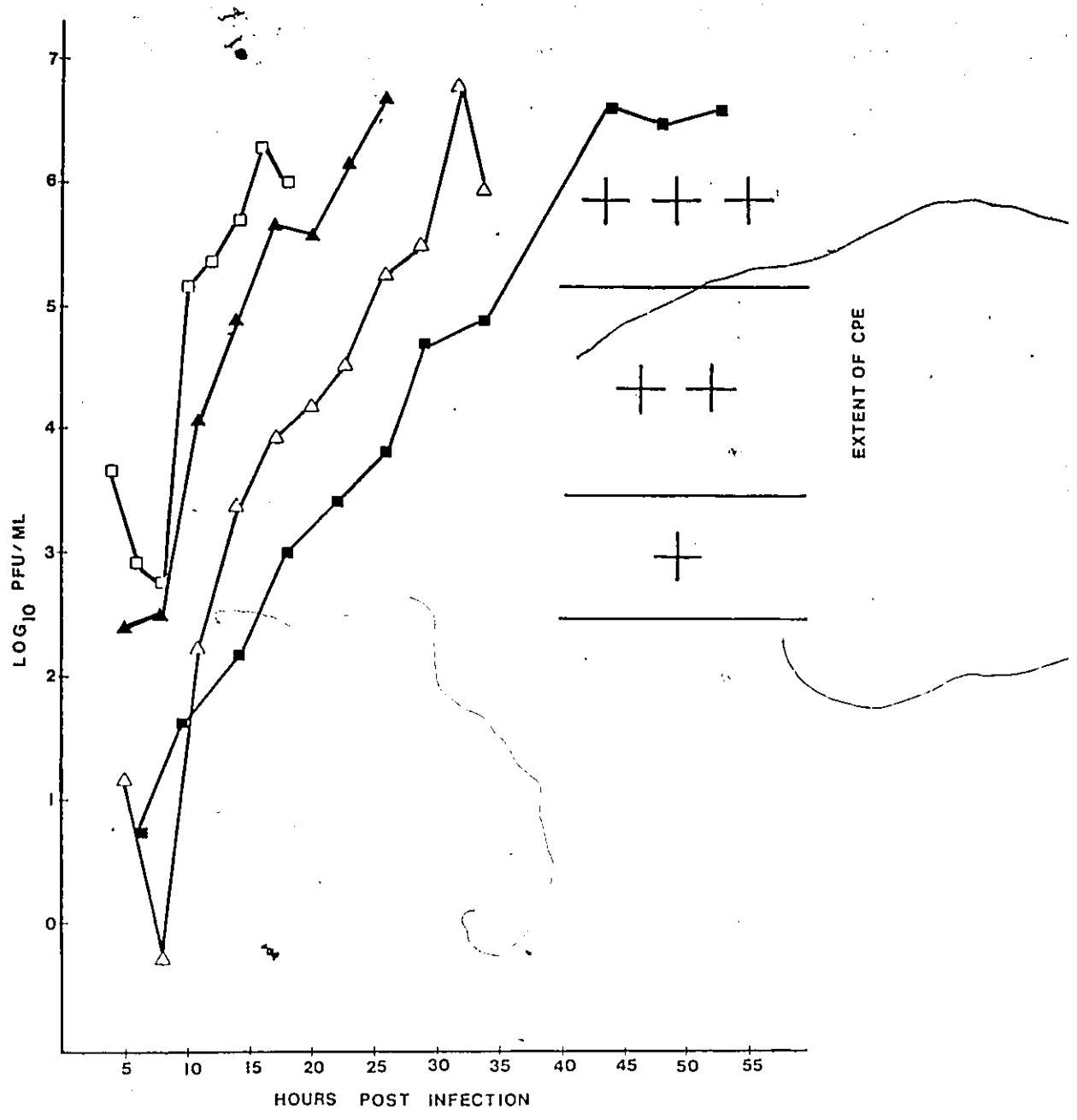
EV70, which has a wide host range in tissue culture (Yoshii et al., 1977), grows readily in the LLC-MK2 continuous monkey kidney cell line which was already in use in the lab for the propagation of human parainfluenza virus type 3. The EV70 cytopathic effect, beginning with retraction and rounding of the cells leading to eventual destruction of the monolayer, appeared within 8-36 hours post infection depending upon the m.o.i. A simple plaque assay procedure was used to quantitate the virus and typical titres ranged from approximately 1.0×10^6-1.0×10^7 pfu/ml depending upon the particular isolate. In general, plaque size was extremely heterogeneous between isolates and even among plaques from a single isolate.

The replication of EV70 in LLC-MK2 cells was further studied by performing a time course experiment (Figure 2). The information obtained from this experiment made it possible to estimate the m.o.i. necessary in future experiments so as to be able to harvest the virus at a suitable

Figure 2. The rates of EV70 production in LLC-MK2 cells at four different m.o.i.'s. Cells were grown to confluency in 100 mm culture dishes, infected with the prototype strain of EV70 (J670) at one of four different m.o.i.'s , then washed to remove non-adsorbed virus. At various time intervals post infection 1 ml aliquots were removed, frozen at -80°C and plaque assayed at a later date. Residual medium was aspirated, fresh medium was added and incubation was continued at 33°C. Also shown in the figure is the extent of CPE at various times post infection.



\+ 25% of cells in monolayer rounded
++ 50% of cells in monolayer rounded
+++ 75% of cells in monolayer rounded


LOG10 PFU/ML
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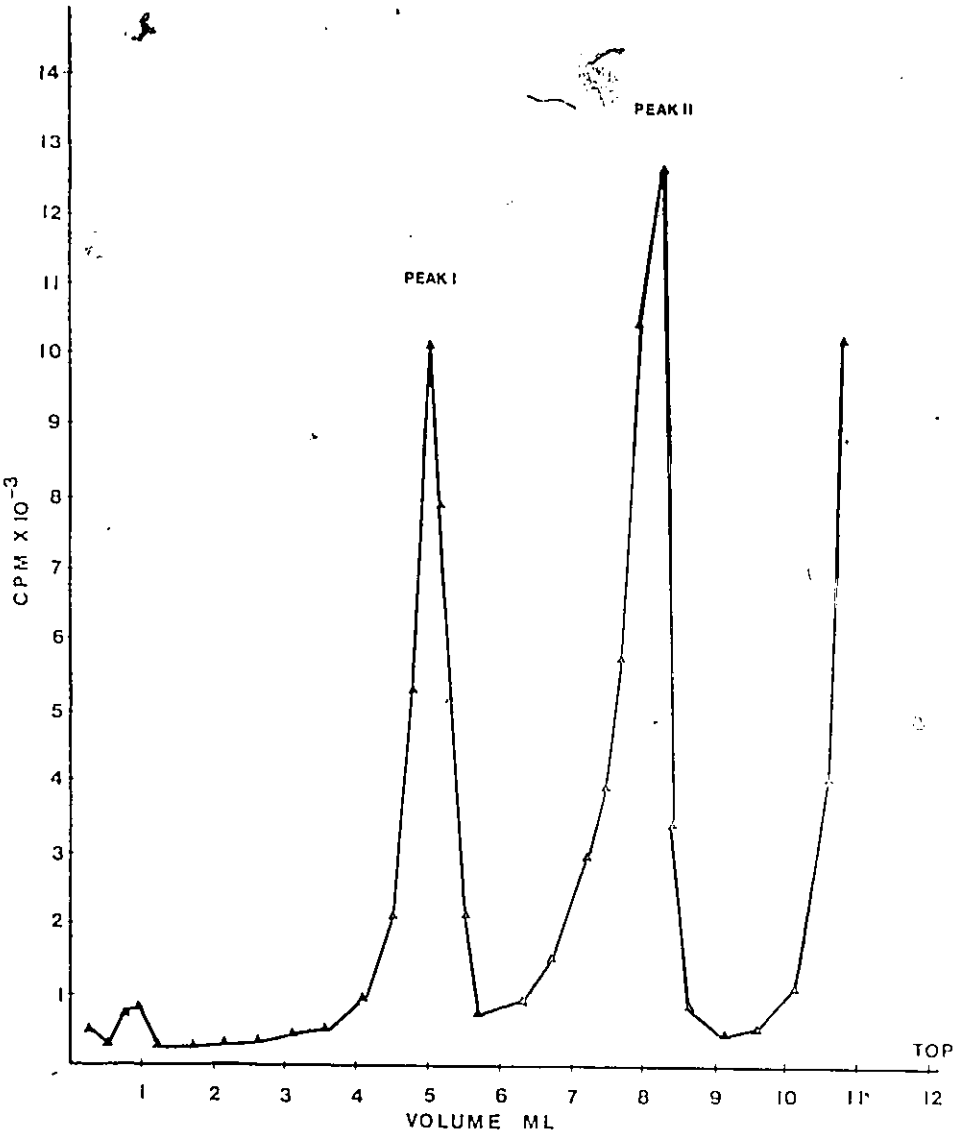
50
55
HOURS POST INFECTION
EXTENT OF CPE

time. For example, (as shown in Figure 2) if newly confluent or subconfluent cells were infected at a m.o.i. of between 0.5-1.0, it was possible to harvest virus approximately 9-12 hours post infection, the point when 50-75% of cells in the monolayer were rounded.

2. Virus Purification

As described in the Materials and Methods section, two different purification protocols were routinely used. Radiolabelled virions prepared in both ways showed the same protein profiles when analysed by SDS-PAGE. In preliminary purification trials using only the ^{35}-methionine labelled prototype J670, it became evident that regions of peak radioactivity often occured in two separate areas of the same sucrose gradient. The lower peak was designated Peak I and the second higher peak, Peak II as shown in Figure 3. Several approaches were used to determine if there were any differences in the protein or RNA composition between virus in the two peaks. First, a plaque assay was performed using aliquots from peak I and peak II. While infectious virus was recovered from both, the titre of virus from peak I was approximately 2 logs greater than that from peak II despite there being more radioactivity in peak II. Secondly, simultaneously prepared and purified prototype virus was labelled with either ^{35}S-methionine or ^{3}H-uridine. By labelling two different viral components, differences in the

Figure 3. Sucrose gradient centrifugation of ^{35}S-methionine labelled EV70 (J670 strain). EV70 infected LLC-MK2 cells were labelled with ^{35}S-methionine for 1 hour and chased for 1.5 hours with excess non-radioactive methionine. The cells were then processed for virion purification as described in the text. PEG-precipitated virus was resuspended in 400 microlitres of TN buffer containing 1 mM each of PMSF and BHCl, layered onto 12 ml 10-40% linear sucrose gradients and centrifuged at 154,000 x g in a SW41 rotor for 3.5 hours at 4°C. Fractions (250 microlitres) were collected by bottom puncture and 50 microlitre aliquots were assayed for radioactivity. The radioactive peak closer to the bottom of the gradient was designated as peak I (viral particles containing RNA) and that closer to the top as peak II (particles lacking RNA, see Figure 4) The top of the gradient is on the right hand side of the figure.


PEAK II
PEAK I
CPM X 10^{-3}
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VOLUME ML

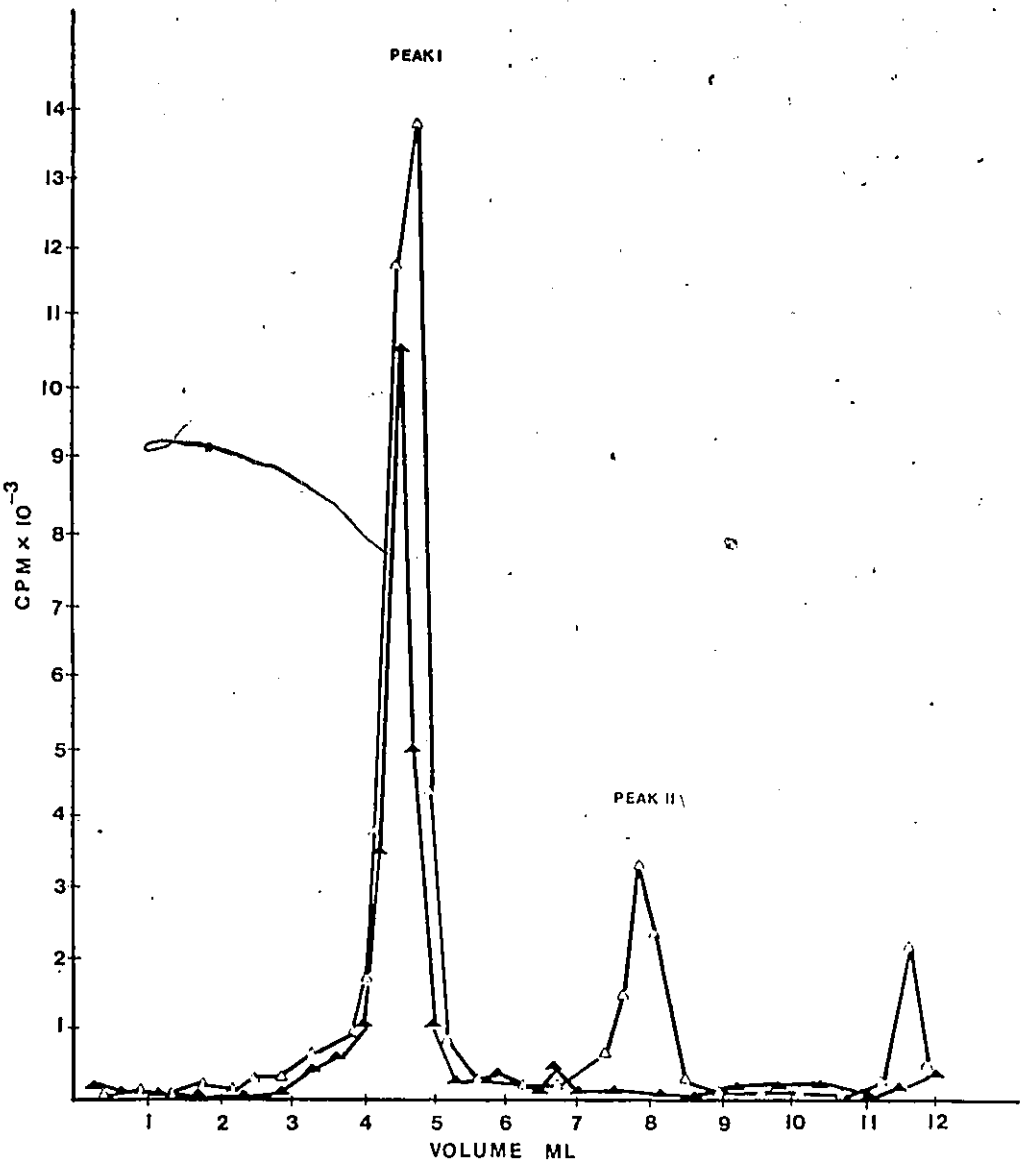
composition of virus in both peaks can be detected, for example, empty capsids lacking RNA could be distinguished from full capsids. The radioactivity obtained from each sucrose gradient was plotted on the same graph (Figure 4). These profiles clearly show that two radioactive peaks were obtained only for the ^{35}S-methionine labelled material. Furthermore, the ^{3}H-uridine peak coincides with only one of the ^{35}S-methionine peaks. In addition, the proteins from both ^{35}S-methionine labelled viral peaks were analysed by SDS-PAGE (Figure 5). The capsid precursor protein 1AB (VP0) as well as the smallest capsid protein 1A(VP4) were not detected in either virion peak. The minor peak of ^{35}S-methionine radioactivity seen at the top of the sucrose gradient may correspond to free methionine. This minor peak was noted each time ^{35}S-methionine labelled virus was purified but was not always of the same magnitude.

3. RNA Purification

^{3}H-uridine labelled viral RNA was extracted from sucrose gradient purified virions, denatured with glyoxal and electrophoresed on a 1% agarose gel. In addition to EV70 RNA, ^{3}H-uridine labelled baby hamster kidney (BHK) ribosomal RNA (kindly provided by D. Murphy) and VSV genomic RNA and defective interfering particle RNA (kindly provided by E. Rud) served as molecular weight markers. Figure 6 shows the resulting autoradiogram. A standard curve was

Figure 4. Sucrose gradient centrifugation of the J670 strain of EV70 labelled with ^{35}S-methionine or ^{3}H-uridine. Ten confluent plates (100 mm) were infected with the J670 isolate of EV70. Five were then labelled with ^{3}H-uridine and 5 with ^{35}S-methionine as described in Materials and Methods. Virions were analysed by centrifugation as described in Figure 3. Fractions were collected by bottom puncture and 50 microlitre aliquots were assayed for radioactivity.

▲ ^{3}H-uridine labelled virus
△ ^{35}S-methionine labelled virus


PEAK I
PEAK II
CPM x 10⁻³
VOLUME ML
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13
14

Figure 5. SDS-PAGE of ^{35}S-methionine labelled EV70 virions and infected LLC-MK2 cell lysates. Lanes 1 and 2: SDS-PAGE of ^{35}S-methionine labelled EV70 virions. Four confluent plates of LLC-MK2 cells were infected with EV70 J670, labelled with ^{35}S-methionine for 1 hour and chased for 1.5 hours with non-radioactive methionine. Peak fractions from a sucrose gradient were pooled, diluted in TN buffer + 1mM each of PMSF and BHCl and virions were pelleted in a SW41 rotor at 210,000 x g for 2.5 hours. Pellets were resuspended in 50 microlitres of 2x SB and heated for 5 minutes at 100^{o}C prior to electrophoresis. Lanes 3 and 4: SDS-PAGE of EV70 infected LLC-MK2 cell lysate. LLC-MK2 cells were infected with the prototype EV70, J670, or mock-infected and labelled with ^{35}S-methionine for 1 hour. The cells were then lysed using RIPA buffer as is described in the text. Each lysate was then added to an equal volume of 2x SB. Lanes 3 and 4 represent equal volumes of cell lysate. All samples were electrophoresed on a 15% polyacrylamide-SDS gel for 16 hours at 20 mA. The gel was prepared for fluorography as described in the text and then exposed to X-ray film for 3 weeks. Lane 1, peak I material (viral particles containing RNA); lane 2, peak II material (viral particles lacking RNA); lane 3, uninfected cell lysate; lane 4, J670 infected cell lysate. Prestained protein molecular weight markers were included in the gel but are not indicated on the figure. The approximate molecular weight of the EV70 polypeptides are indicated.

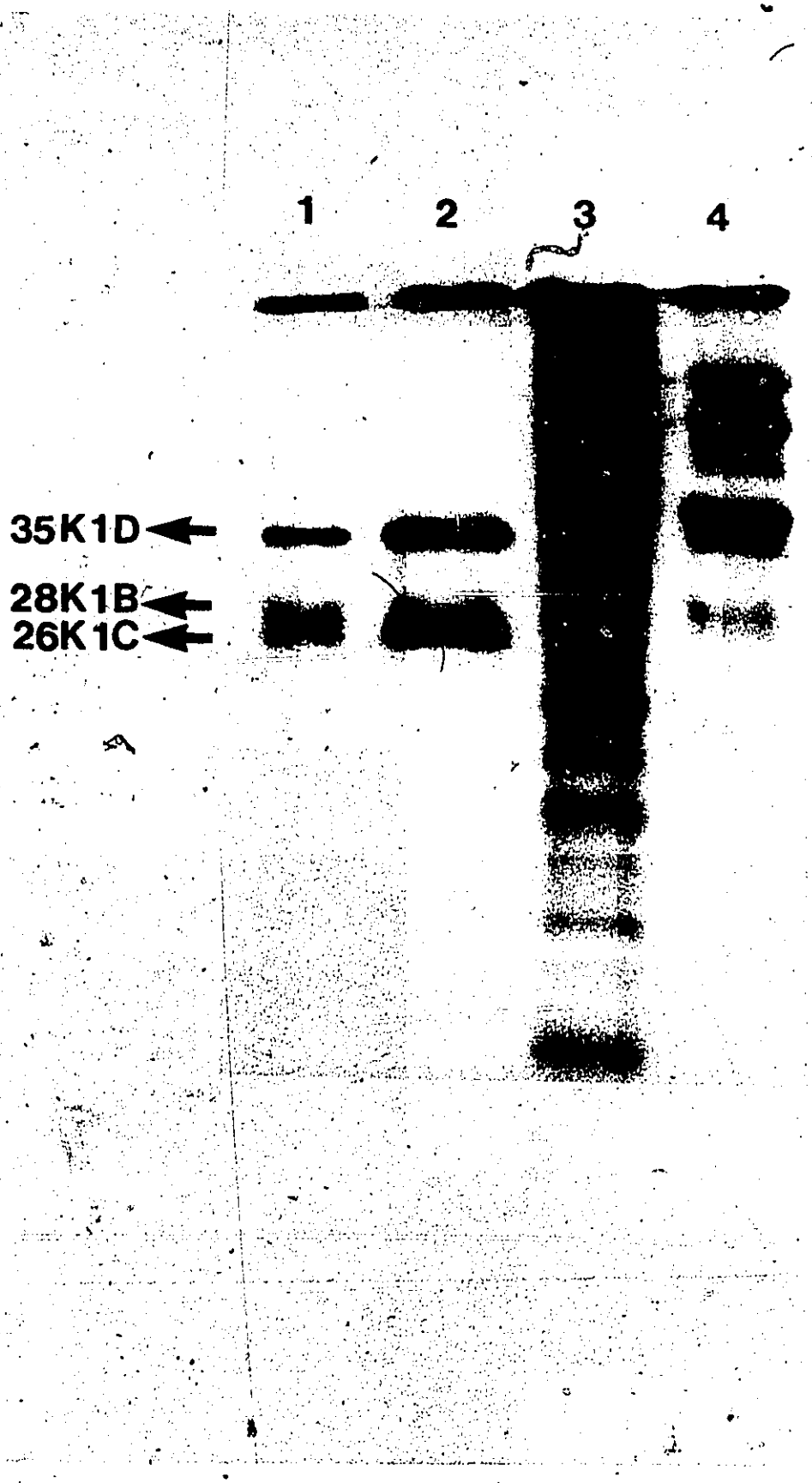
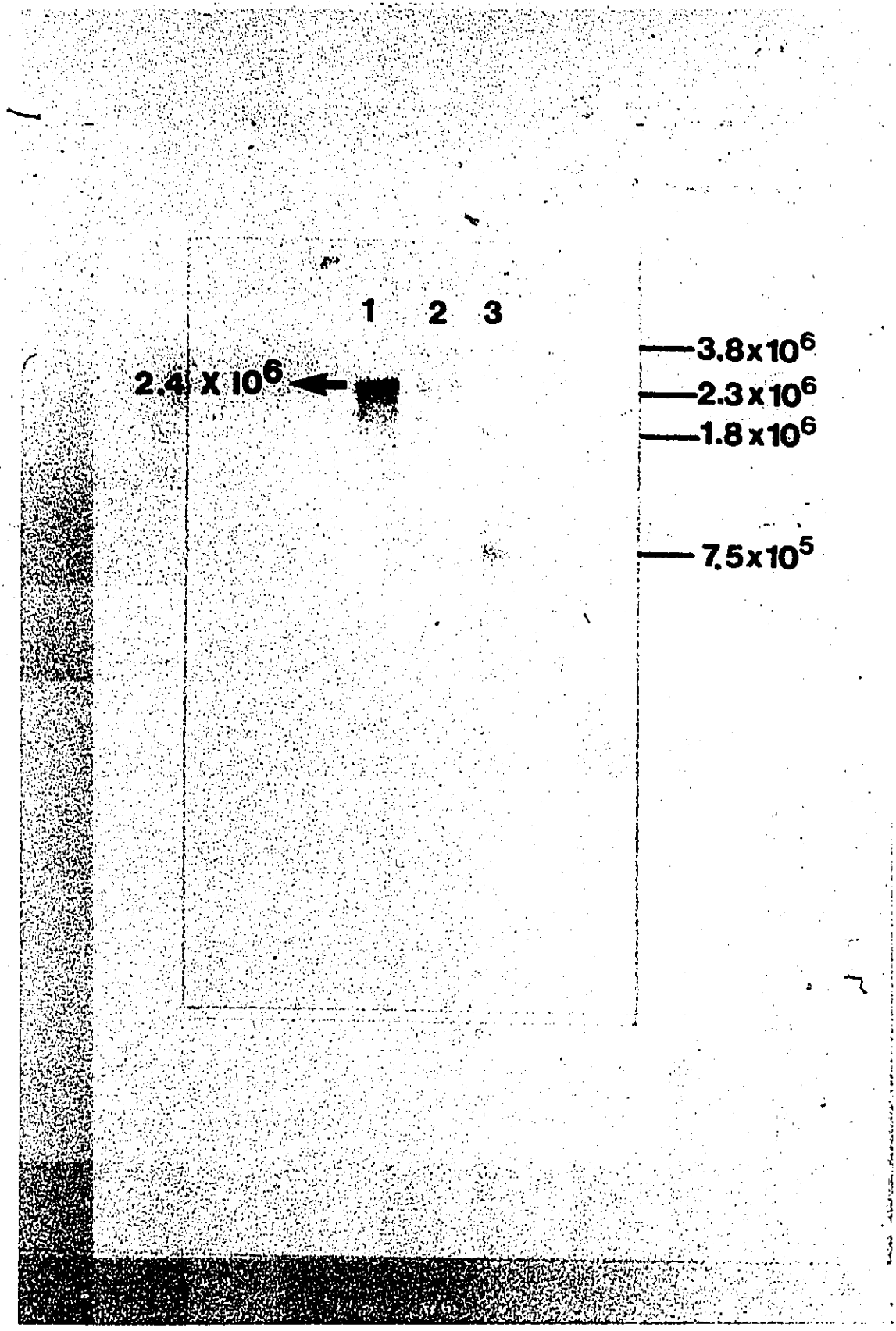

1
2
3
4
35K1D
28K1B
26K1C

Figure 6. Agarose gel electrophoresis of EV70 RNA. RNA was extracted (as described in text) from ^{3}H- uridine labelled virions following sucrose gradient purification, resuspended in RNA sample buffer and electrophoresed for 2 hours at 50 volts on a 1% agarose gel containing 10mM sodium phosphate buffer, pH 7.0. The gel was prepared for fluorography as described in the text and then exposed to X-ray film for 24 hours. Lane 1, ^{3}H-uridine labelled EV70 genomic RNA; lane 2, ^{3}H-uridine labelled vesicular stomatitis virus (VSV) genomic and defective interfering (DI) particle RNA; lane 3, ^{3}H-uridine labelled kidney (BHK) cell ribosomal RNA. The RNA species in lanes 2 and 3 were used as molecular weight markers and their approximate molecular weights are indicated in the figure.

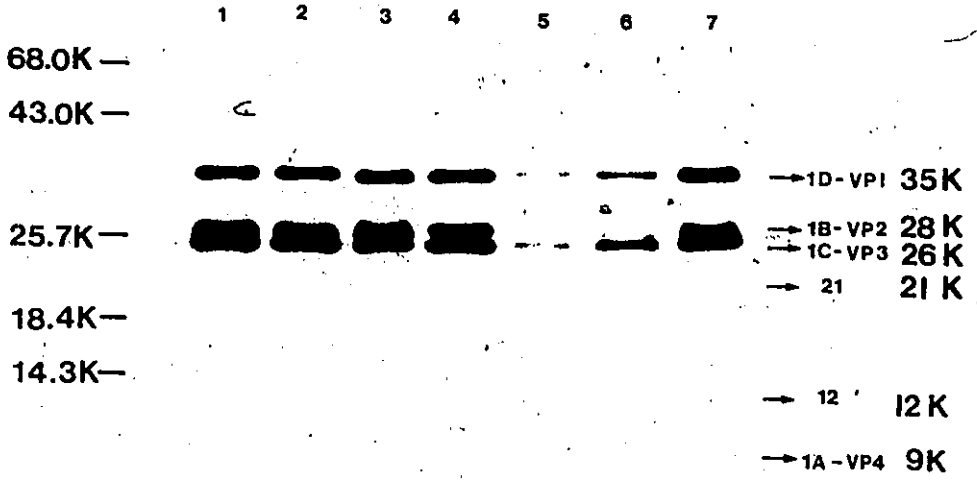

1
2
3
2.4 X 10^6
3.8x10^6
2.3x10^6
1.8x10^6
7.5x10^5

constructed (log MW of markers vs distance migrated) and from it the molecular weight of EV70 RNA was estimated to be approximately 2.4 x 10^6.

4. One Dimensional SDS-PAGE of EV70 Proteins

Sucrose gradient purified virions of each EV70 isolate were analysed on discontinuous 15% polyacrylamide-SDS gels to compare electrophoretic mobilities among the virion structural proteins. The four EV70 capsid proteins have been designated 1A, 1B, 1C and 1D according to the L434 nomenclature of Rueckert and Wimmer (1984) (Figure 1). The position of prestained protein markers from which molecular weights of the viral proteins were estimated are also indicated. As can be seen in Figure 7, the polypeptide pattern is very similar in all the isolates. However there does appear to be a slight difference in the mobility of protein 1D in the J670 isolate; it migrates slower than the corresponding protein in other isolates. In addition to the four capsid proteins, two other unidentified polypeptides with apparent molecular weights of 21 K and 12 K respectively (designated 21 and 12) are also visible. These proteins have been noted in virions purified by sucrose gradient centrifugation (Figure 7) or by the rapid method (Figure 18). The electrophoretic mobilities of proteins specified by EV70 in infected LLC-MK2 cells were also compared. These proteins represent information coded for by

Figure 7. SDS-PAGE of different isolates of EV70. Five 100 mm culture dishes of LLC-MK2 cells were infected with each of 6 isolates of EV70. Virus particles were labelled with ^{35}S-methionine without chasing and purified by sucrose gradient centrifugation. Radioactive fractions representing particles containing RNA (peak I) were pooled, diluted in TN buffer + 1mM each of PMSF and BHCl then virus was pelleted in a SW41 rotor at 210,000 x g for 2.5 hours. Pellets were resuspended in 30 microlitres of 2x SB. All samples were heated for 5 minutes at 100°C prior to electrophoresis. Equal counts were added to each well except for lanes 5 and 6 where the yield of virus was insufficient. Following electrophoresis on a 15% polyacrylamide-SDS gel at 20 mA for 16 hours the gel was prepared for fluorography (as described in the text) and exposed to X-ray film for 2 weeks. Lane 1, J670; lane 2, J670 prepared at a later date than that in lane 1; lane 3, V1250; lane 4, R6; lane 5, RU3573; lane 6, 1604; lane 7, KW97. The location of molecular weight markers are also indicated.


1
2
3
4
5
6
7
68.0K
43.0K
25.7K
18.4K
14.3K
1D-VP1 35K
1B-VP2 28K
1C-VP3 26K
21 21K
12 12K
1A-VP4 9K

the entire genome, in contrast to the structural proteins which are specified by the P1 region only. Infected and mock-infected cytoplasmic extracts were analyzed on discontinuous 12% polyacrylamide gels (Figures 8 and 9). Proteins were designated by comparing the protein profiles and molecular weights to those obtained previously for EV70 and poliovirus (Takeda et al., 1982, Pallansch et al., 1984).There are several differences in electrophoretic mobilities of proteins among the six isolates. Protein 1D (VP1) of the J670 isolate exhibited a slightly reduced mobility compared to the other isolates as was also detected in SDS-PAGE analysis of purified virions. There also appear to be differences in proteins corresponding to molecular weights of 19 K-22 K Preparation of infected cell lysates and analysis by SDS-PAGE were repeated several times. Figures 8 and 9 illustrate two such analyses. We have consistently noted that in the 1981 strains (V1250, KW97 and 1604), the 3C(7c) protein exhibits a slower electrophoretic mobility than in the 1971 (J670 and R6) and 1975 (RU3573) isolates. The two bands seen in the 3C region in lane 3 of Figure 8 (R6) and in lane 1 of Figure 9 (J670) are not consistently present. In both of these isolates, the lower band is always present but the appearance and intensity of the upper band is variable. In addition, there are also slight shifts in the mobility of 3CD (MW of approximately 72 K) a precursor of both 3C (7c) and 3D (4b). This protein

Figure 8. SDS-PAGE of EV70 infected LLC-MK2 cells lysed using swelling buffer. Confluent monolayers of LLC-MK2 cells in 100 mm culture dishes were infected with the 6 EV70 isolates and labelled with ^{35}S-methionine for 1 hour without chasing. The preparation of cell lysates using swelling buffer has been described in detail in the text. After nuclei and other debris had been removed by centrifugation, cell lysates were added to equal volumes of 2x SB, heated for 5 minutes at 100^{o}C and electrophoresed on a 12% polyacrylamide-SDS gel for approximately 16 hours at 20 mA. The gel was prepared for fluorography and exposed to X-ray film for 2 days. UN, uninfected cell lysate; lane 1, J670 infected cell lysate; lane 2, V1250 infected cell lysate; lane 3, R6 infected cell lysate; lane 4, KW97 infected cell lysate; lane 5, RU3573 infected cell lysate; lane 6, 1604 infected cell lysate. The positions of ^{14}C methylated protein molecular weight markers are also shown.

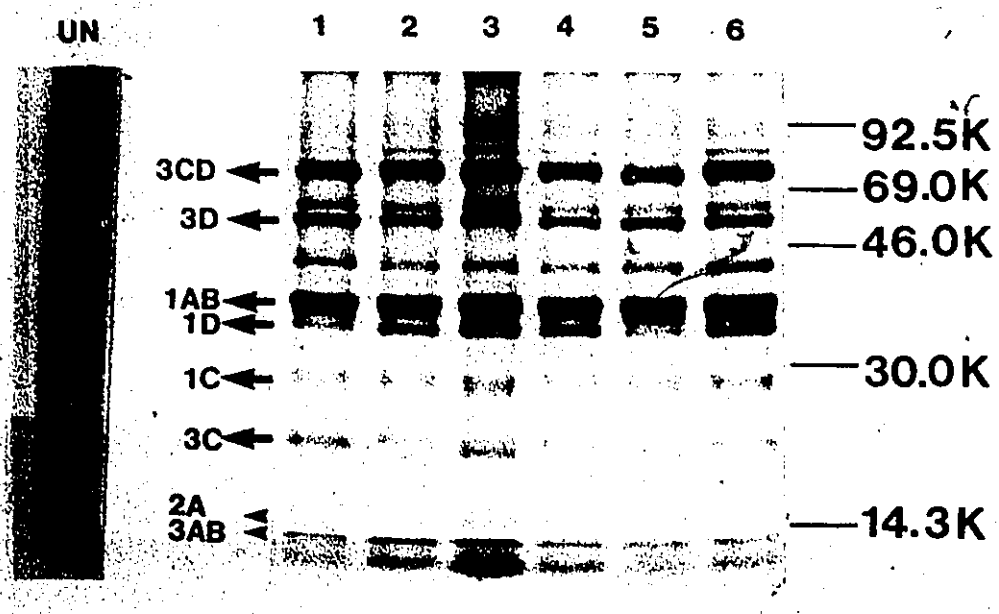
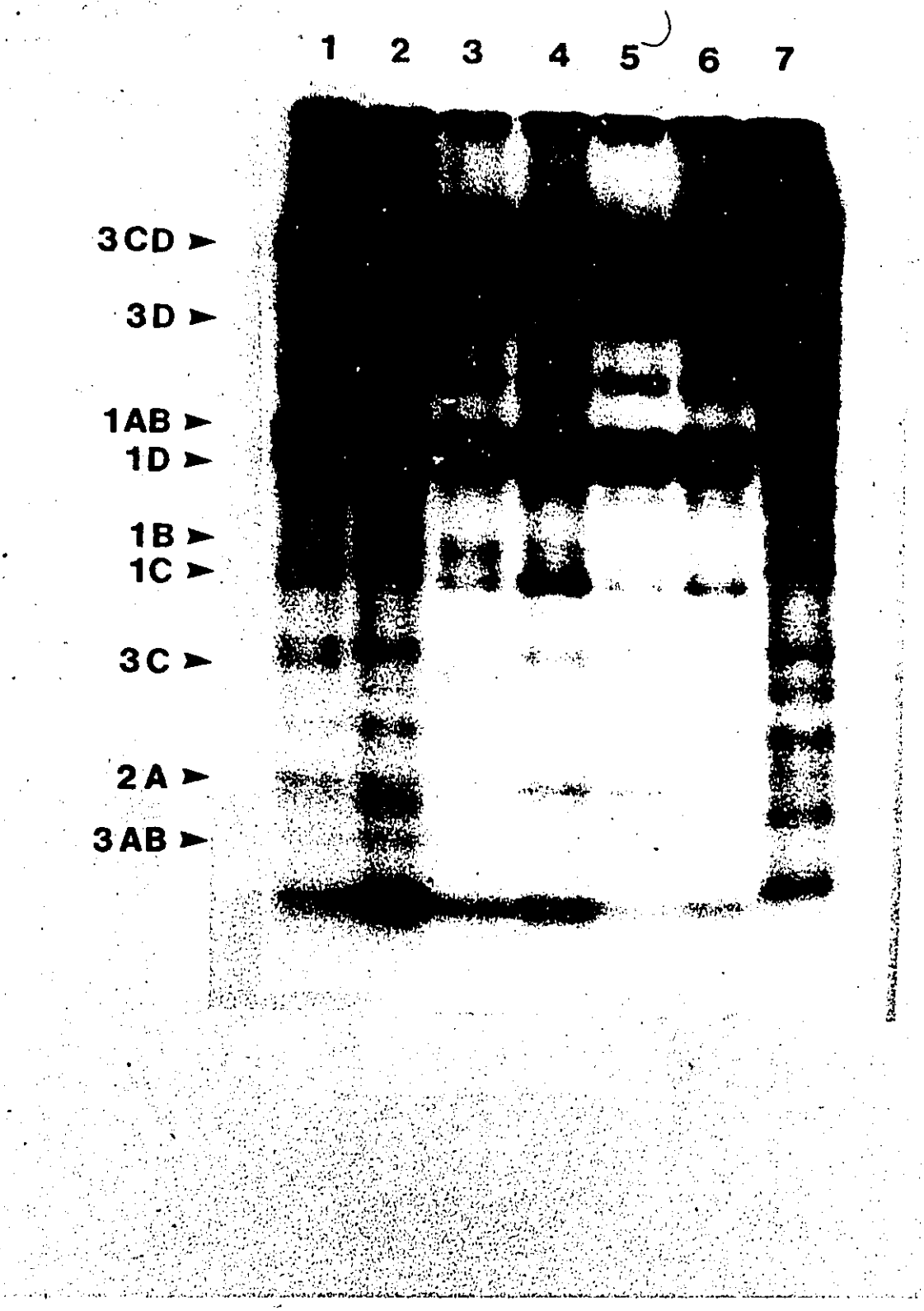

UN
1
2
3
4
5
6
3CD
3D
1AB
1D
1C
3C
2A
3AB
92.5K
69.0K
46.0K
30.0K
14.3K

Figure 9. SDS-PAGE of EV70 infected LLC-MK2 cells lysed by the RIPA buffer method. These lysates were prepared at a different time and using a different lysing buffer than those shown in Figure 8. LLC-MK2 cells were infected with the 6 different EV70 isolates, labelled with ^{35}S-methionine for 1 hour and lysed immediately thereafter using RIPA buffer. This procedure is described in the text. Debris and nuclei were removed by centrifugation and the supernatants (lysates) were prepared for electrophoresis as described in Figure 8. The gel was then prepared for fluorography and exposed to X-ray film for 2 weeks. Prestained protein molecular weight markers were also included in this gel but are not shown. Lane 1, J670 infected cell lysate; lane 2, V1250 infected cell lysate; lane 3, R6 infected cell lysate; lane 4, KW97 infected cell lysate; lane 5 RU3573 infected cell lysate; lane 6, 1604 infected cell lysate; lane 7, uninfected cell lysate.


1
2
3
4
5
6
7
3CD
3D
1AB
1D
1B
1C
3C
2A
3AB

migrates slightly slower in isolates having a protein in the 3C region which also migrates more slowly.

5. Two Dimensional IEF, SDS-PAGE

Viral structural and nonstructural proteins were also examined for differences in charge (pI) and electrophoretic mobility by high resolution two dimensional polyacrylamide gel electrophoresis. Examples of two dimensional profiles obtained for sucrose gradient purified virions are given in Figures 10 and 11. In each isolate two of the four capsid proteins focused and appear as spots on the autoradiogram. A large smear also appears at the acidic end of the pH gradient. The smallest capsid protein, 1A, was not detected in this analysis. It may have been visible with longer exposure of the autoradiograms. Purified J670 virions were run as markers in the SDS-PAGE dimension of the two dimensional profile of J670 (Figure 10). The vertical position of these markers were used to identify the spots. The high molecular weight spot appears to correspond to 1D (VP1) and the larger spot below to 1B (VP2). The streak may correspond to unfocused 1C (VP3). A secondary spot slightly more acidic than the major 1B spot was present in all isolates except J670 for which the two dimensional (2D) profile was more difficult to interpret because of excessive streaking (Figure 11, arrow). As can be seen in Figure 11, the profiles of each isolate were virtually identical, except

Figure 10. High resolution two dimensional electrophoretic profile of J670. ^{35}S-methionine labelled virions were purified by the sucrose gradient method, resuspended in isoelectric focusing lysis buffer and applied to the top of a tube gel. The sample was focused towards the anode for 12 hours at 400 V (first dimension electrophoresis). Second dimension electrophoresis consisted of SDS-PAGE on 15% gels. Following fluorography, the gels was dried and exposed to X-ray film for 3 weeks. The vertical position of the protein 'spots' in the two dimensional analysis were compared to the vertical level of the one dimensional marker proteins and in this way the 'spots' were assigned probable identities. The entire pH gradient is indicated in this figure.

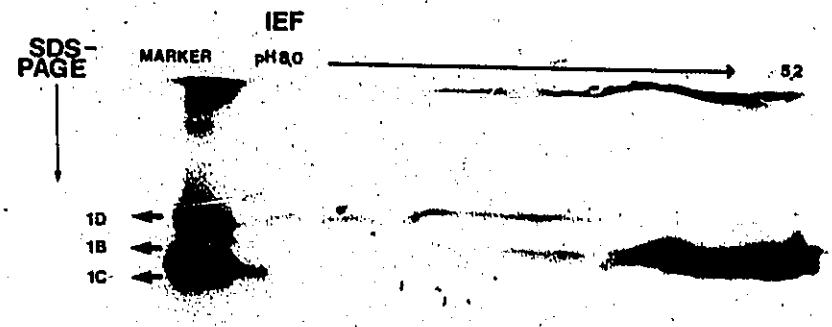
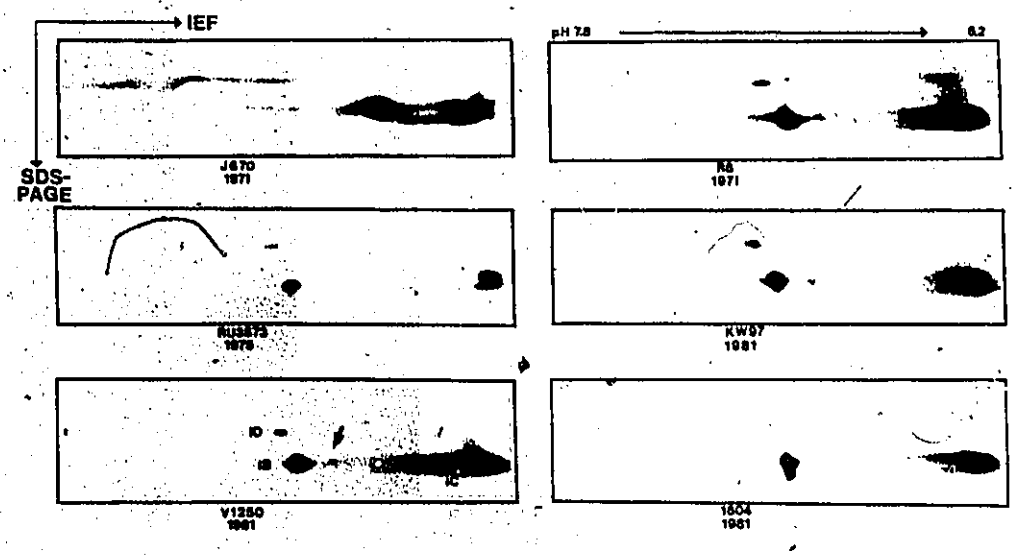

IEF
SDS-
PAGE
MARKER
pH 8,0
5,2
1D
1B
1C

Figure 11. High resolution two dimensional electrophoretic profile of purified EV70 virions. ^{35}S-methionine labelled virions were purified by the sucrose gradient method, prepared for two dimensional analysis and electrophoresed as described in Figure 10. The region of the gels corresponding to a pH gradient of 7.8-5.2 are shown in this figure. The arrow indicates a secondary acidic spot.


IEF
SDS-
PAGE
pH 7.8
6.2
J670
1971
R6
1971
KW57
1981
1504
1981

for J670 which, due to a problem with the second dimension electrophoresis resulted in a poor quality profile. Instead of calculating the pI of each protein, relative positions of the spots were compared. Shifts to the right or left are indicative of charge differences between proteins while vertical position change indicates differences in electrophoretic mobility (O'Farrell, 1975). It was possible to superimpose all isolates except J670 suggesting that the relative position of the spots were very similar in all of these isolates.

Lysates of EV70-infected and mock-infected cells were also subjected to two dimensional analysis. Profiles representing the earliest (J670 and R6), intermediate (RU3573) and most recent (V1250, KW97 and 1604) isolates are shown in Figures 12 and 13 . As for purified virions, infected cell proteins were identified by vertical position in comparison to the position of infected cell lysate proteins run only in the SDS-PAGE dimension (Figure 12). The relative positions of the proteins 3CD, 3D and 1AB were virtually identical between J670, V1250, R6, KW97 and RU35 (they are superimposable) indicating very similar pI and electrophoretic mobility. However, isolate 1604 accidentally ran much further in the second dimension and thus cannot be compared for vertical position (electrophoretic mobility). This should not have affected the horizontal position of the spots. It appears that 3D is slightly more acidic in this

Figure 12. High resolution two dimensional electrophoretic analysis of J670 infected LLC-MK2 cell lysate. Cells were labelled with ^{35}S-methionine and the lysate was prepared using swelling buffer as is described in the text.Urea, NP-40, 2-mercaptoethanol and Ampholines were added to the lysates and these samples were then applied to tube gels and focused towards the anode as described in Figure 10. Second dimension analysis consisted of SDS-PAGE on 12% polyacrylamide gels. The gels were then processed for fluorography and exposed to X-ray film for 3-5 days. Protein 'spots' in the two dimensional profile were identified by comparing their approximate vertical position to that of the one dimensional marker proteins.

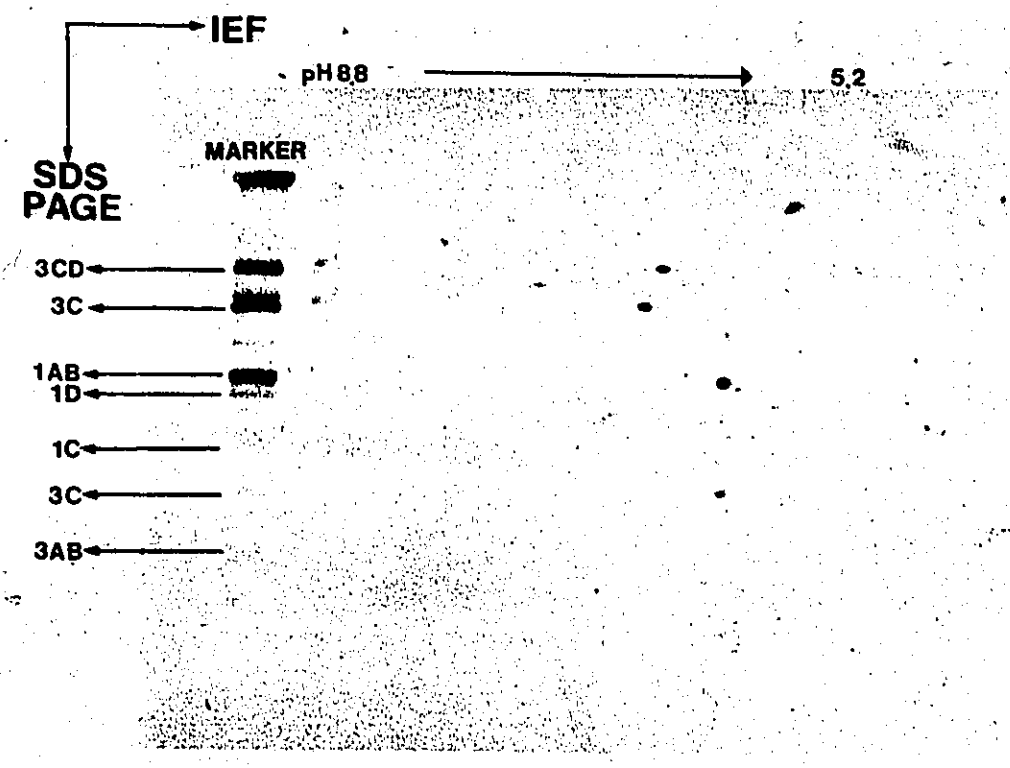
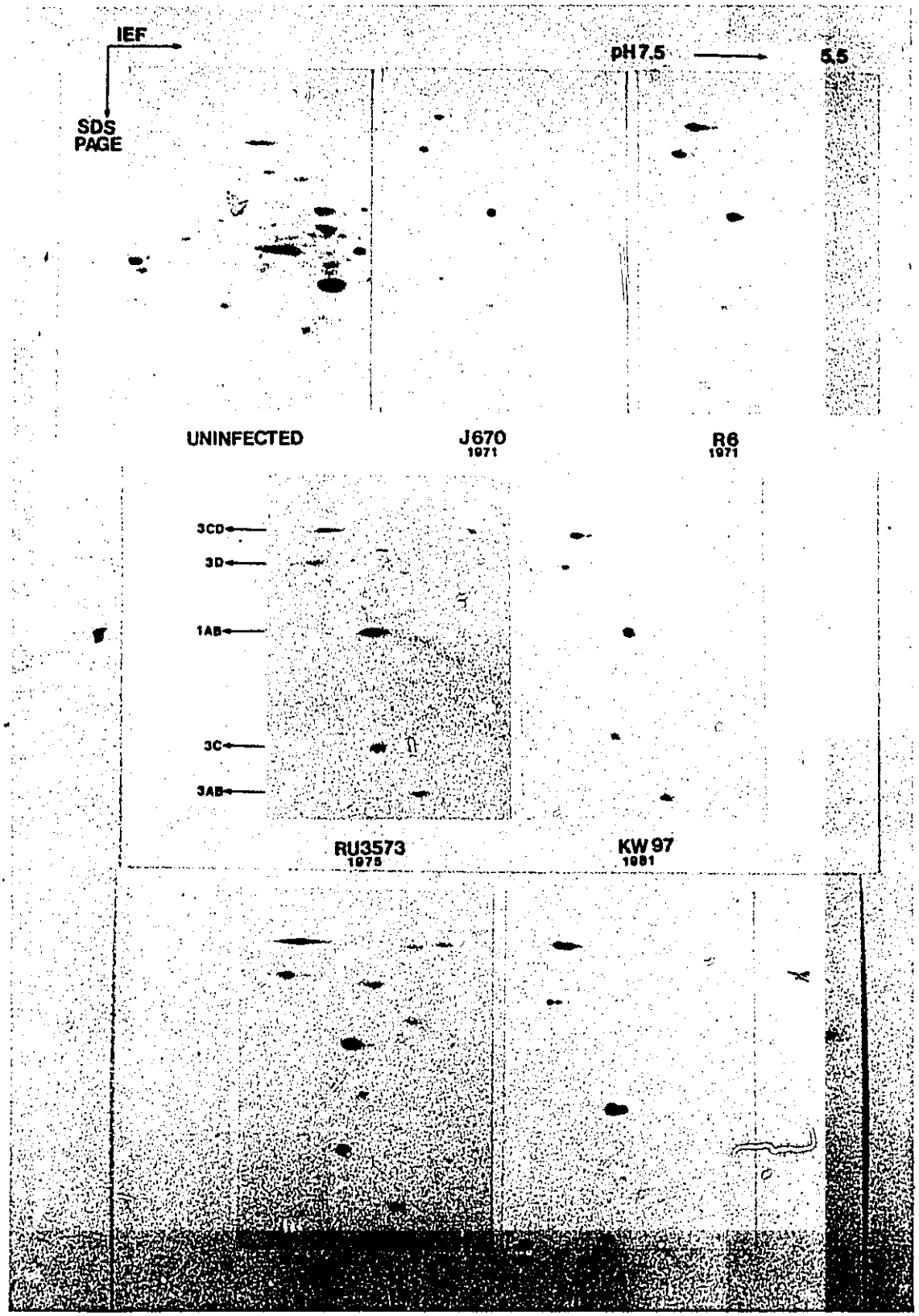

IEF
pH 8.8
5.2
MARKER
SDS
PAGE
3CD
3C
1AB
1D
1C
3C
3AB

Figure 13. High resolution two dimensional electrophoretic analysis of EV70 infected LLC-MK2 cell lysates. Cells were labelled with ^{35}S-methionine and the lysates were prepared using swelling buffer as is described in the text. Refer to Figure 12 for details concerning the two dimensional analysis. For illustration purposes, only the region of the gels corresponding to a pH range of 7.5-5.5 is shown. The complete pH gradient is shown in Figure 12.


IEF
pH 7,5
5,5
SDS
PAGE
UNINFECTED
J670
1971
R6
1971
3CD
3D
1AB
3C
3AB
RU3573
1975
KW 97
1981

isolate than in the others (shift to the right). In addition 3CD, 3D and 1AB show secondary spots (more acidic) which are much more apparent in 1604 than in any other isolate. There may also be pI and mobility differences in 3C and 3AB between the isolates as the relative positions of these spots differ from isolate to isolate. However it was not possible to correlate these differences in 3C with those seen in one dimensional electrophoresis.

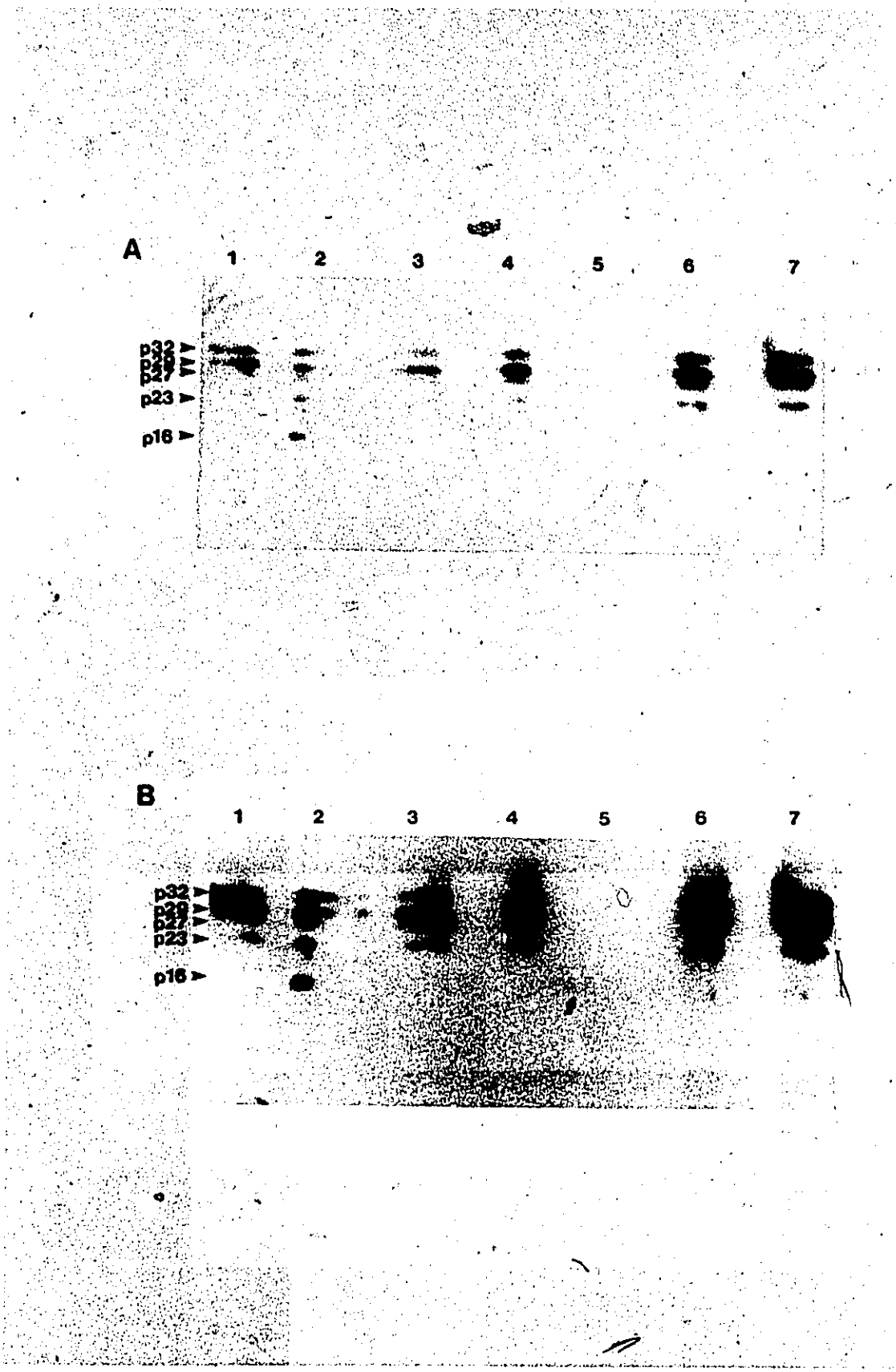
6. Peptide Mapping

Several viral structural proteins were analyzed for change in the amino acid sequence or configuration which might affect the partial proteolytic digestion pattern. Such changes could potentially lead to peptides differing in size or intensity between the isolates of EV70. Capsid protein 1D (VP1), has been shown to play an important role in neutralizing antibody induction, may also contribute significantly to host cell receptor binding properties of the virus and shows low sequence conservation between picornaviruses (Stanway et al., 1984; Rossmann et al., 1985; Skern et al., 1985; Tracy et al., 1985). Thus, it was speculated that evolutionary changes in EV70 might be reflected in this protein. In preliminary experiments, chymotrypsin (Sigma type VII), elastase (Sigma type IV) and Staphylococcus aureus V8 protease (Miles Scientific) were tested for their ability to cleave an EV70 protein (a 50 K precursor) into

peptides detectable by SDS-PAGE. Only V8 protease generated distinct patterns and was used in all subsequent peptide mapping procedures.

Two autoradiograms, a short (A) and a long (B) exposure, representing the peptide banding pattern generated after partial proteolytic digestion of protein 1D from purified virions, are shown in Figure 14. These patterns were very similar from isolate to isolate, however the mobility of a 23 K peptide (p23) appears to be slightly slower in the J670 strain than in others. Upon repeating these peptide maps, the mobility shift in J670 was reproducible. There are also intensity differences in a 16 K peptide (p16) between the isolates. However, two different preparations of J670, lane 1 and 7, also show differences in the intensity of the peptide and when the peptide maps were repeated, these apparent intensity differences between the isolates were not reproducible. It may be that slight variation in the amount of protein substrate or small differences in the amount of protease delivered to a particular well could lead to such intensity differences. As can be clearly seen in lane 5 of Figure 14, which represents RU3573, the amount of substrate (1D) was much less than that for the other isolates because of the low yield of RU3573 when virions were purified for peptide mapping. Thus, the intensity variations in p16 in all probability do not reflect true differences in 1D between the isolates. Attempts to map proteins 1B and 1C

Figure 14. Peptide map of viral capsid protein 1D(VP1). Virions were purified by the 'rapid method'and the structural proteins were separated by SDS-PAGE on 12% gels. Following electrophoresis for 16 hours at 20 mA, the gel was dried without fluorography and exposed to X-ray film for several days. The band corresponding to protein 1D was cut out of the gel, digested with Staphylococcus V8 protease (1 mg/ml) and then electophoresed on a 15% polyacrylamide-SDS gel as described in the text. This gel was subjected to fluorography and exposed to X-ray film for 1 week (A) and then re-exposed for 6 weeks (B). Lane 1, J670; lane 2, V1250; lane 3, R6; lane 4 KW97; lane 5, RU3573; lane 6, 1604; lane 7, J670 (prepared at a later date that in lane 1). The peptides generated by the partial proteolytic digest of 1D have been designated according to their relative molecular weights i.e. p32 indicates a peptide with an approximate molecular weight of 32 K. Molecular weights were estimated from the migration of prestained protein markers which were included in the gel.


A
1
2
3
4
5
6
7
p32
p29
p27
p23
p16
B
1
2
3
4
5
6
7
p32
p29
p27
p23
p16

(VP2, VP3) in a similar fashion were less successful. These two proteins migrate very close together on SDS-polyacrylamide gels, thus the entire 1B/1C region was removed and treated as a single entity. However, the activity of V8 against these proteins appeared to be quite low (Figure 15). The two bands seen in the autoradiogram correspond to undigested 1B and 1C. These same results were obtained when the procedure was repeated a second time.

7. Immunoprecipitation

To probe for differences in antigenicity between isolates, viral polypeptides were immunoprecipitated from EV70 infected cell lysates using non-neutralizing monoclonal antibodies (MAbs) 74-5G (MAb 1) and 73-7H (MAb 2) as well as several polyclonal antisera (Table 3). Results of the immunoprecipitation with monoclonal antibodies (Figure 16) suggested that both MAbs were able to recognize epitopes on capsid proteins 1C (VP3) and 1D (VP1) in all the isolates. Therefore, it was not possible to distinguish between the different EV70 isolates using the two non-neutralizing monoclonals. The fact that monoclonal antibodies, with very restricted specificity, appeared to precipitate two individual structural proteins was puzzling. Thus, it was of interest to determine if both proteins were somehow remaining tightly associated and were being precipitated as a complex or if both MAbs were recognizing epitopes on two

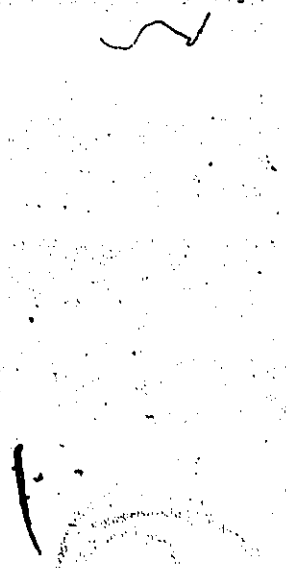


Figure 15. Peptide map of virion proteins 1B(VP2) and 1C(VP3). Viral structural proteins 1B and 1C were isolated as a single entity from purified virions and digested with V8 protease as described in Figure 14. Prestained protein markers were used to estimate the molecular weights of the 2 bands.

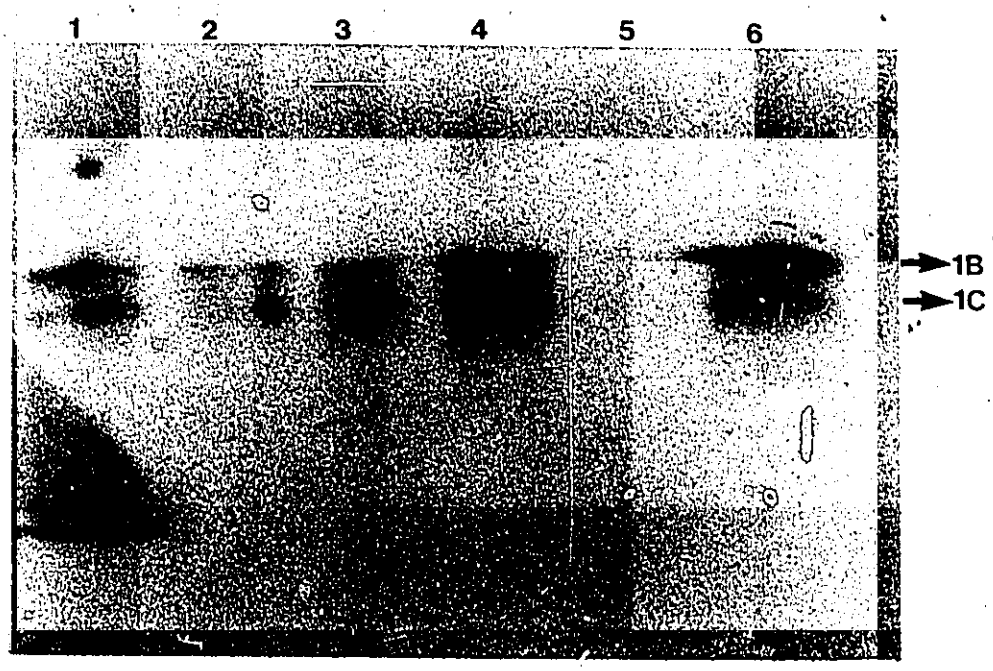
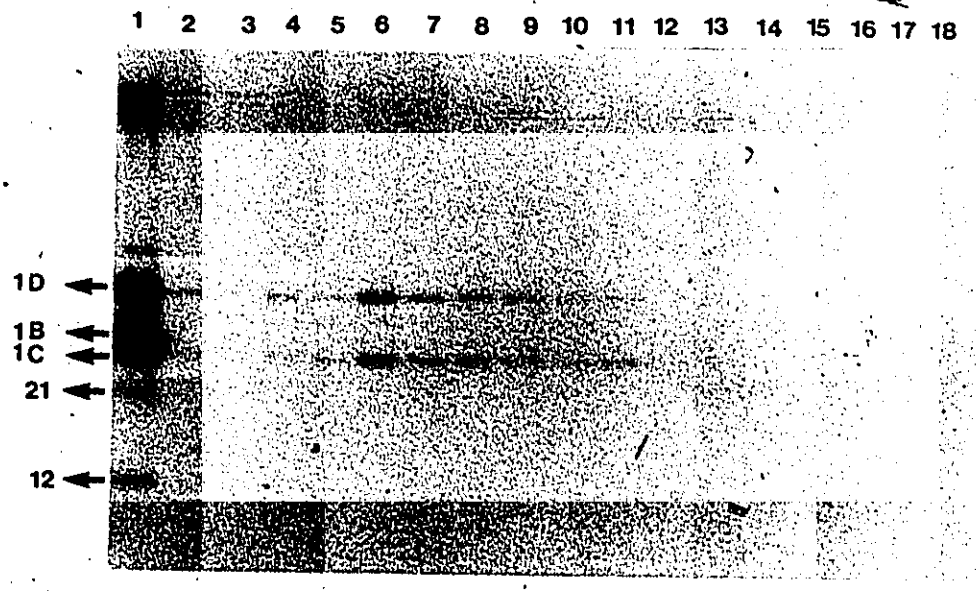

1
2
3
4
5
6
1B
1C

Figure 16. Immunoprecipitation of EV70 proteins with monoclonal antibodies (MAbs). LLC-MK2 cells were mock infected or infected with one of the six different EV70 isolates, labelled with ^{35}S-methionine and lysed in the presence of RIPA buffer. Debris was removed by centrifugation and supernatants (lysates) were incubated with either MAb 1 or MAb 2. A detailed account of immunoprecipitation procedures may be found in the text. 2x SB was added to antigen-antibody complexes and this mixture was heated for 5 minutes at 100^{o}C. Samples were then applied to 12% polyacrylamide-SDS gels and electrophoresed for 16 hours at 20 mA. The gels were processed for fluorography and exposed to X-ray film for 2 weeks.

lane 1, J670 virions;
lane 2, J670 infected cell proteins + MAb 1;
lane 3, J670 infected cell proteins + MAb 2;
lane 4, V1250 infected cell proteins + MAb 1;
lane 5, V1250 infected cell proteins + MAb 2;
lane 6, R6 infected cell proteins + MAb 1;
lane 7, R6 infected cell proteins + MAb 2;
lane 8, KW97 infected cell proteins + MAb 1;
lane 9, KW97 infected cell proteins + MAb 2;
lane 10, RU3573 infected cell proteins + MAb 1;
lane 11, RU3573 infected cell proteins + MAb 2;
lane 12, 1604 infected cell proteins + MAb 1;
lane 13, 1604 infected cell proteins + MAb2;
lane 14, uninfected cell proteins + MAb1;
lane 15, uninfected cells + MAb 2;
lane 16, J670 without MAbs;
lane 17, J670 + rotavirus specific MAb;
lane 18, J670 + normal mouse serum.


1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16 17 18
1D
1B
1C
21
12

different capsid proteins. To this end isolated virions as well as infected cell lysates were heated in 2x SB for 5 minutes at 100°C; the EV70 specific proteins were immunoprecipitated as described previously and the samples were heated a second time just prior to gel electrophoresis. These measures should have been sufficient to thoroughly disrupt any possible protein complexes between 1C and 1D. Results of this experiment (Figure 17) show that both protein 1C and 1D are immunoprecipitated by the monoclonal antibodies under these conditions; suggesting that the MAbs are interacting with epitopes on both 1C and 1D.

Polyclonal antisera were also used to immunoprecipitate EV70 specific proteins from lysates of infected and uninfected cells. Available were mouse, monkey and rabbit antisera, all of which had been prepared against the J670 isolate of EV70. The mouse and monkey sera were tested against all isolates, the rabbit serum only against J670. Figure 18, panels A and B, represent short and long exposure autoradiograms, respectively, of the immunoprecipitation patterns obtained using these antisera. All three sera recognized proteins 1B (VP2), 1C (VP3) and 1D (VP1) of the J670 isolate, as well as precursor polypeptide 1AB (VP0). Mouse serum appeared to immunoprecipitate all of the EV70 proteins better than did the monkey serum. Mouse serum immunoprecipitated polypeptides 1C (VP3) and 1D (VP1) from all strains with approximately equal efficiency. Protein 1B

Figure 17. Immunoprecipitation of denatured EV70 proteins with monoclonal antibodies. In addition to a standard immunoprecipitation test in which infected cell lysates were added to MAb, lysates and purified virions (rapid method) were boiled in the presence of SDS and 2-mercaptoethanol for 5 minutes prior to the addition of MAb in attempt to disrupt any protein complexes. Samples were then diluted and incubated with antibody and beads. Antigen-antibody complexes were released from the beads by resuspending each sample in a small volume of 2x SB and boiling again for 5 minutes. Immunoprecipitated proteins were examined by SDS-PAGE on a 12% polyacrylamide gel. Following fluorography, the gel was exposed to X-ray film for 3 weeks. Lane 1, J670 infected cell proteins; lane 2, J670 infected cell proteins + MAb 1 (standard immunoprecipitation); lane 3, J670 infected cell proteins + MAb 2 (standard immunoprecipitation); lane 4, J670 infected cell proteins (heated) + MAb 1; lane 5, J670 infected cell proteins (heated) + MAb 2;lane 6, J670 virions (heated) + MAb 1; lane 7, J670 virions (heated) + MAb 2; lane 8, J670 virions.

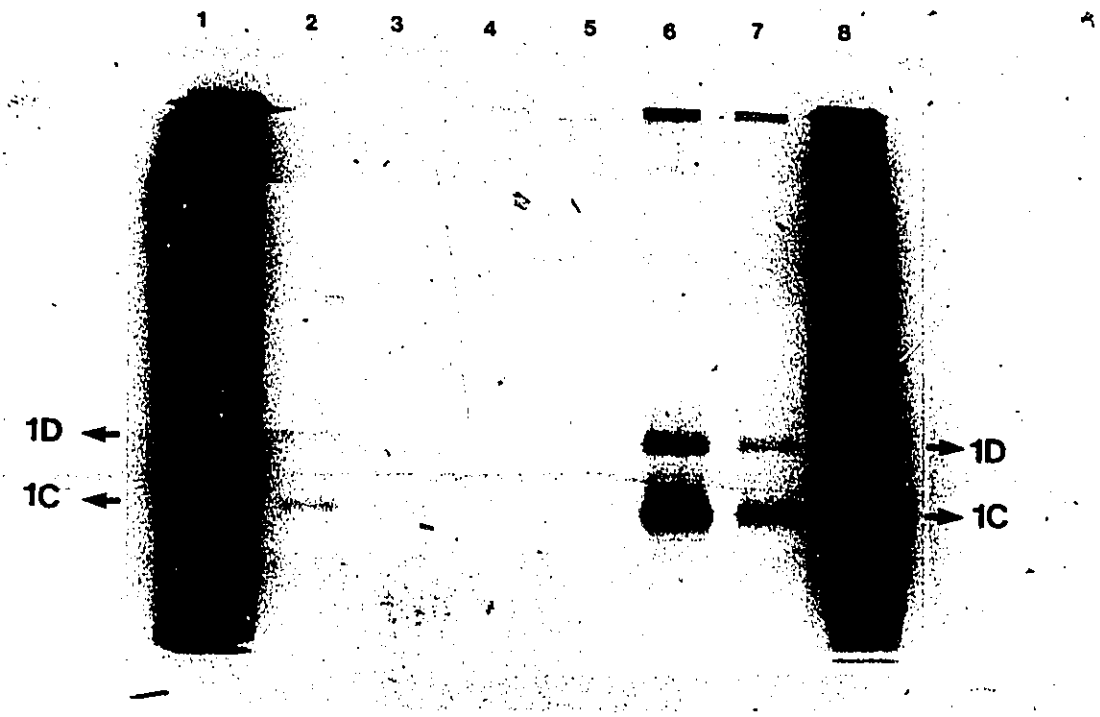
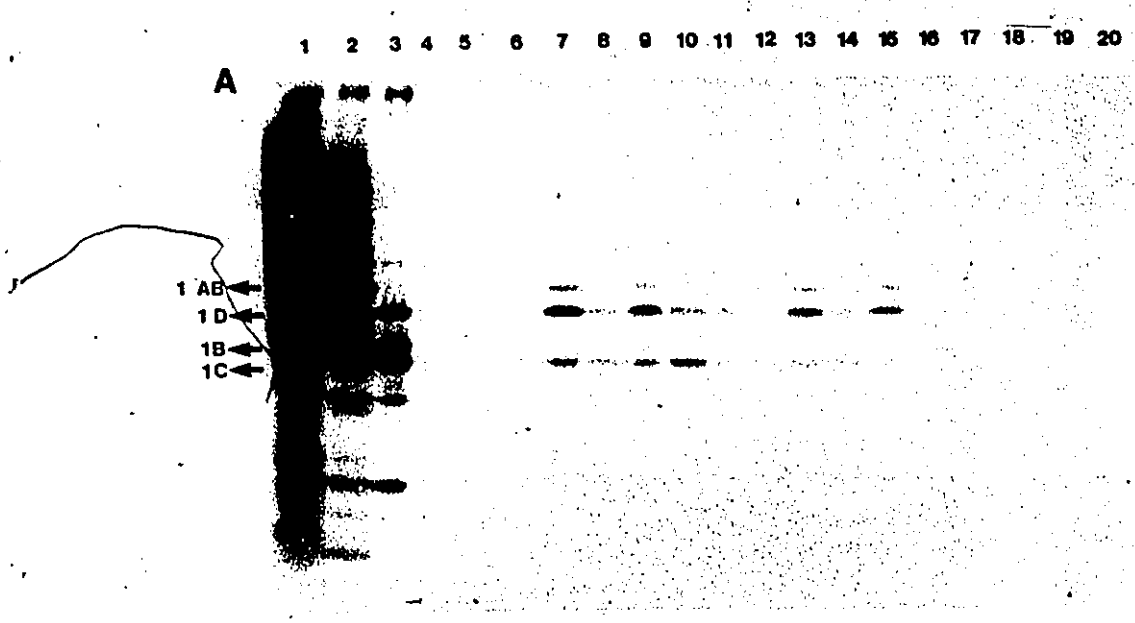
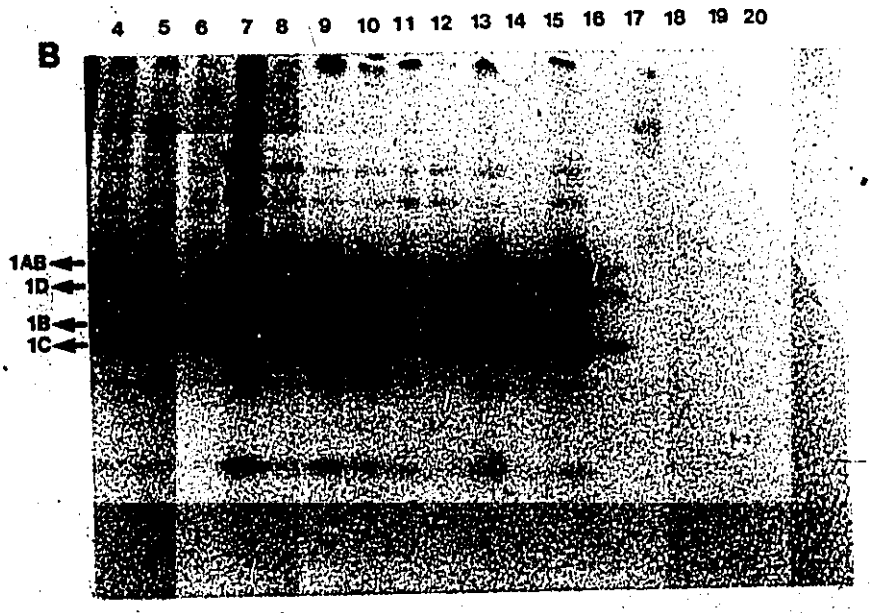

1
2
3
4
5
6
7
8
1D
1C
1D
1C

Figure 18. Immunoprecipitation of EV70 proteins with polyclonal antisera. EV70 infected LLC-MK2 cell proteins labelled with ^{35}S-methionine were prepared by the RIPA buffer method. These proteins were incubated with rabbit, mouse or monkey antisera prepared against the J670 isolate of the virus. A standard immunoprecipitation procedure was carried out as described in the text. Following immunoprecipitation all samples were heated in the presence of SDS and 2-mercaptoethanol prior to electrophoresis on a 12% polyacrylamide-SDS gel. Following electrophoresis, the gel was processed for fluorography and exposed to X-ray film for 4 days (A) and 3 weeks (B).

lane 1, uninfected cell proteins;
lane 2, J670 infected cell proteins;
lane 3, J670 purified virions (rapid method);
lane 4, J670 infected cell proteins + rabbit antiserum;
lane 5, J670 infected cell proteins + mouse antiserum;
lane 6, J670 infected cell proteins + monkey antiserum;
lane 7, V1250 infected cell proteins + mouse antiserum;
lane 8, V1250 infected cell proteins + monkey antiserum;
lane 9, R6 infected cell proteins + mouse antiserum;
lane 10, R6 infected cell proteins + monkey antiserum;
lane 11, KW97 infected cell proteins + mouse antiserum;
lane 12, KW97 infected cell proteins + monkey antiserum;
lane 13, RU3573 infected cell proteins + mouse antiserum;
lane 14, RU3573 infected cell proteins + monkey antiserum;
lane 15, 1604 infected cell proteins + mouse antiserum;
lane 16, 1604 infected cell proteins + monkey antiserum;
lane 17, uninfected cell proteins + mouse antiserum;
lane 18, uninfected cell proteins + monkey antiserum;
lane 19, J670 infected cell proteins + normal mouse serum;
lane 20, J670 infected cell proteins + no antibody.


1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16 17 18 19 20
A
1 AB
1 D
1B
1C


4 5 6 7 8 9 10 11 12 13 14 15 16 17 18 19 20
B
1AB
1D
1B
1C

(VP2) of the J670 isolate was readily immunoprecipitated by mouse serum. However, for the other isolates, as compared to J670, smaller quantities of protein 1B were immunoprecipitated by mouse serum and for KW97, immunoprecipitation of 1B could not be detected, even with prolonged exposure of the autoradiogram (panel B). When monkey serum was used in place of mouse serum, qualitatively similar results in the ability of the serum to immunoprecipitate the EV70 capsid proteins were obtained. It was not possible to detect the presence of the smallest capsid protein, 1A (VP4), in any of the immunoprecipitation tests. Immunoprecipitation of some high molecular weight proteins can also be seen on the long exposure autoradiogram as can several smaller molecular weight proteins.

DISCUSSION

Growth, Quantitation and Purification of EV70

The comparative study of EV70 proteins necessitated growing and purifying the six virus isolates on a regular basis. The initial stages of this project were devoted to establishing these procedures.

Considering the wide host range of EV70 in vitro, it was not surprising that the virus could be easily cultivated in LLC-MK2 cells. A very obvious CPE, notably rounding of cells in the monolayer followed by loss of adhesion, served as a convenient hallmark as to the time of virus harvest. Viral replication in vitro was also studied by performing a time course experiment, results of which were extremely useful because the interval difference between infection at a particular multiplicity and viral harvest could be (roughly) estimated. Cytopathic effect was also recorded and this made it possible to better understand the progress of infection with EV70 in terms of virus yield.

One of the major problems encountered in this study was the relatively low titres (1×10^6-1×10^7) of EV70 in LLC-MK2 cells. To obtain enough virus to extract RNA for example, it was necessary to infect up to 150 culture dishes (100 mm). As this became very labour intensive, roller

bottles (850 cm^2, Falcon Laboratory Products, Oxnard CA.; 490 cm^2 Corning Glass Works,Corning N.Y.) were tested as alternative cell culture containers. However, it was extremely difficult to establish confluent monolayers on the surface of these bottles due to excessive cell clumping which ensued within 5 minutes or so following addition of the monocellular suspension. Repeated attempts to eliminate this problem by changing the pH of the media, the cell passage number, the total length of trypsinization time and/or the speed (rpm) of the roller apparatus failed. Microcarriers (Cytodex 3, Pharmacia Canada) were also tested as potential growth surfaces. However due to problems associated with setting up this type of system such as regulating the pH as well as the time constraints of this project, it was decided instead to continue using the 100 mm culture dishes - a system which, aside from the labour intensiveness, was virtually problem free. The problem of low titre of virus was also approached in another way. A fellow graduate student (Y.Karim) had mentioned that the yield of rhinovirus <u>in vitro</u> could be significantly increased (10 fold) if the virus was grown in an A-5 HeLa cell line instead of L132 cells. Subsequent to this, the growth of EV70 was tested in both HeLa and LLC-MK2 cells and titred in the latter. Results of the plaque assay indicated a slight increase (2 fold) in the titre of virus grown in HeLa cells compared to that from the LLC-MK2 cell line. This

increase was not great enough to warrant switching over to the propagation and quantitation of EV70 in HeLa cells. Another system which may provide a less labour intensive method to routinely cultivate EV70 is the NUNC Cell Factory which provides a surface area of 6000 cm^2. In recent trials using this system (L. Gattinger, 4th year honors project and A. Sheikh, M.Sc. project), EV70 was successfully propagated and purified. The advantages of the Cell Factory include a potential for large scale tissue culture with minimum manipulation and maintenance required.

The plaque assay procedure used for EV70 was the same as that used in the lab for the quantitation of human para-influenza virus type 3 (HPIV3). EV70 plaques were very heterogeneous in size. While plaque assays were repeated many times, it was not possible to categorize or subdivide the isolates on the basis of average plaque diameter except to say that J670 usually gave larger plaques than the others. However, Esposito et al., (1974), compared the plaques sizes of four early (1971) EV70 isolates grown in human embryonic lung fibroblast (HELF) cells. From this study, which included both the J670 and R6 strains, the researchers were able to differentiate the virus isolates into three plaque size groups - large, medium and small. Isolate R6 was found to give larger (5 mm) plaques and J670 medium (2.5 mm) plaques in MEM agarose overlay assays using plaque purified virus. One possible explanation is that EV70

plaque size varies depending upon the cell type being used to propagate the virus.

The purification of EV70 had not been performed in our lab prior to this project and was initially adapted from one being used to purify EV70 for the purpose of RNA extraction and homology studies (Natori et al., 1984). This technique involved PEG precipitation of the virus as well as both sucrose and cesium chloride (CsCl) density gradient centrifugation. In addition to this being a tedious and lengthy procedure, the great number of manipulations also resulted in a large loss of the virus. This exacerbated the problem of low starting titres and therefore the CsCl centrifugation step was eliminated in subsequent viral purifications. To further simplify the purification procedure, a method which involved pelleting the virus from infected cell supernatants in the presence of 0.5% SDS was also used (rapid method). Virions prepared in this manner were ready in a matter of hours as opposed to several days. Furthermore, this procedure involved fewer steps at which viral loss could occur and hence yields of the virus were greater.

In sucrose gradient purification of ^{35}S-methionine labelled EV70 isolates, two regions of high radioactivity were often noted (designated peak I and peak II). When virus containing ^{3}H-uridine labelled RNA was purified in a similar manner, only one peak of radioactivity was detected and this corresponded well with the peak I region of ^{35}S-methionine

labelled material. These findings suggest that virions in peak II lack RNA (empty capsids). This was further substantiated by the plaque assay results using aliquots from both peaks. Although peak I contained less radioactive material, the virus titre was almost two logs greater than the titre of the material in peak II, indicating the presence of more infectious material in peak I. SDS-PAGE of material in both peaks revealed a similar protein profile. The absence of the capsid precursor 1AB (VP0) in peak II was unexpected, assuming that peak II represents empty capsids. One would expect empty capsids to contain 1AB, 1C and 1D since the final maturation step in picornavirion morphogenesis involves the cleavage of 1AB to generate 1A and 1B after the RNA has been added. This cleavage does not occur in empty capsids (Melnick, 1983). Protein 1AB, which can usually be seen as a strong band in the infected cell lysate (Figures 8 and 9), may somehow (perhaps due to an unstable association between 1AB and the other virion structural proteins 1B and 1C) have been lost during the sucrose gradient purification.

Hughes et al., (1984) also observed two regions of radioactivity during ^{125}I labelled hepatitis A virion purification by CsCl gradient centrifugation. Peak I contained the majority of the RNA. In addition, the titre of virus from the peak I material was 3 to 4 logs greater than that from peak II material. SDS-PAGE of material in both

peaks revealed a similar protein content (1D,1C,1B) - the precursor 1AB was not detected. These researchers failed to draw any conclusions from their findings. In poliovirus infected cells, empty capsids occur naturally and are present along with infectious virus in cytoplasmic extracts of infected cells (Sharff and Levintow, 1963). Therefore it seems probable that empty capsids may also be present in EV70 and HAV infected cells and may comprise a great deal of the material in peak II of the sucrose gradient.

EV70 RNA was extracted from sucrose gradient purified virions and analysed by electrophoresis on denaturing agarose gels. The estimated molecular weight of the RNA was 2.4×10^6 which is in close agreement with an earlier estimate of 2.5×10^6 (Yamazaki et al., 1974) and is similar to the average value, for picornaviruses, of 2.5×10^6 (Matthews, 1982).

Protein Comparisons Among EV70 Isolates

1. One Dimensional SDS-PAGE

Purified EV70 virions, when examined by SDS-PAGE exhibited a typical picornavirus protein profile of four capsid proteins 1A, 1B, 1C and 1D with estimated molecular weights of 9 K, 28 K, 26 K and 35 K respectively. Esposito et al., (1976) obtained similar results in an electrophoretic analysis of EV70 proteins. These values are also similar to

those estimated for the capsid proteins of poliovirus (1A, 7 K; 1B, 30 K; 1C, 26 K; 1D, 34 K) (Pallansch et al., 1984). The structural proteins in each isolate exhibit similar electrophoretic mobilities except for protein 1D in J670 which migrates slightly slower than the corresponding protein in the other isolates.

In addition to virion structural proteins, polypeptides representing the entire coding capacity of the EV70 genome were also compared by SDS-PAGE. The slightly reduced electrophoretic mobility of protein 1D in the J670 isolate is also apparent in the protein profiles of EV70 infected cell lysates (Figures 8 and 9). Takeda and co-workers (1984) compared the viral specific intracellular proteins of 15 isolates of EV70 by SDS-PAGE. Their analysis, which included J670, R6, V1250 and 1604 revealed reduced mobility in protein 1D for J670 and G10172, another early Japanese isolate. Similarly, Nottay et al., (1981) noted electrophoretic shifts in protein 1D in an SDS-PAGE comparison of cells infected with wild poliovirus type 1 isolated during epidemic transmission.

The origin of 21 and 12, two proteins which consistently (even with sucrose gradient centrifugation) co-purify with the virus in our hands, is unknown. It seems unlikely that they represent 'new' viral structural proteins. They have not been reported by other groups who have purified EV70 and analysed virions on polyacrylamide gels. However, these two

proteins appear to be present in infected but not uninfected cell lysates (especially noticeable in lanes 2 and 3, Figure 16). Thus, they may be of viral origin. The possibility exists that they represent breakdown products of higher molecular weight structural proteins. However, in all virion purification and cell lysate preparation procedures, protease inhibitors PMSF and BHCl were included so as to avoid or minimize protein degradation. Another possibility is that they are products of alternate cleavage pathways. To our knowledge, alternate cleavage pathways for the processing of picornaviral structural proteins have not been reported (Pallansch et al., 1984). Peptide mapping analysis of these proteins (21 and 12) might help to establish their origin and identify any relationship between them and any other viral proteins.(i.e. precursor/product).

Proteins in the 19 K-22 K molecular weight region of the gel also appear to differ between isolates. The relationship between the two proteins sometimes found in J670 and R6 and the single protein found in the other isolates is unknown. Takeda et al., (1984) noted only one protein (designated 7c = 3C in L434 nomenclature) in this molecular weight range when they compared 15 EV70 isolates by SDS-PAGE. They too observed variation in electrophoretic mobility of 3C among the different isolates. Their results, like those in our study show that 1981 isolates have a 3C protein which

migrates slower than the same protein in the earlier isolates.

In an earlier pulse-chase analysis of EV70 proteins, two bands may be seen in the 3C region following a short chase (10-60 minutes) but, as chase time increases, the intensity of the lower molecular weight protein diminishes while that of the higher molecular weight increases until only the latter can be seen with a chase of 120 minutes (Takeda et al., 1982). These researchers did not discuss the origin or identity of these two proteins; they merely label the higher molecular weight one as 3C. Several possibilities exist as to the nature of the apparent 'differences' between the proteins in the 19 K-22 K molecular weight range. They may reflect inherent differences in the rate of protein processing between the isolates. Perhaps in all isolates two proteins are found in this molecular weight range at some point during infection with subsequent processing eventually producing a single protein. This would suggest that the extent of the infection in each case influences the number of proteins found in this range. In this study, all infections were allowed to proceed until CPE had progressed to the same degree in each isolate in an attempt to minimize the effects that extent of infection might have upon the protein profiles. However, inherently different rates of processing between the isolates may not have been controlled by such measures. It also seems reasonable to speculate that

the proteins in the 3C region arise from alternate cleavage pathways. In poliovirus, there is evidence for multiple potential cleavage sites for P3 region proteins wherein P3 may be cleaved to 3C (20 K) and 3D (52 K) or 3C'(36 K) and 3D'(36 K) (Pallansch et al., 1984). Alternatively, the two proteins sometimes seen in this region of the gel may not be related at all. Interestingly, in those isolates having a slower migrating protein in the 3C region (1981 isolates V1250, KW97 and 1604), a protein with a molecular weight of 72 K (which in poliovirus corresponds to 3CD, the precursor of 3C (the viral protease) and 3D (the viral polymerase)) also migrates slightly faster than the corresponding protein in the other isolates.

These speculations could only be confirmed or refuted by first determining if the proteins in this region are viral specific, and if so, whether or not they are related to one another (i.e. precursor/product). This could be accomplished through peptide mapping studies or by obtaining monoclonal antibodies with specificities for each individual protein and using them to immunoprecipitate proteins from EV70 infected cell lysates. These analyses could also be used to reveal whether the protein (21) which appears in purified virions may also correspond to one of the proteins seen in the 19 K-22 K molecular weight region in EV70 infected cell lysates. Admittedly, SDS-PAGE is a relatively crude tool for protein comparisons - nevertheless it can indicate in which

proteins some mutations may have occurred. For example, oral type 1 poliovirus vaccine strains and the virulent parent Mahoney strain in which electrophoretic changes were observed in 1D, also differed in tryptic peptide mapping analysis of this capsid protein (Kew et al., 1980; Kew et al., 1981).

2. Two Dimensional IEF, SDS-PAGE

High resolution two dimensional electrophoresis is a powerful tool for the analysis of proteins. It permits simultaneous determination of pI and electrophoretic mobility (a molecular weight estimate) and is sensitive enough to resolve proteins differing by only a single charge. If all isoelectric focusing gels are run simultaneously, the SDS-PAGE dimension can be run at different times without affecting reproducibility. Thus, 'spots' representing a particular polypeptide on one separation can be compared with spots on other separations (O'Farrell, 1975).

Several problems were encountered in trying to establish this procedure for the analysis of EV70 proteins. Despite repeated attempts to focus all four virion capsid proteins, only two were adequately resolved. A large streak consistently appeared at the acidic end of the tube gel in the molecular weight range of protein 1C. Unfortunately, the two dimensional profile of the prototype strain is of very poor

quality, making it impossible to compare the profiles of all the isolates. However, the fact that it is possible to superimpose the profiles of the remaining 5 isolates tentatively suggests that pI and electrophoretic mobility of the two capsid proteins 1D and 1B are very similar in each isolate. It is imperative that these profiles be repeated in future studies to verify these preliminary results.

A similar situation exists for the infected cell lysates which were also examined by two dimensional electrophoresis. While it appears that there may be potential differences between the isolates in the pI and electrophoretic mobility of nonstructural proteins 3C, 3D, and 3AB, these profiles would have to be reproduced and compared for all isolates before any definite conclusions could be made.

Other investigators have also reported difficulties in one dimensional IEF and high resolution electrophoresis when analysing picornaviral proteins. King and Newman, (1980) were unable to focus all four virion proteins in FMDV at once due to extreme isoelectric points of 1D (basic) and 1A (acidic). As a result, they had to perform the technique under three different sets of conditions (reverse pH gradient or polarity for example) to accommodate these widely differing isoelectric points. Wiegers and Dernick (1981) analysed lysates of poliovirus infected cells by high resolution electrophoresis and noted that proteins originat-

ing from the P1 region of the genome (Figure 1) (especially 1D and 1C and their precursors) tended to cause streaking in the second dimension by sticking to and precipitating onto the top of the first dimension gel. They speculate that hydrophobic regions in these proteins may be responsible for this 'stickiness'. These investigators also observed that inclusion of NP-40 in the first dimensional analysis as suggested by O'Farrell (1975) reduced the resolution of proteins in infected cell lysates. Using isolated virions, proteins 1C and 1D could not even be detected by this technique (Hamann and Drzenick, 1978). Moreover, when TMEV proteins were examined by high resolution two dimensional electrophoresis, capsid proteins 1B, 1C and 1D appeared in the virion profiles, but were absent from infected cell profiles (Lorch and Friedmann 1983). This same phenomenon was noted in our two dimensional analysis.

In light of these studies, several modifications to the two dimensional analysis of EV70 would be well worth trying. In the present study, electrophoresis in the first dimension was toward the anode. In future experiments, reversing the polarity and changing the pH gradient might lead to better resolution of virion proteins, as might the exclusion of NP-40 from the first dimension. Finally, long exposure autoradiograms of infected cell lysates are highly recommended. It may be that some of the proteins which did not appear in the two dimensional profiles could have been

detected had a longer exposure autoradiograms been available. I feel that high resolution isoelectric focusing, when successfully repeated with some modifications, will yield more information concerning changes in the protein complement of EV70 than were obtained in this study.

3. Peptide Mapping

The peptide banding patterns generated by proteolytic digestion of a protein are characteristic of the protein substrate and the particular proteolytic enzyme used. Thus, relationships between polypeptides can be determined by comparing peptide banding patterns (Hames, 1981). Capsid protein 1D was selected for peptide mapping analysis in this study since it was easily separable from the other capsid proteins. Moreover, it has been shown to play an important role in picornavirus host cell attachment and neutralization and thus may be subject to evolutionary change (Rossmann et al., 1985).

Cleveland peptide mapping analysis of capsid protein 1D revealed a strikingly similar peptide pattern among the six isolates except for slightly slower migration of peptide p23 in J670. Intensity differences in p16 are not felt to reflect real differences between the isolates. The reduced mobility of a peptide in J670 is consistent with the mobility shifts in protein 1D which have been noted in the

one dimensional SDS-PAGE analysis of virions and infected cell lysates.

The fact that peptide banding patterns were so similar between the isolates suggests that there have been very few changes in sequence or conformation of capsid protein 1D which significantly affect partial proteolytic cleavage by V8 protease. This is not to say that changes have not occured in 1D. The peptides generated by V8 protease were quite large, hence changes in 1D could easily have gone undetected. With a technique such as tryptic peptide mapping however, in which the total digestion of a protein results in many small peptides, any changes in 1D would have a better probability of being detected by altered banding patterns (Robson et al., 1980). Potential changes in 1D might also be better detected if the protein was subjected to Cleveland peptide mapping using a battery of different proteases having various specificities instead of a single protease as was done in this study. In our initial peptide mapping procedures we used three different proteases (chymotrypsin, elastase and Staphylococcus aureus V8 protease) to digest a 50 Kd EV70-specific protein and found that V8 was the most active against this protein. It would have been beneficial to test several other different proteases for activity against EV70 structural proteins. In future experiments, it would also be of interest to apply peptide mapping analysis to all four capsid proteins.

Attempts to peptide map 1B and 1C using V8 failed in this study. This was unexpected in view of the preliminary testing of several proteases against EV70 proteins as discussed above. It may be that these two proteins possess very few V8 specific cleavage sites.

4. Immunoprecipitation

EV70 infected cell cytoplasmic extracts were immunoprecipitated with monoclonal or polyclonal antibodies to compare the viral polypeptides at the antigenic level.

Both non-neutralizing MAbs (Table 3) immunoprecipitated capsid proteins 1C and 1D (VP3 and VP1) in all isolates despite vigorous treatment to ensure denaturation of proteins and disruption of protein complexes. These results suggest that both MAbs are directed against epitopes common to 1D and 1C. Prior to obtaining these results, it was suspected that epitope recognition involved a particular protein conformation between 1C and 1D which led to immunoprecipitation of both proteins. It may be that capsid proteins 1C and 1D reassociate during incubation with the antibody or were so tightly associated that they were not affected by measures taken to dissociate them. However, this does not seem likely, especially in view of the results obtained by Hughes et al., (1984) in immunoprecipitation studies with hepatitis A virus, another enterovirus (EV72, see Table 1). They found that murine MAbs and polyclonal

antibodies to HAV consistently immunoprecipitated proteins 1C and 1D if intact virions were used as the antigen. However, when virions were heated in the presence of SDS and 2-mercaptoethanol prior to immunoprecipitation as was done in this study, there was no recognition of any viral polypeptides by either type of antibody. Their findings suggest that virions were successfully disrupted and that protein conformation must have been altered considerably when the capsid proteins were separated away from the whole virus. To differentiate between the two possible reasons for the consistent immunoprecipitation of both 1C and 1D using MAbs, a Western blot analysis would have been very helpful. It was not possible to carry out this procedure due to the lack of sufficient quantity of MAbs. Work is presently underway at the Laboratory Center for Disease Control (LCDC) in Ottawa to develop additional MAbs against EV70 (J. Wiley, Ph.D. project). Preliminary results indicate that five EV70 specific MAbs have been obtained but they have yet to be characterised. When these are made available to us, the antigenic studies can be extended to include Western blot analysis and epitope mapping to probe for antigenic differences between the isolates which may have gone undetected in the present study.

Polyclonal antisera prepared against the prototype strain of EV70, J670, were also used in antigenic comparisons between the isolates. While mouse antiserum appeared to

immunoprecipitate proteins 1AB and 1B to a greater extent than the monkey serum, both sera recognized the same structural proteins (1AB, 1B, 1C, 1D) in each strain. The apparent absence of 1B from KW97 immunoprecipitates with either serum must be interpreted carefully. While it may reflect a reduction in the affinity or recognition of this protein by the antibodies, it may also be due to differences in concentration between the cell lysates. A longer exposure of the autoradiogram might have revealed the presence of 1B in this immunoprecipitation. Kew et al., (1983) mention that neutralization titres are four fold lower for the 1981 EV70 isolates when combined with antiserum to the J670 strain than with homologous antisera, suggesting some antigenic differences between the isolates. However, the immunoprecipitation tests performed in this study indicate that all six isolates of EV70 are antigenically similar enough to be recognized by antisera prepared against the early isolate J670.

CONCLUSIONS

The main objective of this study was to examine changes in EV70 at the protein level. To this end, polypeptides of six isolates of the virus, originating from distant regions of the world and spanning a period of ten years, were compared using a variety of different techniques. We were able to detect only slight differences between the isolates in capsid protein 1D and possibly in the nonstructural proteins 3CD and 3C. Antigenically, all isolates were similar enough to give the same general immunoprecipitation patterns with both MAbs and polyclonal antisera. These results are surprising in view of: 1) the known genomic and biologic differences between the isolates; 2) epidemiological evidence which indicates that EV70 has evolved in a divergent fashion at a constant rate since its emergence in the late 1960's; 3) the enormous numbers of intervening human passages between the 1971 and 1981 strains; and 4) the extensive changes which occur in the proteins of poliovirus upon replication in as few as one or two individuals (Kew et al., 1981; Kew et al., 1983; Takeda et al., 1984, Tanimura et al ., 1985).

However, a similar comparative molecular analysis of TMEV isolates supports our findings, namely that changes at the RNA level may not necessarily be reflected in major changes

at the protein level. TMEV, picornaviral pathogens of mice, although related serologically, have been placed into two subgroups. Subgroup 1 viruses cause a poliomyelitis-like disease in mice and frequently establish persistent CNS infections. Viruses in subgroup 2 are 1000 times more virulent and produce a rapidly fatal encephalitis. In addition to pathological differences, T_1 RNase fingerprinting patterns show only 20% homology between viruses in subgroups 1 and 2 (Lipton, 1978; Lipton 1980; Lorch et al., 1981; Rohzon et al, 1985). SDS-PAGE and pulse-chase experiments revealed that the electrophoretic mobilities of proteins and protein processing were the same for representatives of both groups (Lorch and Friedmann, 1983). High resolution two dimensional electrophoresis of virion proteins and infected cell lysates revealed differences in the pI of proteins 1C, 1D and p10 (nonstructural) between the two subgroups of TMEV in one study, while other investigators have reported no differences in pI or electrophoretic mobility of proteins between the viruses by this same technique (Lorch and Friedmann, 1983; Rozhon et al., 1985). Slight differences were detected in tryptic peptide maps of 1B, 1C and 1D (Lorch et al., 1984). These apparently ambiguous results, that is, extensive differences in viral RNA but very similar proteins between the two subgroups of TMEV can be reconciled. Lorch et al., (1984) have speculated that many of the mutations in the viral RNA may have

produced synonymous genetic codons. If this were the case, such mutations would not necessarily lead to extensive changes in the proteins due to the degeneracy of the genetic code. These investigators feel that it is possible that pathogenic differences between the two groups of TMEV may be explained by minor differences in the amino acid content and secondary structure of the viral proteins.

The results of these comparative protein studies for EV70 and TMEV are in sharp contrast to those obtained in studies aimed at assessing molecular variation in poliovirus upon replication in humans. Vaccine associated isolates of poliovirus type 1, 2 and 3 as well as wild polio type 1 strains were compared at the RNA level by T_1 oligonucleotide fingerprinting and at the protein level by SDS-PAGE. These studies revealed that replication in humans, even passage through as few as one or two individuals, may lead to extensive genome changes and corresponding variation in viral proteins particularly in capsid protein 1D and 1C in poliovirus type 1 and also in 3CD, 3C and 3D in all the serotypes examined (Kew et al., 1981; Nottay et al., 1981). Nottay and co-workers (1981) speculated that changes in the viral structural proteins indicate that selective pressure is primarily exerted on these proteins, most likely by antibodies, while Kew et al., (1981) suggested that changes in nonstructural proteins 3C and 3D (which correspond to the

viral protease and polymerase, respectively) have the potential to alter RNA synthesis and proteolytic processing.

It may be argued that passage of a virus in vitro contributes significantly to the evolution of the genes and hence, gene products. However, experimental evidence suggests otherwise. In poliovirus for example, propagation of the virus in tissue culture is virtually nonselective after adaptation has taken place. On the other hand, extensive genetic changes can occur upon replication in humans (Ghendon, 1963; Nottay et al., 1981). Similarly, in a recent comparative study of eight HAV strains, genomic change as detected by T_1 oligonucleotide mapping was found to be very limited (0.8% sequence difference) in viruses which had been passaged consecutively under constant conditions in vitro. However, adaptation to and passage in marmosets led to the appearance of virus differing by as much as 10% in the sequence of viral RNA (Weitz and Siegl, 1985).

This study was designed to be a first step towards determining which regions of the genome have undergone change as enterovirus 70 has evolved over a ten year period. Although only slight differences were detected between the six isolates of EV70 at the protein level, it does not necessarily follow that few mutations or changes in the proteins have occurred during its brief history as a human pathogen. Experimental evidence has revealed that the RNA

genome of the virus has changed during this time and phylogenetic data suggest that EV70 strains in different regions of the world are genetically polymorphic (Kew et al., 1983; Takeda et al., 1984; Tanimura et al., 1985). Perhaps many of these genomic differences between the isolates have been 'silent' mutations, that is, changes in the RNA which have not altered proteins to a great extent due to the degeneracy in the genetic code as Lorch et al.(1984) have suggested. On the other hand, it is also quite possible that differences in the polypeptides between the various isolates of EV70 were not detected by the methods used in this study.

In future comparative studies, changes in addition to those detected in proteins 1D, 3CD and 3C may be found if high resolution two dimensional electrophoresis is repeated and if other highly sensitive techniques such as tryptic peptide mapping and epitope mapping using MAbs are included. Ultimately, RNA and protein sequence analysis will reveal the exact locations of evolutionary change in EV70. With this information, the origin of this virus and its relationship to other picornaviruses may then become clear. Moreover, it may also be possible to elucidate which areas of the genome or which proteins are responsible for, or contribute to, the pandemic, neurovirulent and temperature sensitive properties of EV70. The recent development of an animal model (rabbit), in which EV70 conjunctival infection

and immunological response closely parallel that found in humans, should enable these aspects of the virus, as well as natural antigenic variation, to be studied (Langford et al., 1986).

EV70 is an interesting virus in many respects. The fact that the emergent strain and subsequent isolates are available make it an attractive model for evolutionary study. The process of evolution in this virus has been likened to that in influenza A, where it has been observed by Buonagurio et al., (1986), that lineages other than the one that gives rise to the future generation have very short life-spans. This pattern of genetic change is apparently distinct from that found in animals and all other viruses, where multiple surviving lineages undergoing slow rates of change co-exist. It is interesting to note that both EV70 and influenza are capable of spreading in a pandemic fashion. However, whether this has anything to do with the way in which these viruses evolve was not addressed by Buonagurio and co-workers (1986). EV70 also possesses many other unique properties which distinguish it from other enteroviruses as have been discussed in this study. Furthermore, the sudden appearance of this previously unknown virus in 1969 and its rapid spread since then to involve millions of people worldwide should serve as a reminder to us that 'new infectious agents' present a continuing threat to our species. It is fortunate for mankind that EV70 acute

hemorrhagic conjunctivitis is not a life threatening disease, as is the case with acquired immune deficiency syndrome (AIDS), another example of a 'new infection' (Grist, 1985). In this regard it is imperative that the origin and evolution of such pathogens be studied so that changes which contribute to the emergence of 'new viruses' can be better understood. Hopefully, an understanding at the molecular level may in turn enable advances to be made at the preventive or therapeutic level.

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