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CLONING AND NUCLEOTIDE SEQUENCE ANALYSIS OF THE P3 REGION OF ENTEROVIRUS 70

Thesis Submitted to the

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In Partial Fulfillment

of the Requirements for the Degree of Master of Science,

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ABSTRACT

Numerous viruses have been found to infect the conjunctival layer of the human eye. In the recent past two new viruses have been identified that cause a syndrome called Acute Haemorrhagic Conjunctivitis. One agent (Enterovirus 70) appears to have a worldwide distribution and has produced two pandemics; 1970-1972 and 1980-1982. Biological and physico-chemical properties suggest that Enterovirus 70 is a member of family Picornaviridae, genus Enterovirus. Enterovirus 70 has unique properties : capacity to spread pandemicly, novel tropism for the conjunctiva, temperature sensitivity, and high evolutionary rate. Serological and molecular studies have suggested that Enterovirus 70 is a new human pathogen.

In this study, nucleotide and amino acid sequence analyses were conducted in the P3 region of the Enterovirus 70 genome. Nucleotide and amino acid sequence alignments show that Enterovirus 70 is a picornavirus and is closely related to polioviruses and coxsackieviruses. The $3D^{pol}$ and 3B(VPg) of Enterovirus 70 show higher similarity to the corresponding regions of Poliovirus 1, while two other regions, 3A and $3C^{pro}$, are more closely related to Coxsackievirus B3 sequences. The 3' untranslated region is also more closely related to Poliovirus 1 sequences. These observations suggest that the P3 region of Enterovirus 70 diverged from poliovirus like sequences. For the two regions which show more similarity to Coxsackievirus B3 like sequences, there is some evidence that this could be the result of recombination, in the P3 region, between an the ancestral sequence to Enterovirus 70 and a coxsackievirus like genome. The importance of the

P3 region in transcription, translation, and replication suggests that these recombinational events may be responsible for some of the biological properties of Enterovirus 70, particularly its temperature sensitive nature and broader host range, as compared to other human enteroviruses.

One night a man had a dream. He dreamed he was walking along the beach with the Lord. Across the sky flashed scenes from his life. For each scene, he noticed two sets of footprints in the sand : one belonging to him, and the other to the Lord.

When the last scene of the life flashed before him, he looked back at the footprints in the sand. He noticed that many times along the path of his life there was only one set of footprints. He also noticed that it happened at the very lowest and saddest times in his life.

This really bothered him and he questioned the Lord about it.

"Lord, you said that once I decided to follow you, you'd walk with me all the way. But I have noticed that during the most troublesome times in my life, there is only one set of footprints. I don't understand why when I needed you most you would leave me."

The Lord replied, "My son, My precious child, I love you and would never leave you. During your times of trial and suffering, when you see only one set of footprints, it was then that I carried you."

Author Unknown

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I would like to express my sincere thanks to Dr. K. Dimock for all his patience, for helping me to learn techniques, and for his critical questions. All this changed my thinking and my perception of science. His extraordinary optimistic view, sometimes combined with humour, was always a sparkle of light, taught me patience and continued the progress towards my goal.

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Special thanks to Dr. Sattar for his occasional guidance. Though discussions with Dr. Tenniswood, Dr. Brown, Dr. Rossier, and Dr. Johnson-Lussenburg were not frequent, I would like to acknowledge their valuable comments, criticisms, and suggestions in latter part of this study. It would be unfair not to admit that my stay in Ottawa would never have been complete if I did not have support from Dr. Dimock and the department. I am deeply in debt for such support.

Though this project is not finished, I would like to thank the Medical Research Council of Canada and the Natural Science and Engineering Research Council of Canada for financial support for this study on the molecular biology of Enterovirus 70.

DEDICATION

This thesis is dedicated to my grandfather,

and to my wife Shabnum Ahsan.

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LIST OF ABREVIATIONS

AHC	Acute Haemorrhagic Conjunctivitis
ATP	Adenosine triphosphate
CDC	Center for Disease Control
cDNA	Complementary DNA
CPE	Cytopathic Effect
CSF	Cerebro-spinal fluid
CVA24	Coxsackievirus A24
CVB1	Coxsackievirus B1
CVB3	Coxsackievirus B3
CVB4	Coxsackievirus B4
DEP	Diethylpyrocarbonate
DMSO	dimethyl sulphoxide
DNA	Deoxynucleic acid
DNase	Deoxyribonuclease
EDTA	Ethylene Diamine Tetraacetic Acid
EMCV	Encephlomyocarditis virus
EtBr	Ethidium bromide
EV70	Enterovirus 70
FMDV	Foot-and-Mouth Disease virus
HAV	Hepatitis A virus
HeLa	Human cervical cancer cell line
HRV2	Human Rhinovirus 2
HRV14	Human Rhinovirus 14
IPTG	Isopropyl-β-D-thiogalactopyranoside

$LLC\text{-}MK_2$	Monkey kidney cell line
MEM	Minimal Essential Medium
MOI	Multiplicity of Infection
MW	Molecular weight
NIH	National Institutes of Health
PFU	Plaque Forming Units
RNase	Ribonuclease
SDS	Sodium Dodecyl Sulphate
TAE	Tris-Acetate-EDTA buffer
TBE	Tris-Borate-EDTA buffer
TBS	Tris Buffered Saline
TE	Tris-EDTA buffer
tRNA	Transfer RNA
U	Unit
v/v	Volume to volume
w/v	Weight to volume
WHO	World Health Organization
X-gal	5-Bromo-4-chloro-3-indolyl-β-D-galactoside

C H A P T E R O N E

INTRODUCTION

1.1 : ACUTE HAEMMORRHAGIC CONJUNCTIVITIS :

The eye(s) can respond to an infection by being painful, having impaired vision and/or exhibiting redness or inflammation of the conjunctiva (conjunctivitis). Redness of the eye(s) brings the most common visitors to emergency departments in hospitals (Howes, 1988).

There are numerous causes of conjunctivitis, infectious or non infectious (Howes, 1988). Notable bacterial agents are Staphylococcus aureus, and Neisseria gonorrhoeae (Alfonso et al, 1983). Chlamydia trachomatis is also a frequent cause of conjunctivitis (Howes, 1988). Conjunctivitis of viral etiology has been attributed to Newcastle disease virus, Adenoviruses 3, 4, 7, 8, 19, 37 (Aoki et al, 1986; Arnow et al, 1977), Herpes simplex virus, Herpes zoster virus, Measles virus, Molluscum contagiosum virus, and picornaviruses. Picornavirus agents which can cause conjunctivitis include Enterovirus 70, a variant of Coxsackievirus A24 (Darougar et al, 1989; Christopher et al, 1982; 1977) and occasionally ECHO viruses 7, 9, 11, Coxsackieviruses B1, B2, and some other enteroviruses (Howes, 1988; Ghendon, 1989; Darouger et al, 1989; Melnick, 1985).

In the early 1970s two viruses were identified as agents responsible for a new type of eye infection, which was named **Acute Haemorrhagic Conjunctivitis** (AHC) (Yin-Murphy and Lim, 1972; Mirkovic et al, 1974). The physico-biological properties of these viruses placed them in the family PICORNAVIRIDAE, genus Enterovirus (Kono, 1978; Cooper et al, 1978). The two agents, Enterovirus 70 and a variant of Coxsackievirus A24, have caused extensive epidemics in Africa, Asia,

and more recently in the American continent (Higgins et al, 1972; Maichuk, 1973; Hierholzer and Hatch, 1985).

1.2 : CLINICAL PICTURE :

AHC caused by Enterovirus 70 and Coxsackievirus A24 cannot be differentiated on the basis of the clinical picture they present to the clinician (Christopher et al, 1977; Hierholzer and Hatch, 1985). The syndrome has also been called Epidemic Kerato-conjunctivitis (EKC), and Singapore Epidemic Conjunctivitis (SEC) (Yin-Murphy and Lim, 1972; Yin-Murphy, 1976). In Bangladesh it was called "Joy Bangla" (Parrott et al, 1971; Pramanik, 1971). AHC caused by Coxsackievirus A24 is seen more in the Far East and appears to be endemic in that region of the world (Higgins, 1985). However, the other picornavirus causing AHC, Enterovirus 70, is much more widely distributed around the world (Hierholzer and Hatch, 1985). The following discussion will be restricted only to AHC caused by Enterovirus 70.

AHC is an acute infection and it has an incubation period of about 24 hours. This is followed by a sudden onset of symptoms starting with pain in the eye(s). Itching, burning, and blurred vision appear to be the next symptoms (Chatterjee et al, 1970a; Nejmi et al, 1974; Roy et al, 1975; Kono et al, 1972; Kono and Uchida, 1977; Patriarca et al, 1983; Sklar et al, 1983; Stansfeild et al, 1984). Constitutional symptoms and respiratory signs during the early phase of the infection; pharyngitis, headache, malaise and fever, have been documented in 5-7% of patients (Roy et al, 1975; Kono et al, 1972; Mitsui et al, 1972; Nabli and Daghfous, 1985; Whitcher et al, 1976; Patriarca et al, 1983; Sklar et al, 1983; Stansfeild et al, 1984; Roy et al, 1975; Yin-Murphy

and Lim, 1972; Jones, 1972; Pramanik, 1971). However, pharyngitis, for example, has been reported consistently in outbreaks and epidemics from India and Thailand (Roy et al, 1975; Yin-Murphy, 1984; Hierholzer and Hatch, 1985). This could be due to properties associated with the circulating strain of AHC virus in a particular geographic area of the world. Foreign body sensation associated with AHC has been observed in 90-100% of the identified cases (Hossain et al, 1983; Patriarca et al, 1983; Sklar et al, 1983). The profuse rubbing of the eye(s) intensifies the symptoms and increased secretion of mucus and other substances results in eyelid edema and palpebral conjunctival follicles (Yin-Murphy, 1984). Subsequently, mechanical friction (rubbing) together with inflammatory response result in petechial haemorrhaging of the conjunctival layer which becomes more confluent with time (Kono et al, 1972; Kono et al, 1977; Chatterjee et al, 1970b; Roy et al, 1975; Whitcher et al, 1976; Patriarca et al, 1983; Sklar et al, 1983; Stansfeild et al, 1984). The intense inflammatory response result in conjunctival congestion in 100% of the patients. This intensifies haemorrhages of sub-epithelial parts of the conjunctival layer of the eye(s) and eyelid(s) (70-100% of cases) (Chatterjee et al, 1970a; Chatterjee et al, 1970b; Stansfeild et al, 1984).

In a few cases of AHC, corneal complications have been reported (Alfonso et al 1983). Secondary bacterial infections with a variety of organisms have also been documented in the literature (Chatterjee et al, 1970a; Roy et al, 1975; Patriarca et al, 1983; Chaturvedi et al, 1975; Wolken, 1974). Regional preauricular lymph node enlargement is noticed in 40-60% of the documented cases (Kono, et al, 1972; Kono and

Uchida, 1977; Chatterjee et al, 1970a; Chatterjee et al, 1970b; Nejmi et al, 1974; Nabli and Daghfous, 1985; Whitcher et al, 1976). Uveitis and keratitis are rare. Most of these complications occur as a result of steroid use (Chatterjee et al, 1970a; Pramanik, 1971; Melselaar et al, 1976).

Laboratory diagnosis of Enterovirus 70 infection is done by testing for sero-conversion, a 4-8 fold rise in neutralizing antibody titres. The frequency of detection of Enterovirus 70 was 25% or lower from conjunctival swabs and 5% from throat swabs (Ishii, 1989a). However, the rise in antibody titre(s) is indicative of recent Enterovirus 70 infection (Editorial, 1982a; Kono et al, 1977; Kono et al, 1975; Kono et al, 1981b; Arnew et al, 1977; Hierholzer et al, 1975). Post epidemic serological studies have shown that children under age 10 have the highest frequency of sero-conversion (Kono et al, 1975; Mather et al, 1977; Stanton et al, 1977). There are a number of different diagnostic assays devised for the quantification of virus antibodies. Haemagglutination, haemagglutination inhibition, as well as complement fixing assays have been used to quantitate antibodies in patient's sera (Hierholzer et al 1984; Kono et al, 1978;). Indirect immunofluorescence assay has successfully been used to detect and monitor Enterovirus 70 antigen(s) in tear samples (Yin-Murphy et al, 1985; Pal et al, 1983). Microneutralization assay for Enterovirus 70 antibodies appears to be the most sensitive detection method (Hierholzer et al, 1984).

Interestingly, hyperimmune sera produced against Enterovirus 69, 70, and 71 showed that AHC virus is relatively weak in eliciting an

immune response in rabbits (Kravchenco et al, 1985). The interferon sensitivity of Enterovirus 70 is also said to be lower than for other virus agents (Stanton and Langford, 1977; Stanton et al, 1977; Langford et al, 1985a; 1979).

Typically, AHC is a self limiting infection and is restricted to the eye(s). In rare instances (1:10,000 cases), neurological complications associated with Enterovirus 70 infection have been documented (Bharucha and Mondkar, 1972; Wadia et al, 1972; Green et al, 1975; Hung et al, 1976; Armstrong and Baker, 1982; Editorial, 1982b; Hung, 1989; Wadia, 1989). The exact pathogenesis of these neurological episodes are poorly understood.

The neurological complications following Enterovirus 70 infection, were first reported in India following the pandemic of 1969-1971 (Bhanucha and Mondkar, 1972; Wadia et al, 1973). A similar type of neurological illness has also been reported from Senegal, Taiwan, and Japan (Kono et al, 1976; Hung et al, 1979; Kono et al, 1974; Higgins, 1982, Aoki et al, 1980; Armstrong and Baker, 1982; DeBeer, 1982, Hoffman, 1982). The symptoms tend to appear between 1-8 weeks after conjunctivitis (Hung et al, 1979; Higgins, 1985). The association of Enterovirus 70 with neurological illness was first demonstrated in Cyanomolgus monkeys by Kono and associates (1973). Serological analysis of cerebrospinal fluids and sera showed high titers of neutralizing antibodies against Enterovirus 70 (Wadia et al, 1983a; Kono et al, 1977; Kono et al, 1981c; John et al, 1981). Until recently, neither virus nor its antigens were shown to be present in nerve cells of patients or experimental animals. Pal and co-workers (1986) have shown

that Enterovirus 70 antigens could be identified in microglial cells of the spinal cord.

The central nervous system (CNS) complications appear to be more frequent in males than in females; 70% of cases are male, with ages between 20-29 years (Hung, 1989). However, the reasons for this selection are not understood (Wolken, 1974). Recently, some cases of a Guillain-Barré like syndrome following Enterovirus 70 infection have also been reported, with one fatal case in India (Chopra et al, 1986).

Complete recovery from neurological complications following Enterovirus 70 infection occurs in 45-50% of affected individuals (Hung, 1976; DeBeer, 1982; Armstrong and Baker, 1982). The remaining patients are left permanently disabled with varying degrees of disability (Hung et al, 1976).

1.3: EPIDEMIOLOGY :

The first epidemic of AHC was recognised in June, 1969 in Ghana (Chatterjee et al, 1970a). This newly recognised eye infection was later identified in different countries along the east (Nigeria, Cameroon) and west (Ivory Coast) coast of Africa (Chatterjee et al, 1970b). This epidemic coincided with the landing of the Apollo mission to the moon and was nicknamed "Apollo 11 disease". By January, 1970, AHC appeared in Sierra Leone and shortly afterwards, in neighboring Gambia, Senegal and Morocco (Bager et al, 1975; Nejmi et al, 1974). In the spring of 1971, AHC was identified in Tunisia, Libya, Saudi Arabia and the Middle East (Whitcher et al, 1976, Yin-Murphy, 1984). Sporadic outbreaks were reported in Egypt, Sudan, Ethiopia, Democratic Yemen, Iraq, and Afghanistan (Ishii, 1989a; Maichuk, 1975). A second focus was

also identified in the Far East in the same year with a mixed etiology of Adenovirus 11 and Enterovirus 70 (Ishii, 1989a). Smaller outbreaks were also reported in Rotterdam, the Netherlands, London, U.K. and the Soviet Union with less intense symptoms (Malchuk, 1975; Jones, 1972; Stanton and Langford, 1977). In 1971, AHC also spread to the Indian subcontinent (Roy et al, 1972). In 1972, an unusual epidemic in Kenya is believed to be the result of transmission of Enterovirus 70 by sailors travelling from India (Metselaar et al, 1976). It became clear by this time that Enterovirus 70 has the ability to spread pandemically (Yin-Murphy, 1984).

The time between 1972-1980 was a relatively quiet period in AHC history. During this period the syndrome occurred as localized outbreaks or small epidemics in India, Japan, Thailand, Bangladesh, Hong Kong, and Taiwan (Aoki et al, 1980; Green et al, 1975; Kaiwar et al, 1983; Yin-Murphy, 1984, Chen and Lin, 1982). Small sporadic outbreaks were also reported during this period in the United Arab Emirates (UAE), Zaire, Egypt, Saudi Arabia, Yugoslavia, France, the Netherlands, and South Africa (Maichuk, 1975; Dawson et al, 1974; Whitcher et al, 1976; Liker et al, 1975; Mirkovic et al, 1973; Golden and Scott, 1974; Metselaar et al, 1976; Hoffman, 1982).

After an interval of eight years, the sporadic outbreaks turned into explosive epidemics in the Indian subcontinent, Singapore, Taiwan, Thailand, Malaysia, Indonesia, Japan, Yemen, UAE, Bangladesh, and Pakistan (Bernard et al, 1982; Thakur, 1981; Hossain et al, 1983; John et al, 1981; Wadia et al, 1983b; Yin-Murphy, 1984; Gangwar et al,1984; Ishii, 1989a). However, it was not possible to trace from where this

epidemic started and spread to other countries (Ishii, 1989a). During this second AHC pandemic a new focus was also established in South America and the Caribbean islands (Hierholzer and Hatch, 1985). The first country to experience this epidemic was Brazil. This epidemic subsequently spread to Surinam, French Guiana, Guyana, Venezuela, Columbia, Trinidad and Tobago, Honduras, Belize, Mexico, the West Indies, Haiti, Puerto Rico, the Virgin Islands, the Dominican Republic, Guatemala, Panama, Cuba, Ecuador, and Argentina (Waterman et al, 1984; Reeves et al, 1986; Hierholzer and Pallansch, 1989). In 1981, AHC was also seen in Florida, and North Carolina in the United States (Sklar et al, 1983; Patriarca et al, 1983; Hatch et al, 1981). During this pandemic, New Zealand also experienced a small outbreak (Ishii, 1989b). In Toronto, Canada, an imported case was responsible for the spread of AHC in a hospital setting (Spence and Vellard, 1981; 1982). In 1984, Antigua, Grenada, and Trinidad and Tobago had epidemics which continued in 1985. AHC due to Enterovirus 70 was identified in 1986 from Taiwan, Japan, American Somoa, India, and Pakistan (Ghendon, 1989). Sporadic outbreaks of conjunctivitis were also reported from the American continent in 1987, and 1988 (Hierholzer and Pallansch, 1989).

AHC is highly contagious and spreads rapidly in crowded, humid coastal areas (Alfonso et al, 1983; Aoki et al, 1980; Arora et al, 1977; Higgins, 1982). In fact, major epidemics of AHC have occurred in a belt between 35^{o} N latitude to 155^{o} E longitude and 10^{o} S latitude to 17^{o} W longitude. This geographic area has a high population density, temperature ranges from 27 ^{o}C to 43 ^{o}C, and annual rainfall is 102-610 cms. (Hierholzer and Hatch, 1985). In this "Acute Haemorrhagic

Conjunctivitis belt", 90% of the large scale epidemics have occurred, involving about 37% of the world's population (Hierholzer and Hatch, 1985; Higgins, 1985; Ishii, 1989a). Laboratory simulation of environmental conditions has shown that at temperatures of 20 ^{o}C, 33 ^{o}C, and 35 ^{o}C, combined with ultrahigh humidity (95+5%), Enterovirus 70 can be recovered from non-porous surfaces even after 24 hours. These results suggest that high relative humidity is an important factor for transmission of Enterovirus 70 (Sattar et al, 1988).

The incidence of AHC in the population during an epidemic ranges from 5 - 25% (Yin-Murphy, 1984). The highest incidence (90%) was recorded in Samoan families (Hierholzer and Pallansch, 1989). However, the Vietnamese refugee families provide us with an example of fast and efficient transmission of Enterovirus 70 within a community (Bager et al, 1982). In another study, 72% of the total number of cases studied were in families, with a higher frequency in 3-5 member families compared to other groups (Arora et al, 1977).

The transmission of Enterovirus 70 is believed to take place by direct person-to-person contact or via fomites (Sattar et al, 1988). There are reports that healthy individuals can be infected with contaminated ophthalmic instruments (Kono, 1978) or ophthalmic solutions (Spence and Vellard, 1981b). Enterovirus 70 has also been isolated from fingertips (Arnew et al, 1977). The rapid spread of the infection can be controlled by stringent cleaning of the hands and hygienic handling of towels, wash basins, pillow covers, clothes, handkerchieves, and other potential fomites (Hung and Kono, 1979; Bernard et al, 1982; Golden and Scott, 1974; Higgins, 1982; Hoffman,

1982; Kono and Uchida, 1977; Kono, 1978; Maichuk, 1975; Pramanik, 1971). The most effective and simplest way to get rid of conjunctivitis is to bathe in sea water (Ray et al, 1975). Laboratory infection(s) with Enterovirus 70 have also been reported in humans as well as in animals (Sasagawa et al, 1976; Langford et al, 1986, 1985b, 1980).

1.4 : ENTEROVIRUS 70 IS A PICORNAVIRUS :

The initial characterization of Enterovirus 70 was carried out in 1971 (Maichuk, 1972). No reactivity with type specific enterovirus sera was established (Melnick, 1974). Resistance to ether, chloroform, and acid (pH 3.0) was sufficient to place Enterovirus 70 in the family Picornaviridae (Yin-Murphy, 1973). The cubic symmetry, with diameter of 29 nm, sedimentation coefficient (S_{20w}) of 155-160, and buoyant density of 1.34 g/mL in CsCl gradients reinforced placing Enterovirus 70 in this group (Esposito et al, 1974; Yamazaki et al, 1974; Kono, 1978). One difference from other enteroviruses, however, was the inability of researchers to isolate Enterovirus 70 from faeces (Hierholzer and Hatch, 1985). The properties of Enterovirus 70 are listed in Table 1.

Here, it would be unfair not to discuss this family in general. The current knowledge about these viruses will enable the reader to comprehend their basic features, as well as their importance in medical virology.

1.5 : FAMILY PICORNAVIRIDAE :

The picornaviruses are among the smallest of RNA viruses; pico - small, rna - RNA as genetic material (Rueckert, 1985). The entire group, one of the largest, contains both human and animal pathogens. Their economic and medical importance is evident from the

TABLE 1

PROPERTIES OF ENTEROVIRUS 70

A. MORPHOLOGICAL :	
- SHAPE	SPHERICAL
- DIAMETER	26 ± 3 nm
B. PHYSIOCHEMICAL :	
- BUOYANT DENSITY, CsCl	1.34 g/mL
- BUOYANT DENSITY, SUCROSE	1.25 g/mL
- VIRION, S_{20w}	150 - 160S
CAPSID PROTEINS	4
- EMPTY CAPSIDS, S_{20w}	80S
CAPSID PROTEINS	3
- STABILITY OF VIRIONS :	
pH	3 - 8.6
UNSTABLE IN DISTILL WATER	
STABLE IN $MgCl_2$ AT 50 ^0C FOR 1 HOUR	
- REACTION TO CHEMICALS :	
SODIUM DEOXYCHOLATE	RESISTANT
CHLOROFORM	RESISTANT
C. VIRUS REPLICATION :	
- SPECTRUM OF INFECTION	PRIMATE & NON-PRIMATE CELL CULTURE
- SITE OF REPLICATION	CYTOPLASM
- OPTIMUM TEMPERATURE	33 ^0C
- NON PERMISSIVE TEMPERATURE	39 ^0C
D. MEDICOBIOLOGICAL PROPERTIES :	
- 1^0 INHABITANT OF THE CONJUNCTIVA	
- CAUSATIVE AGENT OF AHC	
- NEUOVIRULENT TO HUMANS AND MONKEYS	

a) modified from Kono etal, 1978

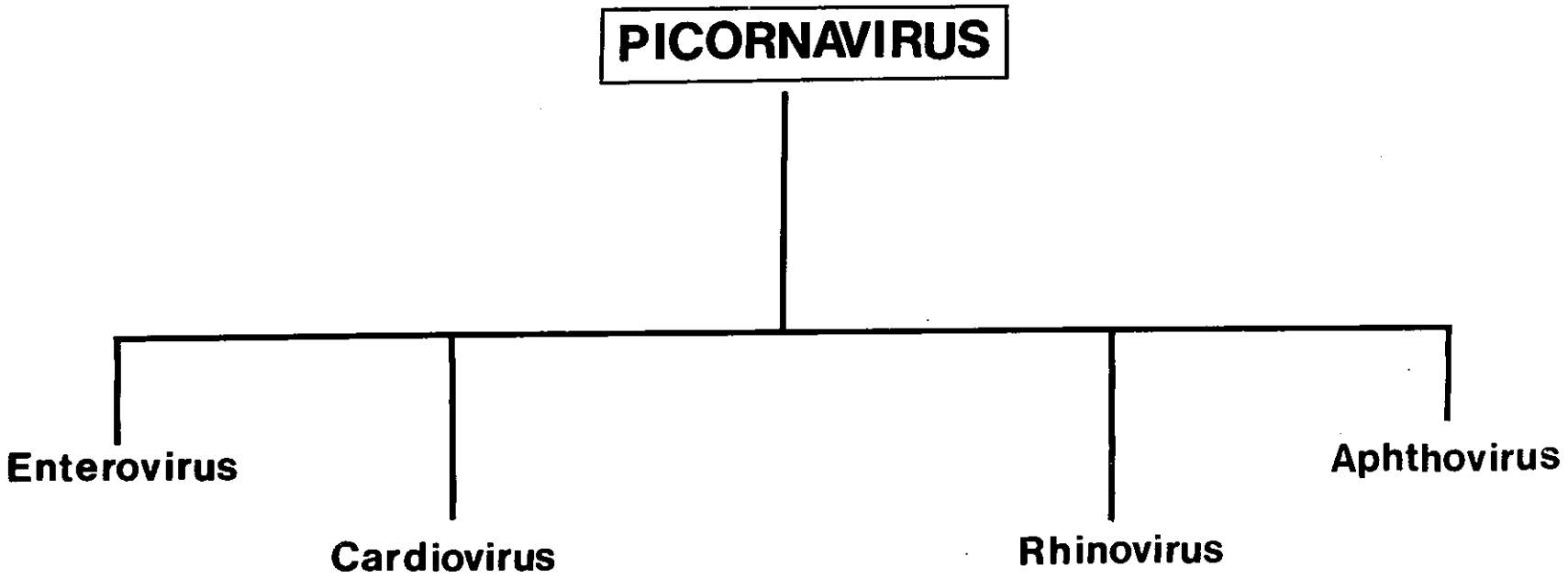
wide range of infections they cause, which can result in the death of animal(s) (Rueckert, 1985).

The family PICORNAVIRIDAE is divided into four genera, Aphthovirus, Cardiovirus, Enterovirus, and Rhinovirus (Figure 1) (Joklik et al, 1980). Throughout the thesis, I will use the general terms aphthoviruses, cardioviruses, enteroviruses, and rhinoviruses to refer to the members of each genus. Poliovirus, an enterovirus, has been considered as the prototype virus to all picornaviruses. Study of this infectious agent had led to numerous discoveries in the past. The influence of these discoveries is evident in other areas of modern virology (Wimmer et al, 1987).

Picornaviruses are 25-30 nm particles. The capsid is made of 60 equivalent subunits of four structural proteins 1A (VP4), 1B (VP2), 1C (VP3) and 1D (VP1) (Wimmer et al, 1987). Picornaviruses do not contain any carbohydrate in their capsids (Rueckert, 1985). The genome of these viruses is single stranded RNA of positive sense, with a covalently linked 5' terminal protein (VPg). The 3'-end is polyadenylated with 35-150 nucleotides. The length of the poly (A) is important for infectivity of the virus particles (Rueckert, 1985). The genomes are approximately $2.3 \times 10^6 - 2.7 \times 10^6$ daltons (Koch and Koch, 1985).

Each genus can be distinguished by a set of characters which includes: pH stability, buoyant density, post translational cleavage patterns, functional variations in the gene products, and other biophysical differences (Rueckert, 1985; Palmenberg, 1987a). Some of these properties are listed in Table 2.

Figure 1. **OLD CLASSIFICATION OF PICORNAVIRUSES:** The family Picornaviridae is divided into four genera: Enterovirus, Cardiovirus, Rhinovirus, and Aphthovirus. The grouping is based on pH stability, optimum temperature for propagation, and biophysical characteristics, such as morphology, buoyant density and sedimentation coefficient.


PICORNAVIRUS
Enterovirus
Cardiovirus
Rhinovirus
Aphthovirus

T A B L E 2

DIFFERENTIATING CHARACTERS OF PICORNAVIRIDAE

Genus[a]	pH stability	Buoyant density (g/mL) virion	shell[b]	S_{w20}[c]
Enterovirus	stable 3-9	1.34	1.30	156S
Cardiovirus	stable 3-9	1.34	1.30	156S
Rhinovirus	labile <6	1.39-1.42	1.29	149S
Aphthovirus	labile <6	1.43-1.45	1.30	142-146S

a) according to the old classification (Rueckert, 1985)
b) empty capsids
c) sedimentation coefficient

The infectious cycle of picornaviruses initiates with the attachment of virions to specific receptors on cells (Colonno, 1987, Crowell et al, 1987). The three dimensional prediction and atomic resolution of the structure of rhinoviruses, polioviruses, FMDV, Hepatitis A virus, and mengovirus (cardiovirus) (Rossmann, 1987; Acharya et al, 1989) have shown that receptor attachment sites of all picornaviruses share a common "canyon" structure, at their fivefold axis of symmetry, surrounded, on the edges of the canyon, by immunodominant residues (Rossmann and Palmenberg, 1988; Rossmann, 1987; Rossmann, 1989). The direct proof of the binding of Rhinovirus 14 to the canyon structure has recently been published (Colonno et al, 1988). The amino terminus of 1A (VP4) is myristylated (Chow et al, 1987) and it is believed that myristylation mediates the transient association of the virus particle to the membrane and may direct virus particles to penetrate the membrane of a cell (Chow et al, 1987; Hogle et al, 1987).

Like for other viruses, uncoating of RNA takes place in the cytoplasm (Rueckert, 1985). The viral RNA (vRNA), with covalently linked protein (VPg), is converted to mRNA by the cleavage of VPg from the genome (Nicklin et al, 1986). It is believed that VPg prevents proper scanning of the vRNA and its removal is essential for translation of the genome (Wimmer et al, 1987). In some other positive stranded RNA plant viruses, however, VPg is required for translation (Hershey and Taylor, 1987). An enzyme has been purified from mouse Krebs ascites cells which specifically cleaves VPg from picornavirus genomes (Drygin et al, 1988). A similar "unlinking enzyme", which cleaves the phosphodiester bond between tyrosine (residue 3) of 3B

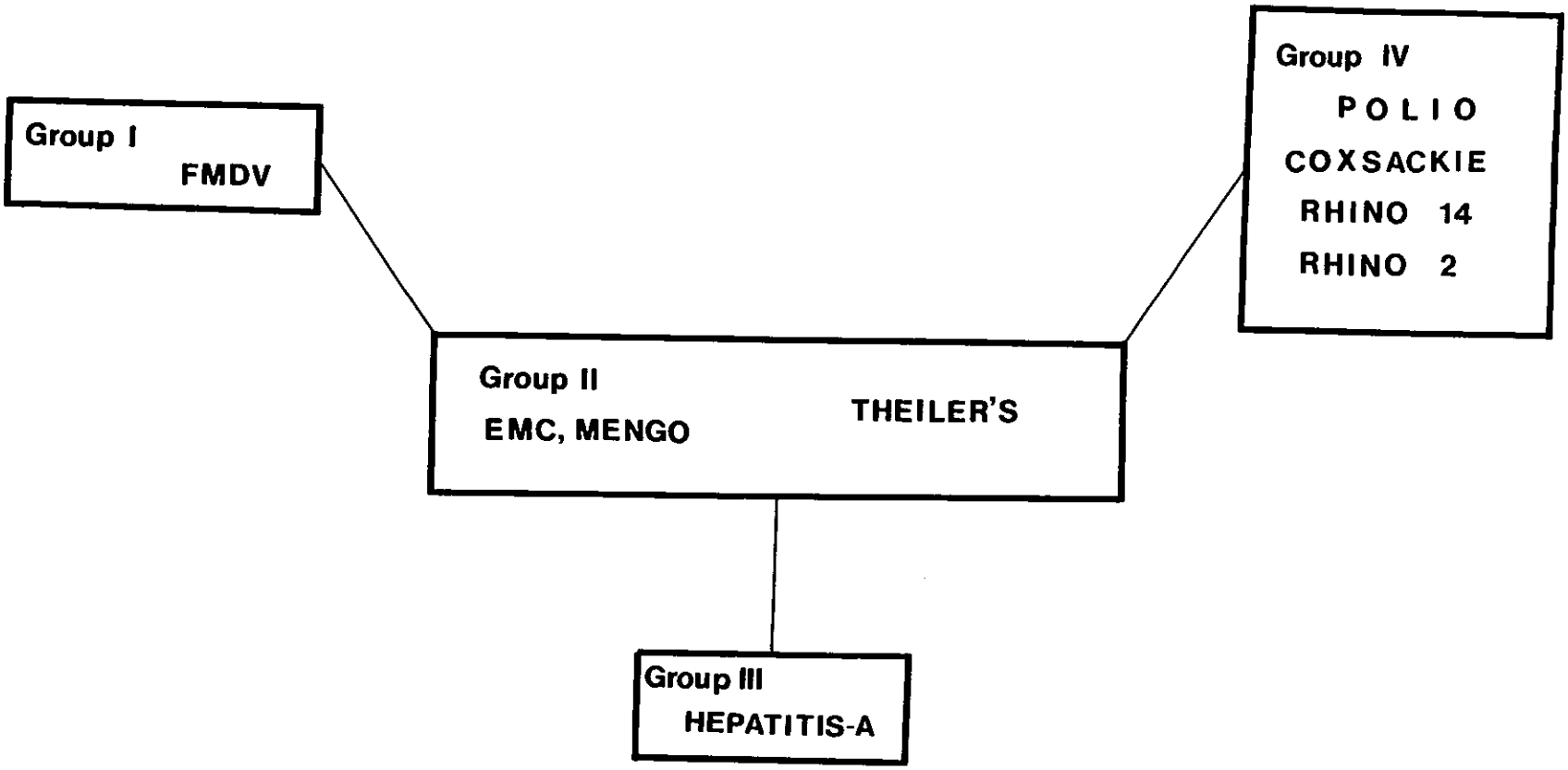
(VPg) and the terminal nucleotide of poliovirus RNA, has also been purified from HeLa cells (Morch et al, 1987).

Picornavirus replication is strictly template-primer-dependent enzymatic catalysis (Flanegan et al, 1987). It has been suggested that oligo (U) priming at the 3' end is required for further events to proceed in replication, and VPg is later covalently attached (Flanegan et al, 1987). In another proposal, the VPg-uridylylate serves as primer for the polymerase ($3D^{pol}$) to initiate replication (Takeda et al, 1987; Toyoda et al, 1987). It has been postulated that either RNA, 3B(VPg), or a combination of both form the catalytic centre during the replication (Tobin et al, 1989). Picornavirus replication takes place in the cytoplasm. It has been shown that the presence of a nucleus is not required for picornavirus replication (Wimmer et al, 1987).

Picornaviruses genomes have been extensively studied at the molecular level. More than 300,000 nucleotides from 32 strains have been sequenced and every day a new virus is added to this list (Palmenberg, 1987a). Recently, sequence comparisons within the family has led to the construction of a phylogenetic tree for the capsid proteins (Palmenberg, 1989). Sequence similarity has also resulted in a new classification scheme for these viruses, which is presented in Figure 2 (Palmenberg, 1987b; Palmenberg, 1989).

Picornavirus genomes vary in size, the Rhinovirus 2 genome being the smallest, with 7,102 nucleotides. On the other end of the scale, the Foot and Mouth Disease virus (FMDV O1K) genome has 8,332 bases (Palmenberg, 1987a). The polioviruses, coxsackieviruses, and rhinoviruses share extensive nucleotide similarity in their 5'-

Figure 2. **NEW CLASSIFICATION SCHEME FOR PICORNAVIRUSES:** In this new proposal for subgrouping picornaviruses, genome organization, capsid structure, and sequence similarity were used to group the viruses (Palmenberg, 1989). These parameters suggest that polioviruses, coxsackieviruses and rhinoviruses should be placed in one group (Group 4). FMDV does not show significant sequence similarity to the above group and forms a separate group (Group 1). EMC, Mengovirus, and Theiler's like viruses form Group 2. Hepatitis A virus (Enterovirus 72) is the most different and forms Group 3 in this scheme.


Group I
FMDV
Group II
EMC, MENGO
THEILER'S
Group III
HEPATITIS-A
Group IV
POLIO
COXSACKIE
RHINO 14
RHINO 2

untranslated region (UTR) and in coding sequences. In fact, the first 600 bases can be aligned very well. The first 10 nucleotides in Poliovirus 1, Coxsackievirus B3, and Rhinovirus 14 are identical. The poliovirus and coxsackievirus genomes have an extra 140 nucleotides which are not present in rhinovirus genomes. However, all of these RNAs form similar stem-loop structures in their 5' -UTRs (Palmenberg, 1987a). In Encephalomyocarditis virus (EMC) (Palmenberg et al, 1984) and FMDV, a 50-400 nucleotide long poly(C) tract has been recognised 150-400 nucleotides from the 5' end. Deletion of this region renders the genome noninfective (Rueckert, 1985). In poliovirus, the 5'-UTR is 743 nucleotides long, whereas in rhinovirus it is 629 nucleotides long. Recently, it has been shown for poliovirus that specific binding of the replicase complex occurs within nucleotides 510-629 and 97-182 in the 5' -UTR (Maria et al, 1989). In Hepatitis A virus, the 5' -UTR of the tissue culture adapted strain has a different predicted RNA secondary structure than the 5' -UTRs of the nonadapted strains. This difference in the secondary structure could be responsible for replication of is virus at a faster rate in cell culture (Paul et al, 1987; Ross et al, 1989).

The 5'-UTR contains several potential start codons (AUGs) (Rueckert, 1985). It has been suggested that nucleotides adjacent to the AUG codon have modulative effects on the choice of the translation initiation site. Most picornaviruses have 11 pyrimidines, interrupted by a purine before the start codon (Pevear et al, 1987). In particular, an AUG codon flanked by purine residues at -3 and +4 (ANNAUGG) is preferred for the start of translation. In aphthoviruses, a leader

sequence (L) has been identified, which is translated from the long 5'-UTR of the genome and appears to take part in replication of these viruses (Sangar et al, 1988).

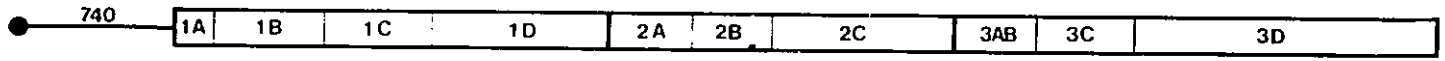
The nucleotide composition for each picornavirus genus is characteristic. The highest GC% has been observed in aphthoviruses (53%), followed by cardioviruses (50%) and enteroviruses (48%). The rhinoviruses and Hepatitis A virus form the most distinct group with 40% GC (Palmenberg, 1987a).

The genomes of these viruses have single open reading frames (ORFs), coding for between 2,150 amino acids for Rhinovirus 2 and 2,332 amino acids for FMDV (Palmenberg, 1987a). The coding sequence can be divided into three regions, the capsid proteins (P1), non structural proteins (P2), and replicase complex proteins (P3) (Rueckert, 1985). The remaining part of the genome consists of the 3' -UTR, which varies from 42-126 nucleotides in length. Small insertion(s) in the 3' -UTR result in temperature sensitive mutant(s), indicating the importance of this region in productive infection (Hershey and Taylor, 1987). Similar temperature sensitivity can also result from alteration of the 5' -UTR (Semlar et al, 1986). The genomes, and gene arrangement of several picornaviruses are given in Figure 3.

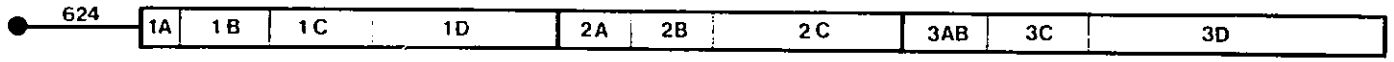
The genomes of picornaviruses are translated into single polypeptides (~247 kdal), which are proteolytically processed by virus specific enzymes (Palmenberg, 1987b). Post translational modifications, such as glycosylation, sulphation, and phosphorylation do not occur in the polyproteins, except for specific myristylation (acetylation) of 1A as discussed above (Urzainqui and Carrasco, 1989).

Figure 3. **GENOME ORGANIZATION OF PICORNAVIRUSES:** Genome organization of poliovirus and other picornaviruses are shown. At the 5' end of the vRNA, VPg is covalently attached (represented as ●). Numbers above the lines show the length of the 5' -untranslated region (UTR), and under the line "CCCC" represents a poly (C) tract in the 5' -UTR, which is only found of cardiovirus and aphthovirus. Leader sequences (L) are also characteristic in cardioviruses and aphthoviruses genomes. The systematic numbering of the products is given as per L-4-3-4 nomenclature (Rueckert and Wimmer, 1984). (Modified from Paul et al, 1987)

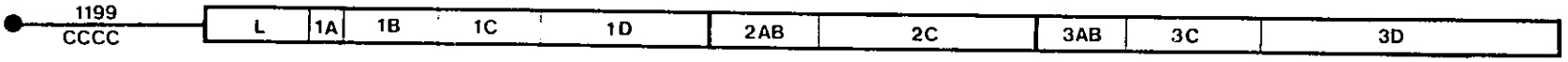
POLIO 1. COXSACKIE



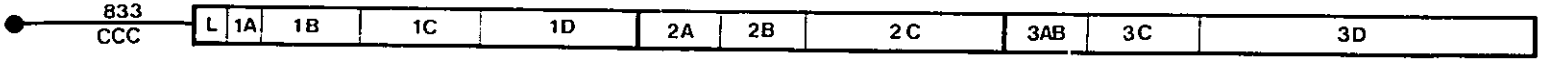
RHINO 14



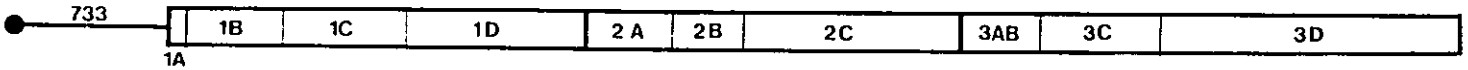
FMDV



EMC. MENGO



HEPATITIS A



The post translational cleavages, which separate all genome products, take place in three steps;

a. **Primary cleavage** : separation of the capsid precursor from the growing polypeptide chain

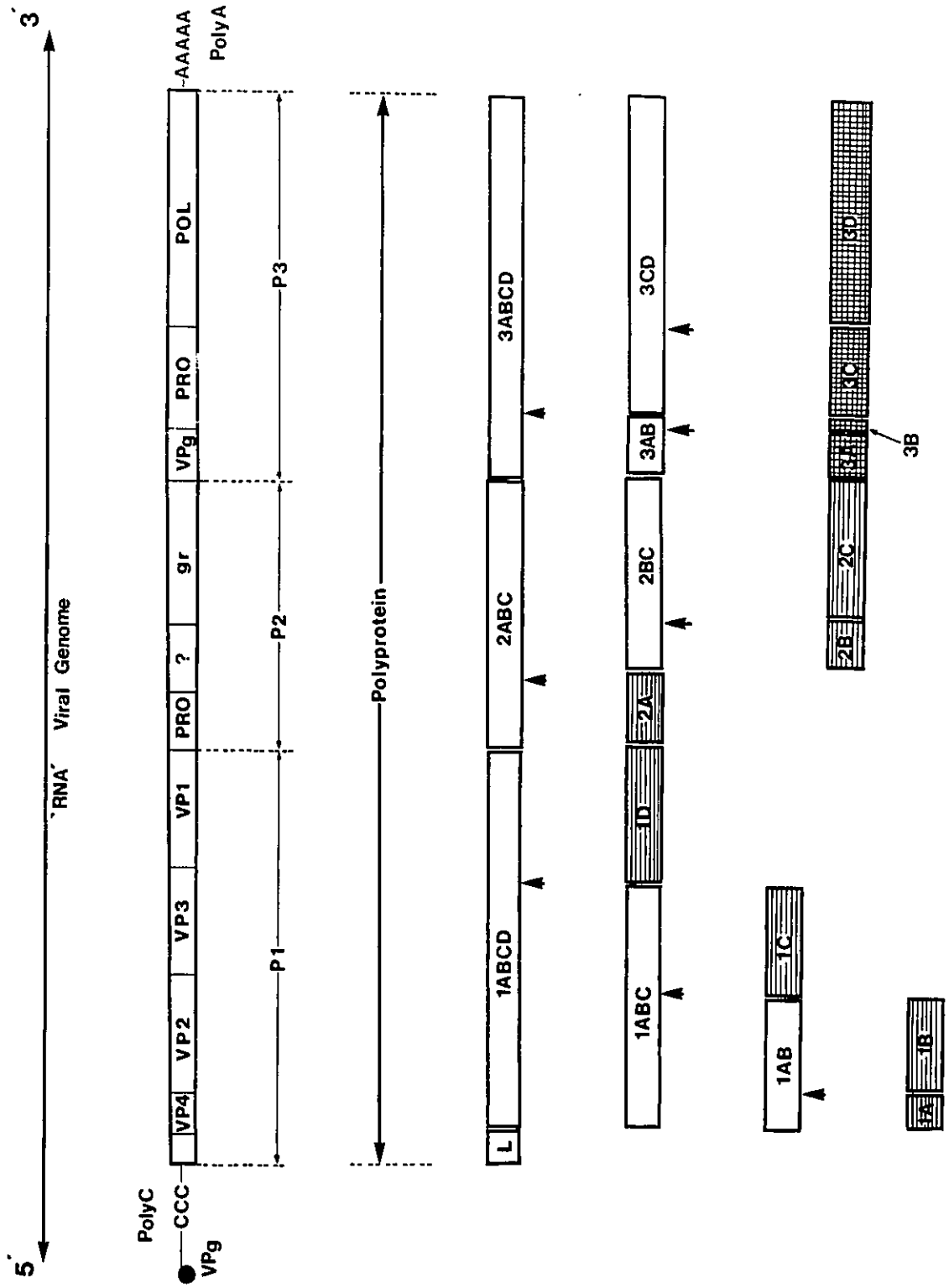
b. **Secondary cleavages** : separation of structural and non structural proteins

c. **Tertiary cleavage(s)** : maturation of virus particles (virions).

In poliovirus, three types of protein cleavages have been identified at three different pairs of amino acids, i.e. Glutamine-Glycine (Q-G), Tyrosine-Glycine (Y-G), and Asparagine-Serine (N-S). It is believed that each cleavage is mediated by the enzymatic activity of a specific protease (Wellink and van Kammen, 1988; Krausslich and Wimmer, 1988). The cleavage patterns for picornaviruses are given in Figure 4.

The primary and secondary cleavages direct the assembly of the virus particles whereas tertiary cleavage(s) result in maturation of the virus particles (Palmenberg, 1987b). In enteroviruses and rhinoviruses, primary cleavage occurs between 1D and 2A, resulting in the release of the P1 precursor protein. Truncation, linker insertion or deletion mutagenesis, in enteroviruses and rhinoviruses, has shown that changes in region 2A block the primary processing of the polyprotein (Krausslich and Wimmer, 1988). The primary cleavages in aphthoviruses and cardioviruses, however, occur between 2A and 2B. It is believed that the L sequence, translated from the 5' end of the genome, has some protease activity which works to release primary

Figure 4. **PROTEOLYTIC PROCESSING OF PICORNAVIRUS POLYPROTEINS:** A systematic representation of the genomes and gene products of picornaviruses is shown. VPg is attached to the 5'-end of the genome and a poly(C) tract is present in cardioviruses and aphthoviruses. The gene arrangement of a typical picornavirus (old nomenclature) is at the top. P1 encodes the structural proteins, P2 and P3 produce non-structural proteins. The arrows indicate cleavage sites. Intermediates of proteolysis are represented with open boxes. Mature products are shown by filled boxes. "L" is the leader sequence found in cardioviruses and aphthoviruses (Palmenberg, 1987).


5'
RNA
Viral Genome
3'
PolyC
CCC
VPg
VP4
VP2
VP3
VP1
PRO
?
gr
VPg
PRO
POL
AAAAA
PolyA
P1
P2
P3
Polyprotein
L
1ABCD
2ABC
3ABCD
1ABC
1D
2A
2BC
3AB
3CD
1AB
1C
2B
2C
3A
3C
3D
3B
1A
1B

products (Sangar et al, 1988; Nicklin et al, 1987). Specific enzyme inhibitors have shown that $2A^{pro}$ is a sulfhydryl protease (Krausslich and Wimmer, 1988).

All Picornaviruses produce $3C^{pro}$, a protease encoded in the P3 region, which carries out most of the proteolytic cleavages. In poliovirus, 8 out of 9 cleavages are carried out by this enzyme (Nicklin et al, 1987). The $3C^{pro}$ cleavages in polioviruses and rhinoviruses occur between amino acids in the pair Q-G (Palmenberg, 1987b). Amino acid substitution of serine (S), threonine (T), alanine (A), valine (V), or methionine (M), for glutamine (Q), does not interfere with the activity of $3C^{pro}$ protease (Wellink and van Kammen, 1988; Krausslick and Wimmer, 1988)· Protease $3C^{pro}$ has been classified as a cysteine protease, and placed within the superfamily of papain-like proteases (Palmenberg et al, 1984).

The asparagine-serine (N-S) tertiary cleavage (between 1A and 1B) results in the process of maturation of the virus particles. In most virion preparations, capsid proteins contain traces of uncleaved 1AB (Rossmann, 1987). Empty capsids isolated from infected cells always contain intact 1AB, but no 1A (Nicklin et al, 1987). The enzyme responsible for this N-S cleavage has not been identified. Crystallographic resolutions and secondary structure predictions suggest that the cleavage site of the peptide 1AB is analogous to the recognition site for serine proteases. The proton acceptor function required for this type of catalysis is fulfilled by vRNA replacing histidine, which is usually present at the active site in these enzymes (Palmenberg et al, 1987b; Rossmann et al, 1985).

1.6: EVOLUTION IN RNA VIRUSES:

Genetic material, DNA or RNA, has two major mechanisms by which it can evolve into new genotypes. Point mutation(s) can result in progressive evolution of the genetic material, and a much faster and efficient way of adaptive evolution could result by recombination (Goldbach and Wellink, 1988; Birky and Walsh, 1988). Accumulation of single base change(s) has become an accepted model for the modulation of RNA genomes in natural environments. Classical studies with Qβ bacteriophage and with TMV have suggested that the virus replicase complex is the primary culprit for this high mutation rate (Zimmern, 1988). Comparisons of the mutation frequency in RNA viral genomes with mutation rates for DNA has shown that rate for RNA genomes is at least a million times higher than for DNA genomes (Holland et al, 1982). It has also been postulated that genome size and mutation rate are inversely related, i.e., the larger the genome, the lower the mutation rate and vice versa (Zimmern, 1988). The high rate of point mutation in RNA genomes also suggests that there is microheterogeneity (quasispecies) in RNA viruses. Such variation in the virus population may not necessarily be responsible for alteration of phenotype. However, such changes may be silent within a population for a certain period of time but can become advantageous in following generations. Further, population genetics has also shown that disadvantageous changes can be removed from the population over several replicative cycles and some consensus sequence could be reached (Zimmern, 1988). The selective pressure exerted on a particular region of a genome may result in modular evolution which could carry a particular region in a

divergent or convergent path (Goldbach and Wellink, 1988). There is evidence for strong selective pressure on viral products involved in replication (Zimmern, 1988). For example, the polymerases of positive stranded RNA viruses show much more conservation of sequence than other regions (Strauss and Strauss, 1988). This high degree of conservation is the result of tremendous selective pressure exerted on the genomes to retain polymerase function. The conservation of polymerase gene sequence similarity is unmatched in other proteins of this group of viruses.

Genetic recombination, another mode of genetic change, in RNA viruses is the process by which parental information can be exchanged between two virus genomes. This results in new and unique genetic information (King, 1988a). RNA recombination was first observed in picornaviruses, about 20 years ago, and has proven to be the model for other viruses (King, 1988b).

The crossovers in genetic material can occur in homologous as well as in non-homologous regions (King, 1988a). In the first model, the two sequences align at homologous regions and with the help of enzymatic breakage and joining events two heterologous genomes are formed (King, 1988b). This type of recombination is analogous to DNA recombination. In RNA viruses this is rarely seen. In a recently proposed model, recombination in positive stranded viruses occurs by a mere switch in the template during replication by RNA polymerase. This is also referred to as the "Copy Choice" model for recombination (Blinov et al, 1986; Kirkegaard and Baltimore, 1986).

In picornaviruses, the frequency of recombination is high between viruses of the same strain, and crossover sites are located at many different loci all along the genome (Kirkegaard and Baltimore, 1986; Blinova et al, 1986). Intertypic recombinants of Poliovirus 3 and Poliovirus 1 recombinants have shown that crossover regions are predominantly located in the 3' conserved half of the genome (Tolskaya et al,1987; Agut et al, 1987; King et al, 1987). The interesting work of Kirkegaard and Baltimore (1986), using two markers separated by 189 nucleotides, has shown that the size of crossover sites can be as small as 2 nucleotides or as large as 32 nucleotides. It has been speculated from this and other work, that regions surrounding the crossover sites may play a role in recombination between genomes. The role of such sequences in crossovers is reinforced by the fact that there is always a high degree of predicted secondary structure within or near recombinational sites (Blinova et al, 1986; Wilson et al, 1988; Kirkegaard and Baltimore, 1986). However, the significance of secondary structure, and its importance in crossover is not fully comprehended (King, 1988b; Blinova et al, 1986). Interestingly, the free energy changes that accompany template switching during recombination (for the recombinants originally characterized by Kirkegaard and Baltimore, 1986), suggest that the recombination events are energetically favourable (29 kcal or less) (King, 1988a).

In poliovirus, it has been estimated that 1.2% of the genome takes part in inter- or intratypic recombination with frequencies of $2x10^{-4}$ to 10^{-5} recombinants/cycle of replication. In FMDV about 5% of the genome may be involved in recombination (King, 1988b).

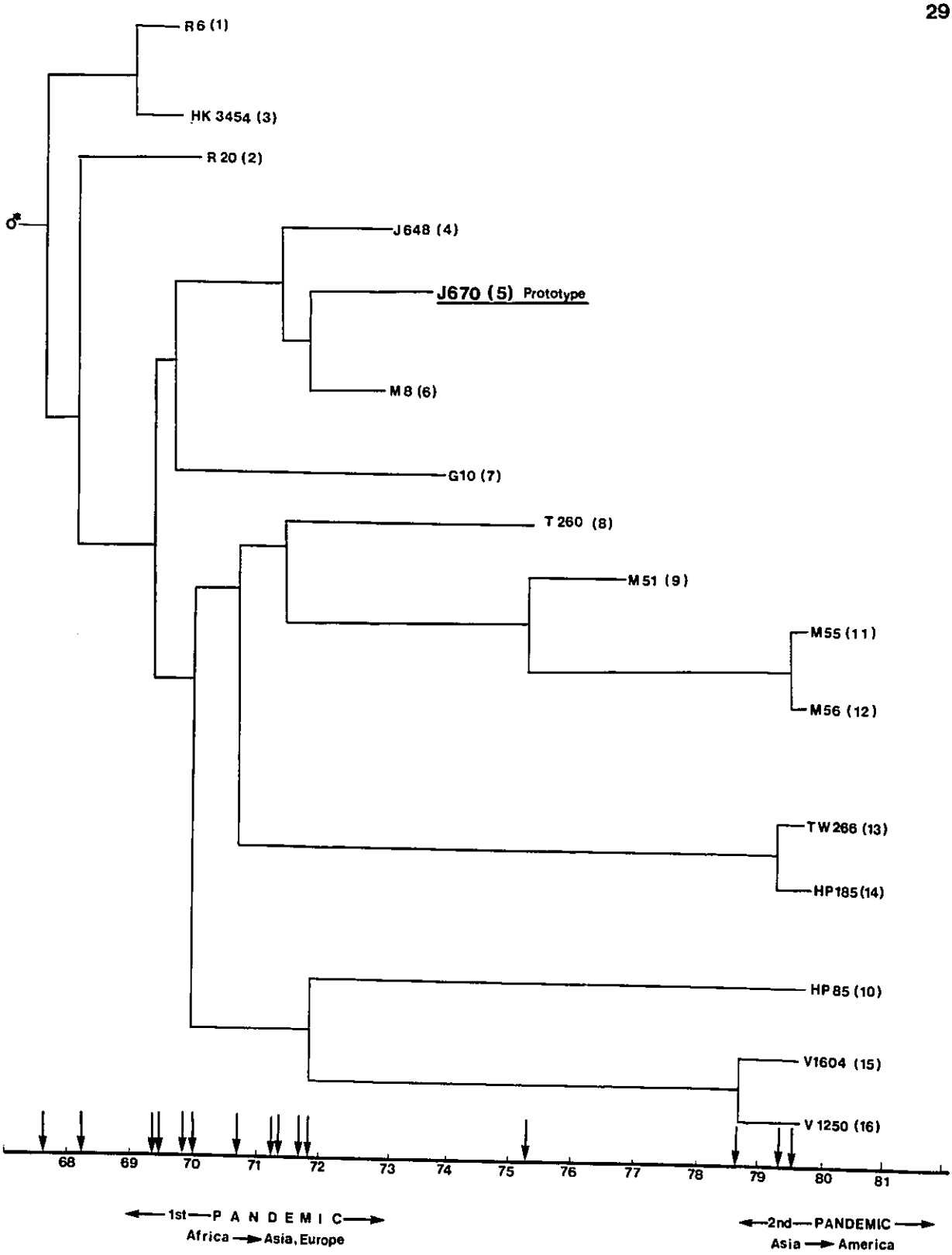
1.7 : MOLECULAR BIOLOGY AND EVOLUTION OF ENTEROVIRUS 70:

The evolution of new viruses which can cause high morbidity and mortality in humans is a poorly understood phenomenon. In recent history, two such examples stand out because of their efficient spread in communities: Human Immunodeficiency Virus, which has caused fear in the general public; and the relatively unfamiliar AHC viruses, which cause benign infections of the eye (Steinhauer and Holland, 1987). In both cases the origins of these agents are obscure.

Serological analysis has shown that neutralizing antibodies specific for Enterovirus 70 were not present in the human population before 1969 (Kono et al, 1981b). In another study, sera from a number of animals prior to the first epidemic, showed low levels of reactivity to Enterovirus 70 proteins (Kono et al, 1981a; Sasagawa et al, 1982; Minami et al, 1981).

Serological studies with different isolates of Enterovirus 70 (1971-1981) have shown that the neutralization epitopes differ (Kawamoto, 1980; 1979; Hierholzer and Hatch, 1985). The T_1 finger prints have shown that all 16 isolates of Enterovirus 70 were derived from a common ancestor (Figure 5) which emerged in West Africa around 1967 (Takeda et al, 1984; Kew et al, 1983; Miyamura et al, 1986). However, the origin of this virus, and its adaptation to become a human pathogen in such a way that it can cause pandemics is not understood. Above all, the biological properties associated with this infectious agent are such that they make it unique among picornaviruses. For example, Enterovirus 70 :

Figure 5. **PHYLOGENETIC TREE OF ENTEROVIRUS 70:** The phylogenetic tree of Enterovirus 70 was constructed using the UPGMA (Unweighted Pair Group Method Using Arithmetic Average) method. On each branch the isolate number is given and in parentheses the order of the isolation. Enterovirus 70 (J670) is considered the prototype. The star indicates the hypothetical ancestral strain. On the X-axis, arrows represent divergence from the parent strain on a time scale (years). The first and second pandemics are marked below the time scale, as is the route of spread from Africa → Asia and Europe and from Asia → Americas (Tanimura et al, 1985).


R6 (1)
HK 3454 (3)
R20 (2)
♂*
J648 (4)
J670 (5) Prototype
M8 (6)
G10 (7)
T260 (8)
M51 (9)
M55 (11)
M56 (12)
TW266 (13)
HP185 (14)
HP85 (10)
V1604 (15)
V1250 (16)
68
69
70
71
72
73
74
75
76
77
78
79
80
81
1st PANDEMIC
Africa → Asia, Europe
2nd PANDEMIC
Asia → America

1. can cause pandemics (Hierholzer and Hatch, 1985),
2. can cause an acute infection of the conjunctiva,
3. can infect a wide range of primate and non-primate cells in vitro (Yoshii et al, 1977).

Enterovirus 70 is also a temperature sensitive virus, like rhinoviruses, and does not replicate at 39 ^{o}C (Miyamura et al, 1976; Melnick, 1985). At this nonpermissive temperature the shutdown of cellular macromolecular synthesis, typical of picornaviruses, is complete (Esposito et al, 1976; Miyamura et al, 1984), however, the highest yield of infective virus is obtained at 33 ^{o}C (Miyamura et al, 1974; Miyamura et al, 1978; Takeda et al, 1982).

Interestingly, the microheterogeneity seen in the neutralization epitopes of the Enterovirus 70 isolates, discussed above, is not like the classic case of Influenza virus A, where only one pandemic strain in usually identified (Gibbs, 1987; Buonagurio et al, 1986). Within one pandemic a number of different sero-strains of Enterovirus 70 have been identified throughout the world (Miyamura et al, 1986; Gibbs, 1987). The divergent nature of Enterovirus 70 was also demonstrable from the T_1 RNase fingerprinting (Takeda et al, 1984; Miyamura et al, 1986; Natori et al, 1984). These experiments have shown that about 320 nucleotides changed over an 11 year period. The evolutionary rate of Enterovirus 70 genome was calculated to be 1.83×10^{-3} substitutions per year per nucleotide, which is extraordinarily high compared to most other viruses (Miyamura et al, 1986). This rate of evolution is comparable to the rates seen for Human Immunodeficiency Virus (HIV)

(Steinhauer and Holland, 1987) and Influenza A viruses (Buonagurio et al, 1986; Gibbs, 1987; Li et al, 1988; Hope-Simpson and Golubev, 1987).

Hybridization results obtained using a number of enteroviruses, including Coxsackievirus A24, show that the Enterovirus 70 genome has unique sequences (Hyypia et al, 1987; Kew et al, 1983; Natori et al, 1984). A similar conclusion was drawn earlier when this new virus was initially characterized using standard pooled sera for enteroviruses (Mirkovic, 1974).

These unique features of Enterovirus 70 are of great interest. A number of investigators have tried to explain the biological properties and the origin of this virus (Kono et al, 1981a; 1981b). It can be considered that the progenitor of Enterovirus 70 may have been :

a. a non pathogenic human virus which became a human pathogen,

b. an animal enterovirus which has adapted to humans,

c. an insect picornavirus which has adapted to humans.

Possible mechanisms which could explain the evolution of Enterovirus 70 are :

a. accumulations of point mutations,

b. a more drastic event, like recombination,

c. a combination of the two.

From the above discussion, it is clear that Enterovirus 70 is a good model for evolutionary studies. The well documented histories of the isolates and the recent origin of Enterovirus 70 provide major advantages over most other viruses.

Analysis of Enterovirus 70 proteins (Anweiler, 1988) was not directed to the question of the origin of Enterovirus 70. The evolution

of this new virus needs a more sophisticated approach, such as nucleotide sequencing, to scrutinize its molecular origin(s). The molecular biology of Enterovirus 70, in light of this technology, will probably unravel the puzzle of this virus. The data gathered from these analyses will enable us to determine its origin(s) and its relationship to other picornaviruses.

OBJECTIVES

The major objectives of this study are :

1. To develop a protocol for large scale propagation and purification of Enterovirus 70,
2. To construct a genomic cDNA library of Enterovirus 70,
3. To determine the nucleotide sequence of Enterovirus 70 cDNA,
4. To determine the relationship of Enterovirus 70 to other members in the family Picornaviridae.

C H A P T E R T W O

MATERIALS AND METHODS

2.1 : CELL CULTURE:

The prototype strain of Enterovirus 70 (J670/71) was propagated in monolayers of the continuous cell line LLC-MK_2 (Flow Laboratories, Rockville, MD) and was used throughout this study. Monolayers of LLC-MK_2 cells were grown in 60 mm or 100 mm diameter tissue culture dishes (Corning Glass Works, Corning, NY) or NUNC^{TM} cell factories (Gibco Canada, Montreal, Quebec). The monolayers were grown in a Shell-Lab incubator (Johns Scientific, Toronto, Ontario) at 37 ^{o}C in presence of 5% CO_2. Cells were provided with essential nutrients from Earle's Minimum Essential Medium (MEM; cat.# 410-1700EG, Gibco) supplemented with 5% Fetal Bovine Serum (FBS; Bocknek Organic Materials, Rexdale, Ontario), 0.15% sodium bicarbonate ($NaHCO_3$; Cellgro Mediatech, Washington, D.C.), 2 mM L-glutamine (Cellgro Mediatech) and 50 μg/mL of gentamycin sulphate (Roussel Laboratories, Wembley, England). Monolayers were washed with Tris Buffered Saline (TBS; 137 mM NaCl, 5 mM KCl, 0.7 mM Na_2HPO_4, 5.6 mM glucose and 25 mM Tris-HCl, pH 7.2) twice and then trypsinized with 0.05% Trypsin - 0.02% EDTA (Gibco) for 2-5 minutes at 37 ^{o}C. Monolayers were disrupted by pipetting up and down and cells were recovered by centrifugation (Minifuge, Heraeus Christ, GmbH, Osterode am Harz, West Germany) at 3k rpm for 5 minutes at 4 ^{o}C. Cells were resuspended in complete medium and seeded in petri plates or cell factories. Each cell factory was seeded with cells from 15-20 plates (100 mm) with a split ratio of 1:10. Petri dishes were routinely split at a ratio of 1:5 to 1:10.

2.2 : PROPAGATION OF ENTEROVIRUS 70:

The prototype strain of Enterovirus 70 was kindly provided by Dr. M. Hatch, Centre for Disease Control, Atlanta, GA. The passage history of the virus was not provided. Newly confluent monolayers of LLC-MK_2 were washed twice with either serum free medium or TBS, and infected with virus at a multiplicity of infection (MOI) of 0.01 - 1.0. Adsorption was carried out at 33 ^{o}C for 90 minutes in serum free MEM. After adsorption, medium was removed and replaced with 3 mL of serum free medium and infection was allowed to proceed at 33 ^{o}C. Infections to monolayers in cell factories were carried out without washes. After cytopathic effects were evident, petri dishes, as well as cell factories, were subjected to two cycles of freezing and thawing. Cellular debris was removed by centrifugation (Model J2-21M, Beckman Instruments, Inc., Palo Alto, CA,) in a JA 14 rotor (Beckman) for 10 minutes at 4 ^{o}C and 3k rpm (1380xg). Supernatants from petri dishes were aliquoted and stored at -80 ^{o}C until needed. Later, supernatants from petri dishes and cell factories were concentrated by ultracentrifugation or ultrafiltration.

2.3 : PLAQUE ASSAY:

Newly confluent monolayers in 60 mm petri dishes or 12 well dishes (Corning) were infected with 0.1 mL of serial dilutions of virus stocks in serum free medium. After adsorption, monolayers were overlaid with 3-4 mL of overlay medium; a 1:1 mixture of 2X serum free medium and 1.6% Agarose (# 44302, BDH Chemicals Ltd., Toronto, Ontario). After solidification of the agarose, monolayers were incubated for three days at 33 ^{o}C. Cells were fixed with 10% formal-saline (10% v/v Formalin,

(BDH) in 0.85% w/v NaCl) overnight at room temperature. Agarose was removed and monolayers were stained with aqueous 1.0% (w/v) crystal violet (Fisher Scientific, Ottawa, Ontario) for 2 minutes at room temperature. Plaques were counted, and virus titres were calculated.

Virus Neutralization Assay: Virus neutralization was carried out using sera collected from an individual suspected of having had conjunctivitis in 1986 and a known seronegative individual. Enterovirus 70 (J670/71) positive rabbit hyperimmune serum was kindly provided by Dr. R. Kono, NIH, Tokyo, Japan. Serial two fold dilutions of sera were made. Neutralization was carried out with 100 PFU of Enterovirus 70 for 60 minutes at room temperature and 50 μL of each virus-antiserum mixture was inoculated into freshly prepared monolayers of LLC-MK_2 cells in 96 well plates (NUNC/Gibco). After adsorption, medium was removed and fresh serum free medium was added to each well. Cytopathic effects were observed over the next 3 days.

2.4 : VIRUS PURIFICATION:

2.4.1: RADIOACTIVE LABELLING:

^{3}H-Labelling: LLC-MK_2 cells were infected with Enterovirus 70 at a MOI of 0.1 - 1.0. Ninety minutes post infection, 20 μCi of [5,6-^{3}H]- uridine (45 Ci/mmol; Amersham Canada Ltd., Oakville, Ontario) was added to each plate. In some experiments 5 μg/mL of Actinomycin D (Boehringer Mannheim, W.Germany) was added and ^{3}H-uridine labelling was delayed for an additional hour. Plates were incubated at 33 ^{o}C until complete degeneration of the monolayer was evident.

^{35}S-labelling: Infectious virus was labeled with [L-^{35}S]-Methionine (400-500 mCi/mmol; Amersham Canada Ltd., Oakville, Ontario) in methionine free, serum free media (Flow laboratories, McLean, VA.). The cells were starved for methionine for 60 minutes in serum free medium. Infections were carried out in 3 mL methionine free medium at a MOI of 0.1-1.0, in the presence of 1/30 volume of serum free medium containing methionine. One hour later, 25 μCi of ^{35}S-methionine was added. Plates were incubated until complete degeneration of the monolayer was observed.

2.4.2 : ULTRACENTRIFUGATION:

Pelleting: Clarified supernatants were mixed with SDS to a final concentration of 0.5% and centrifugations were carried out in SW 28 or SW 27 rotors (Beckman) for 4 hours at 24 ^{o}C and 24k rpm (76,314xg). Sometimes a 20% sucrose cushion (5 mL) (Sigma Chemical Co. St. Louis, MO) in 20 mM phosphate buffer, pH 7.2 (Scotti, 1987) was placed at the bottom of the centrifuge tubes to prevent SDS contamination of the pelleting material(s). Medium was removed by suction and tubes were drained well after completion of the centrifugations.

CsCl Gradient: Viral pellets were resuspended in a volume of 100-200 μL of 20 mM sodium phosphate buffer, pH 7.2. Cesium chloride (4.5 mL) was made a with final density of 1.34 g/mL. Resuspended virus preparations were layered over the CsCl-phosphate buffer solution and samples were centrifuged in the SW 50.1 (Beckman) at 40k rpm (149,632xg) and 24 ^{o}C for 22 hours. Fractions (120-180 μL) were collected by bottom puncture. Counts were determined for each fraction

by liquid scintillation counting and fractions containing the virus peak were pooled. Purified virus was pelleted at 35k rpm (149,548xg) and 20 ^{o}C for 3 hours and 30 minutes in the SW 41 rotor (Beckman).

2.4.3 : ULTRAFILTRATION:

After clarification, Enterovirus 70 was concentrated from the culture supernatant by tangential flow ultrafiltration using the Minitan^{TM} unit with type PTHK 100k filters (Millipore Corporation, Mississauga, Ontario) according to instructions provided by the supplier. Supernatants containing virus and concentrated virus were later plaque assayed.

2.5 : RNA EXTRACTIONS:

2.5.1 : TOTAL CYTOPLASMIC NUCLEIC ACID RNA:

LLC-MK_2 cells were infected with Enterovirus 70 at a MOI of 1.0. Mock infected cells were used as negative control. After cytopathic effects in the test plates were evident, cells were harvested and centrifuged in a table top centrifuge (Heraeus Christ, West Germany) at 0 ^{o}C for 5 minutes and 3k rpm. Pellets were washed in 5 mL of TBS and then resuspended in 1 mL of ice cold RNase free 1X TNMS buffer (100 mM Tris-HCl; pH 7.1, (Boehringer Mannheim), 50 mM NaCl (BDH Chemicals), 10 mM $MgCl_2$ (BDH Chemicals), 500 mM sucrose (Sigma Chemical Co., St. Louis, MO) for every 2-3 plates of cells. The suspended cells were mixed with 10 μL each of 25 mg/mL polyvinylsulphate (Sigma Chemical Co.) and 30 mg/mL spermine (Sigma Chemical Co.) for each millilitre of cell suspension. Triton X-100 (Sigma Chemical Co.) and sodium deoxycholate were added to 1% (v/v and w/v respectively) and cells were disrupted by Dounce homogenization. The lysates were

immediately centrifuged at 5k rpm (3024xg) in a JA 20 rotor (Beckman) for 5 minutes at 4 °C. Supernatants were mixed with equal volumes of RNase free 2X NENS (50 mM Na Acetate; pH 5.1 (BDH Chemicals), 10 mM EDTA (Sigma Chemical Co.), 100 mM NaCl (BDH Chemicals), 0.5% SDS (BioRad Laboratories, Richmond, CA)), containing 500 μg/mL of proteinase K (Boehringer Mannheim). After 30 minutes of digestion at 37 °C, the lysates were extracted twice with phenol (BioRad Laboratories) -chloroform-isoamyl alcohol (25:24:1; v/v) (BDH Chemicals) and once with chloroform-isoamyl alcohol (24:1; v/v). Nucleic acids were precipitated overnight with 2.5 volumes of EtOH (BDH Chemicals) and 0.25 M NaAc (Fisher Scientific).

2.5.2 : VIRAL RNA : Purified virus (pelleted) was resuspended in 400μL of 1X NENS buffer containing 200 μg/mL proteinase K and was digested for 30 minutes at 37 °C. RNA was extracted and precipitated as described above in section 2.5.1.

2.6 : cDNA SYNTHESIS:

The RNA pellets were washed twice with 70% EtOH and then dissolved in DEP-H_2O. RNA concentration was determined by absorbance at 260 nm and adjusted to 1 mg/mL. cDNA was synthesized using 5 μg of RNA as template and oligo $(dT)_{12-18}$ as primer, as described in the Amersham cDNA synthesis system (Amersham Canada Ltd., Oakville, Ontario). The cDNA was passed through a Sephadex G50 (Pharmacia LKB Biotechnology, Montreal, Quebec) column to remove unincorporated nucleotide triphosphates. Sepharose 4B (Pharmacia) column chromotography was used to fractionate the cDNA by size (Maniatis et al, 1982). One drop fractions were collected and Cerenkov radioactivity in each fraction

was recorded. Fractions containing the largest fragments of cDNA were pooled and the cDNA was precipitated with EtOH.

2.7 : CLONING

The plasmid pGEM™ 4Z (Promega Corporation, Madison, WI) DNA was kindly provided by Dr. M.P.R. Tenniswood, Department of Biochemistry, University of Ottawa. Library Efficiency™ *E. coli* DH5α cells (Bethesda Research Laboratories, Burlington, Ontario) were used for transformations as per the protocol provided by the supplier.

Plasmid containing cells were grown for Maxi prep (section 2.8.2) and plasmid DNA was used to prepare the vector for cloning. A total of 10 μg of plasmid DNA was digested with the restriction endonuclease Sma I (Boehringer Mannheim) at 30 °C for 1 hour. The 5'-ends were dephosphorylated with calf intestinal phosphatase (Boehringer Mannheim) according to the conditions recommended by the supplier. The fractionated cDNA and dephosphorylated vector were mixed in a molar ratio of 1:5 to 1:10 and ligated with T_4 DNA Ligase according to the conditions suggested by the supplier (Amersham Canada Ltd.) either for 4 hours at room temperature or overnight at 10 °C. Ligation mixes were diluted and used to transform *E. coli* DH5α competent cells. The transformants were screened on L.B. agar (5 g yeast extract, 10 g tryptone (Difco Laboratories, Detroit, MI) 5 g NaCl (BDH Chemicals) and 15 g Bacto-agar, Difco Laboratories, Detroit, MI in 1L) plates containing 50 μg/mL ampicillin. 10 μL of IPTG (stock 200 mM, Boehringer Mannheim, Dorval, Quebec) and 50 μL of a 2% solution of either X-gal (Boehringer Mannheim) or Bluo-gal (BRL) in N, N - dimethylformamide (Sigma Chemical Co.) were spread on each plate and allowed to dry.

Typically 50 - 150 μL of the transformation mix was spread on the agar surface and plates were incubated overnight at 37 °C. Colonies were picked and grown in 2 mL L.B. broth (5 g yeast extract, 10 g tryptone, 5 g NaCl per Litre) or Terrific broth (12 g Bacto-tryptone, 24 g Bacto-yeast extract (Difco Laboratories), 4 mL glycerol (BDH Chemicals) and water up to 900 mL, plus 100 mL sterile solution of 0.17 M KH_2PO_4 (BDH Chemicals) and 0.72 M K_2HPO_4 (BDH Chemicals)) containing 50 μg/mL ampicillin (Boehringer Mannheim). The cells were frozen in L.B. broth containing 50% Glycerol (BDH Chemicals).

2.8 : PLASMID DNA ISOLATION:

2.8.1 : MINI PREP: 2-5 μL of frozen cells were inoculated into 2-5 mL of either L.B. broth or Terrific Broth containing 50 μg/mL ampicillin in 100 mL Erlenmeyer flasks and incubated overnight on an orbital shaker (Lab line Instruments, Inc., Melrose Park, IL). Cells were pelleted in microfuge tubes (Sarstedt Canada, Mississauga, Ontario) for 30 seconds in a microcentrifuge (Fisher Scientific). Medium was removed, cells were resuspended in 50 μL of Solution A (50 mM glucose, 10 mM EDTA, 25 mM Tris-HCl, pH 8.0; Maniatis et al, 1982) and 50 μL of 10 mg/mL Lysozyme (L-6876, Sigma Chemical Co.) prepared in Solution A were added. Cells were left on ice for 5 minutes. 100 μL of Solution B (200 mM NaOH, 1% (w/v) SDS; Maniatis et al, 1982) was added and the samples were mixed gently until the lysate became viscous and clear. After 5 minutes at room temperature, 100 μL of Solution C (60 mL 5 M KAc, 11.5 mL glacial HAc, 28.5 mL H_2O; Maniatis et al, 1982) was added. Samples were incubated on ice for 30 minutes and then samples were centrifuged at 10k rpm in a refrigerated microfuge (Model RC-25,

Savant Instruments, Farmingdale, NY) at 4 °C for 15 minutes. Supernatants were transferred to new tubes and extracted with phenol-chloroform-isoamyl alcohol (25:24:1; v/v) (Maniatis et al, 1982). The aqueous phase was extracted once with chloroform-isoamyl alcohol (24:1; v/v) and nucleic acids were precipitated overnight with 3 volumes of absolute ethanol and half volume of 8 M NH_4Ac (BDH Chemicals). Nucleic acids were pelleted at 10k rpm for 15 minutes, washed twice with cold 70% (v/v) EtOH and dried in a Speed Vac (Savant Instruments). Nucleic acids were redissolved in 50 µL of DEP-H_2O. RNA was digested with 50 µg/mL DNase free RNase A (Pharmacia) for 30 minutes at 37 °C (Maniatis et al, 1982). Samples were analyzed by horizontal gel electrophoresis (Model HE33, Model HE99, Hoefer Scientific Instruments, San Francisco, CA) in 1-1.5% agarose gels (International Biotechnologies, Inc., New Haven, CT) prepared in TBE or TAE buffers (Maniatis et al, 1982). DNA was stained in buffer containing 0.5 µg/mL ethidium bromide (Boehringer Mannheim) and photographed under ultraviolet light.

2.8.2 : MAXI PREP : Cells (5µL) from preserved cultures were inoculated into 1L of L.B. broth or Terrific broth containing 50 µg/mL ampicillin. Cultures were grown for 18 hours on an orbital shaker at 37 °C. Broths were chilled on ice water for 15 minutes and centrifuged in a JA 10 rotor (J2-21M centrifuge) (Beckman) for 20 minutes at 3k rpm and 4 °C. Pellets were washed in 100 mL of SET buffer (100 mM NaCl (BDH Chemicals), 1 mM EDTA (pH 8.0) (Sigma Chemical Co.), 10 mM Tris-HCl (pH 8.0) (Boehringer Mannheim)) resuspended in 9 mL of Solution A (Maniatis et al, 1982). The suspension was transferred to SW 28 polyallomer tubes (Sarstedt Canada Ltd.) and 1 mL of 10 mg/mL lysozyme was added. The

tubes were left at room temperature for 10 minutes with occasional mixing. 10 mL of solution B was added and the contents were mixed well. Tubes were again left at room temperature for 10 minutes with occasional mixing. 10 mL of Solution C was added and tubes were transferred to ice for 30 minutes. The lysates were centrifuged for 25 minutes at 20k rpm (52,954xg) in the SW 28 rotor at 4 °C. The supernatants were transferred to cold Corex™ tubes (Corning Glass Works) and nucleic acids were precipitated with an equal volume of 2-propanol (BDH Chemicals) on ice for 30 minutes and pelleted at 13k rpm (20,442xg) in a JA 20 rotor (Beckman) for 20 minutes at 4 °C. Pellets were dissolved in 2 mL of DEP-H_2O and then an equal volume of 5 M LiCl (Fisher Scientific) was added. Tubes were then allowed to sit on ice for 30 minutes and centrifuged for 20 minutes at 4 °C and 13k rpm in the JA 20 rotor (Beckman). Supernatants were collected and nucleic acids were precipitated with 3 volumes of EtOH for 30 minutes on ice. Pellets were dissolved in 3.5 mL of TE buffer (10 mM Tris-HCl, pH 8.0 (Boehringer Mannheim), 1 mM EDTA, pH 8.0 (Sigma Chemical Co.) and the final volume was adjusted to 3.8 mL. Exactly 4.0 g of CsCl (Boehringer Mannheim) was added. The volume was determined and 80 µL/mL of 10 mg/mL ethidium bromide (Boehringer Mannheim) was added. Occasionally samples were centrifuged at 10k rpm (12,096xg) in the JA 20 rotor for 10 minutes at 4 °C. Supernatants were pipetted into 4.5 mL polyallomer Quick seal™ tubes (Beckman) and centrifuged for at least 14 hours at 50k rpm (220,360xg) in the VTi65 (Beckman) rotor at 20 °C. The supercoiled DNA band was collected (0.5-0.8 mL) and extracted several times with 2-propanol (BDH Chemicals) saturated with 5 M NaCl (Maniatis

et al, 1982). The aqueous phase was diluted to 5 mL with DEP-H_2O and plasmid DNA was precipitated overnight with 3 volumes of EtOH (BDH Chemicals). Plasmid DNA was collected by centrifugation in a JA 20 rotor for 30 minutes at 12k rpm (17,418xg) and 4 ^oC. Pellets were dissolved in 200 μL of DEP-H_2O and precipitated for the second time with 100 μL 8 M NH_4Ac (BDH Chemicals) and 600 μL of EtOH (BDH Chemicals) in microfuge tubes overnight. DNA was pelleted, washed twice with 70% (v/v) EtOH (BDH Chemicals), vacuum dried, and dissolved in DEP-H_2O. The concentration of DNA was determined by absorbance at 260 nm and adjusted to 1 mg/mL.

2.9 : HYBRIDIZATIONS:

2.9.1 : TOTAL CYTOPLASMIC RNA - cDNA HYBRIDIZATION:

Several tenfold dilutions of total cytoplasmic nucleic acids were made. To each dilution, 39 μL of denaturing buffer (25 μL DMSO (BDH Chemicals), 7.5 μL glyoxal (BDH Chemicals), 5 μL 100 mM NaH_2PO_4; pH 7.0 Fisher Scientific), 1 μL 10 mg/mL tRNA (Sigma Chemical Co.) and 0.5 μL 10% SDS (BioRad Laboratories) was added to each sample. Samples were heat denatured at 65 ^oC for 15 minutes. The samples were then dot blotted with the help of Bio-Dot^{TM} apparatus (BioRad Laboratories) onto Zeta probe^{TM} membrane (BioRad Laboratories). The filter was removed and dried at 37 ^oC. Prehybridization was done in 100 mL of 6X SSC (Maniatis et al, 1982), 10X Denhardt's solution (0.2% bovine serum albumin (U.S. Biochemical Co., Cleveland, OH), 0.2% Ficoll 400 (Pharmacia) 0.2% polyvinylpyrolidone (U.S. Biochemicals)), 0.1% heat denatured single stranded salmon sperm DNA (Sigma Chemical Co.), and 0.1% SDS (BioRad Laboratories) at 67 ^oC-69 ^oC. The prehybridi-

zation buffer was removed, and 40 mL hybridization buffer (6X SSC, 5X Denhardt's solution, 0.1% heat denatured single stranded salmon sperm DNA, 0.1% SDS) and radiolabelled single stranded cDNA probe (cDNA synthesis kit; Amersham Canada Ltd.) was added to the buffer. Hybridization was done at 67-69 ^{o}C for 18 hours. Hybridization buffer was carefully poured off and the membrane was washed twice with 200 mL of 2X SSC, 0.1% SDS at 67 ^{o}C for 25 minutes on a rocking platform (type 54131, Heidolph, West Germany). This was followed by two more washes with 2X SSC, 0.1% SDS at room temperature. The four washes were repeated with 0.2X SSC, 0.1% SDS and a final wash was done in water for 5 minutes. Blots were dried and exposed to X-Ray film (Cronex^{TM} 4, Du Pont de Nemours & Co., Wilmington, DE) in cassettes with intensifying screens (Cronex^{TM}, Du Pont)

2.9.2 : PLASMID - RNA HYBRIDIZATION :

Enterovirus 70 RNA (8 μg) was mixed with 1.4 μL of 250 mM Tris-HCl; pH 9.5. The RNA was hydrolysed at 90-95 ^{o}C for 45 minutes. The 5' end labelling reaction was carried with 5 μL [τ-^{32}P]-dATP (3000 Ci/mmol; Amersham Canada Ltd.), 2 μL PK buffer (500 mM Tris-HCl; pH 7.6, 100 mM $MgCl_2$, 50 mM DTT, 1 mM spermidine, and 1 mM EDTA) and 1 μL 5' T_4 polynucleotide kinase (5 U/μL) (Amersham Canada Ltd). After 30 minutes of incubation at 37 ^{o}C, the reaction was stopped with 0.5% SDS and 20 mM EDTA. The 5' end labelled RNA was passed over a Sephadex G50 column and, after Cerenkov counts were determined, it was added to the hybridization buffer.

Plasmid DNA from mini preps were denatured with an equal volume of 4 M NaOH for 15 minutes at room temperature. Each sample was blotted onto Zeta Probe^{TM} membrane (BioRad Laboratories) using the Bio-Dot^{TM} blotting apparatus (BioRad Laboratories). Membranes were removed from the apparatus and air dried at 37 ^{o}C. Prehybridization was carried out in 100 mL of prehybridization buffer at 67-69 ^{o}C overnight. The prehybridization buffer was removed and hybridization was carried out in 40 mL hybridization buffer (section 2.9.1). After 14-24 hours of hybridization, buffer was poured off from the membranes, and they were washed as described in section 2.9.1. Blots were dried and exposed to X-Ray film (Cronex^{TM} 4, Du Pont) using cassettes (Cronex^{TM}, Du Pont).

2.9.3 : CROSS HYBRIDIZATION:

Six of the largest clones were selected as probes in cross hybridization analyses. The plasmids were digested with Eco RI and Hind III and, after gel electrophoresis, the desired fragments were sliced from the gel and elecroeluted using the Unidirectional Electroeluter (Cat. # 15620, IBI, New Haven, CT). Elecroeluted fragments were precipitated directly from 7.5 M NH_4Ac with 3 volumes of EtOH. DNA pieces were recovered and dried in a Speed Vac (Savant Instruments). The radioactive probes were made using the Multiprime Labelling Kit (RPN 1600Y & RPN 1601Z, Amersham Canada Ltd.) with [-^{32}P]-dCTP (3000 Ci/mmol; Amersham Canada Ltd) for 3 hours at room temperature. Unincorporated isotope was removed using spin columns (Maniatis et al, 1982). Cerenkov counts were determined, probes were denatured as per kit instructions, and were added to the hybridiazation buffer.

2.10 : RESTRICTION ENDONUCLEASE ANALYSIS:

Restriction endonuclease digestions were carried out according to recommendations of the suppliers and physical maps were made for each clone.

2.11 : SUBCLONING:

Two clones (pEV70-L and pEV70-122) were chosen for complete nucleotide sequencing. Serial subclones of pEV70-L were made by the method provided in the Erase-a-Base™ kit (Promega Corp.). One end of the digest was protected by adding thio-nucleotides, while the other end was digested with Exonuclease III (Exo III) (Figure 14). The amount of Exo III and the temperature were reduced to 150 U and 35 °C, respectively.

Subclones of pEV70-122 were made by removing fragments from the parental DNA clone and fragments were subcloned in pGEM™ 4Z and pGEM™ 7Zf+ (Promega Corp.) vectors. A total of four subclones were constructed. Sequencing was done on these subclones. Schematic subcloning strategies for both clones are given in Figure 16.

2.12 : DIDEOXY NUCLEOTIDE SEQUENCING:

Typically, 2-3 μg of CsCl-purified plasmid DNA were used for each set of sequencing reactions. The volume of solution was brought to 18 μL with DEP-H_2O and the samples were denatured with 2 μL of freshly made 2 M NaOH (BDH Chemicals)-2 mM EDTA (Sigma Chemical Co.) at room temperature for 5 minutes. Samples were neutralized with 2 μL of 3 M NaAc (BDH Chemicals) and 6 μL of DEP-H_2O were added. Single stranded plasmid DNA was precipitated with 75 μL of EtOH on dry ice for 10 minutes. After centrifugation for 15 minutes at 4 °C at 10k rpm pellets

were washed twice with 70% (v/v) EtOH. Pellets were vacuum dried in a Speed Vac. DNA was dissolved in 6 μL of DEP-H_2O and 2 μL of 5X Sequencing buffer (Sequenase^{TM} sequencing kit, U.S.Biochemicals) and 3 μL (16 ng/μL) of the appropriate primer (SP6 or T_7) were added to the annealing mixture. Annealing was carried out for a minimum of 2 hours at 37 ^{o}C. Extensions and terminations of the reactions were carried out for 10 minutes and 15 minutes, respectively. Double stranded sequencing was also tried with Mini prep DNA using a protocol described in the literature (Aysubel et al, 1987).

2.13 : SEQUENCING GEL ELECTROPHORESIS:

Standard gels were used to separate the products of sequencing reactions as described in the literature (Aysubel et al, 1987; Davies, 1982) with 0.2 mm thick spacers or Wedge spacers (IBI).

Glass plates, 6 mm thick (IBI), were washed with a 1% solution of 7X (Flow Laboratories) and then wiped with EtOH. Plates were immediately dried with Kim Wipes^{TM} (Kimberly-Clark Corporation, Toronto, Ontario). Both plates were coated with a 2% (v/v) solution of dimethyldichlorosilane (BDH Chemicals). Sometimes the small plate was treated with silane A-174 (BDH Chemicals). PAM^{TM} (Boyle-Midway Canada Ltd., Toronto) was also used occasionally. Repcon II^{TM} (Integon^{TM}, Hamilton, Ontario) was also tested for its efficiency to separate the two plates. Plates and spacers were assembled with clamps and were taped (3M Canada Inc., London, Ontario) all around (sides and bottom).

2.13.1 : SEQUENCING APPARATUS AND POWER PACK: The IBI model STS 45 (IBI) sequencing apparatus with thermoplate was used throughout this study. The IBI model MBP 3000 (IBI) power pack was used to

electrophorese the samples. Normally, electrophoresis was done at 50W constant power.

2.13.2 : POLYACRYLAMIDE GEL: A 6% polyacrylamide gel was made by mixing 2.85 g acrylamide, (BioRad Laboratories), 21 g ultra pure urea (ICN Biochemicals, Inc., Cleveland, OH), 0.15 g N,N' methylene-bis-acrylamide (Sigma Chemical Co.), 5 mL of 10X TBE, and 25 mL of sterile water. The solution was filtered through a 0.2 µm, 150 mL disposable Millipore filter (Millipore Industries Ltd., Mississagua, Ontario). 25 µL of N,N,N',N'-tetramethylene-ethylenediamine (TEMED, Sigma Chemical Co.) and 60 µL of 10% ammonium persulphate (BioRad Laboratories) were added and mixed in. The solution was poured immediately using 10 mL disposable pipette (Kimble™, Division of Owens-Illinois, Toledo, OH) by holding plates at a 45° angle. Plates were laid on a flat surface and a shark tooth comb (inverted) was inserted and clamped in place. The gels were allowed to polymerise for 2 hours at room temperature or left overnight. With wedge spacers (IBI) double the volume of acrylamide solution was required for each sequencing gel. Modified sequencing gels were also tried as described by Wang (1988).

Pre-electrophoresis was carried out with 1X TBE running buffer for 30 - 60 minutes. Sequencing reactions were heated for 3 minutes at 90 °C and then loaded in prerinsed wells. Typically, samples were electrophoresed for approximately 4 hours or until the xylene cyanol dye reached the bottom of the gel. A second load of sequencing samples were run for the next two hours. Gels were lifted on filter paper (3MM Chr, Whatman International Ltd., Maidstone, England), covered with all-purpose laboratory wrap (Fisher Scientific Co.) and dried in a gel

dryer (Model 583, BioRad Laboratories) for 45 minutes on the sequencing cycle. The wrap was peeled off and the gels were exposed to X-ray film (Cronex™, 35.6x43.2 cms, DuPont) for 24-48 hours at -80 °C in large cassettes (Cronex1™, Du Pont). X-ray film was developed with GBX developing kit (Kodak Canada Inc., Toronto, Ontario) and the nucleotide sequences were recorded. Gels were also read with the help of an IBI Gel Reader (IBI).

2.14 : COMPUTER /SEQUENCE ANALYSIS:

Throughout this study an IBM Personal Computer (XT) was used for sequence analysis. The DNA program obtained from IBI (IBI) was useful in recording sequences and handling other functions concerning cloning and sequencing. Sequence alignments were done with both the IBI Pustell DNA program™ (IBI) and MicroGenie™ (Beckman) kindly made available by Dr. M.P.R. Tenniswood.

The Protein Data Base, NBRF - PIR™, version #10 (IBI), was used for comparison of protein sequences of picornaviruses.

C H A P T E R T H R E E

RESULTS

3.1 : LARGE SCALE PROPAGATION AND PURIFICATION OF ENTEROVIRUS 70 :

The first objective of this study was to develop methods for large scale propagation and purification of Enterovirus 70. Protocols for propagation of Enterovirus 70 in LLC-MK_2 cells on a small scale were already in use in our laboratory. Enterovirus 70 shows typical enterovirus like cytopathic effects (Rueckert, 1985) which peak 18-30 hours post infection, depending upon the MOI. Lysates obtained from these infections, were titrated by plaque assay. Virus titres were typically $5x10^6$ to $1x10^7$ PFU/mL. This was an increase over previous results. These higher titres were due in part to the reduced volume of the medium used for infection and may also reflect adaption of Enterovirus 70 to LLC-MK_2 cells with passage. The titres, however, were still $\sim10^3$ times lower than reported for some other enteroviruses (Miller et al, 1988; Rueckert, 1985).

Plaque size heterogeneity was routinely observed in this study. The heterogeneity of plaques has been used to categorize different isolates of Enterovirus 70 (Esposito et al, 1974). In this study, the ratio of the small, medium and large plaques was fairly constant in each plaque assay. Virus was not plaque purified because earlier studies have shown that plaque purification did not prevent plaque size heterogeneity (Anweiler, 1988).

The microneutralization assay with Enterovirus 70 showed that the virus particles were neutralized by Enterovirus 70 specific serum as well as by serum from an individual who has recently experienced AHC. The neutralization titres are given in Table 3.

The low virus titres of Enterovirus 70, however, was a major

T A B L E 3

MICRONEUTRALIZATION ASSAY OF ENTEROVIRUS 70

SERUM	TITRE[a]
rabbit[b]	1:160
human[c]	1:16
human[d]	NA[e]

a) dilution at which no cytopathic effects were observed
b) rabbit anti-Enterovirus 70 serum
c) serum from an individual with a history of AHC
d) serum from a known Enterovirus 70 sero-negative individual
e) no neutralizing activity detected

concern at the beginning of this project. Methods previously used for propagation and purification of Enterovirus 70 (Anweiler, 1988) were modified to meet the requirements of large scale application. Enterovirus 70 was propagated in NUNC^{TM} cell factories. Initially, the virus was pelleted from cell lysates by repeated ultracentrifugations lasting 36 hours and then virus was purified on CsCl gradients. Subsequently, ultrafiltration was used to concentrate virus from the cell lysates prior to purification by CsCl centrifugation. Ultrafiltration required considerably less time and effort (2 hours for concentration), and infectious virus recovery was virtually complete (Table 4, Appendix I).

3.2 : RADIOACTIVE LABELLING :

Enterovirus 70 proteins and RNA were labelled with ^{35}S-methionine and ^{3}H-uridine respectively. Radioactive virus was used to trace the virus peak in the CsCl gradients (Figure 6). The refractometric analysis of each fraction showed that virus peak corresponds to 1.37g/cm^3. ^{3}H (Figure 6) and ^{35}S labelled virus (data not presented) showed minimal background.

3.3 : RNA EXRACTIONS :

RNA was recovered from virions, as described in Maniatis et al (1982), and the virus RNA yield was calculated from the UV absorption. Approximately 16 μg of RNA was recovered from 1x10^9 PFUs.

TABLE 4

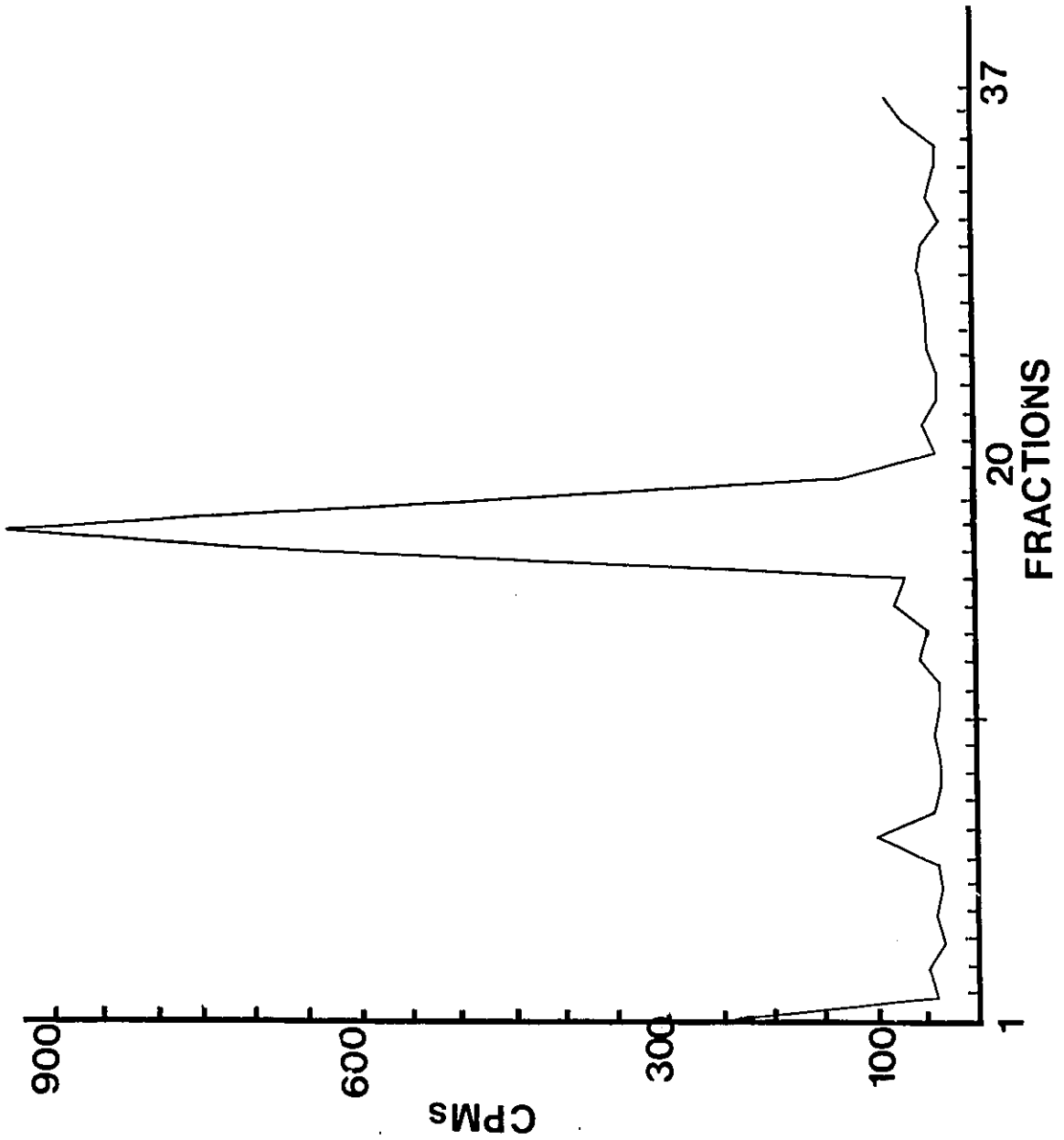
CONCENTRATION OF ENTEROVIRUS 70 BY ULTRAFILTRATION

SAMPLE	VOLUME	PFU/mL	TOTAL VIRIONS
EV70[a]	~550 mL	4.2×10^6	2.3×10^8
EV70[b]	43 mL	2.8×10^7	2.2×10^8
EV70[c]	20 mL	3.2×10^5	6.4×10^6

RECOVERY : $\frac{2.2 \times 10^8}{2.3 \times 10^8} \times 100 = 95\%$

a) clarified supernatant after low speed spin
b) concentrated by ultrafiltration
c) medium retained in the ultrafiltration unit, washed out with MEM

Figure 6. CESIUM CHLORIDE DENSITY PURIFICATION OF ENTEROVIRUS 70: Concentrated virus from repeated ultracentrifugations was mixed with ^{3}H -labelled Enterovirus 70. Virus particles were pelleted at 35k rpm for 4 hours. Pellets were resuspended and layered over a CsCl solution with a density of 1.34 g/mL. Samples were centrifuged for 22 hours at 40k rpm in a SW 50.1 rotor. The gradient was fractionated by bottom puncture and the virus peak was identified by liquid scintillation counting (1μL of each fraction).


900
600
300
100
CPMs
1
20
37
FRACTIONS

3.4 : cDNA SYNTHESIS :

The second objective of this study was to construct a cDNA library of Enterovirus 70 sequences. The genomic RNA was used as a template for synthesis of double stranded cDNA (Gubler and Hoffman, 1983). The cDNA was fractionated using a Sepharose 4B column, and fractions were pooled as shown in Figure 7. The cDNA fractions A-C were used to construct the library.

The specificity of the cDNA was determined by hybridization of single stranded cDNA to total cytoplasmic RNA from Enterovirus 70 infected and uninfected LLC-MK_2 cells. Hybridization results are presented in Figure 8 and show that the cDNA hybridized specifically to RNA from infected cells. Hybridization to large amounts of RNA from uninfected cells suggests a low level of contamination with cellular DNA sequences.

3.5 : CLONING :

Double stranded cDNA was cloned into the Sma I site of pGEM^{TM} 4Z. Recombinant DNA was used to transform E. coli DH5α competent cells. Colourless, ampicillin resistant colonies were selected for further characterization. Theoretical calculations showed that approximately 9x104 clones could have been obtained from 1μg of cDNA. Ampicillin resistant clones were grown in either L.B. broth or Terrific broth and plasmids were analysed for inserts by agarose gel electrophoresis. Fifty clones containing plasmids with the largest inserts were selected for further analysis. The plasmid yield of cells grown in Terrific broth was twice the yield from L.B. broth. Plasmid

Figure 7. **SEPHAROSE 4B FRACTIONATION OF cDNA:** Double stranded cDNA was made using the Amersham cDNA kit and radioactive dATP (3000 Ci/mmol). Unincorporated nucleotides were removed by Sephadex G50 (Maniatis et al, 1982) column chromatography. A Sepharose 4B column was prepared in a 1mL disposible plastic pipette with a siliconized glass wool plug. The column was packed repeatedly with Sepharose 4B until the pipette was full. The column was equilibrated with TE buffer. cDNA was layered on the top and one drop fractions were collected. Each fraction was counted in a scintillation counter.

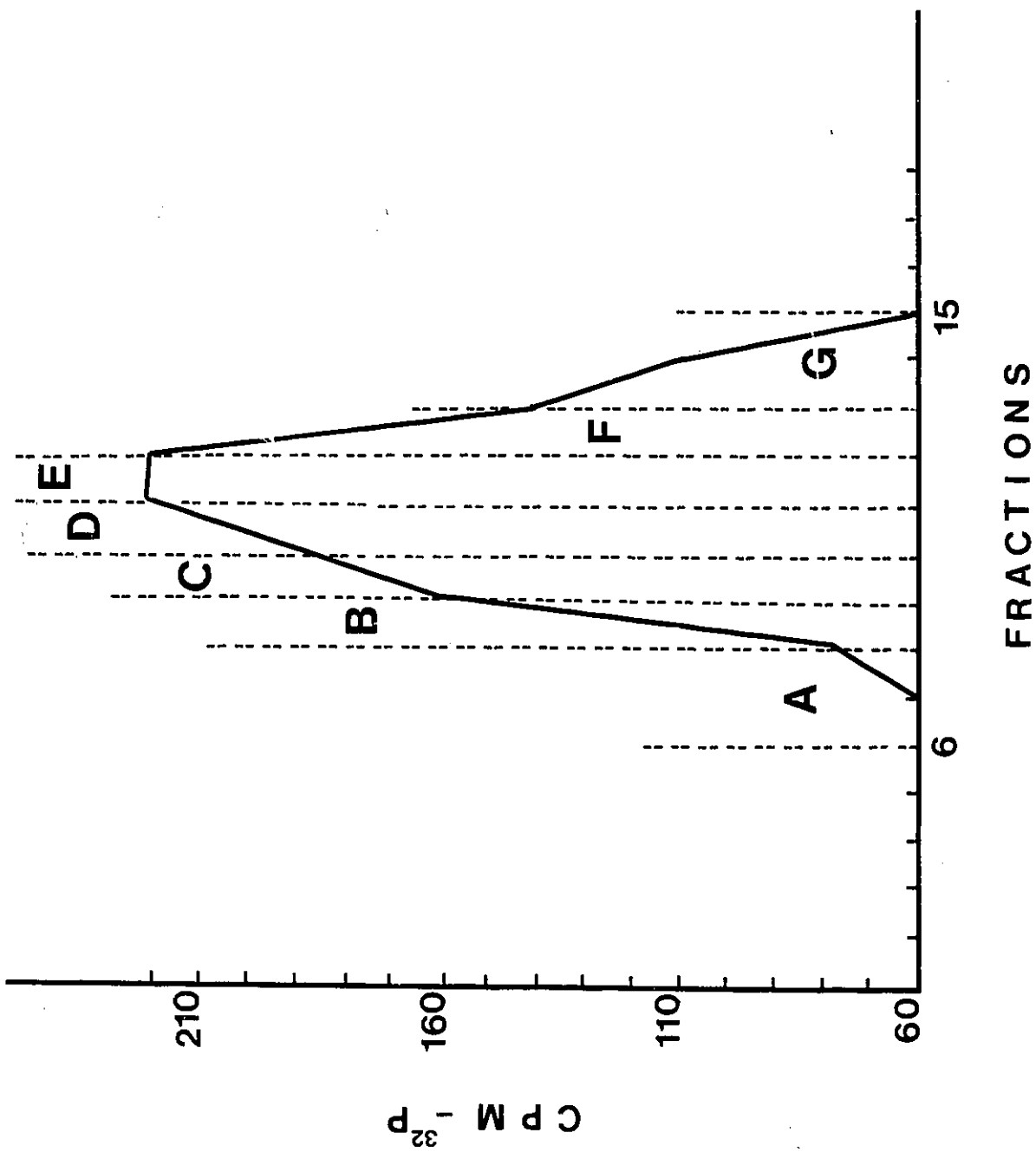
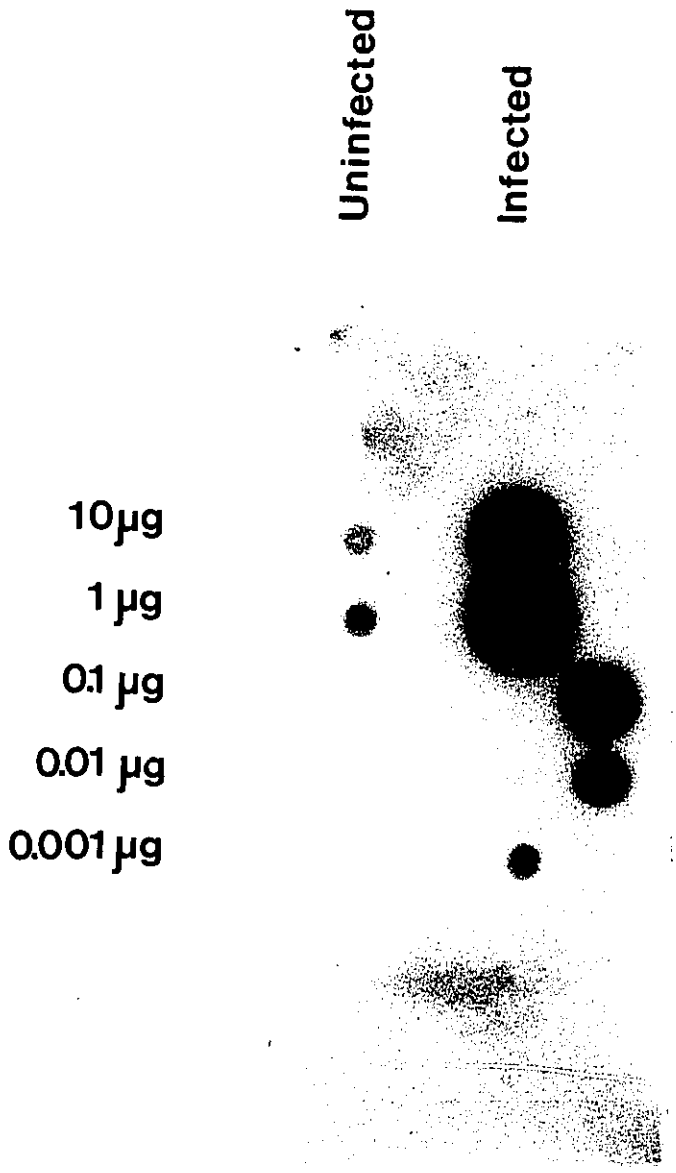

C P M ^{32}P
210
160
110
60
6
15
FRACTIONS
A
B
C
D
E
F
G

Figure 8. **cDNA HYBRIDIZATION WITH TOTAL CYTOPLASMIC RNA FROM UNINFECTED AND ENTEROVIRUS 70 INFECTED CELLS:** Total cytoplasmic RNA was extracted from uninfected and Enterovirus 70 infected LLC-MK_2 cells. RNA samples were treated with glyoxal and were dot blotted onto Zeta probe™. The membrane was dried and prehybridized in 6X SSC, 10X Denhardt's solution, 0.1% salmon sperm DNA, and 0.1% SDS. ^{32}P- end labelled RNA was made using T_4 polynucleotide kinase and was added to hybridization buffer (6X SSC, 5X Denhardt's solution, 0.1% salmon sperm DNA, 0.1% SDS). Hybridization was carried out at 67-69 ^{o}C. The membrane was washed 3 times with 2X SSC, 0.1% SDS and 3 times with 0.2X SSC, 0.1% SDS. The blot was dried and exposed to X-ray film using intensifying screens.


Uninfected
Infected
10µg
1 µg
0.1 µg
0.01 µg
0.001 µg

DNA isolated by the mini prep procedure was subsequently found to be suitable for sequencing.

3.6 : CHARACTERIZATION OF cDNA CLONES :

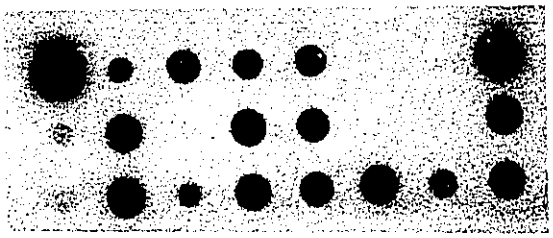
Enterovirus 70 RNA was labelled with polynucleotide kinase and used as a probe to identify clones containing Enterovirus 70 specific inserts by dot blot hybridization. 41 of 50 clones were positive for Enterovirus 70 sequences (82% positive) (Figures 9 and 10). Several clones showed strong positive hybridization signals. Later, these and some other clones were analysed by restriction endonucleases. Insert size, cross hybridizations (data not shown), and restriction endonuclease analysis showed that pEV70-L and pEV70-122 cover about 2,500 nucleotides of the Enterovirus 70 (~34%) genome. Restriction endonuclease analyses, cross hybridization and oligo (dT) hybridization (data not shown) showed that most of the Enterovirus 70 specific inserts were derived from the 3' -end of the genome.

Clones pEV70-L (~1.5 kb) and pEV70-122 (~2.5 kb) cross hybridized with each other but only pEV70-L hybridized with oligo (dT). Restriction endonuclease analysis also suggested a large degree of overlap (Figures 11-13). The relative positions of pEV70-L, pEV70-122 and the third clone, pEV70-15, are shown in Figure 11.

3.7 : SUBCLONING :

The subcloning and sequencing strategy for pEV70-L was devised according to the method of serial Exonuclease III (Exo III) digests (Erase-A-Base^{TM}). Fifteen time points (20 seconds each) were chosen. A decreased quantity of Exo III (1-2 U per μL of reaction mix containing 0.1 μg/μL of DNA) and a lower temperature (35 ^{o}C) achieved expected

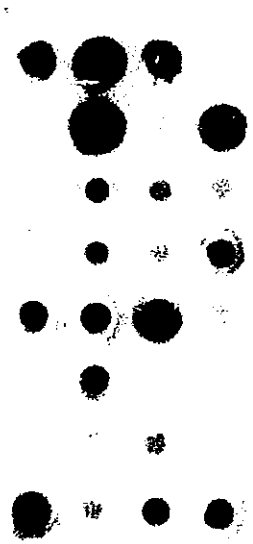
Figure 9. **RNA HYBRIDIZATION OF SELECTED CLONES:** Mini prep DNA from clones with the largest inserts was dot blotted on to Zeta probe™ membrane. Plasmids were denatured on the membrane with 0.5M NaOH. Prehybridization was carried out in 6X SSC, 10X Denhardt's solution, 0.1% salmon sperm DNA, 0.1% SDS. Hybridization was done with 5' -end labelled Enterovirus 70 RNA to identify clones with Enterovirus 70 sequences.



1	30	L
4	31	E_7
7	32	E_{11}
9	34	F_5
14	38	G_7
15	47	pGEM
26	53	Blank
28	63	cDNA

Figure 10. **RNA HYBRIDIZATION OF SELECTED CLONES:** Mini prep DNA from clones with the largest inserts was dot blotted on to Zeta probe™ membrane. Plasmids were denatured on the membrane with 0.5M NaOH. Prehybridization was carried out in 6X SSC, 10X Denhardt's solution, 0.1% salmon sperm DNA, 0.1% SDS. Hybridization was done with 5' -end labelled Enterovirus 70 RNA to identify clones with Enterovirus 70 sequences.

O	64	104	119
2	65	105	122
AB	73	110	126
AE	74	111	128
AF	81	113	133
AG	96	114	pGEM
AK	99	115	Blank
AV	102	118	cDNA



results. Agarose gel electrophoresis analysis of DNA samples showed that about 180-250 nucleotides were removed between each time point. A thionucleotide mix was used to fill in recessed 3'ends was very effective in blocking one end of the DNA (Figure 12).

The subcloning of pEV70-122 was done with four different constructions, which are summarized in Figure 13.

3.8 : DOUBLE STRAND SQUENCING :

Double stranded dideoxynucleotide sequencing (Sanger et al, 1977) was done on clones pEV70-L, pEV70-122 and their subclones (Figure 12 and 13). Initially, DNA polymerase I (Klenow fragment) was used for sequencing, but eventually Sequenase™ (modified T_7 DNA polymerase) became the enzyme of choice. An average of over 400 nucleotides were recorded from sequencing reactions. Sometimes the "I" mix was used to sequence difficult areas.

Modified buffering conditions (Wang, 1988) were useful for further extension of sequencing ladders to 600 plus nucleotides. Wedge spacers, used for the same purpose, did not give us expected results and their use was discontinued. Different procedures for coating glass plates were tested for their efficiency to separate the gel and the plates. Repcon II™ appears to be most effective agent. The complete nucleotide sequence and predicted amino acid sequence of Enterovirus 70 - P3, and 161 nucleotides into 2C is given in Figure 14.

3.9 : SEQUENCE COMPARISONS :

The P3 region and different gene products encoded in the P3 region were compared with the corresponding sequences of other picornaviruses with the help of computer programs. Two different programs showed

Figure 11. **RELATIVE POSITIONS OF ENTEROVIRUS 70 cDNA CLONES:** The length and relative positions of selected cDNA clones generated during this study are shown. Three clones which cover the entire P3 region of the Enterovirus 70 genome were sequenced using Sequenase™. Solid lines represent the cDNA, and open boxes represent plasmid (vector) sequences. Plasmid pEV70-L contains ~100 A residues (poly(A) tail). pEV70-122 does not have a poly(A) tract. The third clone, pEV70-15, has a very short poly(A) tail.

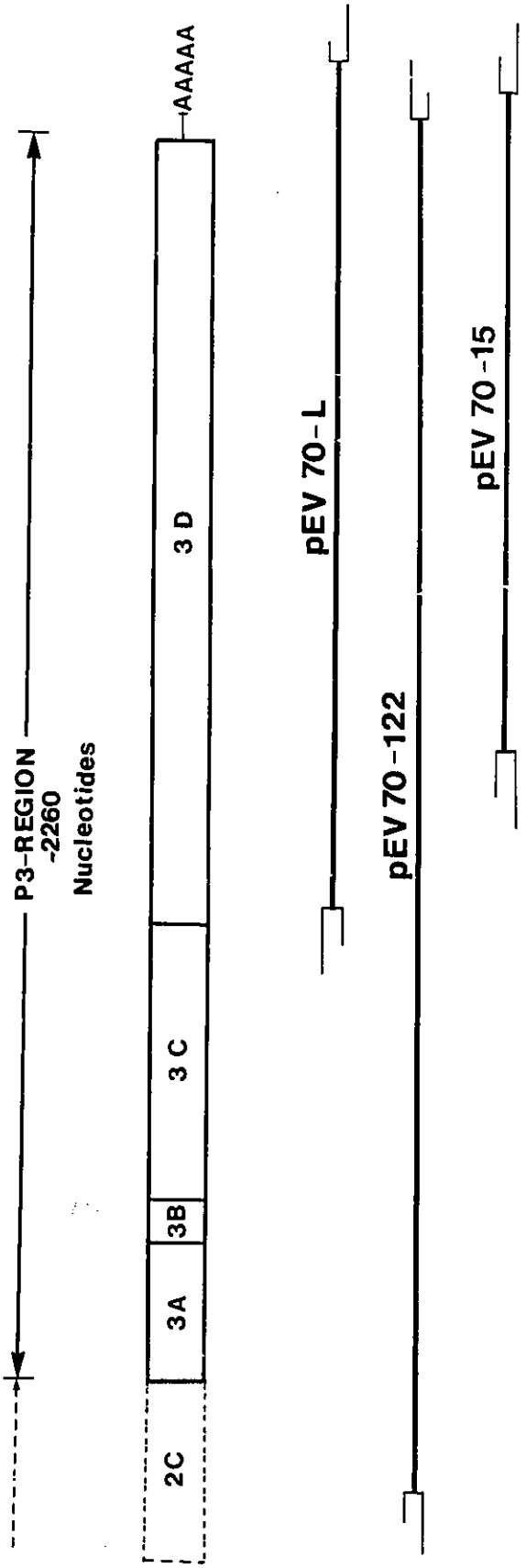
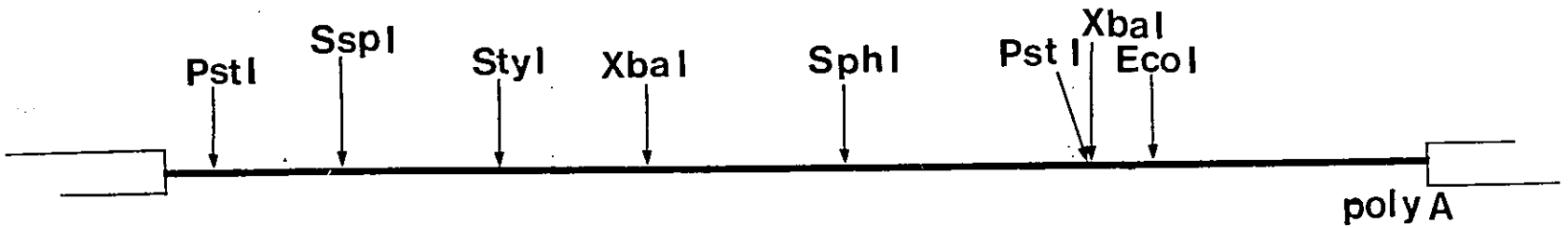

P3-REGION
-2260
Nucleotides
2C
3A
3B
3 C
3 D
AAAAA
pEV 70-L
pEV 70-122
pEV 70-15

Figure 12. **RESTRICTION ENDONUCLEASE MAP AND SEQUENCING STRATEGY FOR pEV70-L.** The positions of restriction endonuclease digestion sites in Enterovirus 70 clone pEV70-L were determined with respect to sites located in the vector (pGEM™ 4Z). Clone pEV70-L was digested to generate serial Exo III nuclease (Erase-a- base kit) subclones. Several clones were used to sequence the pEV70-L subclones (represented in this figure). The gap in the sequencing was determined using clone pEV70-15.



SEQUENCING DIRECTIONS :-

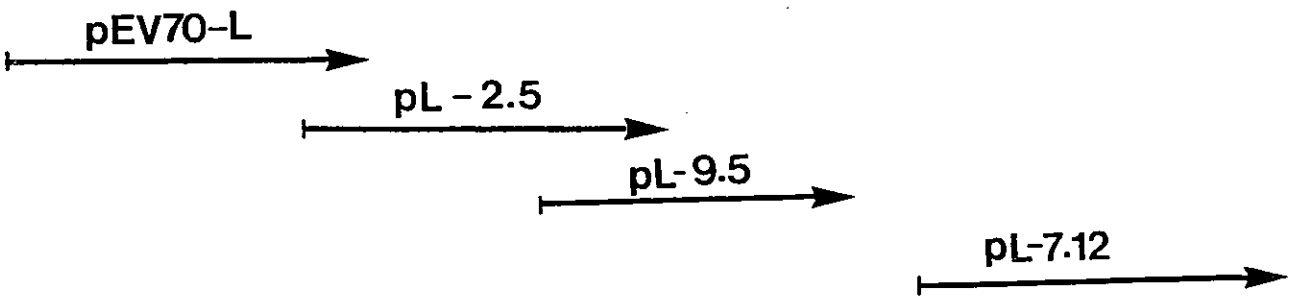
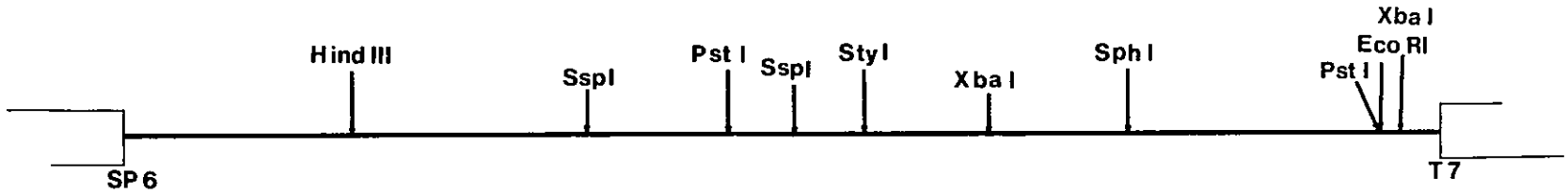


Figure 13. **RESTRICTION ENDONUCLEASE MAP AND SEQUENCING STRATEGY FOR pEV70-122:** The positions of restriction endonuclease digestion sites in Enterovirus 70 clone pEV70-122 were determined with respect to sites located in the vector (pGEM^{TM} 4Z and pGEM^{TM} 7Zf^{+}). Three subclones were constructed. The Pst I - Pst I fragment (one site in the vector) was cloned in pGEM^{TM} 7Zf^{+} (p122-PH-Zf). Later, p122-PH-Zf was used to construct p122-H-Zf containing only the Hind III fragment. The third subclone constructed from the two internal Pst I sites in pEV70-122 (p122-2-4Z). The gap in pEV70-122 which could not be sequenced was covered by clone pEV70-15. The sequencing directions are shown in the figure.



SEQUENCING DIRECTIONS:-

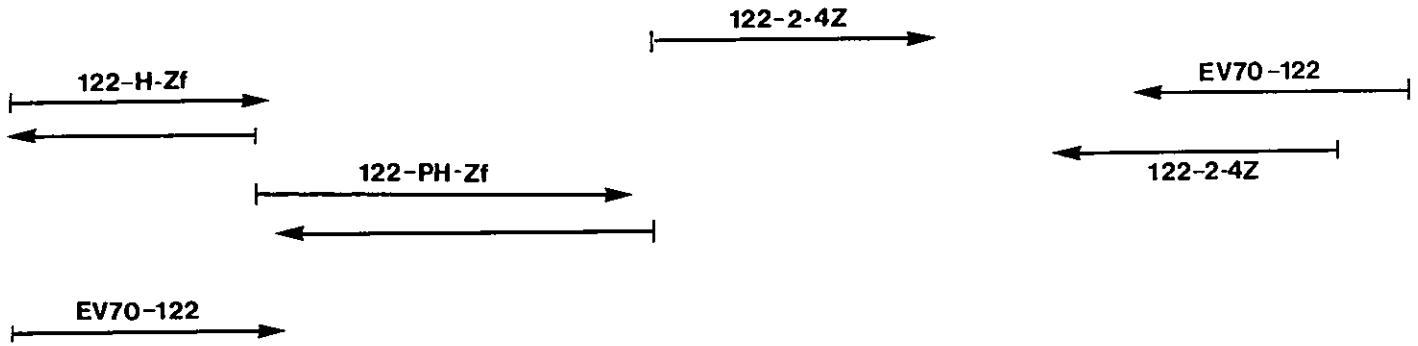
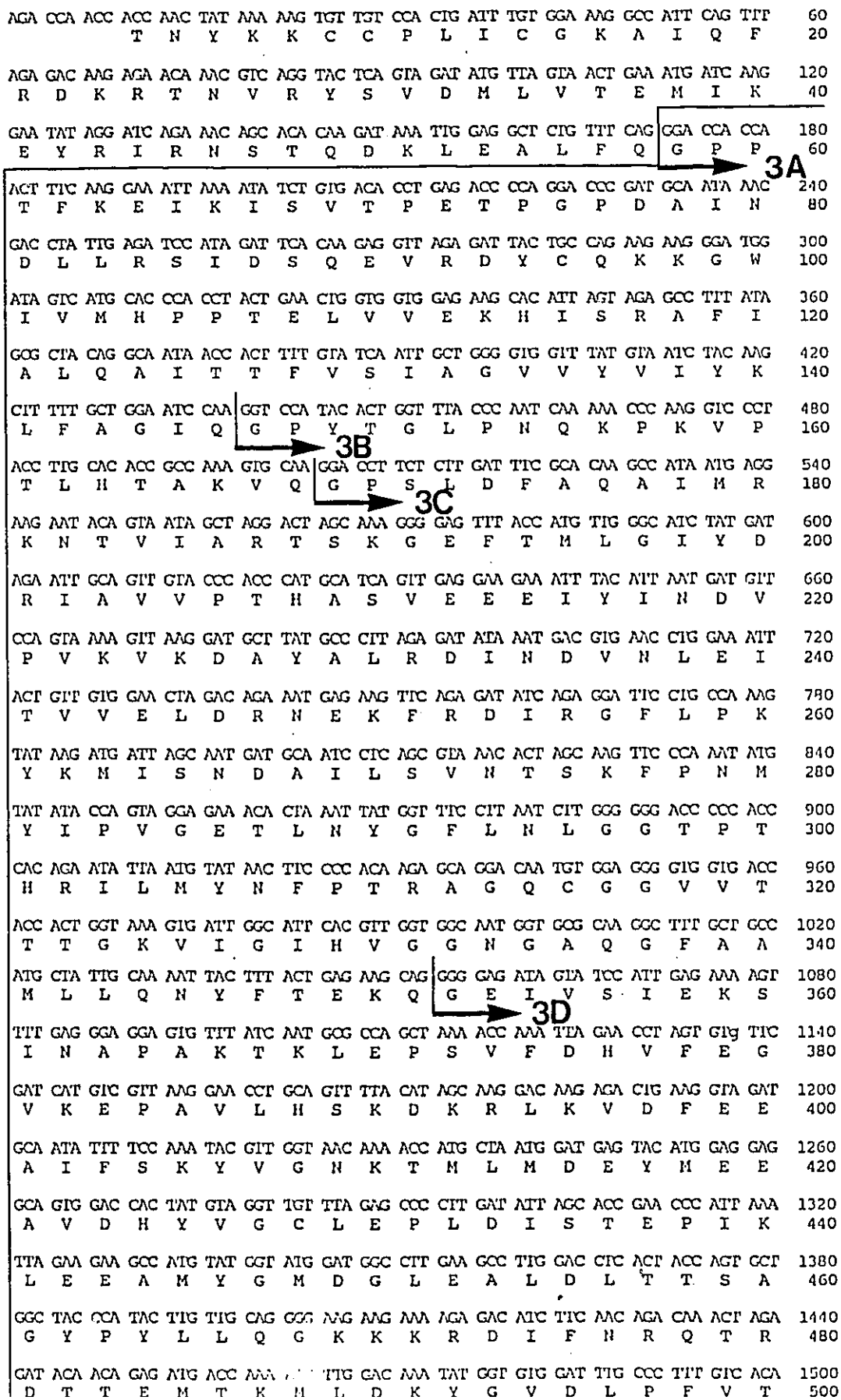


Figure 14. **COMPLETE NUCLEOTIDE AND DEDUCED AMINO ACID SEQUENCE OF THE P3 REGION OF ENTEROVIRUS 70:** Complete nucleotide sequence (codons), and deduced amino acid is presented in this figure. The proteolytic cleavages (from sequence similarity analysis) are marked with vertical line and arrows represents each proteolytic product in the P3 region (boxed) of Enterovirus 70.


3A
3B
3C
3D

```
TTT GTC AAA GAT GAA CTC AGA AGC AGA GAG AAA GTA GAG AAG GGC AAG TCT AGA TTG ATT  1560
 F   V   K   D   E   L   R   S   R   E   K   V   E   K   G   K   S   R   L   I   520

GAA GCT AGT TCT TTA AAT GAT TCA GTT GCC ATG CGA GTC GAG TTT GGA AAC CTT TAT GCA  1620
 E   A   S   S   L   N   D   S   V   A   M   R   V   E   F   G   N   L   Y   A   540

ACA TTC CAC AAA AAT CCA GGA GTC GCC ACT GGG AGC GCT GTT GGA TGT GAT CCA GAT TTG  1680
 T   F   H   K   N   P   G   V   A   T   G   S   A   V   G   C   D   P   D   L   560

TTT TGG TCC AAA ATC CCA GTT ATG TTA GAT GGA AAA ATT TTT GCA TTT GAC TAC ACA GGT  1740
 F   W   S   K   I   P   V   M   L   D   G   K   I   F   A   F   D   Y   T   G   580

TAT GAT GCC AGT CTC TCA CCA GTC TGG TTT GCA TGC CTA AAG AAA ACT CTG GTA AAA TTA  1800
 Y   D   A   S   L   S   P   V   W   F   A   C   L   K   K   T   L   V   K   L   600

GGT TAT ACT CAT CAA ACA GCA TTT GTT GAT TAC TTG TGT CAC TCG GTT CAC TTG TAC AAA  1860
 G   Y   T   H   Q   T   A   F   V   D   Y   L   C   H   S   V   H   L   Y   K   620

GAC AGA AAA TAC ATA GTG AAC GGT GGA ATG CCA TCA GGC TCA TCT GGC ACC AGT ATC TTC  1920
 D   R   K   Y   I   V   N   G   G   M   P   S   G   S   S   G   T   S   I   F   640

AAC ACT ATG ATC AAC AAC ATT ATA ATC AGA ACC CTT TTA CTT AAA GTT TAC AAA GGT ATT  1980
 N   T   M   I   N   N   I   I   I   R   T   L   L   L   K   V   Y   K   G   I   660

GAT TTA GAT CAG TTC AAA ATG ATA GCA TAT GGT GAT GAT GTA ATT GCT AGT TAC CCA CAT  2040
 D   L   D   Q   F   K   M   I   A   Y   G   D   D   V   I   A   S   Y   P   H   680

GAG ATA GAC CCT GGC CTA TTG GCA AAG GCC GGG AAA GAG TAT GGA TTA ATA ATG ACC CCA  2100
 E   I   D   P   G   L   L   A   K   A   G   K   E   Y   G   L   I   M   T   P   700

GCA GAC AAG AGT AGT GGT TTT ACT GAG ACT ACT TGG GAG AAT GTT ACC TTT TTG AAG AGG  2160
 A   D   K   S   S   G   F   T   E   T   T   W   E   N   V   T   F   L   K   R   720

TAT TTC AGA GCA GAT GAA CAG TAT CCC TTT TTG ATC CAC CCA GTT ATG CCA ATG AAG GAA  2220
 Y   F   R   A   D   E   Q   Y   P   F   L   I   H   P   V   M   P   M   K   E   740

ATC CAT GAG TCA ATT AGA TGG ACA AAA GAT CCG AGA AAC ACA CAA GAT CAT GTG AGA TCC  2280
 I   H   E   S   I   R   W   T   K   D   P   R   N   T   Q   D   H   V   R   S   760

TTG TCG CTA CTG GCC TGG CAC AAT GGT GAA GAA ACT TAC AAT GAA TTC TGC AGG AAA ATC  2340
 L   S   L   L   A   W   H   N   G   E   E   T   Y   N   E   F   C   R   K   I   780

AGA ACT GTA CCT GTA GGA AGA GCT TTA GCT TTA CCA GTA TAT TCT AGT TTG AGG CGC AAG  2400
 R   T   V   P   V   G   R   A   L   A   L   P   V   Y   S   S   L   R   R   K   800

TGG TTA GAC TCA TTC TAG ATAACTCTACTTAGGAACCCAAGAACCCCTCCCTGGGGCATTAGATTTTGTAGAG  2464
 W   L   D   S   F   -

GTAAATTTTGGTTAATTGGGAATCAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAA
                                                                          2488
```

similar alignments of the sequences. MicroGenie™ gave overall better results (Tables 4, 5 and 6) because of its greater versatility. The P3 region of Enterovirus 70 was compared, at the nucleotide level (Table 5), with P3 regions of other picornaviruses. Four regions in the Enterovirus 70 - P3 sequences were identified as 3A, 3B(VPg), $3C^{pro}$, and $3D^{pol}$. The individual regions were analysed for their similarities to the corresponding sequences of other members in the family Picornaviridae. The percentage similarity at both the nucleotide and amino acid level are given in Tables 6 and 7.

The overall similarity of the Enterovirus 70 - P3 sequence with P3 sequences of other picornaviruses clearly indicates that Enterovirus 70 belongs to picornavirus group 4 (Palmenberg, 1987) along with other enteroviruses and rhinoviruses (Tables 5-7).

3.10 : SEQUENCE ALIGNMENTS :

The amino acid sequence alignments for 3A, 3B (VPg), $3C^{pro}$, and $3D^{pol}$ are given in Appendix II. Pairwise alignments were done using Pustell™ and/or Microgenie™ programs. The composite alignments were done manually because the available software could not handle all the sequences. Manual and computer alignments matched very well.

T A B L E 5

SIMILARITY OF P3 REGION OF ENTEROVIRUS 70 TO THE P3 REGION OF OTHER PICORNAVIRUSES

(NUCLEOTIDE LEVEL)

GROUP 4:[a]	
POLIOVIRUS 1	64.7%
COXSACKIEVIRUS B3	63.8%
RHINOVIRUS 14	58.9%
RHINOVIRUS 2	53.0%
BOVINE ENTEROVIRUS	60.5%
GROUP 2:[b]	
ENCEPHLOMYOCARDITIS VIRUS	44.6%
GROUP 1:[b]	
FOOT AND MOUTH DISEASE VIRUS	38.4%
GROUP 3:[b]	
HEPATITIS A (ENTEROVIRUS 72)	38%

a) nucleotide alignments were done with Pustell™
b) nucleotide aligements were done with MicroGenie™

T A B L E 6

NUCLEOTIDE COMPARISONS WITHIN P3 REGIONS

	3A[a]	3B[a]	3C[a]	3D[a]	3'-UTR[b]
PUSTELL™					
EV70 : PV1	56%	72%	61%	68%	48%
EV70 : CVB3	60%	65%	63%	67%	31%
PV1 : CVB3	52%	68%	61%	67%	31%
MICROGENIE™					
EV70 : PV1	61%	76%	62%	67%	51%
EV70 : CVB3	62%	68	63%	67%	28%
PV1 : CVB3	58%	58%	61%	68%	31%

a) 3A, 3B, 3C and 3D are proteolytic products from P3 regions
b) 3' -UTR is 3' untranslated regions

T A B L E 7

PROTEIN[a] COMPARISONS WITHIN P3 REGIONS

	3A	3B	3C	3D
PUSTELL™				
EV70 : PV1	58%	77%	60%	75%
EV70 : CVB3	64%	77%	61%	73%
PV1 : CVB3	48%	77%	59%	73%
MICROGENIE™				
EV70 : PV1	58%	77%	61%	73%
EV70 : CVB3	64%	77%	61%	74%
PV1 : CVB3	51%	77%	60%	74%

a) protein comparisons are given in percentage of amino acid identity of aligned sequences

C H A P T E R F O U R

DISCUSSION

The first objective of this study was to develop reliable procedures for large scale propagation and purification of Enterovirus 70. Previously described methods for the propagation, purification, and quantitation of Enterovirus 70 were well outlined by Anweiler (1988). In this study, modifications were made to these procedures to improve the yield of Enterovirus 70 during purification.

Enterovirus 70 is a cytocidal infectious agent in LLC-MK_2 cells. The typical cytopathic effects were rapid, distinct, and similar to those of other enteroviruses; cells become round and more refractile, shrink and show displacement of the cell nucleus. Finally, the cells die and lift from the surface of the culture plate (Rueckert, 1985).

Though the propagation of Enterovirus 70 was not difficult, virus titres were consistently lower than the titres obtained for other enteroviruses (e.g. poliovirus). However, it was noted that, over several virus passages, the titre of Enterovirus 70 did improve as compared to the initial working stock. This improvement in titre could be due in part to the lower volumes of medium used for each infection. In addition to this, Enterovirus 70 may have adapted, with passage in LLCMK_2 cell culture, to give higher yields. A similar phenomenon has been seen with other viruses in cell culture (Miller et al, 1988; Rueckert, 1985). The improved titres of Enterovirus 70 with passage, however, did not reach the titres obtained for poliovirus or some other enteroviruses (Miller et al, 1988; Rueckert, 1985). These low titres were the major obstacle for this project.

To obtain sufficient virions and a good yield of RNA for cDNA

synthesis and cloning, it was decided to propagate Enterovirus 70 in NUNC™ cell factories. The genomic RNA obtained from these factories would be sufficient not only for cDNA synthesis, but also for other related experiments. Initially, culture medium was concentrated by continuous ultracentifugations lasting 36 hours. Results indicated that approximately 50% of infectious virus was lost during these centrifugation steps (Appendix I). Subsequently, the Minitan™ ultrafiltration unit was used to concentrate virus. Ultrafiltration improved recovery and greatly reduced the time taken to concentrate each virus preparation. Recovery of virions from ultrafiltration was 95% (Table 4; Appendix I). Recently, similar results have been reported for HAV (Divizia et al, 1989).

The identity of Enterovirus 70 was confirmed by serological assays. Virus neutralizations were performed with standard Enterovirus 70 specific rabbit anti-serum, obtained from Dr. R. Kono (NIH, Tokyo, Japan), and with sera from an individual who had experienced AHC and from a known sero-negative individual. The presence of relatively low levels (1:32) of neutralizing antibodies in the individual with a history of AHC is similar to observations made in other serological studies (Kono et al, 1981b). Similar findings were made by Kravchenko and co-workers (1985) while preparing diagnostic hyperimmune sera for Enterovirus 70 and two other enteroviruses. It is not clear why Enterovirus 70 does not result in high antiboby titres as other members of this group do.

The density of virions observed in CsCl gradients was consistently slightly higher (1.37g/mL) than previously reported (1.34g/mL) (Kono,

1978). In the literature, various factors have been shown to influence the buoyant densities of virus particles (Scotti, 1985). The fractional increase (0.03g/mL) in the density could be due to the solution used in these gradients. Other possible reasons include error in the refractometer or the presence of contaminating nucleic acids in the purified virion preparation.

After purification of virions, genomic RNA was isolated and used as the template for cDNA synthesis, as described by Gubler and Hoffman (1983). Previous work has shown that the Enterovirus 70 genome is approximately 7.4 kb long (Anweiler, 1988), which is in accord with other picornavirus genomes.

The yield of cDNA was about 20% compared to the starting nucleic acid. Similar results have been achieved by others (Frankel and Friemann, 1987; Rutledge et al, 1988). The preliminary test for specificity of the cDNA showed that cDNA hybridized to total cytoplasmic RNA from infected but not from uninfected cells. Double stranded cDNA was subsequently cloned into the Sma I site of pGEM^{TM} 4Z. The clones were picked and grown in liquid culture medium. Recombinant plasmids were screened by size and then by hybridization with Enterovirus 70 RNA.

Hybridization of selected clones with Enterovirus 70 RNA showed that 82% were virus specific. Of 41 virus specific clones, 5 clones (12%) were positive for oligo $(dT)_{12-18}$ hybridization. It was interesting to note that even after gradient purification of enterovirus 70, 18% of the clones (with inserts) were negative for Enterovirus 70 sequences. A probable explanation for these nonspecific

clones is contamination of virus RNA with cellular DNA. The RNA hybridization results also showed that the cDNA collected from the first few fractions (pool A) of the Sepharose 4B column generated a higher proportion of nonspecific clones. This supports the idea that the density of virions observed in CsCl gradients reflects the presence of cellular DNA (data not presented).

The estimated number of clones ($9x10^4/\mu g$ of cDNA) indicates that the annealing of oligo (dT) and cDNA synthesis were very efficient. However, the low number of oligo $(dT)_{12-18}$ positive clones, on the other hand, suggests that cDNA second strand synthesis was not always complete. This was confirmed by sequence analysis of clones : i) pEV70-122 had no poly (A) tail and the sequence in this clone ends one base before the poly (A) tract; ii) pEV70-L had a very long poly (A) tail (70-100 As).

The Enterovirus 70 specific inserts (41) range from 200 - 2,500 nucleotides long, most between 200 - 1200 nucleotides. Restriction endonuclease analysis, oligo (dT) hybridization and cross hybridizations suggested that most of the inserts represent sequences from the 3' end of the Enterovirus 70 genome.

In this study, dideoxynucleotide sequencing protocols were used to analyse Enterovirus 70 specific clones (Sanger et al, 1977). Initially, DNA Polymerase I (Klenow fragment) was used for sequencing with dideoxynucleotide mixes made in our laboratory. Later, it was decided to switch to Sequenase™ (modified T_7 DNA polymerase). The Sequenase™ kit gave very clean autoradiograms, and the readability was increased to approximately to 400 nucleotides using double stranded plasmid DNA

(Wang, 1988). Modifications in the buffer conditions increased readability a further 200 nucleotides (Wang, 1988; Tsang and Bentley, 1988)).

Sequential subclones of pEV70-L were prepared using a modification of the protocol provided in the Erase-a-Base™ kit. Clone pEV70-122 was digested with several restriction endonucleases and fragments were subcloned into pGEM™ 7Zf+. A gap in pEV70-L was covered by a third clone, pEV70-15. Sequencing was done in both directions to confirm a sequencing ladder. There were 2,475 nucleotides (excluding 70-100 As) which were identified in this study. pEV70-L and pEV70-122 cover the entire P3 region and 162 nucleotides of the P2 region of the Enterovirus 70 genome.

The nucleotide sequence and the deduced amino acid sequence of the P3 region of Enterovirus 70 were aligned with P3 regions of other picornaviruses using two different DNA programs (IBI Pustell program™ and Beckman MicroGenie™). Both programs showed similar alignments. MicroGenie™ software was more useful for two reasons : it introduced gaps into both nucleotide and amino acid sequences which increased the percentage identity in some regions (Tables 4-6); it also allowed for comparisons of similar as well as identical amino acids. Protein alignments were also done manually (Appendix II).

Nucleotide and amino acid sequence comparisons of the Enterovirus 70 - P3 region to the P3 regions of other picornaviruses show that Enterovirus 70 belongs to picornavirus Group 4 (Palmenberg, 1987a). The alignments also indicate that the Enterovirus 70 - P3 region is most like Poliovirus 1 (64.7%) and Coxsackievirus B3 (63.8%) - P3 sequences

(Tables 5 and 6). The comparisons do not allow us to predict whether Enterovirus 70 shows a closer evolutionary relationship to Poliovirus 1 or Coxsackievirus B3. However, the data do suggest that the ancestral sequence to Enterovirus 70 diverged from poliovirus and coxsackievirus sequences at about the same time as poliovirus and coxsackievirus sequences diverged from each other. In the following discussion, by examining specific regions of P3 (divided into 3A-3D and 3'UTR), I will try to answer the question of the evolutionary relationships among these viruses.

The 3' untranslated region (3'-UTR) of the Enterovirus 70 genome is 81 nucleotides long. Comparison with the 3'-UTR of other picornaviruses showed that Enterovirus 70 - 3' -UTR is intermediate in length between the 3'-UTRs of Poliovirus 1 (72 nucleotides) and Coxsackievirus B3 (101 nucleotides). The stop codon is similar in all three viruses. However, the GC content in the 3' -UTR of Enterovirus 70 is more similar to that of Poliovirus 1 and the nucleotide sequence of the Enterovirus 70 3'-UTR aligned best with the sequence of the Poliovirus 1 3'-UTR (Table 5).

The $3D^{pol}$ RNA-dependent-RNA polymerase of Enterovirus 70 has been compared with other picornavirus polymerase sequences. Protein alignments and percentage similarity suggest that Enterovirus 70 $3D^{pol}$ is most closely related to the corresponding region of Poliovirus 1 (75%) (Koch and Koch, 1985). Next in line is Coxsackievirus B3 (73%) (Lindberg et al, 1987). Nucleotide sequence comparisons gives similar results, i.e. 68% similarity with Poliovirus 1 and 67% with Coxsackievirus B3 sequences. The Enterovirus 70 sequence compared to

Rhinovirus 14 and Rhinovirus 2 sequences (Callahan et al, 1985; Skern et al, 1985), shows less homology than to $3D^{pol}$ sequences of the enterovirus subgroup. The length of the Enterovirus 70 sequence, at both nucleotide and amino acid levels, is very close to the length of $3D^{pol}$ of the poliovirus/coxsackievirus subgroup. The cleavage site between $3C^{pro}$ and $3D^{pol}$ is also identical to that of the group 4 viruses (Figure 15).

The protein $3C^{pro}$ is a protease which cleaves the QG pair of amino acids (Palmenberg, 1987a). The Enterovirus 70 3C region contains 549 nucleotides and $3C^{pro}$ is the second largest product of the P3 region of the genome. At the amino acid level the Enterovirus 70 $3C^{pro}$ is 60% similar to the poliovirus protease and 61% similar to the corresponding sequence of coxsackievirus. Nucleotide alignments and similarity showed that Enterovirus 70 $3C^{pro}$ is more closely related to Coxsaxckievirus B3 sequences (63%) than to Poliovirus 1 $3C^{pro}$ sequences (61%) (Table 6).

The 3B (VPg) proteins of different picornaviruses are very similar and align very well (Vartapetain and Bogdanov, 1987). The 3B sequence of Enterovirus 70, at the nucleotide level, is closest to the Poliovirus 1 sequence (75%) (Table 6). In the amino acid alignment, the percent similarity is the same for both Poliovirus 1 and Coxsackievirus B3 (Table 7). The sites for cleavage (between 3A/3B and 3B/3C) are the same in Enterovirus 70 as in group 4 viruses (Figure 15). The size of the peptide and the number of nucleotides in Enterovirus 70 - 3B (VPg) aree also the same as in poliovirus/coxsackievirus sequences.

The gene product of the 3A region of Enterovirus 70 was compared with the corresponding sequences of other picornaviruses from different

Figure 15. **CLEAVAGE SITES IN DIFFERENT PICORNAVIRUSES.** The table represents dipeptides at cleavage sites in the polyprotein of different picornaviruses. In group 4 viruses (poliovirus/coxsackievirus), the cleavage site is QG. Eight of the nine cleavages are carried out by $3C^{pro}$. , tentative, based on published sequence ; possible cleavage site based on protein alignment; possible site based on protein alignment.

	L-1A	1A-1B	1B-1C	1C-1D	1D-2A	2A-2B	2B-2C	2C-3A	3A-3B	3B-3C	3C-3D
EV 70	-							QG	QG	QG	QG
Polio (all)	-	NS	QG	QG	YG	QG	QG	QG	QG	QG	QG
CoxsackieB3	-	NS	QG	QG	TI	QG	?	?	?	?	QG
Hepatitis A	-	AD	QM	QV	QA	QG	QM●/QS	QG●/QS▲	HF●/EG▲	QV●/ES▲	QA●/ES▲
Rhino 14	-	NS	QG	EG	YG	QG	QA	QG	QG	QG	QG
Rhino 2	-	QS	QG	QN	YV	QG	ES	QG	QG	QG	QG
EMC	QG	AD	QS	QG	ES	QG	QS	QG	QG	QG	QG
FMDV A10	GQ●/KG▲	AD	EG	QT	LN	ES	QL	QI	EG	ES	EG
FMDV A12	GQ●/KG▲	AD	VG	QT	LN	ES◆	QL	QI	EG	ES	EG
FMDV O1K	GN●/KG▲	AD	EG	ET	LN	ES◆	QL	QI	EG	ES	EG

groups (Palmenberg, 1987a). The total number of nucleotides in this region is constant i.e. 249 nucleotides. The cleavage sites (2C/3A and 3A/3B) in Enterovirus 70 show that they are consistent with poliovirus and coxsackievirus cleavage patterns (Figure 15). The comparison among 3A genes showed that Enterovirus 70 - 3A is more similar to Coxsackievirus B3 (60%) than to Poliovirus 1 (56%) (Table 6). At the amino acid level a similar relationship was observed (64% similarity with Coxsackievirus B3 and 58% with Poliovirus 1) (Table 7).

The nucleotide and protein comparisons in $3D^{pol}$, 3B, and the 3'-UTR have shown that they are most closely related to Poliovirus 1 sequences. The high level of similarity in $3D^{pol}$, 3B and in the 3'-UTR with Poliovirus 1 sequences suggests that the P3 region of Enterovirus 70 has evolved from poliovirus like sequences. The interactions among the 3' -UTR, $3D^{pol}$, and 3B during formation of an initiation complex for replication support this conclusion (Sarnow, 1989; Flanegan et al, 1987; Paul et al 1987). Potyviruses (plant picorna-like viruses) have recently been classified according to their similarity in 3' -UTR (Frenkel et al, 1989). If this simple classification scheme is applied to picornaviruses, Enterovirus 70 would be grouped with the polioviruses.

For the other two regions, 3A and $3C^{pro}$, the similarity profiles suggest that these regions are more closely related to Coxsackievirus B3 sequences. Scrutiny of these region showed that short stretches of sequence with a high degree of similarity to Coxsackievirus B3 sequences acount for these observations.

Closer analysis showed that at the 3' terminus of 3A, a stretch of nucleotides with 75% similarity (80% at amino acid level) to the corresponding Coxsackievirus B3 sequence is present (Figure 16). At the amino acid level the same stretch has four differences from the coxsackievirus peptide. Sequence comparisons at the amino acid or nucleotide level on either side of this stretch show more similarity to Poliovirus 1 sequences than to Coxsackievirus B3 sequences. The overall similarity in 3A is affected by this region. The pattern of similarity in P3, and in and around this stretch, suggests that this highly conserved sequence has been acquired from the Coxsackievirus B3 genome during the evolution of Enterovirus 70, possibly by recombination.

The $3C^{pro}$ sequence can be divided into three regions. The 5' -end of this gene (240 nucleotides) is more similar to the Poliovirus 1 sequence (58%) than to the Coxsackievirus B3 sequence (57%). The central region (9/30 nucleotides; 30%) is not conserved at the nucleotide level. On the other hand, the 3' -end (68%) of $3C^{pro}$ is more similar to the Coxsackievirus B3 sequence and may be another region of Enterovirus 70 acquired by recombination.

Does this close examination of the Enterovirus 70 - P3 sequence provide any other evidence that would support the idea that recombination has been involved in the evolution of this region of the genome? The middle part of 3C has a high AT content. On either side of the coxsackievirus like sequence in 3A, a similar high AT content is observed. A high AT content, as shown by Hughes and associates (1988), lowers the melting point for RNA secondary structure; it is believed that this is a prerequisite for recombination. Another interesting

Figure 16. **NUCLEOTIDE AND AMINO ACID ALIGNMENT OF COXSACKIEVIRUS B3 LIKE SEQUENCE OF ENTEROVIRUS 70:** A stretch of ~60 nucleotides, presented here, shows 75% nucleotide sequence similarity (80% at the protein level) with the corresponding Coxsackievirus B3 sequence. The same stretch is also more similar to Coxsackievirus B1 (Iizuka et al, 1987) and Coxsackievirus B4 (Jenkins et al, 1987) sequences as compared to the corresponding Poliovirus 1 sequence. Around this stretch, at both the nucleotide and amino acid level, the sequence shows more similarity to Poliovirus 1 sequences. Positions of amino acids are marked as given in the amino acid alignment (3A; Appendix II)

NUCLEIC ACIDS :

```
EV70  GAGAAGCACATTAGTAGAGCCTTTATGCGCCT-ACAGGCAATAACCACTTTTGTATCAAT
CVB3  GAGAAACATGTCAGTCGGGC-TTTCATTTGCTTACAGGCATTGACCACATTTGTGTCAAT
CVB1  GAGAAGCATGTCAGTAGG-CCTTTATC-TGTTTGCAAGCATTGACTACTTTTGTC--AGT
CVB4  GAAAAGCACGTCAGTAGAGCATTTATC-TGCCTCCAAGCACTGACAACTTTCGTGTCTCT
PV1   GTCAACATCACCAGCCAGG--TTCAAACAGAAAGGAACAACAGGGCAAT-----GACAAT
```

AMINO ACIDS :

```
          57                                   79
EV 70     E K H I S R A F I A L Q A I T T F V S I
CV B3     E K H V S R A F I C - Q A L T T F V S M
CV B1     E K H V S R A F I C L Q A L T T F V S V
CV B4     E K H V S R A F I C - Q A L T T F V S V
PV 1      E R N I N R A M T I L Q A V T T F V - -
```

observation is that the dinucleotide "AA" occurs with high frequency in or adjacent to recombination sites (King, 1988a). This dinucleotide is found on both sides of the Coxsackievirus B3 like stretch (Figure 15) in Enterovirus 70 - 3A. Recently, King (1988a) suggested that the copying of "AA" or "UU", which serve as primer attachment sites or as primers for initiation of replication (Takeda et al, 1987; Toyoda et al, 1987; Flanegan et al, 1987), may function as enter/exit signals for replication. Such enter/exit signals could result in the release of the replicase complex from the template; the complex may then enter and begin to copy another template. The model of Romanova and co-associates (1986) suggests a similar conclusion.

In poliovirus, it has been shown that recombination can occur between two markers separated by 189 nucleotides (Kirkegaard and Baltimore, 1986). In another study, intertypic recombinants of poliovirus, isolated from vaccinees, show that recombination can lead to acquisition of small RNA pieces (~90 nucleotides) and result in the formation of mixed genomes (King, 1988b). In FMDV inter- and intratypic recombination has also been reported (Wilson et al, 1988) and double crossover recombinants of FMDV O1 and O6 serotypes have been isolated (King, 1988b). The mechanism behind such recombination events is not fully understood, however, it is believed that these events occur during negative strand synthesis (Romanova et al, 1986; Kirkegaard and Baltimore, 1986; King, 1988a).

Some other features of the Enterovirus 70 - P3 region also suggest that recombinational events may have occurred. Careful nucleotide analysis shows that at the junction of $3C^{pro}$ and $3D^{pol}$ in Enterovirus

70 there are two short sequences (Appendix II) that appear to be deleted, as compared to the sequences of Poliovirus 1 and Coxsackievirus B3. Previous work has shown that such deletions are found near recombination sites and are formed as a result of a switch in template (Keck et al, 1987; Zimmern, 1988).

Recombination appears to be a mechanism common to all positive stranded RNA viruses. However, recombinants occur rarely, even if selected under laboratory conditions (Kirkegaard and Baltimore, 1986, King, 1988b). A recently recognized example of a natural recombinant virus, Western Equine Encephalitis Virus (WEEV), appears to be the result of recombination between a Sindbis like virus and an Eastern Equine Encephalitis like virus, resulting in a virus with novel serological properties (Hahn et al, 1988). Three recombinants of Mouse Hepatitis Virus (MHV; a murine coronavirus) with multiple crossovers in their genomes have been isolated. One of these recombinants has undergone at least three recombination events (Keck et al, 1987). Isolation of multiple crossover recombinants is compatible with the idea that multiple transcriptional pausing as a result of secondary structure of RNA plays a role in recombination (Keck et al, 1987; Wilson et al, 1988). Recombination events, and possibly multiple recombination events, could have occurred during the evolution of the Enterovirus 70 genome. The examples described above show that recombination can lead to genetic diversity and altered phenotypic (biological/antigenic) properties. Can any of the unique biological characteristics of Enterovirus 70 be explained by such recombinational events, e.g. in 3A or $3C^{pro}$?

Recent work of Bellocq and co-associates (1987) have shown that mutations in the central region of Poliovirus 1 $3C^{pro}$ could produce a small plaque mutant. Further characterization of this mutant showed that $3C^{pro}$ was a defective enzyme which resulted in temperature sensitivity of the mutant Poliovirus 1. This resulted from altered protein processing by $3C^{pro}$ and a decreased amount of $3D^{pol}$, which indirectly affected the synthesis of progeny virus, and led to temperature sensitivity. This also indicates that conditional lethal (ts) mutants may be much more complex in their phenotypic expression then previously thought. The temperature sensitivity in Enterovirus 70 could also be due to a defect in this enzyme. Characterization of temperature sensitivity in Enterovirus 70 has shown that RNA synthesis occurred at the nonpermissive temperature, but virus production did not (Miyamura et al, 1984). This temperature sensitivity could be due to inefficient protein processing, which may also explain the low titres of Enterovirus 70 and plaque size heterogeneity.

In summary, the P3 sequence of Enterovirus 70 resembles the sequences of polioviruses and coxsackieviruses. Three regions show a higher degree of similarity (nucleotide and amino acid) to Poliovirus 1 than to Coxsackievirus B3 sequences; 3' -UTR, $3D^{pol}$, and 3B. However, $3C^{pro}$ and 3A show more similarity to Coxsackievirus B3 sequences; this appears to be the result of recombination between the genome of an ancestor to Enterovirus 70 and a Coxsackievirus B3 like sequence. Some of the biological properties of Enterovirus 70 may be explained by the unique nature of $3C^{pro}$ and 3A (which is believed to be a component of the replicase complex) (Bernstein et al, 1986; Semler et al, 1988). The

natural temperature sensitivity of Enterovirus 70 could also be due to properties of $3C^{pro}$ (Ballocq et al, 1987). Structural proteins are believed to be the major players in host range and virulence of viruses (Yoshii et al, 1977). However, nonstructural proteins, e.g. 3A and $3C^{pro}$, may have much to say in determining the phenotypic characteristics of an infectious agent, like Enterovirus 70.

The sequence comparisons showed that, although Enterovirus 70 is a newcomer as a human pathogen, its sequence is old enough to have a close relationship to both polioviruses and coxsackieviruses. This brings us to the question of whether Enterovirus 70 is a direct descendent of polioviruses or coxsackieviruses. Analysis of the P3 region of Enterovirus 70 suggests that the Enterovirus 70 lineage probably diverged from the poliovirus lineage at about the same time as polioviruses and coxsackieviruses diverged from each other. The recombination that appears to have occurred during evolution of Enterovirus 70, between an ancestor to Enterovirus 70 and a coxsackievirus, was probably a relatively recent event(s). Phylogenetic analysis of this region (P3 region) will probably give us the answer to that question.

Finally, future investigations into the sequences of Enterovirus 70 that impart neurovirulence (poliovirus like syndrome), temperature sensitivity (rhinovirus like), receptor binding properties (cardiovirus like), and most importantly the pandemic nature of Enterovirus 70 could be helpful in understanding the molecular biology of this outstanding candidate for the study of the origin and evolution of new viruses.

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APPENDIX I

COMPARISON OF YIELDS OF VIRUS PARTICLES FROM ULTRACENTRIFUGATION AND ULTRAFILTRATION METHODS.

ULTRACENTRIFUGATION :

A. NUNC™ cell factory surface area = 6,000 cm^2

Number of plates in one cell factory = 120 plates

Approximate number of cells in subconfluent monolayer, 100mm plate = 10^7 cells

B. Virus titre obtained from one plate, 3mL culture medium (total) = $(5x10^6).3$
= $1.5x10^7$ PFUs

Expected titre from one cell factory = $(1.5x10^7).120$
= $1.8x10^9$ PFUs

Total (expected) virus particles from six cell factories (total) = $(1.8x10^9).6$
= $\underline{1.8x10^{10}}$ PFUs

C. Plaque assay result, just prior to start of CsCl gradient, 4.5 mL (total) = $(2x10^9).(4.5)$
= $\underline{9x10^9}$ PFUs

$$\text{RECOVERY} : \frac{9x10^9}{1.8x10^{10}} \times 100 = 50\%$$

ULTRAFILTRATION :

A. Lysate titre from 95, 100mm plates = $4.5x10^5$ PFU/mL

Total PFU in 550 mL of supernatent = $2.3x10^8$ PFUs

Concentrated virus (total) = $2.2x10^8$ PFUs

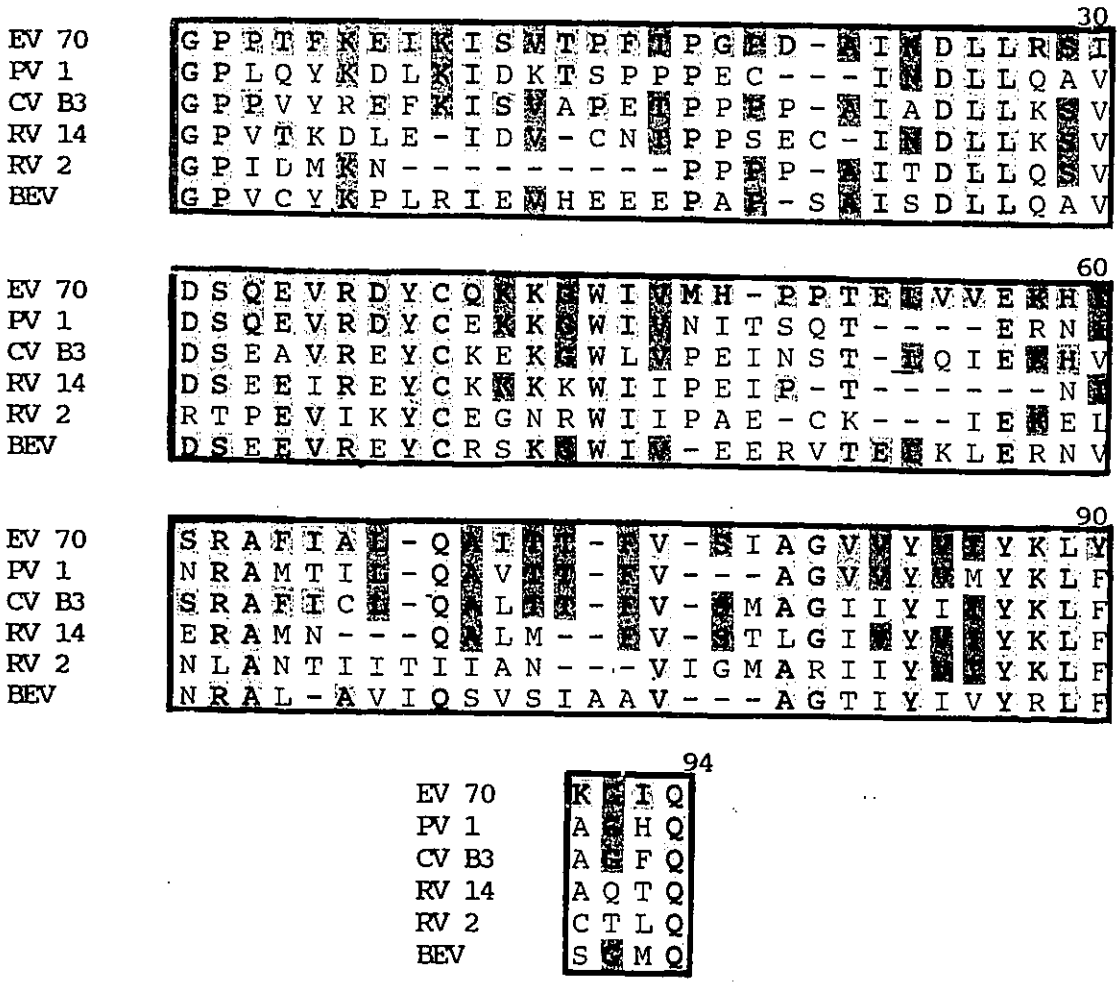
$$\text{RECOVERY} : \frac{2.2x10^8}{2.3x10^8} \times 100 = 95\%$$

APPENDIX II

AMINO ACID SEQUENCE ALIGNMENTS WITH IN PICORNAVIRUSES:

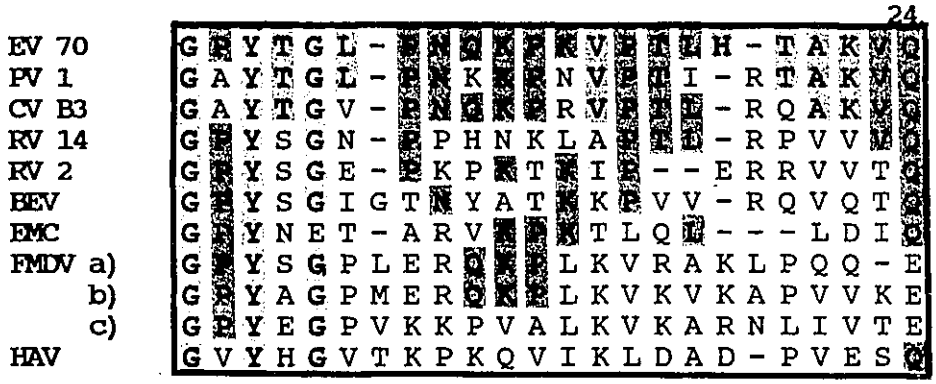
The P3 region of Enterovirus 70 was divided into 5 areas, 3A, 3B, $3C^{pro}$, $3D^{pol}$ and 3' -UTR. Amino acids for four coding regions of different picornaviruses; Poliovirus 1:PV1 (Koch and Koch, 1985), Coxsackievirus B3:CVB3 (Lindberg et al, 1987), Rhinovirus 14:RV14 (Callahan et al, 1985), Rhinovirus 2:RV2 (Skern et al, 1987), Bovine Enterovirus:BEV (Earle et al, 1988), Encephalomyocaditis virus:EMC (Palmenberg et al, 1984), Foot and mouth disease virus:FMDV (Forss et al, 1984), and Hepatitis A virus (Enterovirus 72):HAV (Paul et al, 1987) are given in these sequences. All these alignments were done manually. Green represents conservation of amino acids in picornaviruses, orange in group 4 viruses, and yellow in subgroups. Gaps in the alignments are represented by "-".

PICORNAVIRUS 3A GENE.



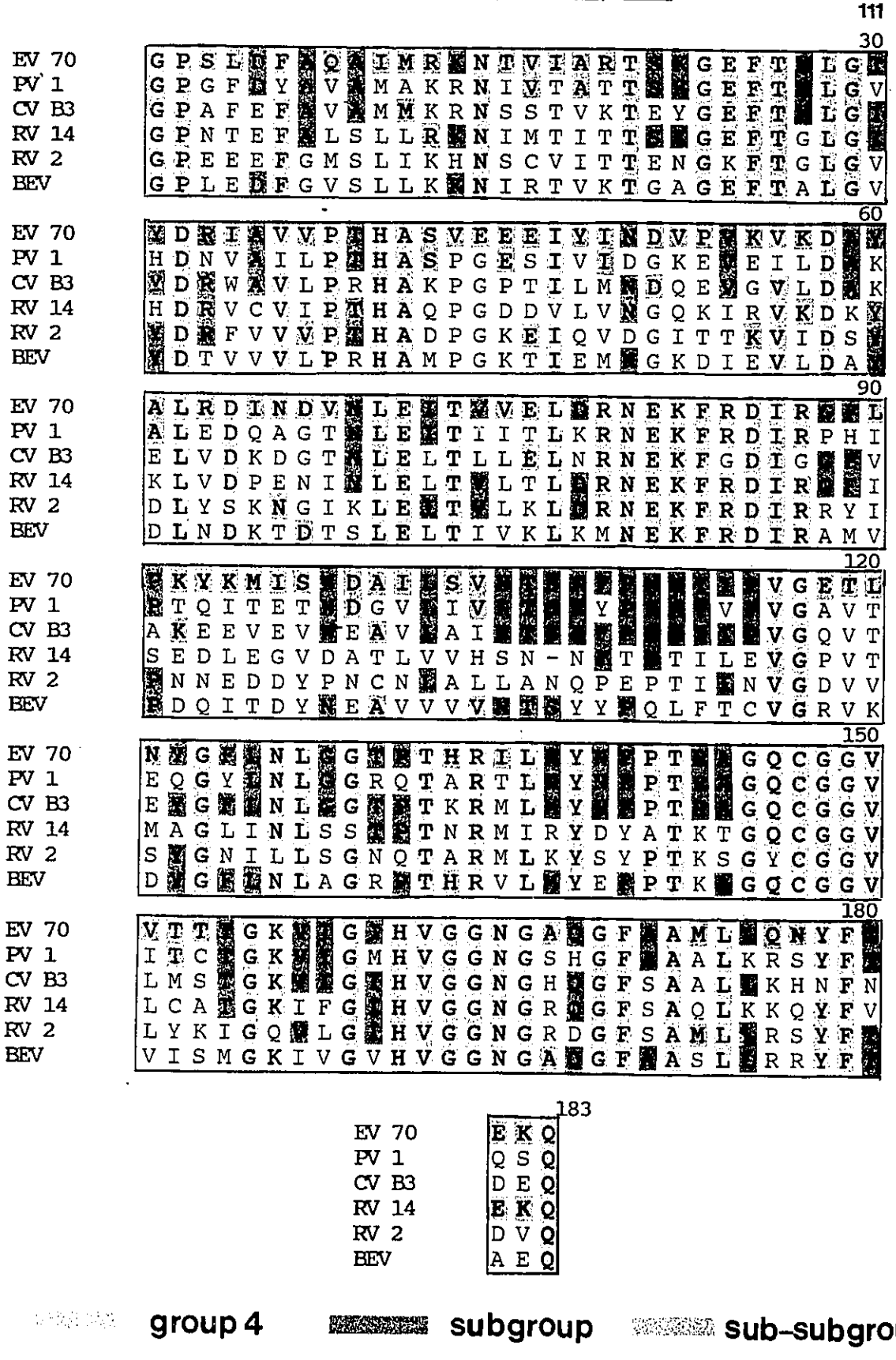
group 4 sub-group sub-subgroup

PICORNAVIRUS 3B (VPg) GENE.

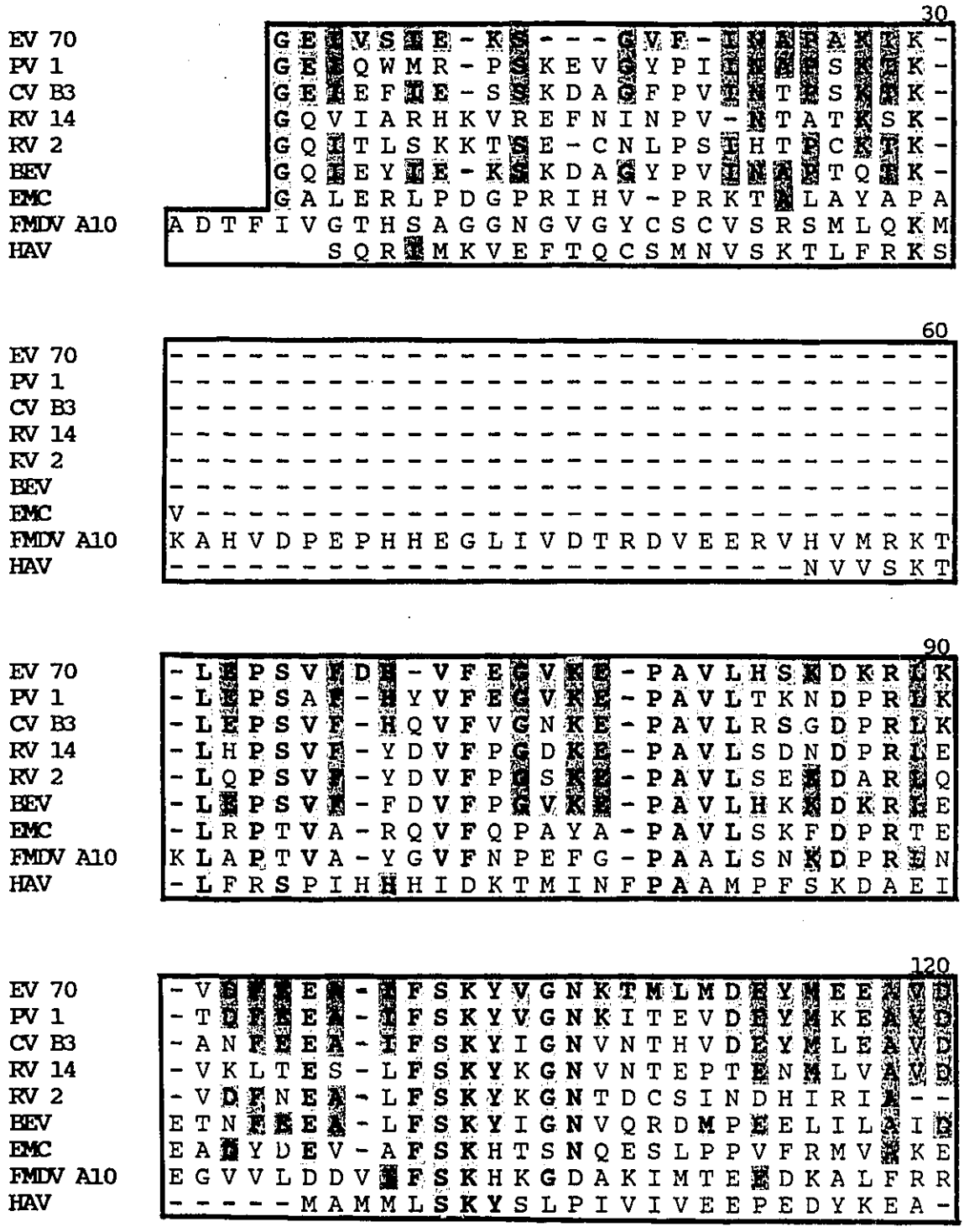


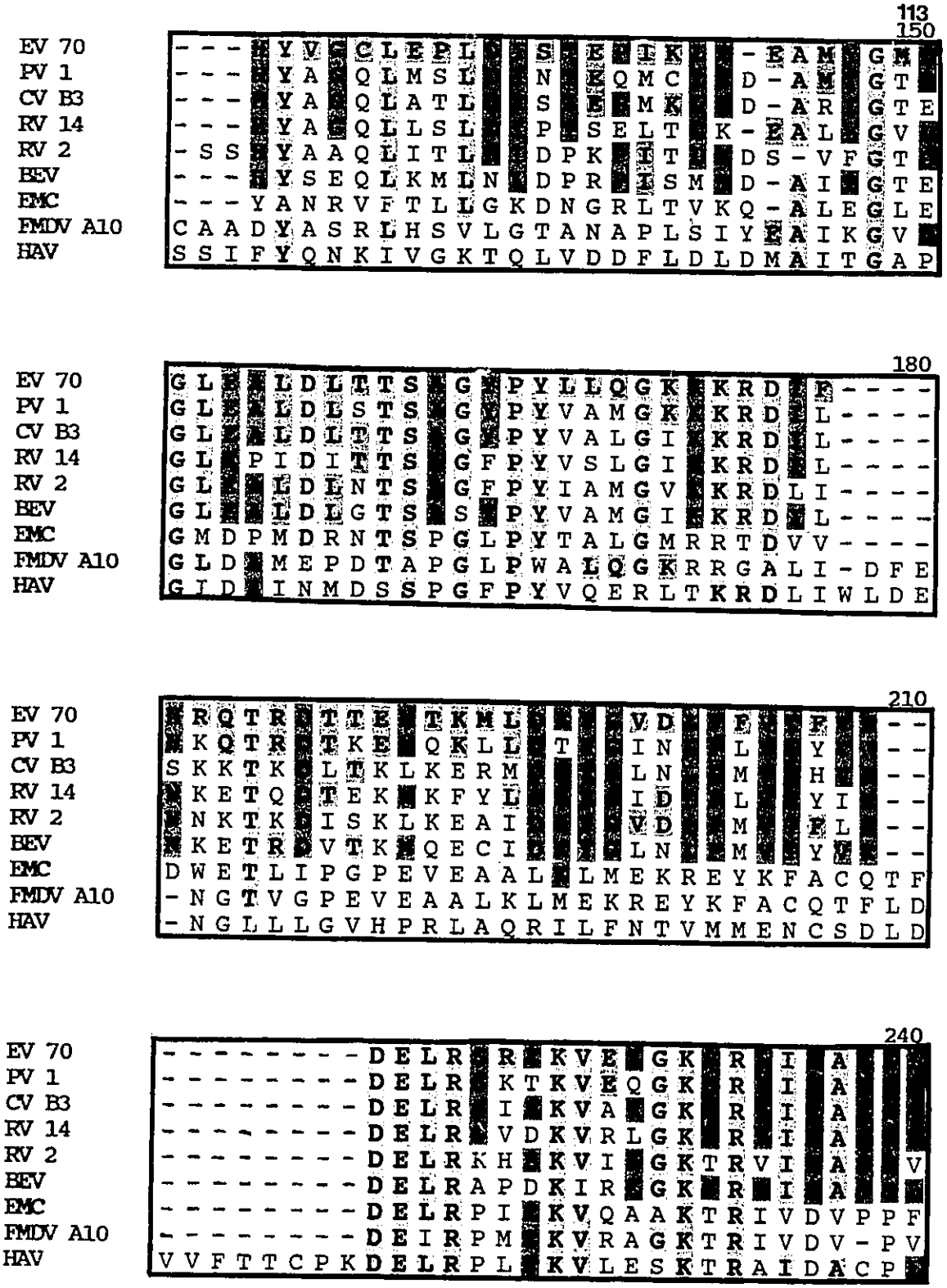
Picorna group 4 subgroup

PICORNAVIRUS 3C (PROTEASE) GENE.



PICORNAVIRUS 3D POLYMERASE GENE.

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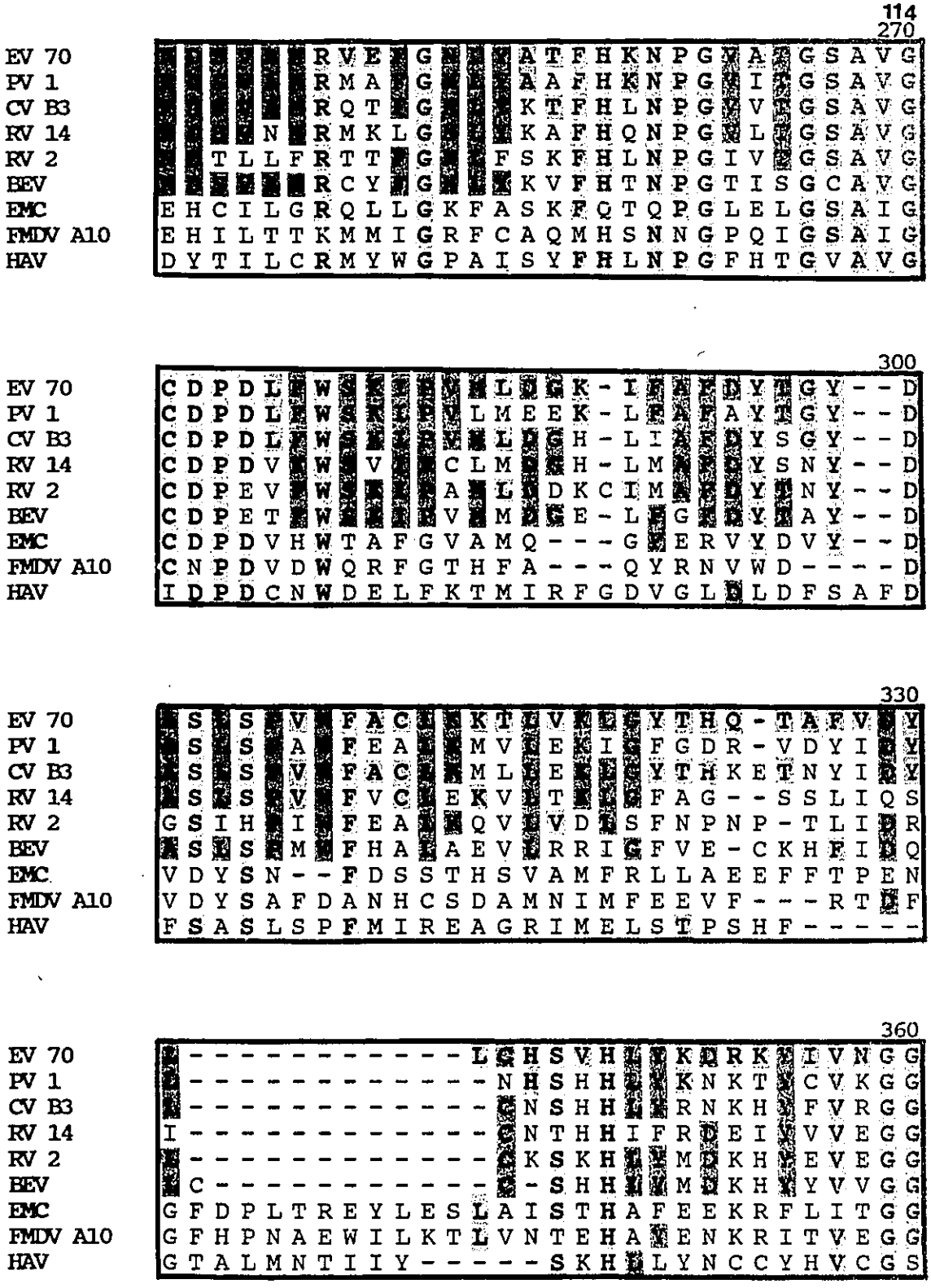
PV 1
CV B3
RV 14
RV 2
BEV
EMC
FMDV A10
HAV
180
210
240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CV B3 RQT G KTFHLNPGVVTGSAVG
RV 14 NRMKLG KAFHQNPGVLTGSAVG
RV 2 TLLFRTT G FSKFHLNPGIVTGSAVG
BEV RCY G KVFHTNPGTISGCAVG
EMC EHCILGRQLLGKFASKFQTQPGLELGSAIG
FMDV A10 EHILTTKMMIGRFCAQMHSNNGPQIGSAIG
HAV DYTILCRMYWGPAISYFHLNPGFHTGVAVG
300
EV 70 CDPDL WSKSPVMLDGK-IFAFDYTGY--D
PV 1 CDPDL WSKSPVLMEEK-LFAFAYTGY--D
CV B3 CDPDL WSRSPVLDGH-LIAFDYSGY--D
RV 14 CDPDV WSVCLMSH-LMAFDYSNY--D
RV 2 CDPEV WSASLDKCIMAFDYTNY--D
BEV CDPET WSVMDGE-LFGFDYTAY--D
EMC CDPDVHWTAFGVAMQ---GFERVYDVY--D
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PV 1 SSAFEAMVEKIGFGDR-VDYIDY
CV B3 SSVFACMLEDIGYTHKETNYIDY
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RV 2 GSIHIFEAQVVDTSFNPNP-TLIDR
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PV 1 C----------NHSHHLVKNKTMCVKGG
CV B3 ----------GNSHHLIRNKHMFVRGG
RV 14 I----------GNTHHIFRDEIMVVEGG
RV 2 ----------GKSKHLIMDKHMEVEGG
BEV C----------G-SHHLMMDKHMYVVGG
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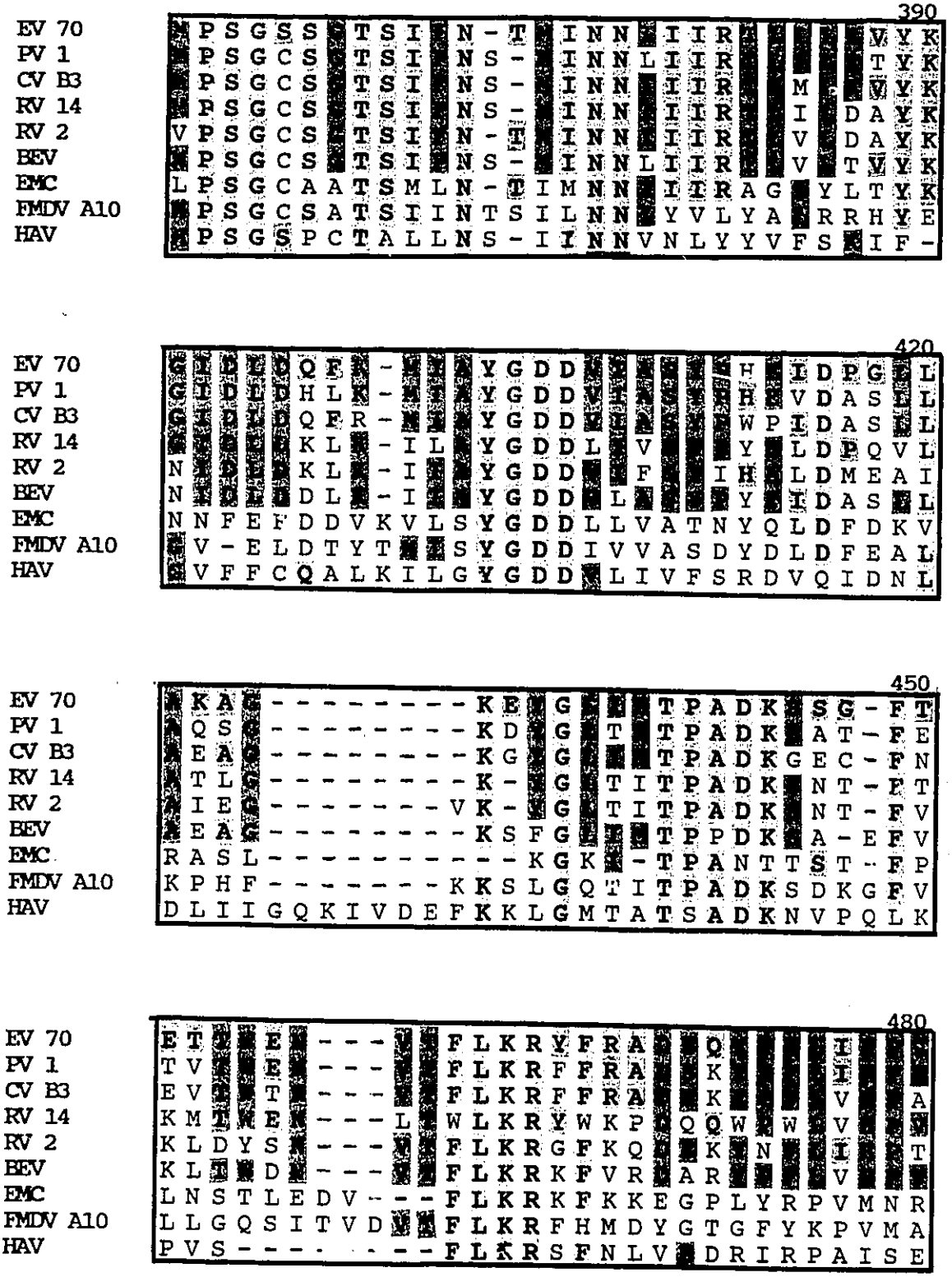
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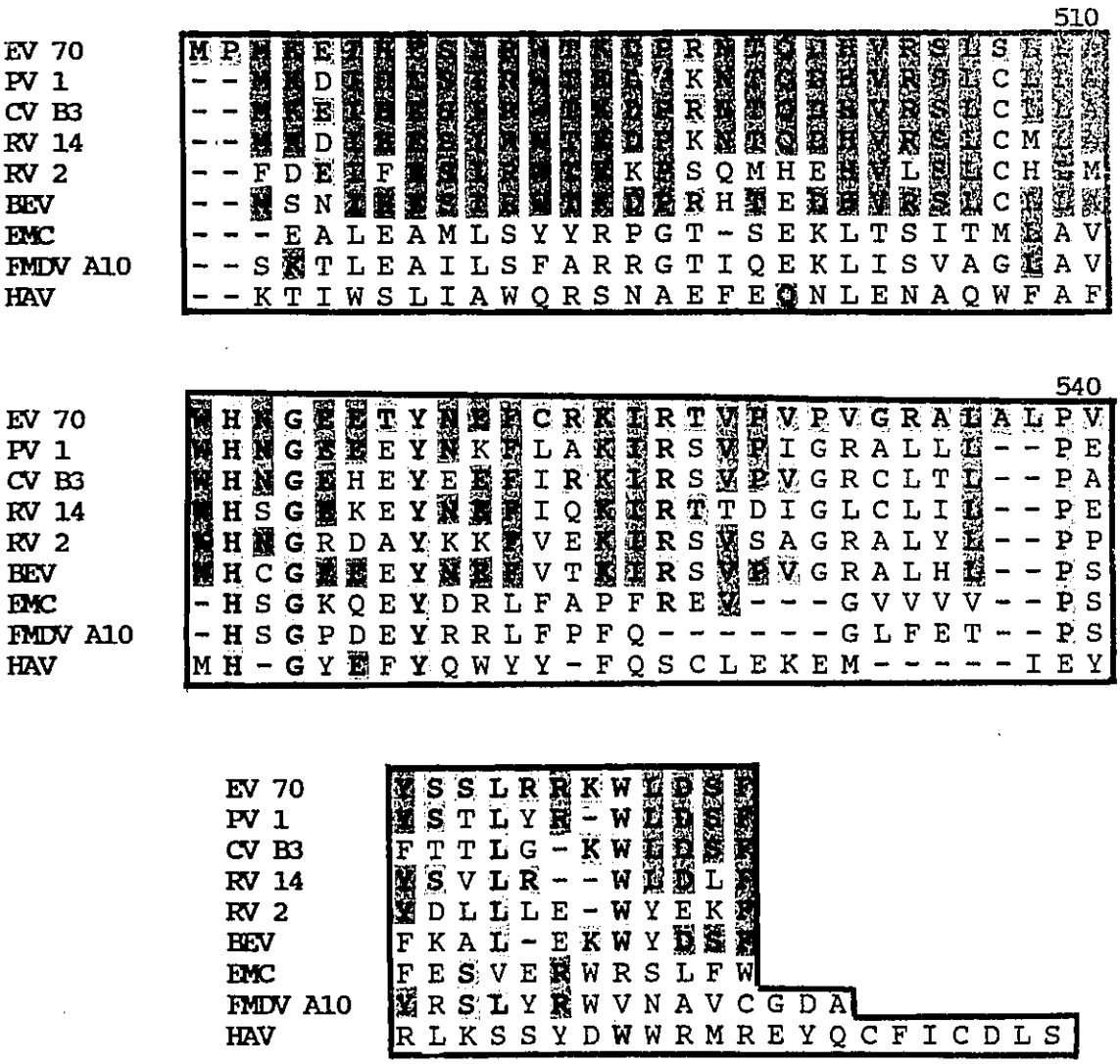
RV 2
BEV
EMC
FMDV A10
HAV
420
450
480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540
EV 70
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RV 2
BEV
EMC
FMDV A10
HAV