IDENTIFICATION OF NEUTRALIZATION ASSOCIATED SITES OF ENTEROVIRUS 70

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ABSTRACT

Enterovirus 70 (EV70), the causative agent of acute haemorrhagic conjunctivitis (AHC) is a picornavirus with a number of unique biological and pathogenic properties. *In vivo*, EV70 shows tropism for the conjunctiva and in rare cases, can infect the central nervous system, while *in vitro*, it has the ability to infect a wide range of mammalian cells in culture. The main objective of this study was to identify sites associated with neutralization of EV70. Identification of neutralization-associated sites is an initial stage for producing subunit vaccines, and neutralizing monoclonal antibodies produced against these sites may have direct application as therapeutic agents. Several different monoclonal antibodies which have neutralizing activity against the prototype EV70 J670 were tested. Two antibodies yielded resistant virus plaques. The nucleotide sequence of the capsid protein-coding region of each mutant was determined following cDNA synthesis, PCR amplification and cloning. The deduced amino acid sequences of the capsid protein-coding regions were compared with the corresponding sequences from the EV70 prototype and several wild isolates of EV70. Amino acid residues that correlated with escape from neutralization were identified. Two neutralization associated determinants were identified on VP1 and VP2. The locations of these sites were compared with neutralization associated sites of other picornaviruses.

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DEDICATION

This work is dedicated to my grandparents and to my husband

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LIST OF ABBREVIATIONS

AHC	Acute Haemorrhagic Conjunctivitis
ATP	Adenosine Triphosphate
BEV	Bovine Enterovirus Strain VG-5-27
BSA	Bovine Serum Albumin
CA24V	Coxsackievirus A24 Variant
cDNA	Complementary DNA
CPE	Cytopathic Effect
DEP	Diethylpyrocarbonate
DNA	Deoxyribonucleic Acid
dNTPs	Deoxyribonucleotide Triphosphates
DTT	Dithiothreitol
EDTA	Ethylene Diamine Tetraacetic Acid
EMC	Encephalomyocarditis Virus
EtBr	Ethidium Bromide
EV70	Enterovirus 70
FBS	Fetal Bovine Serum
FMDV	Foot-and-Mouth Disease Virus
GM	Growth Medium
HA	Hemagglutination Activity

HAV	Hepatitis A Virus
hr	Hour
hrs	Hours
HRV	Human Rhinovirus
ICAM-1	Intercellular Adhesion Molecule 1
IgA	Immunoglobulin A
IgG	Immunoglobulin G
IgM	Immunoglobulin M
IPTG	Isopropyl-β-D-thiogalactopyranoside
LLC-MK_2	Lilly Laboratories Culture - Monkey Kidney Cell Line
Log	Logarithm
M	Molar
mA	Milliampere
mAb	Monoclonal Antibody
MEM	Minimal Essential Medium
Mini-Prep	Small-scale Preparation of Plasmid DNA
μg	Microgram
μl	Microliter
ml	Millilitre
mm	Millimeter
mM	Millimolar

MOI	Multiplicity of Infection
NENS	Buffer containing NaAc, EDTA, NaCl,
ng	Nanogram
Nims	Neutralization Immunogens
NT	Neutralization Test
Nt	Nucleotide
ORF	Open Reading Frame
PCR	Polymerase Chain Reaction
PFU	Plaque Forming Units
RNA	Ribonucleic Acid
RNase	Ribonuclease
RPM	Revolutions per Minute
SDS	Sodium Dodecyl Sulphate
SOC	Medium containing Bacto-tryptone, Yeast extract, NaCl and $MgSO_4$
STET	Medium containing Sucrose, Triton X-100, EDTA and Tris-HCl
TBS	Tris-Buffered Saline
TE	Tris-EDTA buffer
TSB	Tryptic-Soy Broth
UTR	Untranslated Region
V	Volt

V/V	Volume per Volume
W/V	Weight per Volume
W	Watt
W/W	Weight per Weight
X-Gal	5-Bromo-4-Chloro-3-Indolyl-β-D-Galactoside

CHAPTER 1

INTRODUCTION

1.1. Acute Haemorrhagic Conjunctivitis (AHC)

AHC was first recognized as a new disease entity in 1969 in Africa. Enterovirus 70 (EV70) and a coxsackievirus A24 variant (CA24V) (Ghendon 1989) were identified as the etiologic agents of this disease. Similar symptoms are associated with infection by these two viruses and the differential diagnosis is mainly based on serological tests such as neutralization test, haemagglutination inhibition test, complement fixation test and enzyme-linked immunosorbent assay (Natori *et al.*, 1984).

CA24V was first determined to be a cause of AHC during an outbreak in Singapore in 1970. Other than its ability to cause ocular infection, CA24V has all the characteristics typical of other enteroviruses (Mirkovic *et al.*, 1974).

The first identification of EV70 as a cause of AHC was during the 1969-1971 pandemic introducing in Africa, Southeast Asia, Japan, and India (Mirkovic *et al.*, 1973).

During this pandemic, EV70 was isolated in Japan from conjunctival scrapings and was identified as a new type of enterovirus (Kono *et al.*, 1972). It was originally named AHC virus (Kono *et al.*, 1972), but later was renamed enterovirus 70 as a result of studies done in collaboration with the WHO International Reference Centre for Enteroviruses (Kono *et al.*, 1972; Mirkovic *et al.*, 1973).

Unlike CA24V, EV70 is not a typical enterovirus. It has many unique features in addition to the characteristics which it shares with other enteroviruses. These unique features include its target site in the host and its mode of transmission, the temperature sensitivity of its growth, its wide host range and its haemagglutination (HA) activity (Yamazaki and Miyamura, 1989). The primary site of infection for most of the enteroviruses is the intestinal tract, however, the replication site of EV70 is the conjunctiva. It has been shown that EV70 can be successfully isolated from the conjunctival swabs of AHC patients, but it can only rarely be isolated from feces of patients (Higgins, 1982). Transmission of the disease takes place through hand contact directly and indirectly. The temperature sensitivity of the virus is evident by the fact that EV70 grows well at 33°C (the optimal temperature), does not grow as well at 37°C, and does not grow at all at 39°C. This feature provides supporting evidence for the replication of the virus in the conjunctiva (Miyamura *et al* ., 1974). The wide host range is demonstrated by the fact that EV70 not only replicates in primate cells but also in some non-primate cell lines, such as murine, hamster, rabbit, porcine and bovine (Yoshii *et al.*, 1977). The HA activity of EV70 is supported by the fact that human "O", guinea pig, and chicken erythrocytes can be haemagglutinated by EV70. It has also been found

that EV70 receptors on the erythrocyte surface are sensitive to neuraminidase (Utagawa *et al.*, 1982).

1.2. Clinical Picture

The prime site of EV70 infection is the conjunctiva. One of the main reasons for this may be the temperature sensitivity of the virus. The optimum growth temperature of EV70 is 32 - 34°C which is probably the temperature range for this site in the body (Miyamura *et al.*, 1974; Stanton *et al.*, 1977; Hung and Kono, 1979). The incubation period for AHC is approximately 24 hrs and is followed by onset of symptoms. These symptoms include ocular redness, pain, epiphora, eye lid swelling and photophobia. Recovery is usually complete within a week (Uchida, 1989; Mirkovic *et al.*, 1973).

AHC is usually limited to the eye and no specific treatment is needed. In 1/10,000 - 1/17,000 cases, however, poliomyelitis-like disease may result from EV70 infection (Hung, 1989). This includes impairment of spinal nerves, cranial nerves or a combination of both (Wadia *et al.*, 1983). Spinal nerve involvement is characterized by acute asymmetrical motor paralysis of one or more limbs followed by muscle dystrophy, while acute cranial motor nerve impairment leads to palsy of some of the facial muscles (Vejjajiva, 1989).

Since isolation of EV70 from infected conjunctivae is fairly difficult, the diagnosis of AHC is mainly based on serological tests, such as haemagglutination inhibition, complement fixation, enzyme immunoassay, and neutralization tests (NT) (Hierholzer *et al.*, 1984; Kono *et al.*, 1978; Ishii and Hikiji, 1989). Among these diagnostic tests, NT

appears to be the most useful. A recent EV70 infection can be demonstrated by a 4 - 8 fold increase in specific antibody titer (Kono *et al.*, 1975; 1977).

At present, there are no specific drugs or vaccines available and treatment is mainly supportive. Vasoconstrictor eye drops can be used for subjective relief and antibiotic eye drops can be used to prevent secondary bacterial infection (Santos and Linhares, 1989).

1.3. Epidemiology

The history of EV70 as a recognized human pathogen is brief. The first outbreaks of AHC due to EV70 were identified in June of 1969 in Ghana, West Africa (Chatterjee *et al.*, 1970). The disease spread across West Africa and reached North Africa by December, 1970. In 1970 - 71, outbreaks spread rapidly over Southeastern Asia and India (Mirkovic *et al.*, 1973). Japan was also affected but to a lesser degree (Kono *et al.*, 1972). In 1971 - 72, AHC occurred in the Middle East and Europe and in 1974 - 75, widespread epidemics were reported in Thailand, Singapore and India (Reeves *et al.*, 1986). After a quiet period of several years, during which only occasional cases were observed, a second pandemic of AHC occurred in 1980 - 82 involving India, Pakistan, Nigeria and, for the first time, the Americas. In the Americas, the disease first appeared in Brazil and quickly spread to the Caribbean, the northern part of South America, Central America, Florida and more than 2 million cases of AHC were reported. (Morgan *et al.*, 1981; Maliso *et al.*, 1984; Waterman *et al.*, 1984; Asbell *et al.*, 1985; Reeves *et al.*, 1986). There were also small clusters of cases

in North Carolina, Minnesota, California, New York and Ontario (Patriarca, 1989). Since the 1980 - 82 pandemic, there have been outbreaks of AHC in various parts of the world. In 1983, an outbreak of AHC occurred in Singapore (Goh, *et al.*, 1990). In 1984, AHC was reported in Beijing, P. R. China (Mu, 1989) and in Sao Paulo, Brazil (Waldman *et al.*, 1990). AHC occurred in Kaduna, Nigeria (Babalola *et al.*, 1990), Saudi Arabia (Moustafa *et al.*, 1989; Ramia *et al.*, 1990) in 1985, and there was an epidemic of AHC in Delhi, India (Kishore *et al.*, 1989) in 1986. In 1988 and 1991, epidemics of AHC were reported in Guangzou, P. R. China (Song, 1992) and in American Samoa (Bern *et al.*, 1992), respectively.

AHC is very contagious under crowded and unhygienic conditions and in warm, humid, coastal climates. Spread between family members is common (Melnick, 1989) with transmission believed to occur directly via person-to-person contact or indirectly via fomites (Sattar *et al.*, 1988). Ophthalmic instruments and solutions contaminated with eye discharges have also been implicated as vehicles for the spread of the disease (Kono *et al.*, 1978; Spence and Vellard, 1981).

1.4. Evolution of EV70

EV70 can be considered a recently "emerged" virus. Although the origin of EV70 is not clearly understood, T_1 RNase fingerprinting and phylogenetic studies have shown that all isolates of EV70 have derived from a common ancestral virus. It has been postulated that the original strain emerged in West Africa around 1967 and has since spread among human beings (Takeda *et al.*, 1984; Miyamura *et al.*, 1986). Prior to the

first outbreak of AHC in West Africa in 1969, few individuals in the world were positive for antibody to EV70 (Kono 1985). Three possible sources for a progenitor of EV70 have been proposed (Kono *et al.*, 1981): (i) an animal picornavirus which acquired pathogenicity for humans; (ii) a human virus which gained new pathogenic and infectious properties; or (iii) an insect picornavirus.

The first hypothesis is supported by the observation that sera of cows, horses, sheep and swine from Japan and Africa had neutralizing activity against EV70 before the first large human outbreaks (Kono *et al.*, 1981; Sasagawa *et al.*, 1982). However, the finding that the neutralizing activity was associated with IgM and never with IgG, suggested that the neutralizing activity was nonspecific and may have been due to antibody to an animal virus that cross-reacted with EV70 (Sasagawa *et al.*, 1982). Another peculiar feature of EV70 is its wide host range in vitro. EV70 grows not only in human and simian cells but also in bovine, porcine, leporine, cricetine and murine cell lines (Yoshii *et al.*, 1977). In addition, sequence comparisons between EV70 and other picornaviruses have demonstrated that EV70 is more closely related to bovine enteroviruses (BEV) (Ryan *et al.*, 1990). This also suggests an animal origin for EV70.

In support of the second hypothesis, however, nucleotide sequence analysis of the virus genome and computer-aided homology searches have shown that EV70 sequences are closely related to human enteroviruses such as the polioviruses and coxsackie B viruses (Miyamura *et al.*, 1989). EV70 sequences have less homology to animal picornaviruses such as encephalomyocarditis (EMC) and foot-and-mouth-disease virus

(FMDV) (Palmenberg *et al.*, 1985; Honey *et al.*, 1987; Cann *et al.*, 1984). The least homology is to insect picornaviruses (King *et al.*, 1987).

Whatever its origins, the evolutionary pattern of EV70 is divergent, a pattern also seen for other enteroviruses (Buongurio *et al.*, 1986). EV70 evolves continuously at a constant and very high rate estimated to be 1.84×10^{-3}/ nucleotide substitutions/year. In this situation, a collection of descendants with genetically and antigenically distinct characteristics is produced (Yamashita *et al.*, 1988; Yokoyama *et al.*, 1988).

1.5. General Properties of Picornaviruses and EV70

EV70 has been classified as a member of the genus Enterovirus, family Picornaviridae. Picornaviruses are among the smallest of RNA viruses (Pico - small, rna - RNA as genetic material) (Rueckert, 1985). They are also one of the largest and most important families of viral pathogens. The picornavirus family is currently divided into five genera which differ from one another in physicochemical properties such as buoyant density, acid lability and sedimentation coefficients (Francki, 1991). The five genera are: (i) Enterovirus; (ii) Hepatovirus; (iii) Rhinovirus; (iv) Aphthovirus; and (v) Cardiovirus. Human and animal enteroviruses constitute the genus Enterovirus. The genus Hepatovirus consists of human and simian hepatitis A viruses (HAV). The genus rhinovirus is made up of human rhinoviruses (HRV) (over 100 different serotypes) and bovine rhinoviruses (2 serotypes). The genus Aphthoviruses comprises of 7 serotypes of FMDV which infect cloven hoofed livestock. The genus Cardioviruses includes the rodent viruses, mengovirus, EMC virus and Theiler's encephalomyocarditis virus. In

spite of their diversity, all picornaviruses share similar structural components and use similar replication strategies (Putnak and Phillips, 1981; Rossmann *et al.*, 1985).

Picornaviruses are non-enveloped and have spherical capsids (24 - 30 nm) with icosahedral symmetry (Rueckert, 1985). They possess positive sense single-stranded RNA genomes with molecular weights of 2.5 - 2.9 x 10^6 (Koch and Koch, 1985). The genome has a VPg protein covalently linked to its 5' terminus and a 3' poly (A) tract. The RNA also contains a 5' untranslated region (UTR) 624 - 1200 nucleotides (nt) in length, a single long open reading frame (ORF) which contains over 2000 codons, and a 3' non-coding region of 47 - 126 nt (Stanway, 1990).

The VPg protein may serve as a primer for RNA synthesis and covalent attachment of VPg to plus and minus strands is required for RNA replication and for viral protein synthesis (Putnak and Phillips, 1981; Reuer *et al.*, 1990; Cao *et al.*, 1993).

The sequences of the 5' UTR are more conserved than those of other parts of the genome. The 5' UTR is unusually long and is predicted to have a high degree of secondary structure (Stanway, 1990). Studies of poliovirus 1 and EMC have demonstrated that prior to initiating translation, eukaryotic ribosomes bind to a region in the UTR. The region which is required for internal ribosome binding was named the ribosome landing pad (RLP) in entero/rhinovirus UTR and the internal ribosome entry site (IRES) in cardio/aphthovirus UTR (Meerovitch and Sonenberg, 1993; Pelletier and Sonenberg, 1988). Despite dissimilarity of the RLP and the IRES in their primary and secondary structures, two features, a polypyrimidine tract and an AUG triplet approximately 15 - 20 nt downstream from the polypyrimidine tract, are conserved

among all picornaviruses. It is predicted that 40S ribosome subunits bind directly to the region containing the polypyrimidine tract-spacer-AUG motif (Pestova *et al.*, 1991; Nicholson *et al.*, 1991; Jang *et al.*, 1989; Trono *et al.*, 1988; Pelletier and Sonenberg 1988; Pestova *et al.*, 1989).

The long open reading frame encodes a polyprotein. It has been shown that the polyprotein is cleaved to produce structural (P1) and non-structural proteins (P2 and P3). The structural proteins consist of VP1, VP2, VP3 and VP4, while the non-structural proteins include the P2 proteins, 2A (protease), 2B and 2C (function unknown), and P3 proteins, 3AB (precursor for VPg), 3C (protease) and 3D (RNA-dependent RNA polymerase) (Bazan and Fletterick, 1988).

The role of the 3' non-coding region is not clearly understood, however, it may be involved in polymerase binding (Stanway, 1990; Pilipenko *et al.*, 1992). The function of the poly (A) tract is also not known. It has been suggested that poly (A) tract seems to prime (-) strand synthesis (Sarnow, 1989).

Like other picornaviruses, EV70 has a capsid of icosahedral symmetry approximately 30 nm in diameter (Ryan *et al.*, 1990). The virus possesses a positive sense single-stranded RNA (7390 nt) and a 5' linked VPg protein (Yamazaki *et al.*, 1974). The genome contains a 5' UTR (727 nt), single open reading frame (6582 nt) (2194 codons), a 3' UTR (82 nt) and a 3' poly (A) tract (Takeda *et al.*, 1989; Ryan *et al*, 1990).

It has been shown that VPg of EV70 could play a role in determining the temperature sensitivity of EV70 replication (Takeda *et al.*, 1989). The 5' UTR of EV70

is homologous with the 5' UTR's of other picornaviruses. When a number of different enterovirus specific DNA probes were used, only the 5' UTR of EV70 was recognized in hybridization studies (Auvinen *et al.*, 1989). Sequence analysis has confirmed this result. This implies that the 5' UTR of EV70, like the 5' UTR's of other picornaviruses, is involved in the translation process.

Translation of EV70 RNA is believed to start from the initiation codon at nt 727 - 729 and to proceed to the termination codon at nt 7309 - 7311, encoding a polyprotein of 2194 amino acids (Takeda *et al.*, 1989; Ryan *et al.*, 1990; Dimock, 1990). The polyprotein is then further cleaved to give the structural (P1) and non-structural proteins (P2 and P3). The structural proteins include VP1, VP2, VP3 and VP4 and the VPg is also considered a structural protein but comes from the P3 region. The non-structural protein contains 2A, 2B, 2C, and 3AB, 3C and 3D. The functions of the non-structural proteins of EV70 are predicted to be similar to those of other picornaviruses (Bazan and Fletterick, 1988; Takeda *et al.*, 1989; Ryan *et al.*, 1990).

1.6. Picornavirus Capsid Structure

The capsid proteins of picornaviruses have at least four important functions: (i) they shield the RNA from nucleases in the environment; (ii) they interact with specific cell-coded receptors on the surface of susceptible cells and as a result, to a large extent, determine host range and tissue tropism; (iii) they direct the packaging of the viral genome and include proteases which are involved in maturation of the virion; and (iv)

they are active vehicles for penetrating the plasma membrane and are responsible for delivering the RNA genome into the cytoplasm of susceptible cells (Rueckert, 1985).

The shell of all picornaviruses has icosahedral symmetry and consists of 12 pentamers. The pentamer is the basic building block of the icosahedral capsid and is composed of 5 identical protomers. The protomer is the smallest structural unit of the virion shell and contains 1 copy of each of the capsid proteins VP1, VP2, VP3 and VP4. While the sizes of the three major capsid proteins, VP1, VP2, and VP3, are similar among all picornaviruses, the size of VP4 is different and usually much smaller. The size ranges of these proteins are 209 - 302 amino acids (VP1), 218 - 272 amino acids (VP2), 221 - 246 amino acids (VP3) and 17 - 85 amino acids (VP4) (Rueckert, 1990).

The sequences of the picornavirus capsid proteins, while homologous, exhibit considerable sequence dissimilarity. However, detailed structure analysis (Rueckert, 1990) has demonstrated that their secondary and tertiary structures are very similar. It has been found that each of the proteins is wedge-shaped with 8 stranded anti-parallel β-barrels forming a core domain of the viral shell. The narrowest end of the wedge is directed towards the five-fold or three-fold axis of the symmetry. At the surface, while VP1 is positioned in the region surrounding the five-fold axis of the pentamer, VP2 and VP3 are located in the area around the three-fold axis (Hogle *et al.*, 1985). Since the N- and C-terminal regions of the major capsid proteins are not parts of the β-barrel 'core', they may extend from the core at the internal (the N termini) or external (the C termini) surface of the shell. These termini can interact with each other and/or they can interact with other proteins, including VP4, which is situated internally beneath the other

proteins. The pentamer can be stabilized by these interactions (Boeyé and Rombaut, 1992). Adjoining pentamers can hold together by hydrogen bonds formed between parts of VP2 and VP3 (Stanway, 1990).

The loops which link the strands of the β-sheets project from the virion surface. These loops are hydrophilic and their amino acid sequences vary considerably among picornaviruses. Studies using monoclonal antibody escape mutants have demonstrated that neutralization epitopes can be found in these regions (Minor *et al.*, 1986; Ferguson *et al.*, 1985; Sherry *et al.*, 1986). Of the neutralizing antigenic sites present on poliovirus serotypes 1, 2 and 3 (Minor *et al.*, 1986), HRV 14 (Sherry *et al.*, 1986), mengovirus (Luo *et al*, 1987), hepatitis A virus (Ping *et al.*, 1988) and FMDV (Kitson *et al.*, 1990), greater than 50% are conformational in nature.

The surface features of picornaviruses have been well studied. X-ray diffraction studies on HRV 14 have shown the presence of a canyon structure on the virion surface. The canyon is located at a constant distance around the five-fold axis and its wall is lined with amino acid residues from VP1 and VP3 (Rossmann *et al.*, 1985). The depth of the canyon is approximately 25 Å. The widths of the canyon at the entrance and at the canyon floor are 30 Å and 12 Å, respectively. The narrowness of the canyon is predicted to prevent the F(ab) arms of antibodies from penetrating to the canyon floor since the diameter of F(ab) arms is approximately 35 Å (Rossmann *et al.*, 1985). As a result, the amino acids of the canyon floor are shielded from antibody, and thus the amino acids at the viral attachment site are conserved (Rossmann *et al.*, 1985; Rossmann and Johnson, 1989). Mutagenesis studies have provided direct evidence for the location

of the viral attachment site on the canyon floor. In the case of HRV 14, infectious cDNA clones were used and mutants which possessed single amino acid changes at positions located on the canyon floor were selected. Most of these mutants resulted in an altered phenotype with decreased binding affinity for Hela cell receptors (Colonno *et al.*, 1988). In addition, the attachment between the virus and its cell receptor can be blocked by using drugs such as antiviral "WIN" compounds which are second generation antivirals derived from arildone (McSharry *et al.*, 1979). These drugs bind to a hydrophobic cavity located below the canyon floor and produce conformational changes in this area (Smith *et al.*, 1986; Pevear *et al.*, 1989). Similar canyon structures have also been found on poliovirus types 1, 3 and mengovirus, which provides further evidence that the canyon is functionally active in a number of picornaviruses (Hogle *et al.*, 1985; Luo *et al.*, 1987).

When the sequences of different rhinovirus and enteroviruses were compared, it was found that the sequences of amino acids that line the canyon were much more conserved than those of other surface residues. While noncanyon residues are predicted to experience continuous selective pressure from neutralizing antibodies, the canyon residues are under no such pressure since they are hidden from antibodies (Rossmann and Palmenberg, 1986). Placing the essential amino acids in an inaccessible location may be one of the strategies used by viruses to protect themselves from immune intervention.

Based on the receptors that they recognize, picornaviruses can be classified into several categories (Colonno, 1987). While FMDVs and cardioviruses make up two groups, the classification among the entero- and rhinoviruses is more complex. A

minority of rhinoviruses (10-15%) recognize a receptor identified as a 120 KD protein with an unknown function (Abraham and Colonno, 1984; Mischak *et al*, 1988). The majority of rhinoviruses, as well as some enteroviruses (coxsackieviruses A13, A18 and A21), bind to another receptor, identified as intercellular adhesion molecule 1 (ICAM-1). The ICAM-1 is a 95 KD glycoprotein that can be found on the surface of many kinds of cells (Greve *et al.*, 1989; Staunton *et al.*, 1989). ICAM-1 belongs to the immunoglobulin superfamily, a family of proteins having structures similar to immunoglobulin molecules. The poliovirus receptor is a 45 KD glycoprotein that is also a member of the immunoglobulin superfamily. While ICAM-I contains five predicted Ig-like domains, the poliovirus receptor possesses only three (Mendelsohn *et al.*, 1989; Racaniello, 1990). Recently, the attachment between HRV16 and its receptor, ICAM-1 was visualized directly by cryoelectron microscopy (Olson *et al.*, 1993). This should provide strong evidence that the virus-receptor interface lies within the canyon.

1.7. Picornavirus Neutralization Associated Antigenic Sites

The antigenic structure of picornaviruses has been extensively studied over the past few years using a variety of approaches. These include: (i) the isolation of mutants which escape neutralization by monoclonal antibodies; (ii) the study of interactions between monoclonal antibodies and synthetic peptides from a regions believed to be located in an antigenic site; (iii) the stimulation of immune responses using synthetic peptides from possible antigenic sites; (iv) the identification of hydrophilic nonconserved sequences predicted to be situated at the virion surface and

subject to immune pressure; (v) the identification of prominent features of the viral capsid that may be accessible to antibody using crystallographic techniques (Minor, 1990). Information on antigenic structures and sites is available for several picornaviruses, including the poliovirus, rhinovirus, bovine enterovirus, mengovirus, FMDV and HAV. Since poliovirus, rhinovirus and BEV are more closely related to EV70 than the other picornaviruses in this list, the antigenic structures of these three viruses will be discussed in some detail.

1.7.1. Neutralizing Antigenic Sites on Polioviruses

The antigenic sites on polioviruses 1, 2 and 3 have been studied using variants that escape neutralization by mouse monoclonal antibodies (Ferguson *et al.*, 1984; Minor *et al.*, 1983; 1985; Evans *et al.*, 1983). Four neutralizing antigenic sites were defined for all 3 serotypes, while a fifth was defined for types 1 and 2 only (Table 1) (Boeyé and Rombaut, 1992; Uhlig *et al.*, 1990). Site I is located on VP1, between residues 91 - 102 and is ordinarily thought of as a continuous antigenic site. However, it was shown (Wiegers *et al*, 1989) that mutants isolated by a single monoclonal antibody were altered either in amino acids 99 - 101 or 144. These results cast doubt on the continuous nature of this site since amino acid 144 is located in a different VP1 loop than amino acids 99 - 101. Site I is immunodominant, but only in poliovirus type 3. Site II is a discontinuous neutralization site and is positioned not only on VP1 (residues 221 - 226), but also on VP2 (residues 164 - 170 and 270) (Page *et al.*, 1988). Site IIIa (Page *et al.*, 1988) or

TABLE 1 MUTATIONS IN ANTIGENIC VARIANTS OF POLIOVIRUSES SELECTED WITH NEUTRALIZING mAbs

Sites	Mutated residues
I	VP1 91 - 102 [1, 2, 3, 4]
	VP1 144[5]
II	VP1 221 - 226 [6, 7]
	VP2 164 - 170, 270 [7]
IIIa (III)	VP3 58 - 60 [7]
	VP3 71 - 73 [6]
IIIb (IV)	VP2 72[6, 7]
	VP3 76[6, 7]
V	VP2 239 - 245[8]
	VP3 195 - 207[8]

Data from: [1] Wiegers *et al.* (1988); [2] Minor *et al.* (1986a); [3] Minor *et al.* (1985); [4] La Monica *et al.* (1987); [5] Wiegers *et al.* (1989); [6] Diamond *et al.* (1985); [7] Page *et al.* (1988); [8] Uhlig *et al.* (1990)

III (Minor, 1990) includes residues 58 - 60 and 71 - 73 of VP3. Site IIIb (Page *et al.*, 1988) or IV (Minor, 1990) contains residues 72 of VP2 and 76 of VP3. It is evident that both antigenic sites III (IIIa) and IV (IIIb) are also conformational. Site V (Uhlig *et al.*, 1990) is composed of amino acids 239 - 245 of VP2 and amino acids 195 - 207 of VP3 and is also predicted to be conformational. It is important to note that all of the amino acid substitutions that influence the antigenic structures of poliovirus are sited in the loops which protrude from the viral surface (Minor, 1990).

1.7.2. Neutralizing Antigenic Sites on HRV 14

The antigenic sites on HRV 14 have also been studied following the isolation of mutants that escape neutralization by monoclonal antibodies (Sherry and Rueckert, 1985; Sherry *et al.*, 1986). Four groups of mutants were defined by cross-neutralization analyses. These analyses suggest that there are four neutralization sites, called neutralization immunogens (Nims, Table 2). Nim Ia, the most commonly recognised site, is identified by mutations in VP1 at residues 91 or 95. Nim III, the next most frequently identified site, includes residues 72, 75, 78 and 203 of VP3 and residue 287 of VP1. Nim II is located on VP2 (residues 136,158, 159, 161 and 162) and on VP1 (resisue 210), while Nim Ib includes residues 83, 85, 138 and 139 of VP1. Of the four immunogenic sites, Nim Ib, Nim II and Nim III, are conformational. Nim II and Nim III are comprised of noncontinuous sequences located on the same coat protein and at least one amino acid residue on a second capsid protein, whereas Nim Ib is made up of

TABLE 2 MUTATIONS IN ANTIGENIC VARIANTS OF HRV 14 SELECTED WITH NEUTRALIZING mAbs

Site	Mutated residues*
Nim Ia	VP1 91, 95
Nim Ib	VP1 83, 85, 138, 139
Nim II	VP2 136,158, 159, 161, 162
	VP1 210
Nim III	VP3 72, 73, 75, 78, 203
	VP1 287

* Sherry *et al.* (1986)

noncontinuous sequences on the same capsid protein. Nim Ia on the other hand, appears to be a linear epitope (Sherry *et al.*, 1986). In spite of their close location, Nim Ia and Nim Ib are functionally different.

Studies on the three-dimensional structure of HRV 14 demonstrate that each of the substitution clusters is located in a protrusion from the virus surface with the side chains of the altered amino acids directed outwards (Rossmann *et al.*, 1985). The neutralization sites of HRV 14 are structurally similar to those of poliovirus, although there are some differences. For example, in HRV 14, the region which is similar to site I of poliovirus has two distinct sites, Nim Ia and Nim Ib, only the Nim Ia component has a counterpart in poliovirus (Minor, 1990). In addition, a synthetic peptide of the poliovirus VP2 sequence which corresponds to HRV 14 Nim II elicits a low level neutralizing response; however, this site on the poliovirion is poorly immunogenic (Emini *et al.*,1983a; 1984). Nim III may also be used in poliovirus neutralization, as a neutralization antiserum raised against a mixture of poliovirus VP3 and VP4, binds a poliovirus synthetic peptide corresponding to HRV 14 Nim III (Emini *et al.*, 1983a).

1.7.3. Neutralizing Antigenic Sites on HRV 2

The neutralization sites on HRV 2 have been studied using the isolation of antigenic mutants escaping neutralization by mAbs (Appleyard *et al.*, 1990). Three antigenic sites were identified (Table 3). Site A is located on VP1, contains amino acids 85, 86 and 92. Site B includes residues 264, 267-269, 276 and 278 of VP1, 163-164

TABLE 3 MUTATIONS IN HRV 2 ANTIGENIC VARIANTS SELECTED WITH NEUTRALIZING mAbs

Site	Mutated residues*
A	VP1 85, 86, 92
B	VP1 264, 267-269, 276, 278 VP2 163-164, VP3 59
C	VP2 236, 238

* Appleyard *et al.* (1990)

of VP2 and 59 of VP3. Site C is situated on VP2 and consists of amino acids 236 and 238. All of these antigenic sites are conformational and predicted to be located on the surface of HRV 2 virions.

1.7.4. Neutralizing Antigenic Sites on BEV

The antigenic sites of BEV have been studied using synthetic peptides which correspond to sequences of other picornavirus antigenic sites and which represent regions of BEV. These peptides can induce antibodies reacting with the virus in a number of assays (Smyth *et al.*, 1990 ; 1992). Two antigenic sites were identified (Table 4). Site I is made up of amino acids 152 - 161 of VP2, while site II includes residues 54 - 63 of VP3. Both of these sites are linear and predicted to be located on the surface of the virions.

1.7.5. Neutralizing Antigenic Sites on EV70

Since EV70 is a recently identified virus with so many unique features, it would be of interest and of importance to understand the molecular nature of these characteristics. Such knowledge may improve our understanding of the pathogenesis of AHC and lead to new approaches for prevention or treatment of the disease. Studies to identify the EV70 receptor and to characterize the antigenic sites of EV70 have been initiated in our laboratory. One of the goals of this work was to define antigenic sites of EV70 using escape variants which are resistant to neutralization by monoclonal antibodies. Identification of antigenic sites associated with neutralization on the surface

TABLE 4 BEV ANTIGENIC SITES IDENTIFIED WITH SYNTHETIC PEPTIDES

Site	Synthesized peptides*
I	VP2 152 - 161
II	VP3 54 - 63

* Smyth *et al.* (1990; 1992)

of the virion is important to an understanding of mechanisms of neutralization and provides a basis for studying antigenic variation. Finally, it is an initial stage for producing subunit vaccines, since isolated proteins or genetically engineered constructs could be used to stimulate protective immune responses. As well, in the case of EV70, neutralizing monoclonal antibodies could possibly be used directly as therapeutic agents (Rueckert, 1990).

OBJECTIVES

The overall objectives when this work began were to identify neutralization associated sites of EV70 and to test the hypothesis that neutralizing antibodies do not play a role in selection of EV70 in nature (Miyamura *et al.*, 1986). It was hoped that a panel of mAbs would be available. As it turned out, the number of mAbs available made it impossible to address the second objective in a meaningful way. However, it was still possible to identify sites associated with neutralization of EV70. The specific goals established during the course of this project therefore were:

(i) to test the ability of several mAbs to neutralize the prototype EV70 J670.

(ii) to use the neutralizing mAbs to select mutants which were resistant to neutralization.

(iii) to characterize the escape mutants.

(iv) to clone and sequence the capsid protein coding region of the escape mutants and several wild isolates of EV70. The purpose of this approach was to identify amino acid differences that correlated with escape from neutralization.

(v) to compare the location of amino acid residues associated with EV70 neutralization (escape) by analogy with similar sites for other picornaviruses.

CHAPTER 2

MATERIALS and METHODS

2.1. Cells

The Rhesus monkey kidney cell line, LLC-MK_2, was originally obtained from Flow Laboratories (Rockville, MD) and was used throughout the work described in this thesis. Cells were grown as monolayer cultures in growth medium (GM) consisting of autoclavable minimal essential medium (MEM; Cat.# 410-1700EG, Gibco/BRL, Burlington, Ont), 5% heat inactivated fetal bovine serum (FBS; Gibco/BRL), 0.2% (w/v) $NaHCO_3$ (Gibco/BRL), 2 mM L-glutamine (Gibco/BRL), and 50 μg/ml of gentamycin sulphate (Gibco/BRL) in 60 mm or 100 mm diameter tissue culture dishes (Canlab, Toronto, Ont) at 37°C in the presence of 5% CO_2 in a Shellab incubator (Johns Scientific, Toronto, Ont).

When cells reached confluency, they were passaged as follows: (i) culture medium was aspirated; (ii) cells were washed with 3 ml of 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) warmed to 37°C; (iii) 3 ml of 0.005% trypsin-0.002% EDTA (Gibco/BRL) in TBS were added; (iv) cells were incubated at 37°C for 3 - 5 minutes; (v) trypsin was inactivated by addition of fresh GM warmed to 37°C; (vi) cells were removed from the plates by gentle pipetting; (vii) cells were diluted in fresh GM and plated in new culture dishes. Normally, cells were split at a ratio of 1:5 to 1:10.

2.2. Virus

Isolates of EV70 listed in Table 5 were obtained from Dr. M. Hatch and Dr. M. Pallansch (Centers for Disease Control and Prevention, Atlanta, GA). All virus stocks were prepared as follows. Confluent plates of LLC-MK_2 cells were washed with TBS and infected with the virus at a multiplicity of infection (MOI) of 0.1 - 0.2 plaque forming units (PFU) per cell in serum-free GM. The cells were incubated at 33°C for 1 hr, with occasional rocking, to allow the virus to adsorb. The inoculum was then replaced with fresh GM. Infected cells were incubated at 33°C for 24 - 36 hrs. When cytopathic effects (CPE) were evident, three cycles of freezing and thawing were carried out. Subsequently, cell debris was removed by centrifugation at 14,000 rpm and 20°C for 15 minutes (Beckman SW28 rotor). Supernatants were divided into 1 ml aliquots and stored at -80°C.

2.3. Plaque Assay

Serial dilutions of virus were made in serum-free GM. Culture medium was removed from confluent monolayers of LLC-MK_2 cells grown in 60 mm dishes and the

TABLE 5 ORIGIN OF EV70 ISOLATES

Isolates	Year of Isolation	Location	Outbreak
J670[1]	1971	Japan	Pandemic
R6	1971	Morocco	Pandemic
Ru3573	1975	S. Vietnam	Localized
V1250	1981	Honduras	Pandemic
KW97	1981	Key West, FL	Pandemic
1604	1981	Pakistan	Pandemic

[1] Prototype strain

cells were washed with TBS and infected with 0.3 ml of the serial dilutions. Adsorption was done at 33°C for 1 hr with occasional rocking. After the inoculum was removed, the cells were overlaid with 4 ml of GM containing 0.8% agarose. Cells were incubated at 33°C for 3 days to allow plaques to develop. Monolayers were then fixed with 10% (v/v) formalin, 0.8% (w/v) NaCl at 37°C for 1 hr. The agarose was removed with running water and plaques were visualized by staining with 0.1% (w/v) crystal violet at room temperature for 10 minutes. The plates were dried and plaques were counted. Assays were routinely performed in duplicate.

2.4. Antibodies

Monoclonal antibodies (mouse ascites fluids) 72-5E (IgG3) and 73-2F (IgG2B) were kindly provided by Dr. L. J. Anderson (Centers for Disease Control and Prevention). Monoclonal antibodies (mouse ascites fluids) EV-13 (IgM), EV-12 (IgG2a) and EV-11 (IgG1) were kindly donated by Dr. B. Brodeur and Dr. J. Wiley (Laboratory Centre for Disease Control, Ottawa, Ont). The neutralization titers of the antibodies were determined using the plaque reduction test (see Section 2.5).

2.5. Plaque Reduction Neutralization Test

Serial 10-fold dilutions of ascites fluids were made in serum-free GM. Approximately 150 PFU of virus in 150 μl of serum-free GM were combined with an equal volume of diluted ascites fluid and incubated for 1 hr at room temperature. The antibody-virus mixture was adsorbed to LLC-MK_2 cell monolayers and plaque assays

were performed as described above in Section 2.3. The 50% neutralization titers were determined by the Reed-Muench method (Reed and Muench, 1938).

2.6. Determination of Neutralization Titers

The 50% neutralization titers were determined as follows. The negative logarithm of the 50% neutralization titer = the negative logarithm of the antibody dilution above the 50% neutralization plus the proportionate distance factor (corrected for dilution series used). The proportionate distance = ((% neutralization at dilution next above 50% neutralization) - (50%)) / ((% neutralization at dilution next above 50% neutralization) - (% neutralization at dilution next below 50% neutralization)). The proportionate distance factor = proportionate distance × dilution factor ($\log_{10}$). Since the serial dilutions were 10-fold in this study, the dilution factor is 1 and the proportionate distance is equal to the proportionate distance factor.

2.7. Isolation of Antibody-Escape Mutants

250 μl of prototype virus J670 (2.5×10^6 PFU) were incubated in serum-free GM with an equal volume of a 10^{-2} dilution of ascites fluid for 1 hour at room temperature and then adsorbed to LLC-MK_2 cell monolayers. A standard plaque assay was carried out except that the agarose overlay contained a 5×10^{-4} dilution of ascites fluid. Resistant plaques were picked for purification following a second overlay with agarose containing 0.2 mg/ml neutral red. Red colour was allowed to develop for 24 hrs at 33°C and virus in individual plaques was isolated by removing an agarose plug and the

cells within and around the edge of each plaque with a sterile Pasteur pipette. The cells and agarose were transferred to fresh LLC-MK_2 cell monolayers which were incubated at 33°C until CPE was extensive. After three cycles of freezing-thawing, cell debris was removed by centrifugation and the virus-containing supernatants were titrated by plaque assay. Virus was plaque-purified twice on LLC-MK_2 cells following the same protocol. Frequency of antibody-resistant mutants was calculated as the number of plaques in the presence of antibody divided by the number of PFU plated (Sherry and Rueckert, 1985).

2.8. Isolation of RNA

2.8.1. Isolation of RNA from EV70-Infected Cells

Newly confluent LLC-MK_2 cells in 100 mm culture dishes were infected at a MOI of approximately 0.2 PFU/cell as described in Section 2.2. Mock-infected cells were used as negative controls. When CPE in the test plates were evident, cells were scraped from the plates with a rubber policeman and pelleted at 5000 rpm for 5 - 10 minutes at 4°C (Heraeus Minifuge 2, Pointe-Claire, Quebec). The cell pellets were washed twice with cold TBS and resuspended in NENS buffer (400 μl/plate; 50 mM NaAc, pH 5.1, 10 mM EDTA, 100 mM NaCl, 0.5% SDS) containing 500 μg/ml of proteinase K (Boehringer Mannheim, Laval, Quebec). The resulting cell lysate was incubated at 37°C for 1 hr. During the 1 hr incubation, the lysate was sonicated at the minimum energy setting using a small microtip for 10 seconds, until the solution was clear (Sonifier model w-350, Branson Sonic Power Company, Shelton, CT). The lysate was extracted with an equal volume of phenol-chloroform-isoamyl alcohol (25/24/1; v/v/v) and then with

an equal volume of chloroform-isoamyl alcohol (24/1; v/v). Nucleic acid was precipitated with 2.5 volumes of ethanol overnight at -20°C. The nucleic acid pellet obtained by centrifugation at 12,000 rpm and 4°C for 30 minutes (Fisher model 235B microfuge, Ottawa, Ont.) was washed with 70% ethanol and dissolved in 250 μl of diethylpyrocarbonate-treated water (DEP-H_2O). 250 μl of 5 M LiCl were added and the samples were incubated at 4°C for 30 minutes. Samples were centrifuged at 12,000 rpm and 4°C for 30 minutes and the pellet was redissolved in 200 μl of DEP-H_2O. A final precipitation was carried out by addition of 20 μl of 3M NaAc (pH 5.2) and 2.5 volumes of ethanol and incubation overnight at -20°C. The RNA obtained by centrifugation at 12,000 rpm and 4°C for 30 minutes was washed with 70% ethanol, resuspended in 20 μl DEP-H_2O and stored at -80°C.

2.8.2. *Isolation of EV70 Genomic RNA*

LLC-MK_2 cells were infected with EV70 at a MOI of 0.2 PFU/cell. The infection was allowed to proceed overnight at 33°C until destruction of the monolayer was complete. The cells and the culture media were transferred to a 15 ml plastic tube and frozen and thawed three times. Cell debris was pelleted at 14,000 rpm for 15 minutes at 20°C (Beckman SW28 rotor). The supernatant was transferred to a second tube and 10% (w/v) SDS was added to a final concentration of 0.1%. The virus was pelleted at 25,000 rpm for 4 hrs at 24°C (Beckman SW28 rotor). After the supernatant was discarded, the pellet was drained well and resuspended in 200 μl NENS buffer

containing 500 μg/ml of proteinase K. The procedures for RNA extraction described in Section 2.8.1 were used to isolate EV70 genomic RNA.

2.9. cDNA Synthesis

cDNA reactions typically consisted of cell RNA or EV70 genomic RNA in 50 mM Tris-HCl, pH 8.3, 40 mM KCl, 6 mM $MgCl_2$, 2 mM DTT, 50 ng each of the (-) sense primers, 5' (5'-CCAGTCTACATAAACTGACC-3'; complementary to nucleotides 3408 - 3427 of the EV70 genome); 2204 (5'-GAGACCCTGAGATCCAAGG-3'; complementary to nucleotides 2186 - 2204 of the EV70 genome) (Ryan *et al*, 1990; Takeda *et al*, 1989), 1 mM of each of the four dNTPs and 20 units of RNasin (Promega/Biotec, Madison, Wisconsin) in a total volume of 19 μl. Samples were heated at 90°C for 5 minutes and placed at 4°C for 5 minutes. 1 μl (200 units) of M-MuLV reverse transcriptase (Gibco/BRL) was added to each sample and cDNA synthesis was carried out at 37°C for 30 minutes. Samples were heated at 90°C for 10 minutes and then immediately placed on ice.

2.10. Polymerase Chain Reaction (PCR)

cDNA was used as template in the polymerase chain reaction. 80 μl of PCR buffer (50 mM KCl, 10 mM Tris-HCl, pH 8.3, 1.5 mM $MgCl_2$, 0.01% (w/v) gelatin, 0.01% nuclease-free bovine serum albumin), 50 ng of each of the (+) sense primers "2^{+BST}" (5'-CGCTAGTGGAGTGGAGAGG-3'; corresponding to nucleotides 1864-1882 of the EV70 genome; 591 (5'- ATGGTGACAATCAGAGATTG -3'; corresponding to

nucleotides 591 - 610 of the EV70 genome (Ryan *et al*, 1990; Takeda *et al*, 1989), and 0.5 μl (2.5 units) of Taq DNA Polymerase (Gibco-BRL) were added to each cDNA sample. The reaction mixture was overlayed with 100 μl of mineral oil and the amplification was carried out using a step-cycle file (Veres *et al*, 1987, Coy Tempcycle model 60) as follows: (i) 3 cycies of 5 minutes denaturation at 95°C, 2 minutes annealing at 51°C and 3 minutes extension at 72°C; (ii) 28 cycles of 1 minute denaturation at 95°C, 2 minutes annealing at 51°C, and 3 minutes extension at 72°C which was increased by 40 seconds for each cycle and; (iii) cooling to 4°C. The bottom aqueous phase was removed from each sample and extracted once with an equal volume of phenol-chloroform-isoamyl alcohol (25/24/1; v/v/v) and then with an equal volume of chloroform/isoamyl alcohol (24/1; v/v). One-tenth of each sample was analyzed by agarose gel electrophoresis (see Section 2.11).

2.11. Agarose Gel Electrophoresis

Electrophoresis of PCR products was carried out in 1% agarose gels using TBE buffer (89 mM Tris, 89 mM boric acid, 2 mM EDTA pH 8.0) at 60 volts for 2 hrs (Minnie submarine agarose unit model HE 33, Hoefer, San Francisco, CA). DNA was visualized and photographed, following staining with 1 μg/ml ethidium bromide (EtBr), using a polaroid MP4 land camera.

2.12. DNA Manipulation and Cloning

2.12.1. Phosphorylation of DNA

PCR DNA products were precipitated by adding 1/10 volume of 3 M NaAc (pH 5.2) and 2 volumes of ethanol at -20°C. After centrifugation, the pellet was resuspended in 14 μl of H_2O and 4 μl of 10 × One-Phor-All buffer (Pharmacia, Baie d'Urfe, Quebec), 1 μl of 100 mM ATP and 1 μl of polynucleotide kinase (10 units/μl, New England Biolabs, Mississauga, Ont.) were added. The reaction was incubated at 37°C for 30 minutes and then heated at 65°C for 15 minutes. Samples were incubated at room temperature for 20 minutes and the DNA was precipitated with 1/2 volume of 7.5 M NH_4Ac and 2 volumes of ethanol at -20°C.

2.12.2. Filling 3' Recessed Termini

DNA was redissolved in 20 μl of H_2O and adjusted to 50 mM Tris-HCl, pH 7.2, 10 mM $MgCl_2$, 0.1 mM DTT, 50 μg/ml BSA, 200 μM dNTPs in a final volume of 25 μl. 2 units of the Klenow fragment of DNA Polymerase I (Pharmacia) were added and the sample was incubated at room temperature for 30 minutes and then heated at 70°C for 5 minutes. Samples were incubated at room temperature for 20 minutes and the DNA was precipitated with ethanol at -20°C as described in Section 2.12.1.

2.12.3. Isolation of DNA from Agarose Gels

Electrophoresis was carried out in 0.8% SeaPlaque GTG agarose (Mandel, Guelph, Ont) gels in TBE buffer at 50 volts for 2 - 2.5 hrs and DNA was stained with

EtBr for 5 minutes. DNA bands were visualized and cut from the gels under long wave UV light. Each agarose slice was placed in a sterile 1.5 ml microfuge tube and stored at 4°C.

2.12.4. Preparation of Vector

2 μg of plasmid pGEM7Zf(+) DNA (Promega) were digested with restriction endonuclease *Sma I* (20 units, New England Biolabs) at room temperature for 1.5 hrs. DNA was dephosphorylated by the addition of 0.8 units of calf intestinal phosphatase (Pharmacia) and incubated for an additional 30 minutes at 37°C. Enzymes were inactivated by heating at 85°C for 15 minutes and the DNA was precipitated with ethanol at -20°C.

2.12.5. In Gel Ligation

Agarose slices containing phosphorylated PCR products were melted by heating to 68°C for 10 minutes and placed at 37°C. Blunt end ligation was carried out in 20 μl reaction mixtures containing 2 μl of 10 $\times$ T_4 DNA ligase buffer (0.5 M Tris-HCL (pH 7.6), 100 mM $MgCL_2$, 10 mM ATP, 10 mM DTT, 50% (w/v) polyethylene glycol-8000, Gibco/BRL), 1 μl of vector (50 ng), 1 μl (6 Weiss units) of T_4 DNA ligase (Gibco/BRL) and 16 μl of a melted agarose gel slice at 16°C for 16 - 24 hrs (Struhl *et al*, 1985).

2.13. Electro-Transformation

Ligation reactions were heated at 68°C for 5 minutes and placed at 37°C. For electrotransformation, an aliquot of competent *E. coli* DH5α was thawed at room temperature and placed on ice. 20 μl of competent cells were mixed with 2 μl of a ligation reaction (1:2 dilution in 10 mM Tris-Cl, pH 7.5) and incubated on ice for 1 minute. The Cell Porator Voltage Booster (Gibco/BRL) was used for electrotransformation. Electrotransformation was done following the protocols recommended by the supplier. The Voltage Booster was set at 4 k Ω and the pulse control unit was set for 330 μF capacitance, low Ω, and fast charge rate. Following electrotransformation, the cell-DNA mixture was removed from each chamber with a micropipette, diluted with 1 ml of SOC (2% Tryptone, 0.5% yeast extract, 0.05% NaCl, 10 mM $MgCl_2$, 10 mM $MgSO_4$, 20 mM glucose) and incubated at 37°C for 1 hr with shaking (200 rpm). 200 μl of each sample were plated onto TSB (Tryptic Soy Broth, 3% w/v) agar (1.5% w/v) plates containing 50 μg/ml ampicillin (Boehringer Mannheim) which were inverted and incubated overnight at 37°C.

2.14. Small-Scall Preparation of Plasmid DNA

Small scale plasmid preparations (mini-preps) were done using a boiling method (Holmes and Quigley, 1981). Single bacterial colonies were transferred into 5 ml of 3% TSB medium containing 50 μg/ml ampicillin in 50 ml Erlenmeyer flasks and grown overnight at 37°C with agitation (200 rpm). 1.5 ml of the culture was pipetted into a microfuge tube and cells were pelleted at 12,000 rpm for 2 minutes in a microfuge. The

medium was aspirated and the pellet was resuspended in 500 μl of STET (8% sucrose, 0.5% Triton X-100, 50 mM EDTA, 10 mM Tris-HCl pH 8.0) containing 10 mg/ml lysozyme (Sigma, St. Louis, MO). The tubes were placed in a boiling water bath for 40 seconds and centrifuged immediately for 20 minutes at room temperature in a microfuge. Supernatants were transferred to fresh tubes and 2 μl of DNase-free RNase A (10mg/ml, Boehringer Mannheim) were added to each sample. Samples were incubated at 37°C for 30 minutes and extracted once with phenol-chloroform-isoamyl alcohol (25/24/1) and once with chloroform-isoamyl alcohol (24/1). DNA was precipitated by addition of 40 μl of 2.5 M NaAc and 420 μl of isopropanol and incubation at -20°C overnight. DNA was recovered by centrifugation at 12,000 rpm for 30 minutes at room temperature and pellets were washed with 70% ethanol then dried for 10 minutes in a Speed Vac (Fisher, Ottawa, Ont.). DNA was resuspended in 100 μl of TE (10 mM Tris-HCl, 1 mM EDTA, pH 8.0) and electrophoresed in 1% agarose gels according to the procedures described in Section 2.11.

2.15. DNA Sequence Analysis

2.15.1. DNA Sequencing

2 - 3 μg of DNA were denatured with 0.2 M NaOH at room temperature for 5 minutes in a final volume of 20 μl. The samples were neutralized with 10 μl of 5 M NH_4Ac (pH 7.5) and DNA was precipitated with 2 volumes of ethanol. DNA was recovered by centrifugation for 30 minutes at 12,000 rpm and 4°C in a microcentrifuge, washed with 70% ethanol, and dried. DNA was annealed with 16 ng of oligonucletide

primer in Sequenase™ buffer (40 mM Tris-HCl, pH 7.5, 20 mM $MgCl_2$, and 50 mM NaCl) in a final volume of 10 μl by incubating for 5 minutes at 65°C and then cooling for 2 - 3 hrs to 25°C. DNA sequences were determined by the dideoxynucleotide chain termination method (Sanger *et al.* 1977) using the Sequenase™ DNA Sequencing Kit (United States Biochemical, Cleveland, OH) and [α-^{35}S] dATP (Du Pont, Mississauga, Ont.).

2.15.2. Sequencing Gels and Electrophoresis

Sequencing samples were electrophoresed on 0.2 mm thick 5% polyacrylamide gels (4.75% acrylamide, 0.25% bis, 7 M urea in 1.2 × TBE) using an IBI model STS-45 sequencing apparatus. Pre-electrophoresis was performed for 30 - 60 minutes in TBE running buffer (1 × TBE in the upper chamber and 1.5 × TBE in the lower chamber) at 55 W constant power (30 mA and 2500V were set as upper limits for amperage and voltage). Sequencing reactions were heated for 3 minutes at 90°C and 2 μl of the sequencing reactions were loaded onto gels. Electrophoresis was carried out at 55 W constant power for another 2 - 5 hrs. After electrophoresis, gels were lifted from the glass plates onto Whatmann 3 MM paper, covered with plastic wrap and dried (Model 583 gel dryer, BioRad, Mississauga, Ont.). Gels were exposed to X-ray film over night at -80 °C.

2.15.3. Analysis of Nucleotide and Amino Acid Sequences

Searches for potential open reading frames, translation of DNA sequences, and alignment of DNA and protein sequences were performed using the Pustell Sequence Analysis Programs (IBI) Version 2.04.

CHAPTER 3

RESULTS

3.1. Neutralization Titers of EV70-Specific Neutralizing Monoclonal Antibodies

An initial objective of this project was to isolate mutants of EV70 that were resistant to neutralization by neutralizing monoclonal antibodies. It was necessary therefore, as a preliminary step, to determine the neutralization titers of the 4 available neutralizing monoclonal antibodies (72-5E, 73-2F, EV-12 and EV-13) and to establish conditions for the selection of neutralization escape mutants of EV70.

Two virus stocks of EV70 J670 were prepared and titrated as described in Chapter 2. The virus titers of the two stocks were 8.0×10^6 PFU/ml and 5.0×10^7 PFU/ml. The neutralization titers of the antibodies were determined using the two stocks

of EV70 J670 and the plaque reduction neutralization test described in Chapter 2. For these tests, approximately 150 PFU of the prototype virus J670 were combined with 10-fold dilutions (10^{-2} - 10^{-7}) of the different antibodies. An EV70 specific monoclonal antibody, EV-11 (Drs. J. Wiley and B. Brodeur, Health and Welfare of Canada) was used as a control antibody. EV-11 was reported to have a low neutralizing activity (Wiley *et al.*, 1990). Monoclonal antibody 3B3.16 (Dr. K. Wright, Department of Microbiology and Immunology, University of Ottawa) which is specific for the nucleoprotein of Pichinde virus, was also used as a negative control.

As shown in Figure 1, these experiments demonstrated that antibodies 72-5E, 73-2F and EV-13 had very good neutralizing activities towards EV70 at room temperature, whereas the neutralizing activities of antibodies EV-11 and EV-12 were much lower. In an attempt to identify conditions under which antibodies EV-11 and EV-12 might neutralize EV70 more efficiently, the neutralization step was carried out at 37°C and 33°C, as well as at room temperature. For these tests, antibody 72-5E and serum-free GM were used as positive and negative controls respectively. Variations in the temperature of the neutralization step did not improve the ability of antibody EV-11 to neutralize EV70 (data not shown), however, as shown in Figure 2, the neutralization temperature did affect the ability of EV-12 to neutralize EV70. At 37°C, EV-12 had its highest neutralizing activity, while at room temperature, neutralization was low. Temperature had no effect on the viability of EV70 during the course of the neutralization test (data not shown) nor on the neutralizing activity of antibody 72-5E.

Figure 1. Neutralization of EV70 J670 by monoclonal antibodies

LLC-MK_2 cells were grown to confluency in 60 mm culture dishes. 10-fold dilutions of ascites fluid 72-5E, 73-2F, EV-11, EV-12 and EV-13 were made in serum-free GM. Antibody 3B3.16 was used as a negative control. Approximately 150 PFU of prototype EV70 J670 in 150 μl of serum-free GM were mixed with an equal volume of each diluted ascites fluid (in duplicate) and incubated for 1 hr at room temperature. The antibody-virus mixtures were used to infect LLC-MK_2 cell monolayers and plaque assays were carried out as described in Chapter 2. The percent reduction indicates the percent reduction in the number of plaques as compared to control samples which contained no antibody. (○) 72-5E, (▽) 73-2F, (□) EV-13, (◆) EV-12, (●) EV-11, (▲) 3B3.16.

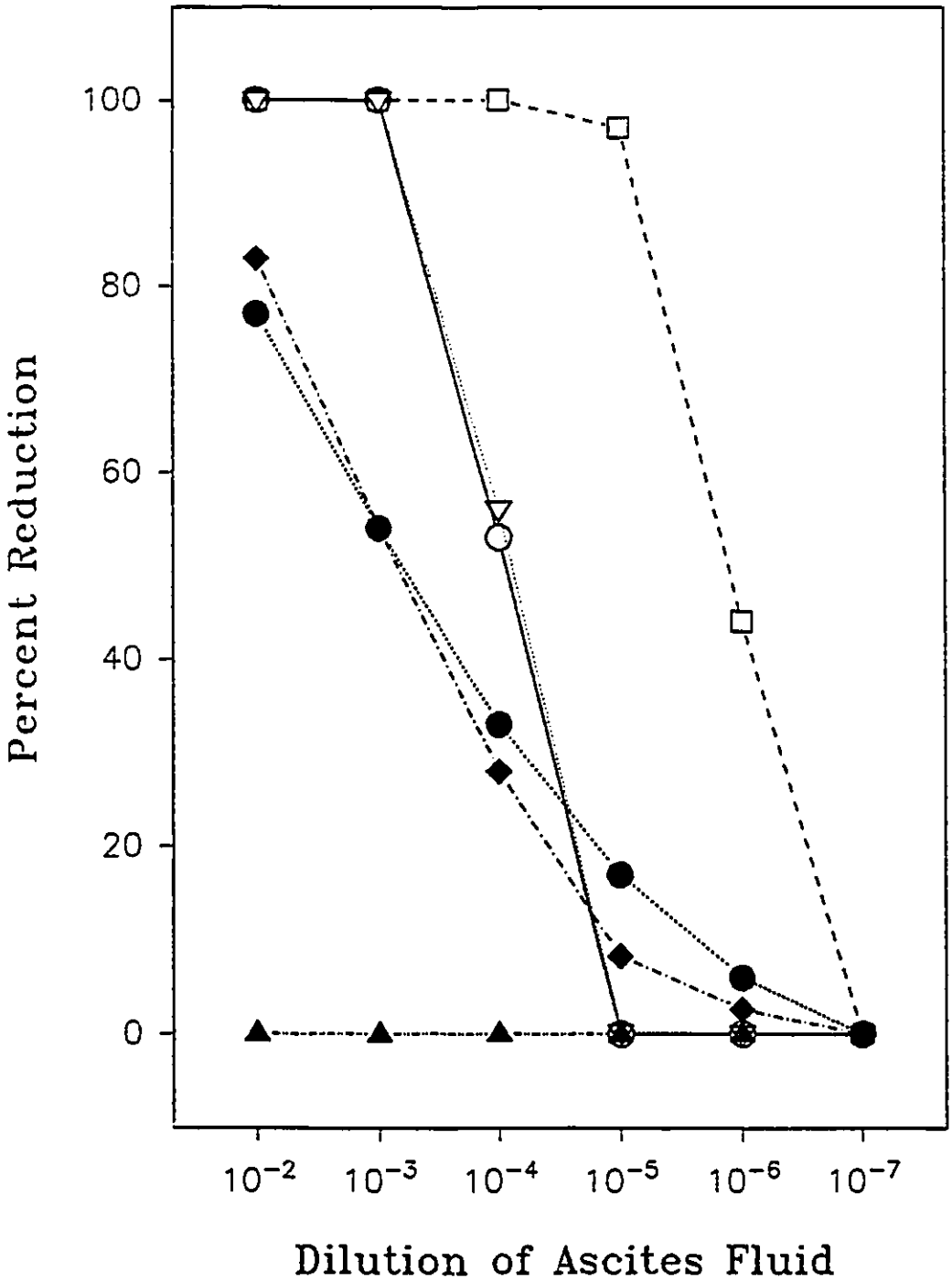
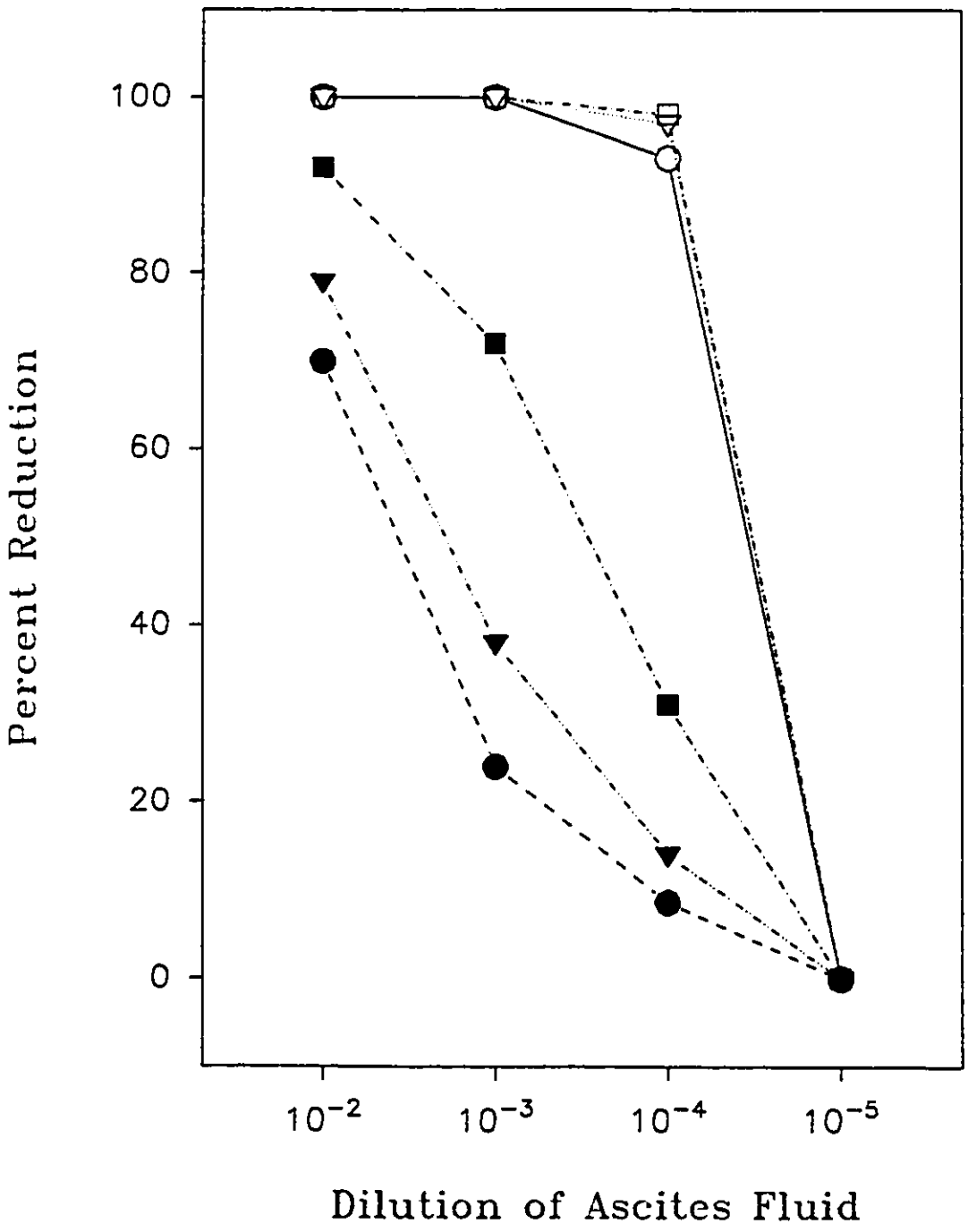

100
80
60
40
20
0
Percent Reduction
10^{-2}
10^{-3}
10^{-4}
10^{-5}
10^{-6}
10^{-7}
Dilution of Ascites Fluid

Figure 2. Effect of temperature on neutralization of EV70 J670 by mAb EV-12

The experiments were done as described in the legend to Figure 1 except that: (i) the ascites fluid was either antibody EV-12 or antibody 72-5E (as a positive control) and (ii) the neutralizing step was performed at three different temperatures, 37°C, 33°C and room temperature (RT). 72-5E: (○) RT, (▽) 33°C, (□) 37°C; EV-12: (●) RT, (▼) 33°C, (■) 37°C.


100
80
60
40
20
0
Percent Reduction
10^{-2}
10^{-3}
10^{-4}
10^{-5}
Dilution of Ascites Fluid

Additional attempts were made to improve neutralization by antibody EV-11. The effects of a 10-fold increase in antibody concentration and doubling neutralization time were tested both individually and together. There was still no improvement in the neutralizing ability of EV-11 and it was not used in subsequent experiments.

Following the plaque reduction tests, the 50% neutralization titers of the antibodies were determined (Table 6) using the Reed-Muench method as described in Chapter 2. The 50% neutralization titers of 72-5E and 73-2F were both calculated to be 4.1 (Log_{10} neutralization titer). The 50% neutralization titer of antibody EV-13 was approximately 60 times higher than for the average titer of 72-5E and 73-2F and the titer of antibody EV-12 was approximately 30 times lower. The 50% neutralization titer of antibody EV-12 at different temperatures is shown in Table 7. At 37°C, the neutralization titer of EV-12 was approximately 40 times greater than at room temperature.

3.2. Isolation of Antibody-Escape Mutants

Isolation of antibody-escape mutants was carried out using two stocks of prototype EV70 J670 and the four neutralizing monoclonal antibodies, 72-5E, 73-2F, EV-12, and EV-13, as described in Chapter 2. For the monoclonal antibodies 72-5E, 73-2F, and EV-13, a working dilution of 10^{-2} was used, while for the antibody EV-12, a working dilution of 10^{-1} was used, i. e., a concentration of antibody at least 100 times greater than the concentration at which 50% neutralization of EV70 J670 was obtained. 250 μL

TABLE 6 50% NEUTRALIZATION TITERS OF mAbs

Antibodies	Log_{10} Neutralization Titer[1]
72 - 5E	4.1
73 - 2F	4.1
EV - 13	5.9
EV - 12	2.6
EV - 11[2]	2.1

[1] The mean of 2 experiments

[2] There was insufficient neutralization to calculate the 50% neutralization titer The value was approximated using the neutralization curve shown in Figure 1

TABLE 7 50% NEUTRALIZATION TITERS OF mAbs TESTED AT DIFFERENT TEMPERATURES

Temperature	Log_{10} Neutralization Titer[1] 72-5E	EV-12
RT	4.5	2.2
33°C	4.5	2.5
37°C	4.5	3.8

[1] The mean of 2 experiments

containing 2.5 x 10^6 PFU of each of the two stocks of EV70 J670 were mixed with an equal volume of 10^{-2} or 10^{-1} dilutions of monoclonal antibody in serum-free GM. The mixtures were incubated for 1 hr at room temperature for antibodies 72-5E, 73-2F, EV-13 and at 37°C for antibody EV-12, and the samples were used to infect LLC-MK_2 cells. Standard plaque assays were carried out using agar overlays containing 5 x 10^{-4} dilutions of the appropriate antibody to prevent plaque formation by surviving wild type virus. Plaques were allowed to develop for 3 days after which the plates were overlaid with 0.8% agarose containing 0.2 mg/ml neutral red. Plaques were counted and for each antibody, 4 plaques (2 for each virus stock) that survived neutralization were picked 24 hrs later and then plaque purified on LLC-MK_2 cells with antibody present in the overlay.

The frequencies of apperance of plaques resistant to neutralization were calculated as the number of plaques which developed in the presence of antibody divided by the number of PFU plated (as determined by the number of plaques which appeared on control plates) and are listed in Table 8. During plaque purification 93% and 95% of the PFU that escaped neutralization by mAbs 72-5E and EV-13, respectively, remained resistant to neutralization. For antibodies 73-2F and EV-12, however, only 2 to 3% of the PFU that escaped neutralization remained resistant during plaque purification. At this time, it was considered that these low numbers for antibodies 73-2F and EV-12 could be due to contamination of the first generation isolates with wild type virus. For this reason, a second round of plaque purification was carried out for all of the isolates. Following the second plaque purification, 94% and 96% of the PFU from plaques

TABLE 8 FREQUENCY OF APPEARANCE OF PLAQUES RESISTANT TO NEUTRALIZATION

Antibody	Virus stock	Frequency of escape mutants
72-5E	1	1.2×10^{-5}
	2	8.6×10^{-6}
73-2F	1	1.22×10^{-4}
	2	6.32×10^{-5}
EV-13	1	6.4×10^{-5}
	2	4.8×10^{-6}
EV-12	1	3.84×10^{-4}
	2	3.92×10^{-4}

isolated using antibodies 72-5E and EV-13 respectively were resistant to neutralization, but no plaques at all appeared for viruses isolated by antibodies 73-2F and EV-12. This confirmed the suspicion that the virus plaques present after neutralization with 73-2F and EV-12 were in fact wild type plaques. The high apparent frequencies of escape from neutralization by antibodies 73-2F and EV-12 therefore were likely due to wild type virus (Table 8). Additional attempts were made to determine conditions that would permit isolation of neutralization resistant mutants for antibodies 73-2F and EV-12, for example: (i) the amount of virus present during the neutralization was varied, (ii) the amount of antibody present in the virus-antibody mixture and/or in the overlay was varied, (iii) neutralization time and temperature were increased. The experiments were repeated several times using several combinations of these parameters. Unfortunately, either no resistant plaques were observed following neutralization or the plaques that were selected were not resistant during a second round of neutralization. Therefore it was necessary to continue the study with the two antibodies 72-5E and EV-13 and with the mutants resistant to neutralization by these two antibodies. Stocks for each of the neutralization escape mutants were prepared as described in Chapter 2.

3.3. Neutralization of Antibody-Escape Mutants and Wild Isolates

Before undertaking sequence analysis, it was important to verify that the isolates were in fact escape mutants. This was done by assaying whether they could be neutralized by the homologous antibody in a standard neutralization test. In addition, it was of interest to determine whether the two neutralizing mAbs, 72-5E and EV-13, were

interacting with similar or different sites on EV70 and if these sites were unchanged on different clinical isolates of EV70.

3.3.1. Neutralization of EV70 Escape Mutants by mAbs Used in Selection

Stocks of prototype EV70 J670 and mutants 72-5E 1-4 and EV-13 1-4 were titrated by the plaque assay as described in Chapter 2. 10-fold dilutions of mAbs 72-5E and EV-13 were made in serum-free GM and 150 μl aliquots of the diluted antibody were incubated with an equal volume of each of the viruses containing approximately 150 PFU, for 1 hr at room temperature. The antibody-virus mixtures were then used to infect LLC-MK_2 cells. Plaque assays were performed and neutralization titers were determined as detailed in Chapter 2. The patterns of neutralization of the various escape mutants by the selecting antibodies, 72-5E and EV-13 are presented in Figures 3 and 4 respectively. In contrast to the neutralization pattern of the prototype EV70 (J670) by mAbs 72-5E and EV-13, the mutants were resistant to neutralization by the antibodies used in their selection.

3.3.2. Neutralization of EV70 Escape Mutants by mAbs not Used in Selection

To test whether or not antibodies 72-5E and EV-13 were interacting with the same or different sites on EV70, standard neutralization tests were carried out on each of the mutants with the antibody that was not used in their selection. As shown in Figures 5 and 6, 72-5E resistant mutants and EV-13 resistant mutants were sensitive to

Figure 3. Neutralization of EV70 escape mutants selected with mAb 72-5E by mAb 72-5E

10-fold dilutions of ascites fluid 72-5E were made in serum-free GM. Approximately 150 PFU of prototype J670 or escape mutants (72-5E 1-4) in 150 μl of serum-free GM were mixed with an equal volume of diluted ascites fluid and incubated for 1 hr at room temperature. The treated virus was used to infect LLC-MK_2 cells and the plaque assays were done as described in Chapter 2. The percent reduction indicates the percent reduction in the number of plaques as compared to control samples which contained no antibody. (●) J670, (▾) 72-5E(1), (■) 72-5E(2), (▲) 72-5E (3), (◆) 72-5E(4).

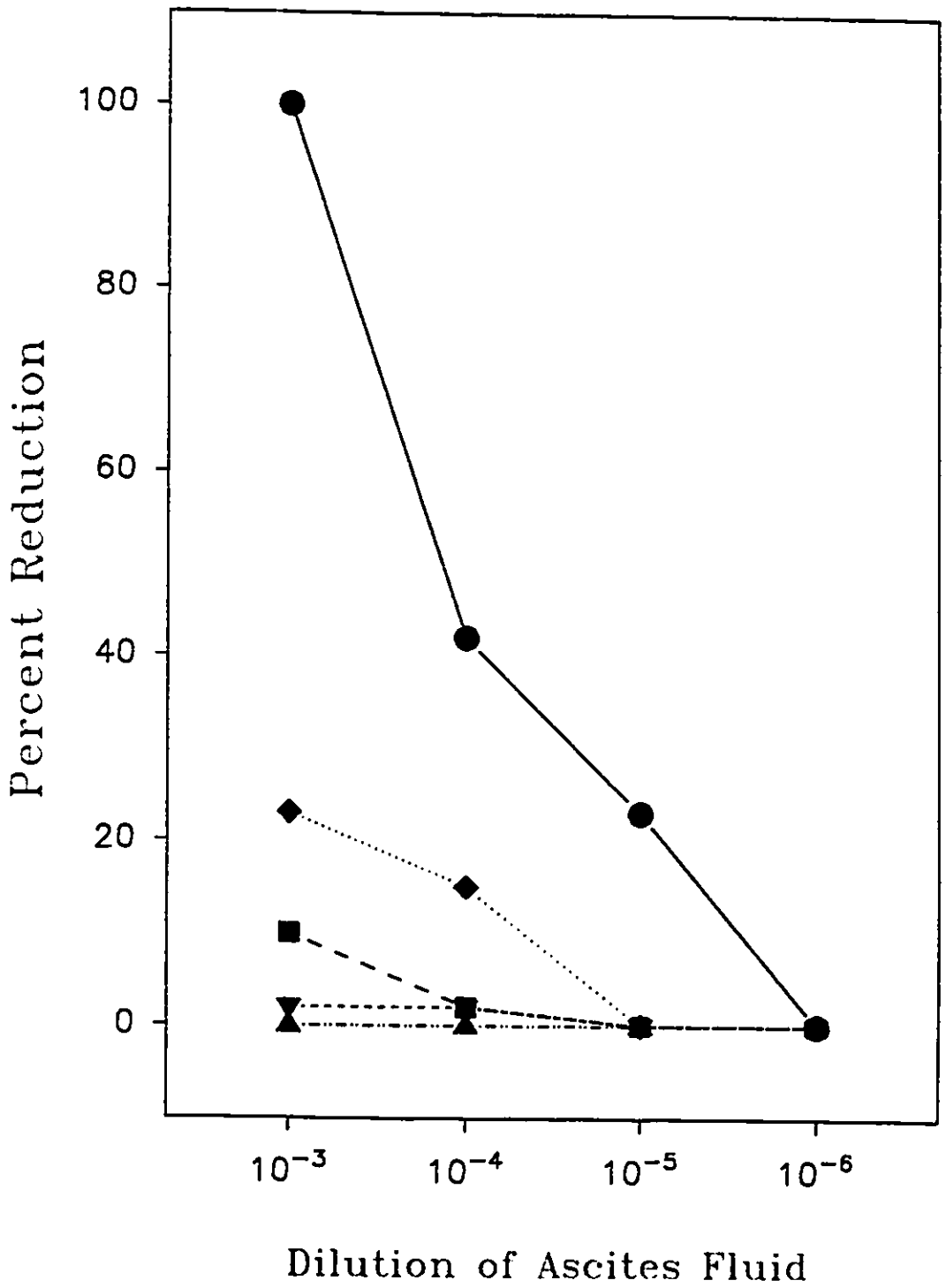

Percent Reduction
100
80
60
40
20
0
10^{-3}
10^{-4}
10^{-5}
10^{-6}
Dilution of Ascites Fluid

Figure 4. Neutralization of EV70 escape mutants selected with mAb EV-13 by mAb EV-13

The experiments were carried out as described in the legend to Figure 3, using escape mutants EV-13 1-4 and the homologous antibody EV-13. (●) J670, (▼) EV-13(1), (■) EV-13(2), (▲) EV-13 (3), (◆) EV-13(4).

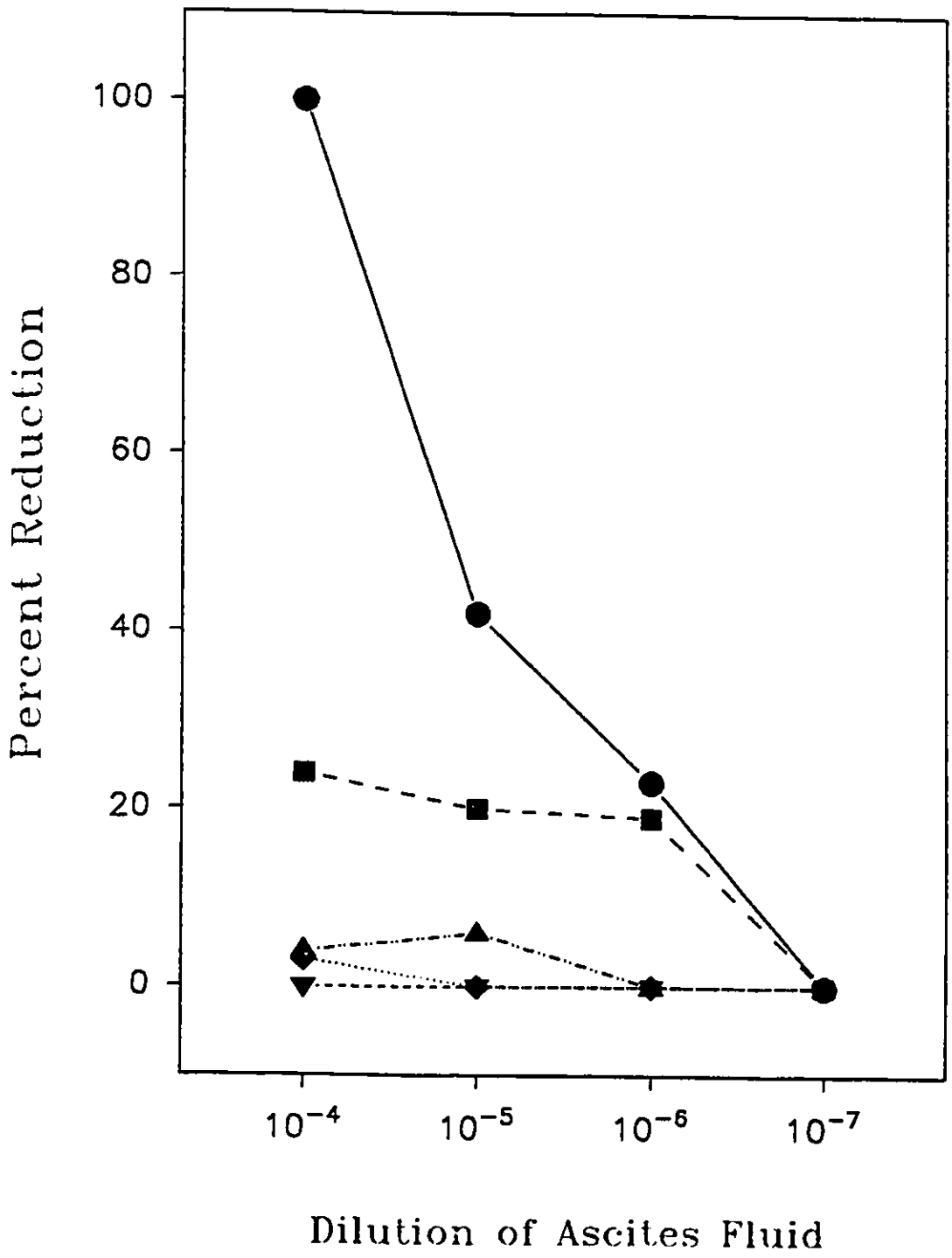

Percent Reduction
100
80
60
40
20
0
10^{-4}
10^{-5}
10^{-6}
10^{-7}
Dilution of Ascites Fluid

Figure 5. Neutralization of EV70 escape mutants selected with mAb 72-5E by mAb EV-13

The tests were done as described in the legend to Figure 3, except that the mAb used in this experiment was EV-13 instead of 72-5E. (●) J670, (▼) 72-5E(1), (■) 72-5E(2), (▲) 72-5E (3), (◆) 72-5E(4).

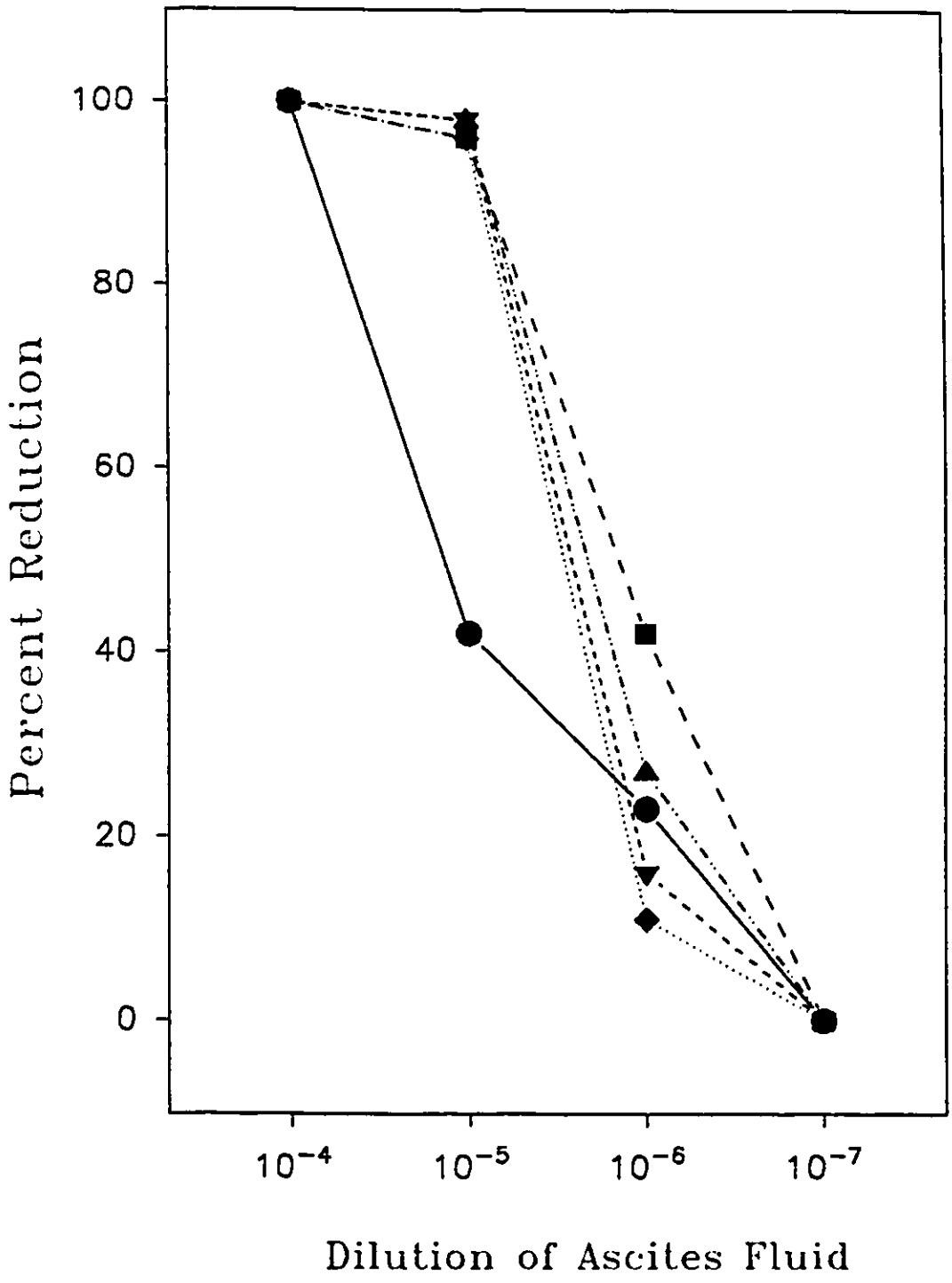
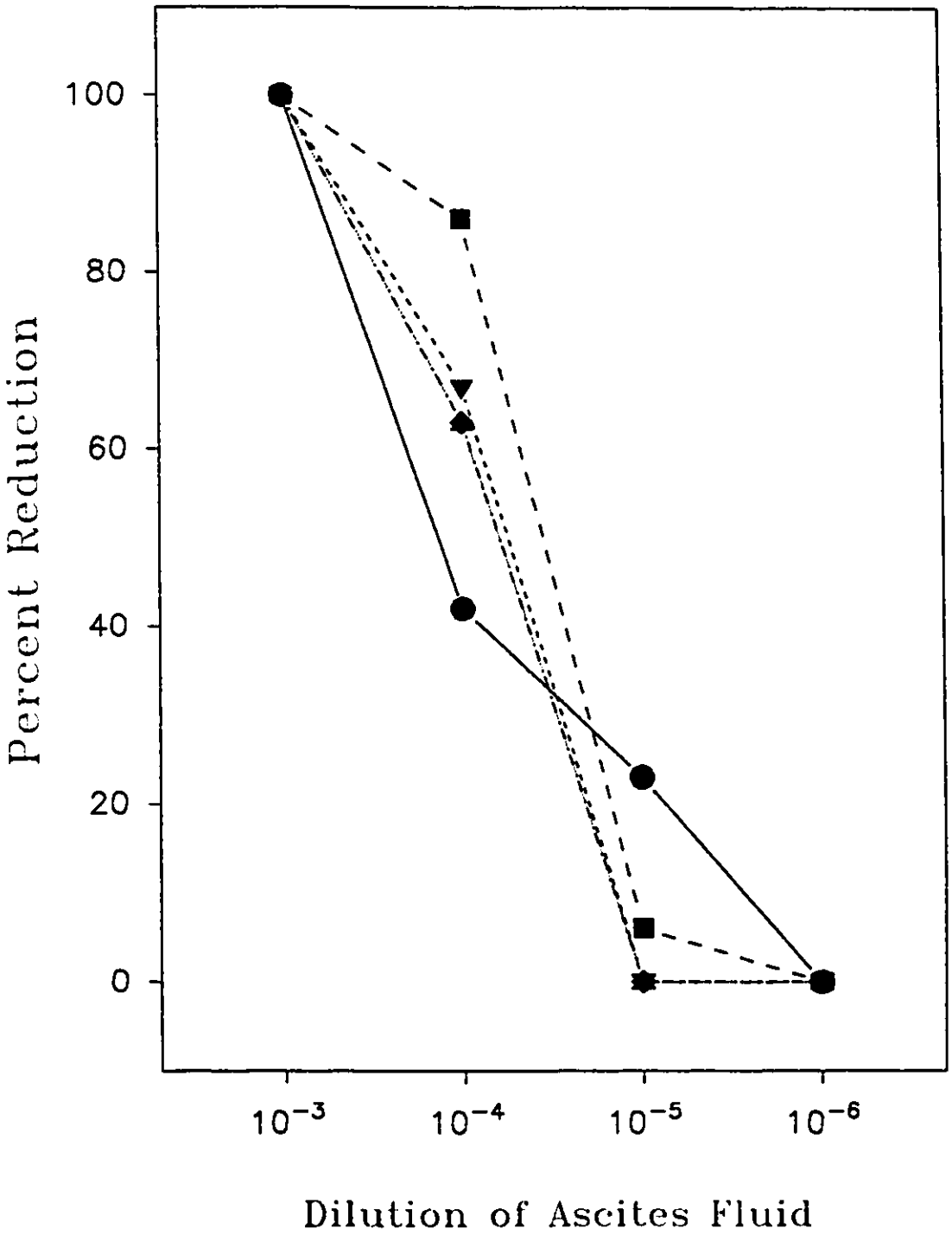

100
80
60
40
20
0
Percent Reduction
10^{-4}
10^{-5}
10^{-6}
10^{-7}
Dilution of Ascites Fluid

Figure 6. Neutralization of EV70 escape mutants selected with mAb EV-13 by mAb 72-5E.

The neutralization tests were performed as described in the legend to Figure 3 using the variants EV-13 1-4 rather than 72-5E 1-4. (●) J670, (▾) EV-13(1), (■) EV-13(2), (▲) EV-13 (3), (◆) EV-13(4).


100
80
60
40
20
0
Percent Reduction
10^{-3}
10^{-4}
10^{-5}
10^{-6}
Dilution of Ascites Fluid

neutralization by the heterologous antibody that was not used in their selection. These results suggest that the two neutralizing mAbs 72-5E and EV-13 were interacting with different sites on EV70. The mutants also appeared to be slightly more sensitive to neutralization than was the prototype EV70 J670. This was particularly evident with the 72-5E mutants.

3.3.3. Neutralization of Wild Isolates of EV70 by mAbs 72-5E and EV-13

One purpose of this project was to test the hypothesis that neutralizing antibodies do not play a role in the selection of EV70 in nature. One of the ways to test this would be to compare wild isolates and escape-mutants in a standard neutralization test with neutralizing mAbs and to determine if wild isolates behaved like the prototype or like the mutants. Neutralization patterns for wild isolates of EV70 by mAbs 72-5E and EV-13 are presented in Figures 7 and 8, respectively. As shown in Figure 7, antibody 72-5E completely inhibited plaque formation for all wild isolates (R6, RU3573, KW97, V1250 and 1604) at a dilution of 10^{-3} and each of the isolates showed a pattern of neutralization similar to that of the prototype (J670) upon antibody dilution. Similar patterns of neutralization were also seen for all the wild isolates when antibody EV-13 was used in the plaque reduction assay, except that the antibody dilution at which 100% inhibition of plaque formation was observed was 10^{-4} (Figure 8). The results suggest that none of the wild isolates have mutations similar to those present in the escape mutants.

Figure 7. Neutralization of EV70 wild isolates by mAb 72-5E

LLC-MK2 cells were grown to confluency in 60 mm culture dishes. 10-fold dilutions of ascites fluid 72-5E were made in serum-free GM. Approximately 150 PFU of prototype J670 and wild isolates R6, RU3573, KW97, V1250, and 1604 in 150 μl of serum-free GM were mixed with an equal volume of the diluted ascites fluid 72-5E and incubated for 1 hr at room temperature. The antibody-virus mixture was used to infect LLC-MK2 cell monolayers. Plaque assays were carried out as described in Chapter 2. (●) J670, (▾) R6, (■) RU3573, (○) KW97, (□) V1250, (▽) 1604.

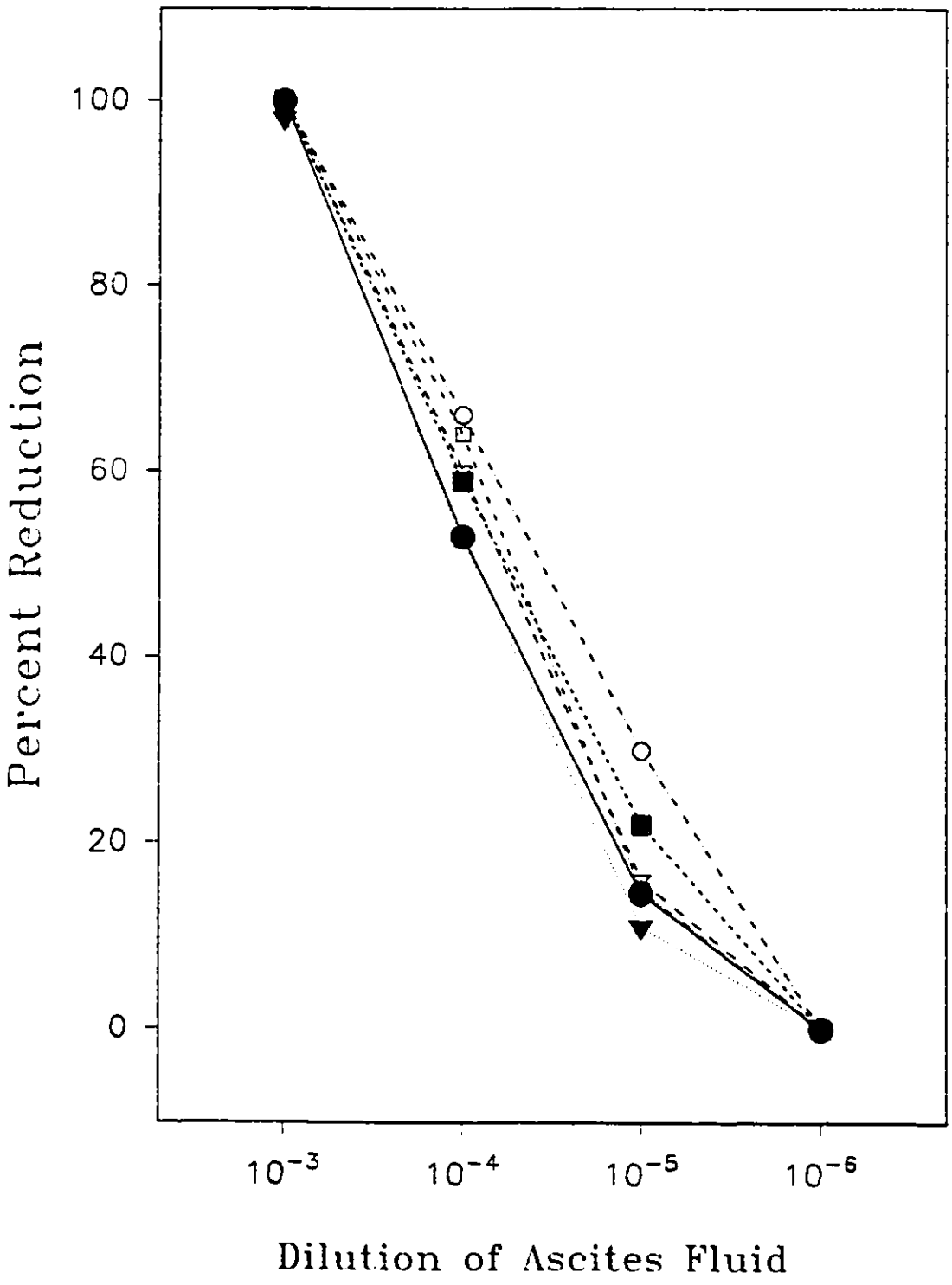
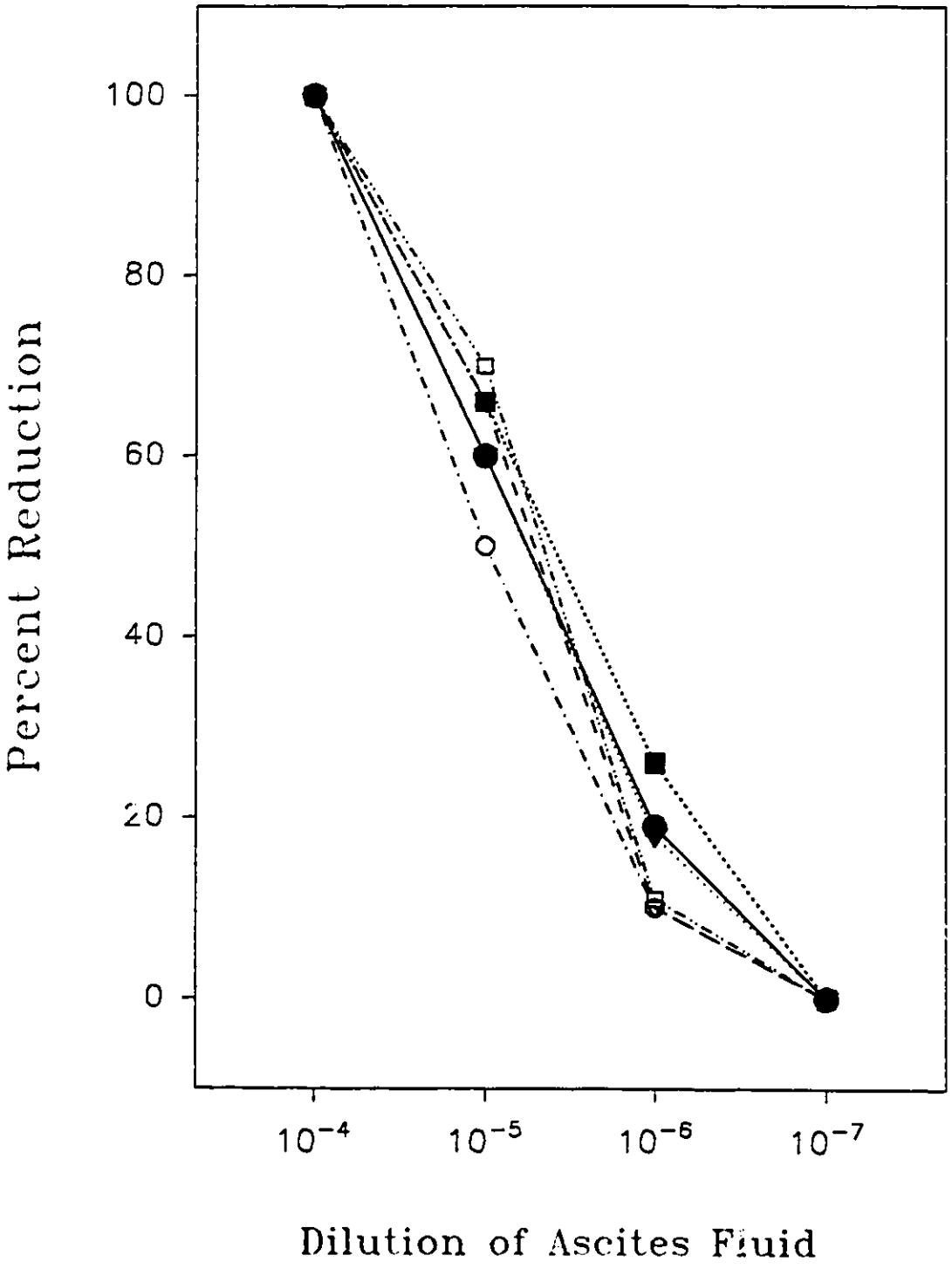

100
80
60
40
20
0
Percent Reduction
10^{-3}
10^{-4}
10^{-5}
10^{-6}
Dilution of Ascites Fluid

Figure 8. Neutralization of EV70 wild isolates by mAb EV-13

The experiments were done as described in the legend to Figure 7, except that the ascites fluid used in the experiments was mAb EV-13. (●) J670, (▾) R6, (■) RU3573, (○) KW97, (□) V1250, (▿) 1604.


Percent Reduction
100
80
60
40
20
0
10^{-4}
10^{-5}
10^{-6}
10^{-7}
Dilution of Ascites Fluid

3.3.4. 50% Neutralization Titers

The 50% neutralization titers of monoclonal antibodies 72-5E and EV-13 were calculated for the EV70 escape mutants (72-5E 1-4 and EV-13 1-4) and the EV70 wild isolates (R6, RU3573, KW97, V1250 and 1604) as described in Chapter 2. They are presented in Tables 9 and 10, respectively. As shown in Table 9, for the mutants 72-5E 1-4 or EV-13 1-4, the 50% neutralization titers of the heterologous antibodies EV-13 or 72-5E were very similar, while for the prototype EV70 J670, the 50% neutralization titers of 72-5E and EV-13 were calculated to be 2 and 2.5 times higher, respectively, than the mutants. In Table 10, for both antibodies, the 50% neutralization titers for the different wild isolates R6, RU3573, KW97, V1250 and 1604 were similar to each other. The average titer of antibody EV-13 was approximately 13 times greater than the titer of antibody 72-5E.

3.4. Cloning and Sequence Analysis of EV70 cDNA

The 8 mutants (72-5E 1-4 and EV-13) selected were confirmed to escape neutralization by the antibody used in their selection. The next objective was to determine how the mutants were different from the prototype virus at the nucleotide and amino acid level. Differences at the amino acid level should explain the resistance of the mutants to neutralization by the selecting antibody. In addition, it was of interest to compare the sequences of the mutants to those wild isolates to identify variable amino acid positions. In order to address these questions, cloning and sequence analysis were carried out.

TABLE 9 50% NEUTRALIZATION TITERS OF mAbs 72-5E AND EV-13 FOR EV70 ESCAPE MUTANTS

Antibodies	Viruses	Log_{10} neutralization titer
72-5E	J670[1]	4.0
	EV-13(1)	4.2
	EV-13(2)	4.4
	EV-13(3)	4.2
	EV-13(4)	4.2
EV-13	J670[1]	5.2
	72-5E(1)	5.6
	72-5E(2)	5.8
	72-5E(3)	5.7
	72-5E(4)	5.5

[1] J670 is the prototype virus of EV70

TABLE 10 50% NEUTRALIZATION TITERS OF mAbs 72-5E AND EV-13 FOR EV70 WILD ISOLATES

Antibodies	Viruses	Log_{10} 50% neutralization titer
72-5E	J670[1]	4.0
	R6	4.0
	Ru3573	4.2
	KW97	4.4
	V1250	4.3
	1604	4.2
EV-13	J670[1]	5.2
	R6	5.2
	Ru3573	5.4
	KW97	5.6
	V1250	5.3
	1604	5.3

[1] J670 is the prototype virus of EV70

3.4.1. Polymerase Chain Reaction (PCR)

Because the yield of EV70 RNA is fairly low, it was decided to use a combination of reverse transcription and PCR (RT-PCR) to synthesize and amplify EV70 cDNA. In parallel experiments, 100 mm plates of LLC-MK2 cells were infected with the prototype J670, the wild isolates R6, RU3573, KW97, V1250 and 1604 and the mutant viruses 72-5E 1-4 and EV-13 1-4 at a M.O.I. of approximately 0.2 PFU/cell. Infected cells were harvested either 16 hrs after infection when 50% - 75% of the cells showed CPE, or two days postinfection when the cell monolayer was completely destroyed. Virus was concentrated from the latter source by ultra-centrifugation and RNA was extracted from both sources as described in Chapter 2. Different amounts of RNA from each source were used as template for cDNA synthesis. Two oligonucleotides were designed as primers for the amplification of a region of the EV70 genome between nucleotides 1864-3427. The capsid protein coding region of EV70 begins with an initiation codon at nucleotide positions 728-730 and ends with a termination codon at position 7310 - 7312 (Takeda *et al.*, 1989). The region amplified using these two primers would include all of the VP1 coding sequence and approximately 75% of the VP3 coding sequence. Oligonucleotide EV70 "5-" (5' -CCAGTCTACATAAACTGACC -3') was complementary to nucleotides 3408-3427 of the EV70 genome and was used as a primer for reverse transcription. PCR was done using the cDNA as a template and oligonucleotide EV70 "$2^{+(BST)}$" (5' -CGCTAGTGGAGTGGAGAGG- 3'), which corresponded to nucleotides 1864-1882 of the virus genome, and oligonucleotide 5- as primers. These primers should yield a product 1563 nucleotides in length. Thirty two

cycles of amplification were carried out as detailed in Chapter 2. One-tenth of the PCR product obtained from each reaction was electrophoresed in a 1% agarose gel and stained with ethidium bromide. The size of the PCR product determined for each virus was approximately 1.6 kb (Figures 9 and 10), corresponding well with the size predicted for the region of the EV70 genome that was to be amplified. When infected cellular RNA was used as the template for reverse transcription, the yield of the PCR product was greater than when virion RNA was used (Figure 9). Therefore, in subsequent experiments, RNA isolated from infected cells was used as the starting material for reverse transcription and PCR amplification (Figure 10).

3.4.2. Cloning

Following PCR amplification, DNA was purified from gels using a modification of the Glass Milk method (Pharmacia) and direct sequence analysis (Winship *et al*, 1989) was performed. The sequencing results were not reliably suitable, therefore, the PCR products were cloned into plasmid pGEM-7Z f(+) prior to sequence analysis. The PCR products were treated with the Klenow fragment of DNA polymerase I to fill in recessed termini and the DNA was ligated into the Sma I site of pGEM-7Z f(+). The ligation products were used to transform *E. coli* DH5α and white colonies were picked and inoculated into TSB containing 50 μg/ml ampicillin. After overnight growth of the cultures at 37°C, mini-prep DNAs were screened for inserts by agarose gel electrophoresis (Figure 11). Plasmid DNAs containing inserts were then used for sequence analysis.

Figure 9. PCR amplification of a region of the EV70 genome from nucleotides 1864 to 3427

RT-PCR was carried out using RNA isolated from EV70 J670 virus particles or from LLC-MK_2 cells infected with EV70 J670. Lane 1, RNA from LLC-MK_2 cells (2×10^5) infected with EV70 J670 was used as template; lane 2, RNA from infected LLC-MK_2 cells (2×10^4) was used as template; lane 3, pGEM DNA marker (Promega) which are made by combining equimolar amounts of pGEM -3 DNA digested separately with Hinf I, Rsa I and Sin I. The sizes of DNA fragments in base pairs are indicated to the right of the figure; lane 4, EV70 genomic RNA isolated from the culture medium corresponding to approximately 2×10^5 infected cells was used as template; lane 5, EV70 genomic RNA recovered from the supernatant of approximately 2×10^4 infected cells was used as template; lane 6, RNA from mock-infected cells (2×10^5) was used as template.

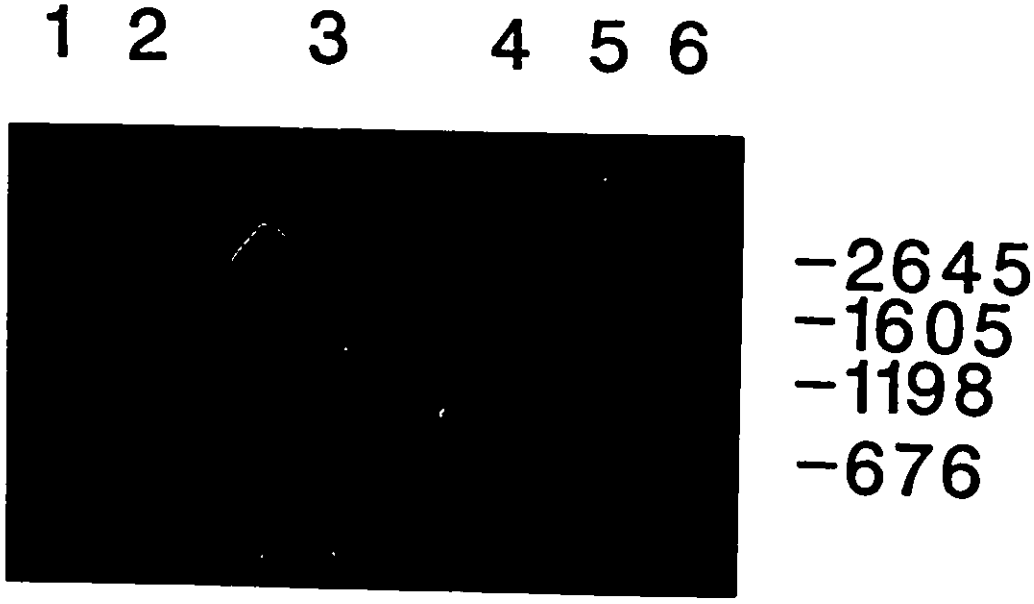

1 2 3 4 5 6
-2645
-1605
-1198
-676

Figure 10. Agarose gel electrophoresis of products amplified with primers EV70 5^{-} and EV70 2$^{+(BST)}$

RT-PCR was carried out as described in the legend to Figure 9 using RNA isolated from LLC-MK_2 cells infected with different EV70 mutants or wild isolates. One-tenth of the product from each PCR was electrophoresed on a 1% agarose gel and visualized after staining with ethidium bromide. (A) lanes 1-4, RNA from cells infected with mutants 72-5E 1-4 respectively; lanes 6-9, RNA from cells infected with mutants EV-13 1-4 respectively; lane 5, pGEM DNA marker as described in the legend to Figure 9. (B) lanes 1-5, RNA from cells infected with wild isolates R6, RU3573, KW97, V1250 and 1604 respectively; lane 6, pGEM DNA marker. The size of the pGEM marker fragments are indicated to the right of the figure. The expected PCR product was 1.563 kb in size.

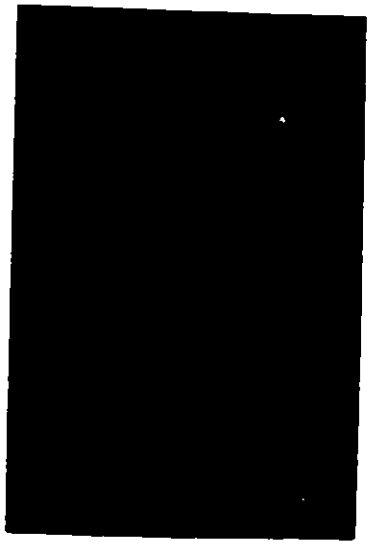
A

1 2 3 4 5 6 7 8 9

-2645
-1605
-1198

B

1 2 3 4 5 6

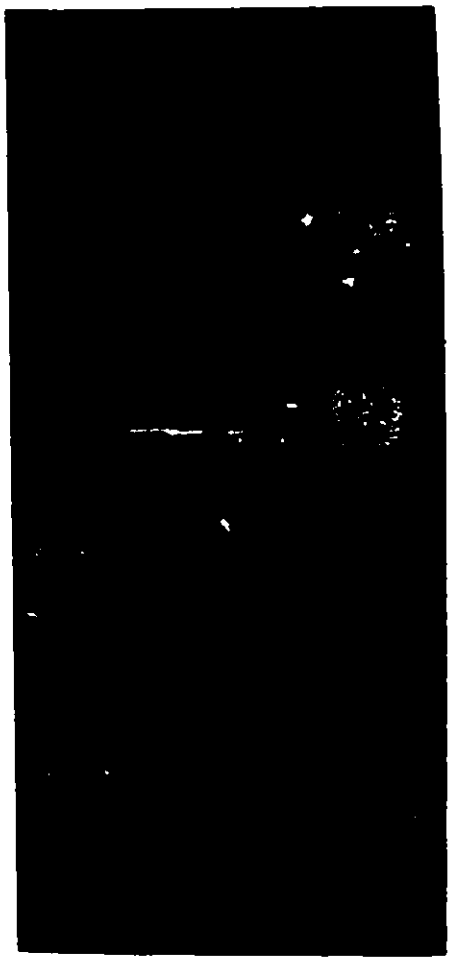


-2645
-1605
-1198

Figure 11. Screening for EV70 inserts by agarose gel electrophoresis of mini-prep DNA

Mini-prep DNA suspected of containing EV70 cDNA inserts was isolated from 1.5 ml cultures of *E.coli*. One-tenth of the mini-prep DNA was electrophoresed in a 1% agarose gel and visualized by ethidium bromide staining. Lane 1, vector pGEM-7Z f(+); lanes 2-4, EV70 J670 mini-prep DNA from 3 different cultures.

1 2 3 4



3.4.3. Sequence Analysis of Cloned EV70 cDNA

Preliminary sequence analysis with SP6 and T7 primers was used to confirm that the termini of the inserts corresponded to the expected EV70 sequences.

A previously published prototype nucleotide sequence for EV70 (Takeda *et al*, 1989) was used to design positive and negative sense oligonucleotide sequencing primers; positive sense primers corresponded to nucleotide positions 2158 - 2176, 2462 - 2480, 2759 - 2777, and 3068 - 3086; negative sense primers were complementary to nucleotide positions 3134 - 3116, 2829 - 2811, 2524 - 2506, and 2204 - 2186. Please refer to Appendix I for the sequences of the various primers. Dideoxynucleotide sequence analysis was done for both the positive and negative DNA strands of each clone to ensure the correctness of the sequences. Initially, the complete sequence of the EV70 sequences between nucletides 1864 and 3427 was determined for 2 clones of EV70 prototype J670 sequences and for 1 clone representing each neutralization-escape mutant and each wild isolate.

Nucleotide sequence comparisons revealed 1 to 11 nucleotide differences between the sequences of the various escape mutants and EV70 prototype; these differences were predicted to result in 0 to 6 amino acid differences from the prototype sequence. Many more differences were noted when the sequences of the wild isolates were compared to the prototype sequence. Amino acid differences in the capsid coding regions, and possibly in the VP1 and VP3 sequences represented in the cDNA clones, were predicted to occur. However, the observed number of differences is probably an overestimate because of nucleotide substitution errors introduced during PCR amplification, a

phenomenon reported extensively in the literature (Mendelman *et al.*, 1989; Pethuska *et al.*, 1988).

In an effort to account for PCR errors, the following strategies were used. It was assumed that: (i) PCR errors would occur randomly and independent cDNA clones for a single escape mutant should have different PCR substitutions; and (ii) substitutions related to escape from antibody neutralization would be identical in independent cDNA clones representing a single escape mutant. Therefore, nucleotide sequence differences observed in the first cDNA clone representing each escape mutant were verified by sequencing a second clone using selected oligonucleotide primers. This approach permitted preliminary identification of amino acid positions associated with neutralization by mAb EV - 13, however, no substitutions that might account for resistance to mAb 72-5E were identified. Therefore, a similar approach (ie. RT-PCR amplification using primers 591(+) and 2204(-) (Appendix I), cloning and sequencing of 2 cDNA clones) was used to analyze the remainder of the capsid protein-coding region (encompassing coding sequences for VP4, VP2 and rest of the VP3) for all of the mutants. The results of the nucleotide sequence determined are presented in Figures 12 - 14.

The nucleotide and amino acid sequences determined for the capsid protein-coding region of EV70 will first be compared to the previously published sequences for EV70 (Takeda *et al*, 1989; Ryan *et al*, 1990). The results of sequence analysis of the capsid protein-coding region of the neutralization escape mutants will then be presented. Complete sequencing of the capsid protein-coding region for the wild isolates was not considered critical to this work but is ongoing in the laboratory. The partial sequences

obtained for the wild isolates will be presented and referred to where appropriate. It should be acknowledged that sequence analysis of the VP4 and VP2 sequences of the neutralization escape mutants and of the second set of clones representing VP3 and VP1 coding sequences of the escape mutants was completed by M-C. Tardif.

3.4.3-1. Comparison of the EV70 Nucleotide Sequence with Published Sequences

The nucleotide sequence determined for the capsid protein-coding sequences of EV70 J670 was compared with the sequences published by Takeda *et al* (1989) and Ryan *et al* (1990). The nucleotide sequence (Figure 12) determined in this work showed 10 and 12 differences when compared to the sequences of Takeda *et al* (1989) and Ryan *et al* (1990), respectively (Table 11). Together, differences among the 3 sequences were found at 13 positions. The sequences determined in this work showed differences from both of the previously published sequences at 9 positions (1253, 2068, 2104, 2883, 2891, 3240, 3278, 3316 and 3338). The sequences determined by Takeda *et al* (1989) differed from those of both Ryan *et al* (1990) and the sequences determined in this work at 1 position (3314), whereas the sequences of Ryan *et al* (1990) differed from Takeda *et al* (1989) and our sequences at 3 positions (3052, 3234 and 3253). All the nucleotide changes shown in Table 11 are predicted to result in amino acid alterations except for those at positions 2068, 2104, 3052 and 3253. At these positions listed in Table 11, the sequences of the wild isolates (Figure 13) had the same nucleotides as the prototype sequence with the following exceptions.

Figure 12. Complete nucleotide and deduced amino acid sequence of the P1 region of EV70 (J670)

Complete nucleotide and deduced amino acid sequence of the P1 region of EV70 J670 are presented in this figure. The nucleotide sequence is presented in positive sense and positions are numbered from the 5' end of the genome. The predicted cleavages between the capsid proteins are marked with vertical lines and arrows. The polyprotein translation initiation codon lies between nt 728 - 730. The P1 region ends at nt 3340.

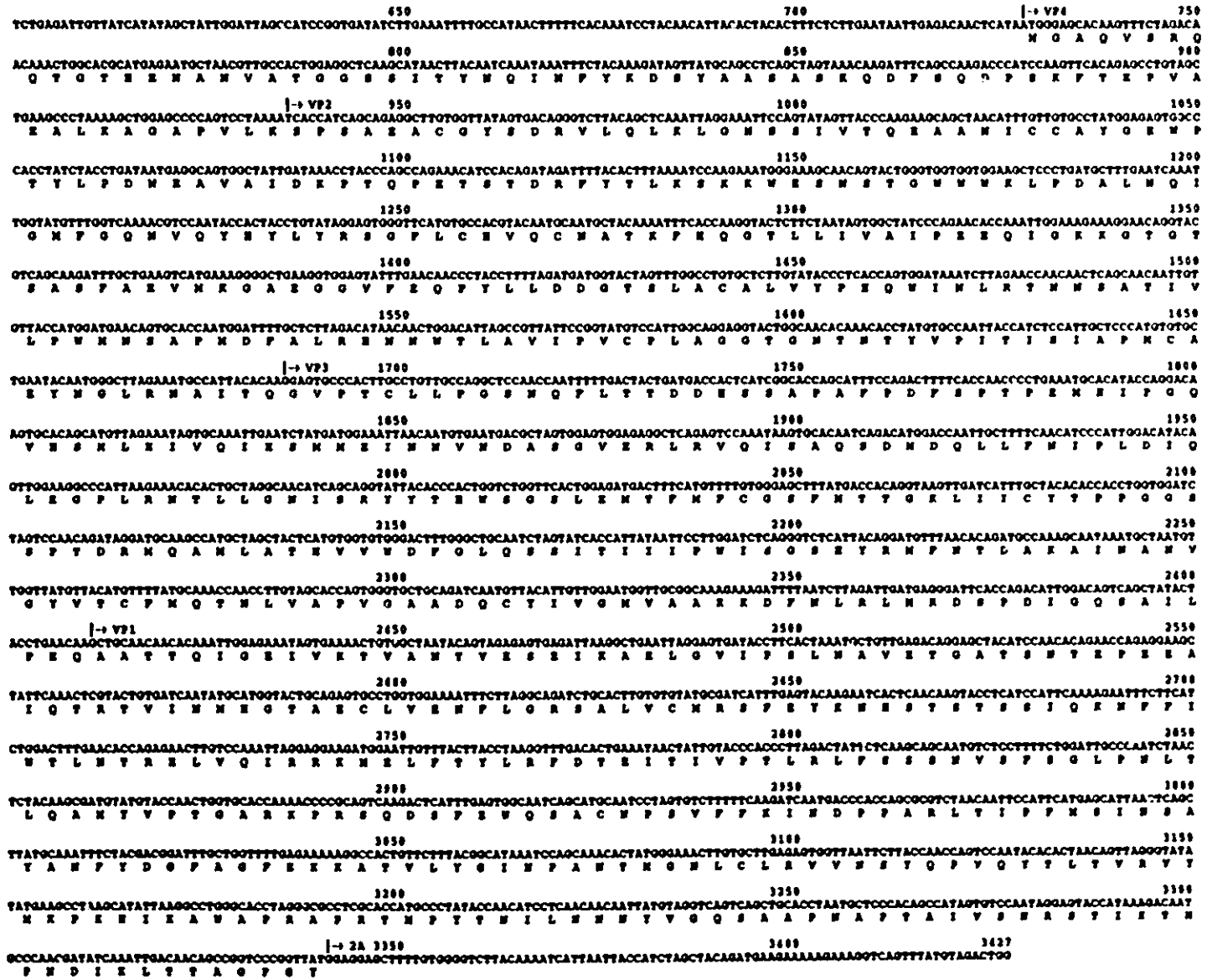


TABLE 11 NUCLEOTIDE AND AMINO ACID DIFFERENCES

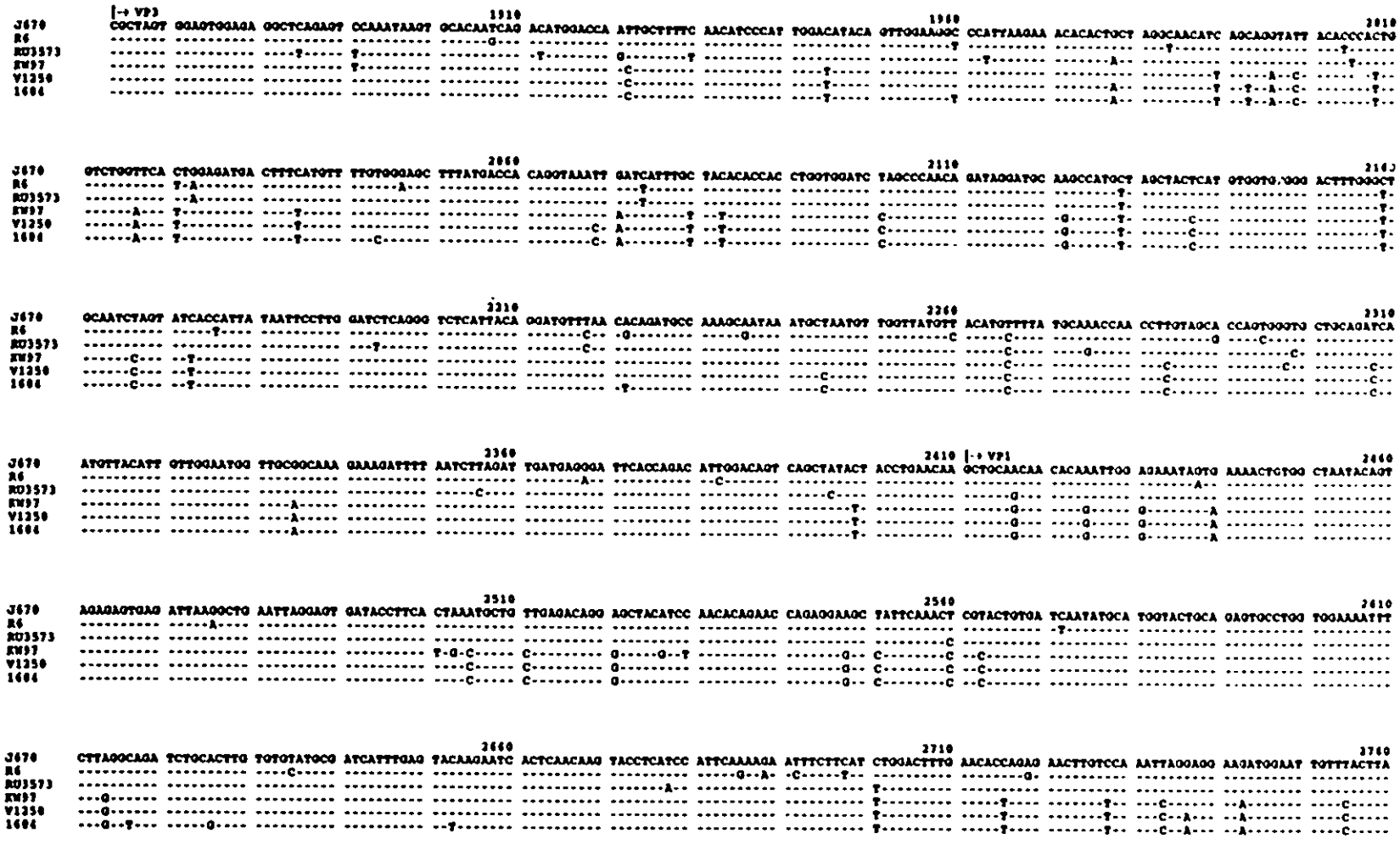
AMONG THREE EV70 J670 SEQUENCES

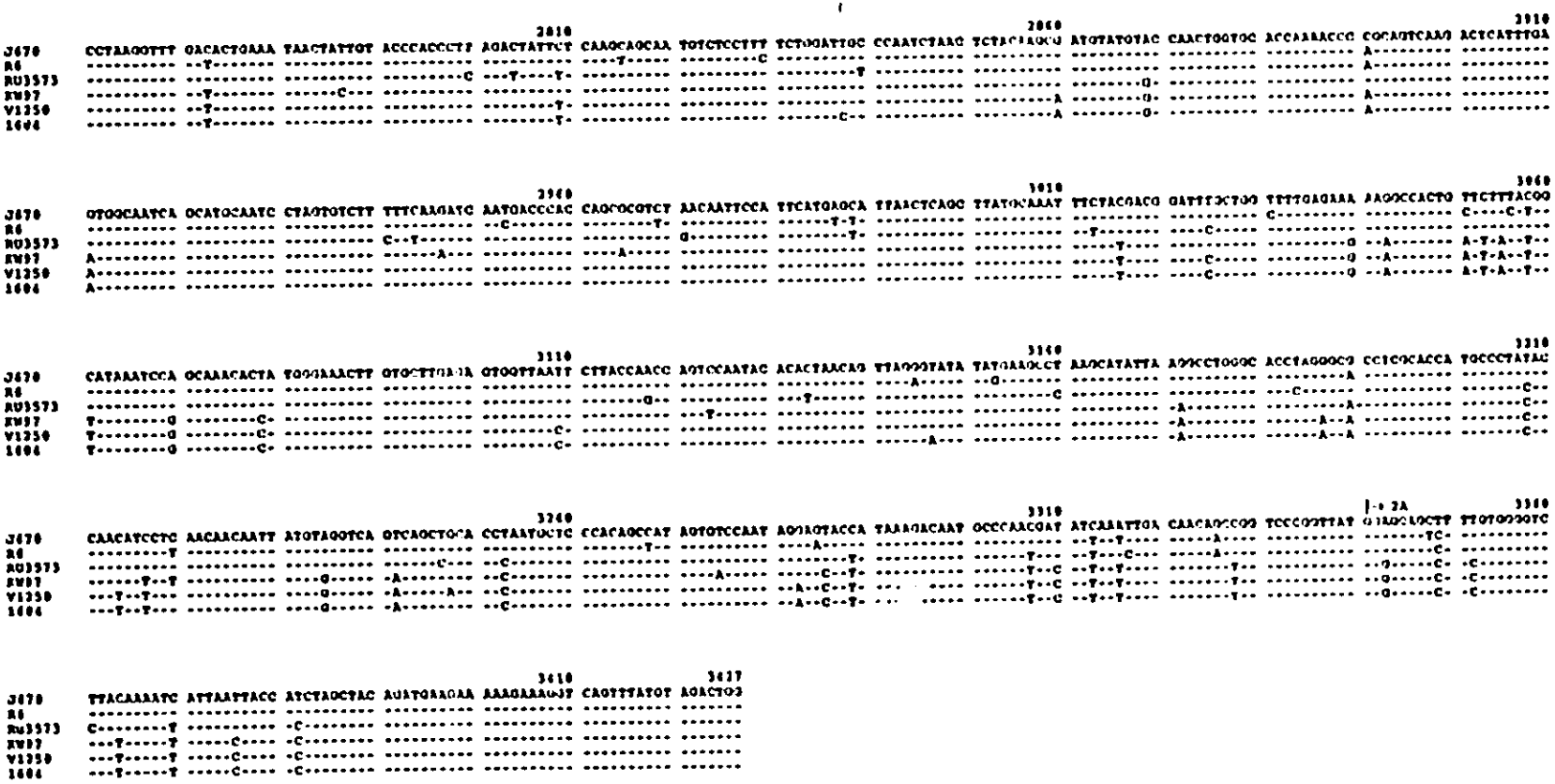
POSITION	Takeda *et al* (1989)	Ryan *et al* (1990)	Fan *et al* (1994)
1253	C (L)[1]	C (L)	A (M)
2068	A (K)	A (K)	G (K)
2104	C (S)	C (S)	T (S)
2883	G (R)	G (R)	C (P)
2891	A (S)	A (S)	C (R)
3052	T (V)	C (V)	T (V)
3234	T (V)	C (A)	T (V)
3240	G (R)	G (R)	A (Q)
3253	T (P)	C (P)	T (P)
3278	G (D)	G (D)	A (N)
3314	G (D)	A (N)	A (N)
3316	T (N)	T (N)	A (K)
3338	T (Y)	T (Y)	C (H)

[1] the predicted amino acid is shown in parenthesis

Figure 13. Nucleotide sequences of EV70 wild isolates from nucleotide position 1864 to 3427

Following RT-PCR, cDNAs representing the region of the EV70 genome (from nucleotide position 1864 to 3427) for each of the wild isolates were cloned into pGEM 7Zf (+) and the nucleotide sequences were determined. Sequences are presented in positive sense and nucleotide positions are indicated above each line. Differences with respect to prototype EV70 J670 are presented below the J670 virus nucleotide sequence. Identical nucleotides are denoted with a dash.





72

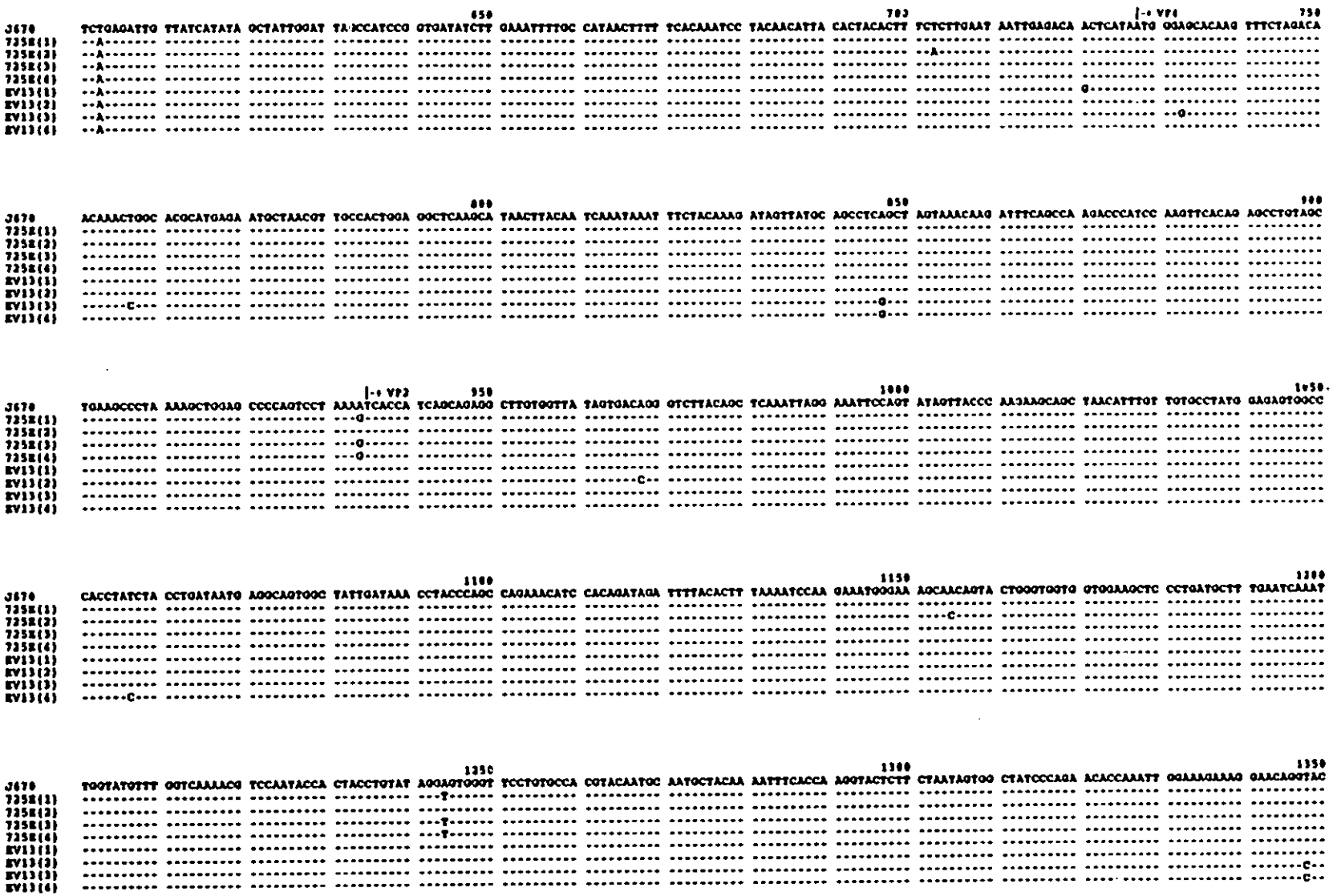
The nucleotides at position 2891 for KW97 and at position 3316 for RU3573 were identical to those published by both Takeda *et al* (1989) and Ryan *et al* (1990) and for KW97, V1250 and 1604, the nucleotides at positions 3052 and 3234 were identical to those published by Ryan *et al* (1990). Those differences could result from the different cell lines used for the propagation of EV70. Hela cells were used by both Takeda *et al* and Ryan *et al*, while LLC-MK_2 cells were used by Fan *et al*. The differences could also reflect true sequence variability at these sites.

3.4.3-2. Sequence Analysis of the Mutants

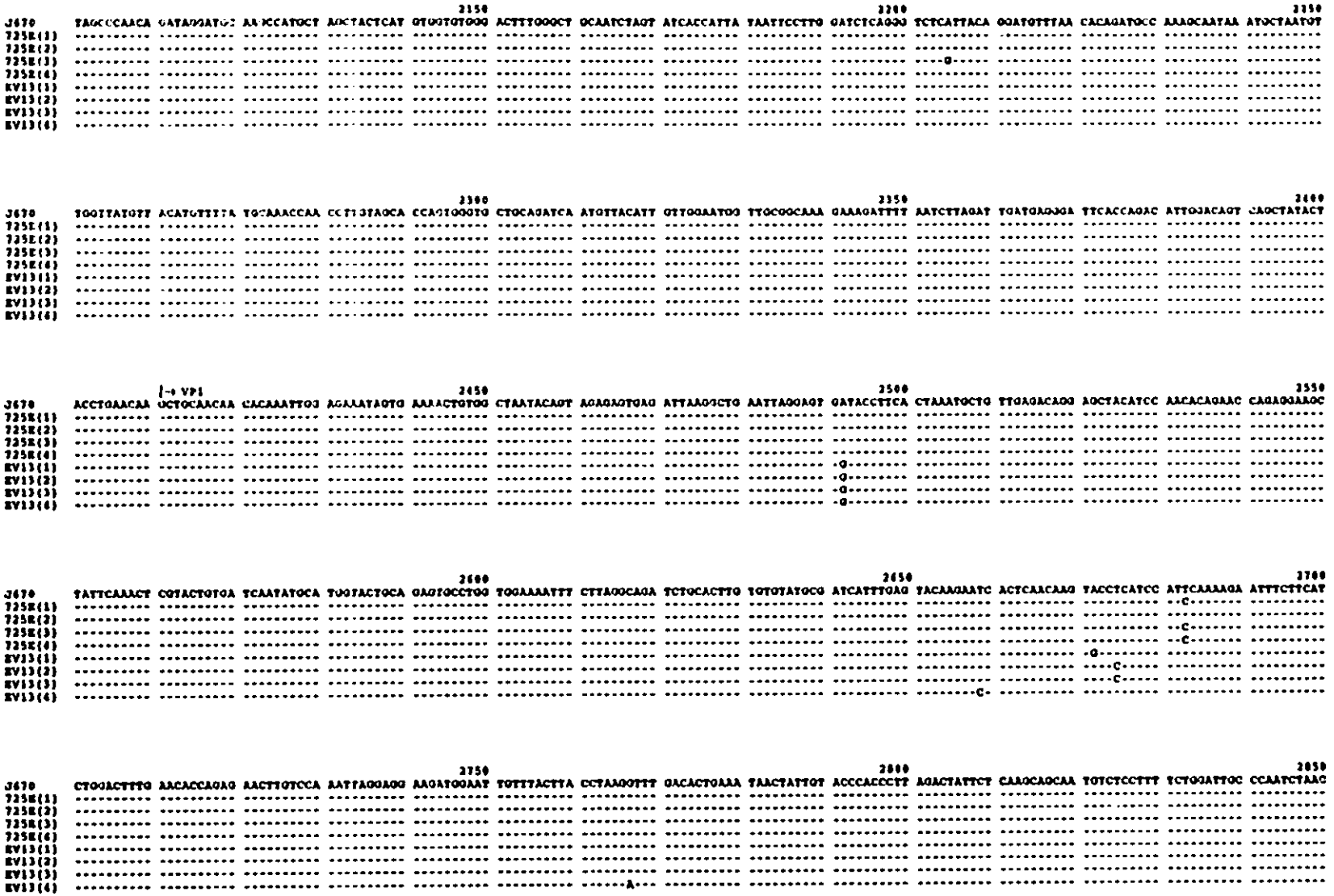
A major objective of this projective was to identify amino acid substitutions which might be responsible for escape from neutralization by mAbs 72-5E and EV-13. Therefore, the complete nucleotide sequence of the capsid protein-coding region of each of the escape mutants was aligned and compared with a consensus sequence determined for the corresponding region of J670 (Figure 14). This consensus sequence was determined from the previously published sequences (Takeda *et al.*, 1989; Ryan *et al.*, 1990) and from data obtained in this study. Nucleotide differences were detected at 30 positions. Of these differences, 22 were base transitions and 8 were transversions. While 17 differences (59%) were in the third position of codons, 9 (30%) and 4 (13%) were in the first and second positions respectively. The deduced amino acid sequences predicted from the capsid protein-coding nucleotide sequences of the mutants are shown and compared with the J670 sequence in Figure 15.

Figure 14. Nucleotide sequences of the capsid protein-coding region of EV70 neutralization escape mutants

Following RT-PCR, cDNAs representing the capsid protein-coding region of the EV70 genome (from nucleotide position 591 to 3427) for each the mutant viruses were cloned into pGEM 7Zf (+) and the nucleotide sequences were determined. Sequences are presented in positive sense and nucleotide positions are indicated above each line. Differences with respect to a consensus sequence for the prototype EV70 J670 are presented below the J670 virus nucleotide sequence. Identical nucleotides are denoted with a dash.



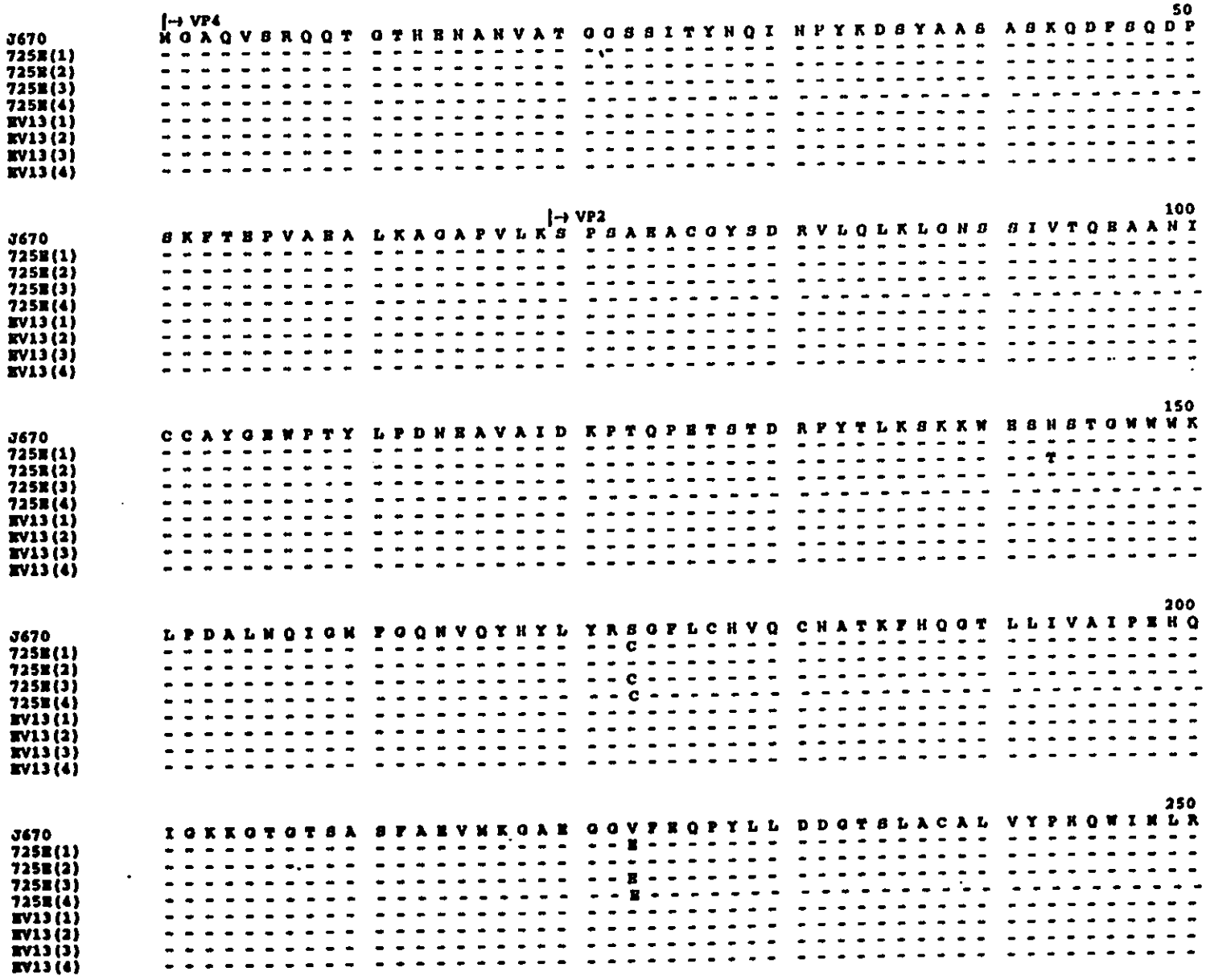
```
                                                       1400                                                       1450                                                       1500
J670     GTCAGCAAGA TTTACTGAAG TCATGAAAGG GGCTGAAGGT GGAGTATTTG AACAACCCTA CCTTTTAGAT GATGGTACTA GTTTGGCCTG TGCTCTTGTA TACCCTCACC AGTGGATAAA TCTTAGAACC AACAACTCAG CAACAATTGT
725K(1)  ---------- ---------- ---------- ---------- ----A----- ---------- ---------- ---------- ---------- ---------- ---------- ---------- ---------- ---------- ----------
725K(2)  ---------- ---------- ---------- ---------- ---------- ---------- ---------- ---------- ---------- ---------- ---------- ---------- ---------- ---------- ----------
725K(3)  ---------- ---------- ---------- ---------- ----A----- ---------- ---------- ---------- ---------- ---------- ---------- ---------- ---------- ---------- ----------
725K(4)  ---------- ---------- ---------- ---------- ----A----- ---------- ---------- ---------- ---------- ---------- ---------- ---------- ---------- ---------- ----------
KV13(1)  ---------- ---------- ---------- ---------- ---------- ---------- ---------- ---------- ---------- ---------- ---------- ---------- ---------- ---------- ----------
KV13(2)  ---------- ---------- ---------- ---------- --------C- ---------- ---------- ---------- ---------- ---------- ---------- ---------- ------G--- ---------- ----------
KV13(3)  ---------- ---------- ---------- ---------- ---------- ---------- ---------- ---------- ---------- ---------- ---------- ---------- ---------- ---------- ----------
KV13(4)  ---------- ---------- ---------- ---------- ---------- ---------- ---------- ---------- ---------- ---------- ---------- ---------- ---------- ---------- ----------

                                                       1550                                                       1600                                                       1650
J670     GTTACCATGG ATGAACAGTG CACCAATGGA TTTTGCTCTT AGACATAACA ACTGGACATT AGCCGTTATT CCGGTATGTC CATTGGCAGG AGGTACTGGC AACACAAACA CCTATGTGCC AATTACCATC TCCATTGCTC CCATGTGTGC
725K(1)  ---------- ------T--- ---------- ---------- ---------- ---------- ---------- ---------- ---------- ---------- ---------- ---------- ---------- ---------- ----------
725K(2)  ---------- ---------- ---------- ---------- ---------- ---------- ---------- ---------- ---------- ---------- ---------- ---------- ---------- ---------- ----------
725K(3)  ---------- ------T--- ---------- ---------- ---------- ---------- ---------- ---------- ---------- ---------- ---------- ---------- ---------- ---------- ----------
725K(4)  ---------- ------T--- ---------- ---------- ---------- ---------- ---------- ---------- ---------- ---------- ---------- ---------- ---------- ---------- ----------
KV13(1)  ---------- ---------- ---------- ---------- ---------- --------C- ---------- ---------- ---------- ---------- ---------- ---------- ---------- ---------- ----------
KV13(2)  ---------- ---------- ---------- ---------- ---------- ---------- ---------- ---------- ---------- ---------- ---------- ---------- ---------- ---------- ----------
KV13(3)  ---------- ---------- ---------- ---------- ---------- ---------- ---------- ---------- ---------- ---------- ---------- ---------- ---------- ---------- ----------
KV13(4)  ---------- ---------- ---------- ---------- ---------- ---------- ---------- ---------- ---------- ---------- ---------- ---------- ---------- ---------- ----------

                                        |-> VP3        1700                                                       1750                                                       1800
J670     TGAATACAAT GGGCTTAGAA ATGCCATTAC ACAAGGAGTG CCCACTTGCC TGTTGCCAGG CTCCAACCAA TTTTTGACTA CTGATGACCA CTCATCGGCA CCAGCATTTC CAGACTTTTC ACCAACCCCT GAAATGCACA TACCAGGACA
725K(1)  ---------- ---------- ---------- ---------- ---------- --CC------ ---------- ---------- ---------- ---------- ---------- ---------- ---------- ---------- ----------
725K(2)  ---------- ---------- ---------- ---------- ---------- ---------- ---------- ---------- ---------- ---------- ---------- ---------- ------G--- ---------- ----------
725K(3)  ---------- ---------- ---------- ---------- ---------- ---------- ---------- ---------- ---------- ---------- ---------- ---------- ---------- ---------- ----------
725K(4)  ---------- ---------- ---------- ---------- ---------- ---------- ---------- ---------- ---------- ---------- ---------- ---------- ---------- ---------- ----------
KV13(1)  ---------- ---------- ---------- ---------- ---------- ---------- ---------- ---------- ---------- ---------- ---------- ---------- ------G--- ---------- ----------
KV13(2)  ---------- ---------- ---------- ---------- ---------- --CC------ ---------- ---------- ---------- ---------- ---------- ---------- ---------- ---------- ----------
KV13(3)  ---------- ---------- ---------- ---------- ---------- ---------- ---------- ---------- ---------- ---------- ---------- ---------- ---------- ---------- ----------
KV13(4)  ---------- ---------- ---------- ---------- ---------- ---------- ---------- ---------- ---------- ---------- ---------- ---------- ---------- ---------- ----------

                                                       1850                                                       1900                                                       1950
J670     AGTGCACAGC ATGTTACAAA TAGTGCAAAT TGAATCTATG ATGGAAATTA ACAATGTGAA TGACGCTAGT GGAGTGGAGA GGCTCAGAGT CCAAATAAGT GCACAATCAG ACATGGACCA ATTGCTTTTC AACATCCCAT TGGACATACA
725K(1)  ---------- ---------- ---------- A--------- ---------- ---------- ---------- ---------- ---------- ---------- ---------- ---------- ---------- ---------- ----------
725K(2)  ---------- ---------- ---------- ---------- ---------- ---------- ---------- ---------- ---------- ---------- ---------- ---------- ---------- ---------- -A--------
725K(3)  ---------- ---------- ---------- ---------- ---------- ---------- ---------- ---------- ---------- ---------- ---------- ---------- ---------- ---------- ----------
725K(4)  ---------- ---------- ---------- A--------- ---------- ---------- ---------- ---------- ---------- ---------- ---------- ---------- ---------- ---------- ----------
KV13(1)  ---------- ---------- ---------- ---------- ---------- ---------- ---------- ---------- ---------- ---------- ---------- ---------- ---------- ---------- ----------
KV13(2)  ---------- ---------- ---------- A--------- ---------- ---------- ---------- ---------- ---------- ---------- ---------- ---------- ---------- ---------- ----------
KV13(3)  ---------- ---------- ---------- A--------- ---------- ---------- ---------- ---------- ---------- ---------- ---------- ---------- ---------- ---------- ----------
KV13(4)  ---------- ---------- ---------- ---------- ---------- ---------- ---------- ---------- -------G-- ---------- ---------- ---------- ---------- ---------- ----------

                                                       2000                                                       2050                                                       2100
J670     GTTGGAAGGC CCATTAAGAA ACACACTGCT AGGCAACATC AGCAGGTATT ACACCCACTG GTCTGGTTCA CTGGAGATGA CTTTCATGTT TTGTGGGAGC TTTATGACCA CAGGTAAATT GATCATTTGC TACACACCAC CTGGTGGATC
725K(1)  ---------- ---------- ---------- ---------- ---------- ---------- ---------- ---------- ---------- ---------- ---------- ---------- ---------- ---------- ----------
725K(2)  ---------- ---------- ---------- ---------- ---------- ---------- ---------- ---------- ---------- ---------- ---------- ---------- ---------- ---------- ----------
725K(3)  ---------- ---------- ---------- ---------- ---------- ---------- ---------- ---------- ---------- ---------- ---------- ---------- ---------- ---------- ----------
725K(4)  ---------- ---------- ---------- ---------- ---------- ---------- ---------- ---------- ---------- ---------- ---------- ---------- ---------- ---------- ----------
KV13(1)  ---------- ---------- ---------- ---------- ---------- ---------- ---------- ---------- ---------- ---------- ---------- ---------- ---------- ---------- ----------
KV13(2)  ---------- ---------- ---------- ---------- ---------- ---------- ---------- ---------- ---------- ---------- ---------- ---------- ---------- ---------- ----------
KV13(3)  ---------- ---------- ---------- ---------- ---------- ---------- ---------- ---------- ---------- ---------- ---------- ---------- ---------- ---------- ----------
KV13(4)  ---------- ---------- ---------- ---------- ---------- ---------- ---------- ---------- ---------- ---------- ---------- ---------- ---------- ---------- ----------
```

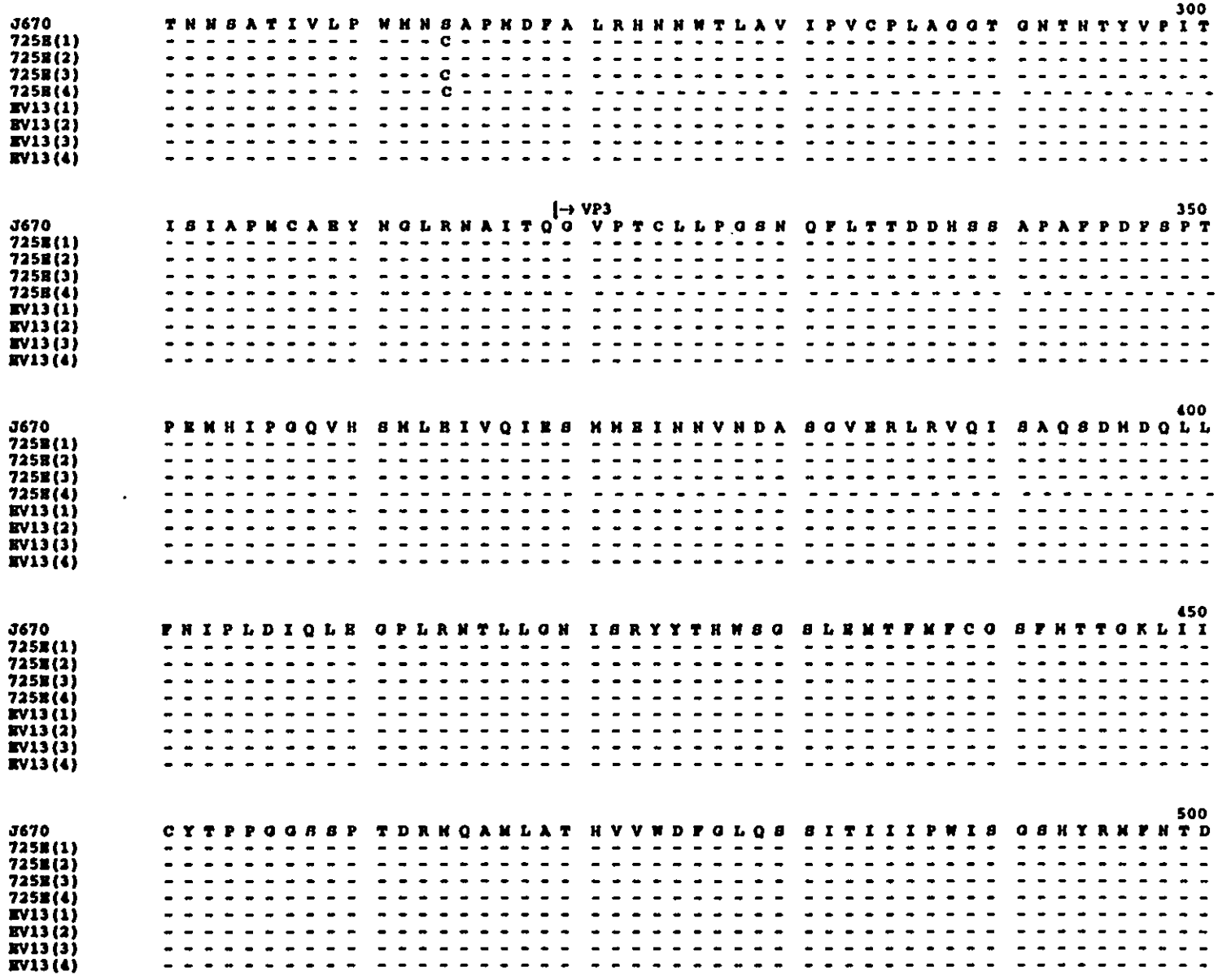

2150
2200
2250
2300
2350
2400
|-- VP1
2450
2500
2550
2600
2650
2700
2750
2800
2850
J670
725E(1)
725E(2)
725E(3)
725E(4)
EV13(1)
EV13(2)
EV13(3)
EV13(4)

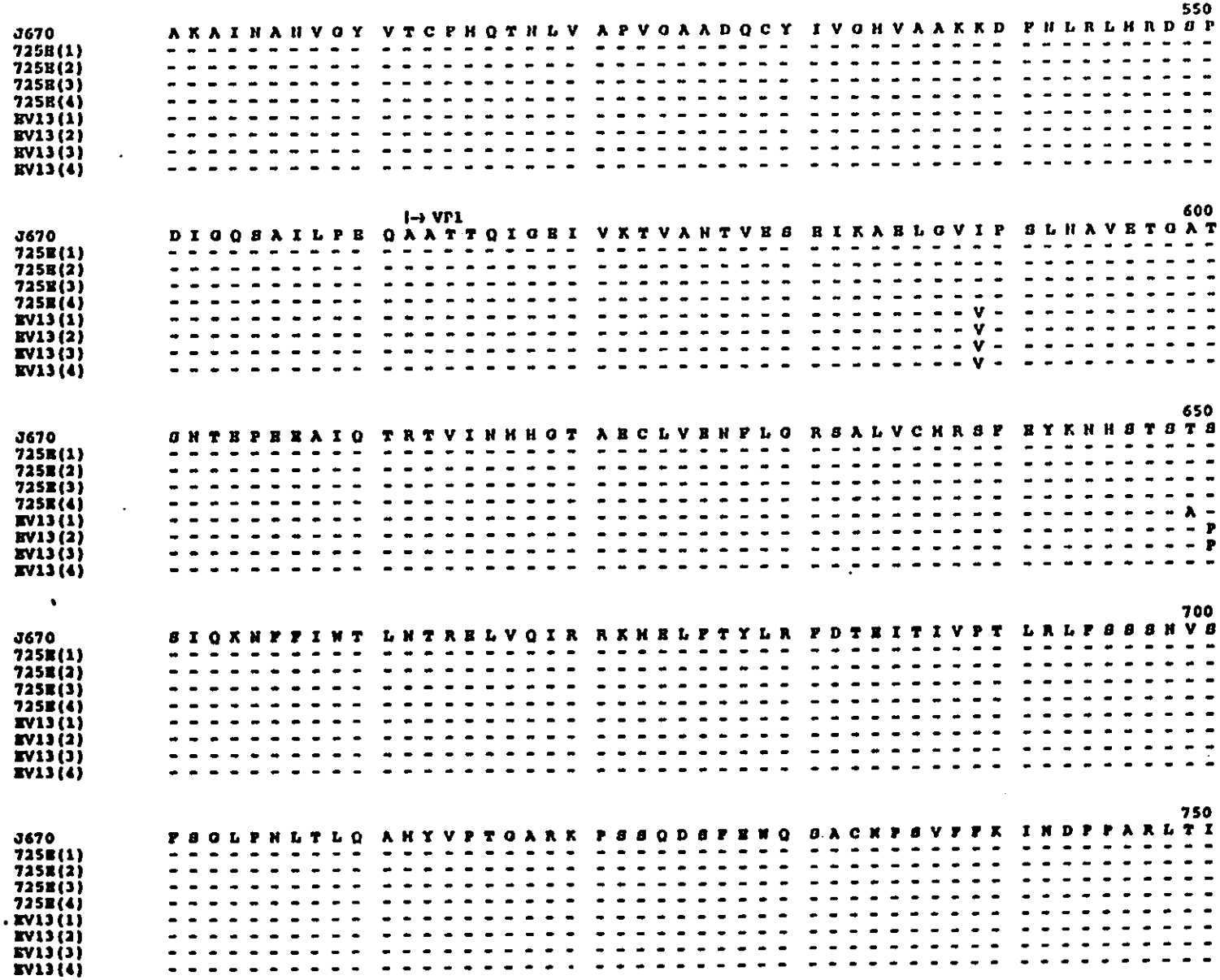
```
                                                         2900                                                        2950                                                        3000
J670      TCTACAAGCG ATGTATGTAC CAACTGGTGC ACGAAAACCC AGCAGTCAAG ACTCATTTGA GTGGCAATCA GCATGCAATC CTAGTGTCTT TTTCAAGATC AATGACCCAC CAGCGCGTCT AACAATTCCA TTCATGAGCA TTAACTCAGC
735E(1)   ---------- ---------- ---------- ---------- ---------- ---------- ---------- ---------- ---------- ---------- ---------- ---------- ---------- ---------- ----------
735E(2)   ---------- ---------- ---------- ---------- ---------- ----G----- ---------- ---------- ---------- ---------- ---------- ---------- ---------- ---------- ----------
735E(3)   ---------- ---------- ---------- ---------- ---------- ---------- ---------- ---------- ---------- ---------- ---------- ---------- ---------- ---------- ----------
735E(4)   ---------- ---------- ---------- ---------- ---------- ---------- ---------- ---------- ---------- ---------- ---------- ---------- ---------- ---------- ----------
EV13(1)   ---------- ---------- ---------- ---------- ---------- ----G----- ---------- ---------- ---------- ---------- ---------- ---------- ---------- ---------- ----------
EV13(2)   ---------- ---------- ---------- ---------- ---------- ----G----- ---------- ---------- ---------- ---------- ---------- ---------- ---------- ---------- ----------
EV13(3)   ---------- ---------- ---------- ---------- ---------- ----G----- ---------- ---------- ---------- ---------- ---------- ---------- ---------- ---------- ----------
EV13(4)   ---------- ---------- ---------- ---------- ---------- ----G----- ---------- ---------- ---------- ---T------ ---------- ---------- ---------- ---------- ----------

                                                         3050                                                        3100                                                        3150
J670      TTATGCAAAT TTCTACGACG GATTTGCTGG TTTTGAGAAA AAGGCCACTG TTCTTTACGG CATAAATCCA GCAAACACTA TGGGAAACTT GTGCTTGAGA GTGGTTAATT CTTACCAACC AGTCCAATAC ACACTAACAG TTAGGGTATA
735E(1)   ---------- ---------- ---------- ---------- ---------- ---------- ---------- ---------- ---------- ---------- ---------- ---------- ---------- ---------- ----------
735E(2)   ---------- ---------- ---------- ---------- ---------- ---------- ---------- ---------- ---------- ---------- ---------- ---------- ---------- ---------- ----------
735E(3)   ---------- ---------- ---------- ---------- ---------- ---------- ---------- ---------- ---------- ---------- ---------- ---------- ---------- ---------- ----------
735E(4)   ---------- ---------- ---------- ---------- ---------- ---------- ---------- ---------- ---------- ---------- ---------- ---------- ---------- ---------- ----------
EV13(1)   ---------- ---------- ---------- ---------- ---------- ---------- ---------- ---------- ---------- ---------- ---------- ---------- ---------- ---------- ----------
EV13(2)   ---------- ---------- ---------- ---------- ---------- ---------- ---------- ---------- ---------- ---------- ---------- ---------- ---------- ---------- ----------
EV13(3)   ---------- ---------- ---------- ---------- ---------- ---------- ---------- ---------- ---------- ---------- ---------- ---------- ---------- ---------- ----------
EV13(4)   ---------- ---------- ---------- ---------- ---------- ---------- ---------- ---------- ---------- ---------- ---------- ---------- ---------- ---------- ----------

                                                         3200                                                        3250                                                        3300
J670      TATGAAGCCT AAGCATATTA AGGCCTGGGC ACCTAGGGCG CCTCGCACCA TGCCCTATAC CAACATCCTC AACAACAATT ATGTAGGTCG GTCAGCTGCA CCTAATGCTC CCACAGCCAT AGTGTCCGAT AGGAGTACCA TAAAGACAAT
735E(1)   ---------- ---------- ---------- ---------- ---------- ---------- ---------- ---------- ---------- ---------- ---------- ---------- ---------- ---------- ----------
735E(2)   ---------- ---------- ---------- ---------- ---------- ---------- ---------- ---------- ---------- ---------- ---------- ---------- ---------- ---------- ----------
735E(3)   ---------- ---------- ---------- ---------- ---------- ---------- ---------- ---------- ---------- ---------- ---------- ---------- ---------- ---------- ----------
735E(4)   ---------- ---------- ---------- ---------- ---------- ---------- ---------- ---------- ---------- ---------- ---------- ---------- ---------- ---------- ----------
EV13(1)   ---------- ---------- ---------- ---------- ---------- ---------- ---------- ---------- ---------- ---------- ---------- ---------- ---------- ---------- ----------
EV13(2)   ---------- ---------- ---------- ---------- ---------- ---------- ---------- ---------- ---------- ---------- ---------- ---------- ---------- ---------- ----------
EV13(3)   ---------- ---------- ---------- ---------- ---------- ---------- ---------- ---------- ---------- ---------- ---------- ---------- ---------- ---------- ----------
EV13(4)   ---------- ---------- ---------- ---------- ---------- ---------- ---------- ---------- ---------- ---------- ---------- ---------- ---------- ---------- ----------

                                             |→ 2A 3350                                                      3400                             3427
J670      GCCCAACGAT ATCAATTTGA CAACAGCCGG TCCCGGTTAT GGAGGAGCTT TTGTGGGGTC TTACAAAATC ATTAATTACC ATCTAGCTAC AGATGAAGAA AAAGAAAGGT CAGTTTATGT AGACTGA
735E(1)   ---------- ---------- ---------- ---------- ---------- ---------- ---------- ---------- ---------- ---------- ---------- ---------- -------
735E(2)   ---------- ---------- ---------- ---------- ---------- ---------- ---------- ---------- ---------- ---------- ---------- ---------- -------
735E(3)   ---------- ---------- ---------- ---------- ---------- ---------- ---------- ---------- ---------- ---------- ---------- ---------- -------
735E(4)   ---------- ---------- ---------- ---------- ---------- ---------- ---------- ---------- ---------- ---------- ---------- ---------- -------
EV13(1)   ---------- ---------- ---------- ---------- ---------- ---------- ---------- ---------- ---------- ---------- ---------- ---------- -------
EV13(2)   ---------- ---------- ---------- ---------- ---------- ---------- ---------- ---------- ---------- ---------- ---------- ---------- -------
EV13(3)   ---------- ---------- ---------- ---------- ---------- ---------- ---------- ---------- ---------- ---------- ---------- ---------- -------
EV13(4)   ---------- ---------- ---------- ---------- ---------- ---------- ---------- ---------- ---------- ---------- ---------- ---------- -------
```

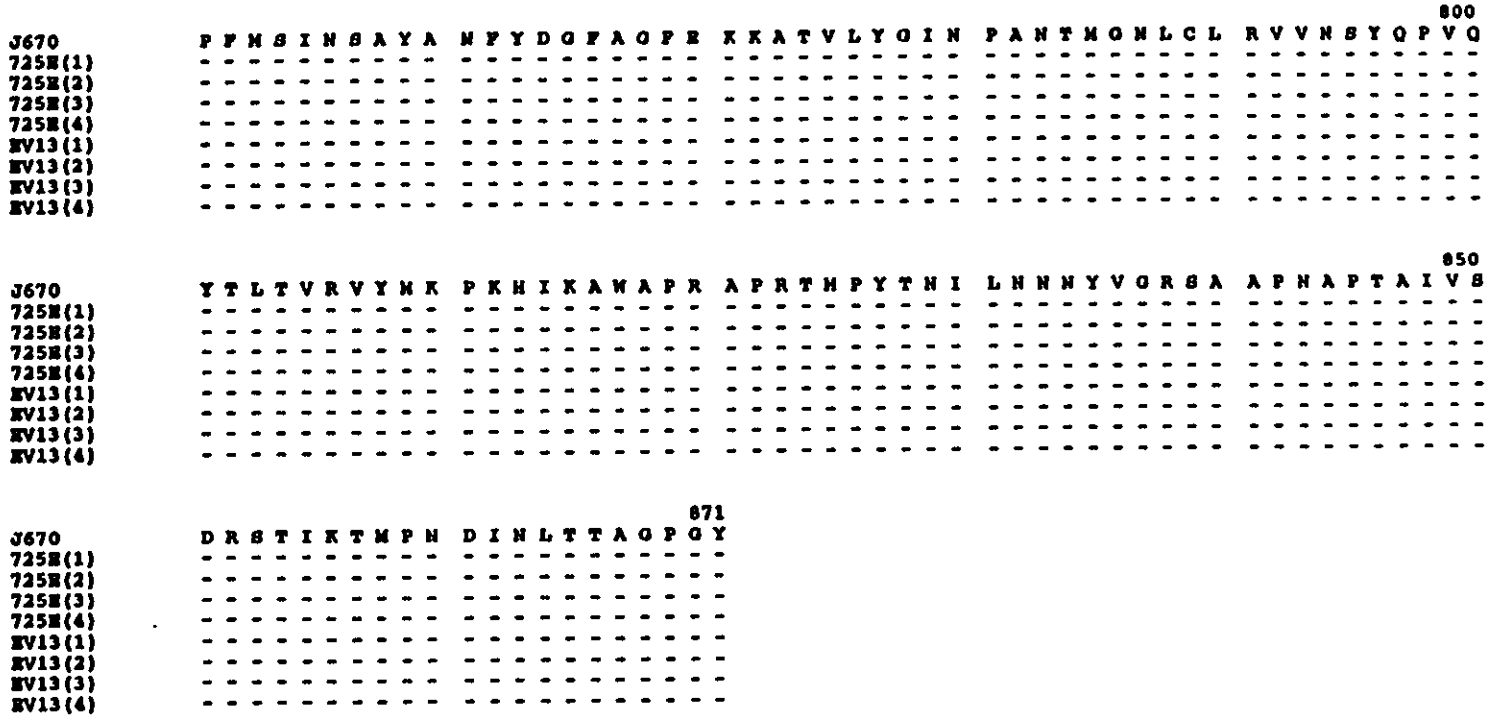
Figure 15. Predicted amino acid sequences of the capsid proteins of neutralization escape mutants of EV70

The predicted amino acid sequences of the capsid protein-coding region of the neutralization escape mutants of EV70 are shown. The amino acid position is numbered on the top of each line. Amino acids that differ from the prototype (J670) sequence are indicated below the J670 amino acid sequence. Identical amino acids are shown by dashes.


|→ VP4
50
J670 M G A Q V S R Q Q T G T H E N A N V A T G G S S I T Y N Q I N P Y K D S Y A A S A S K Q D F S Q D P
725E(1)
725E(2)
725E(3)
725E(4)
EV13(1)
EV13(2)
EV13(3)
EV13(4)
|→ VP2
100
J670 S K F T E P V A E A L K A G A P V L K S P S A E A C G Y S D R V L Q L K L G N S S I V T Q E A A N I
725E(1)
725E(2)
725E(3)
725E(4)
EV13(1)
EV13(2)
EV13(3)
EV13(4)
150
J670 C C A Y G E W P T Y L P D N E A V A I D K P T Q P E T S T D R F Y T L K S K K W E S N S T G W W K
725E(1) T
725E(2)
725E(3)
725E(4)
EV13(1)
EV13(2)
EV13(3)
EV13(4)
200
J670 L P D A L M Q I G M F G Q N V Q Y H Y L Y R S G F L C H V Q C H A T K F H Q G T L L I V A I P E H Q
725E(1) C
725E(2)
725E(3) C
725E(4) C
EV13(1)
EV13(2)
EV13(3)
EV13(4)
250
J670 I G K K G T G T S A S F A E V M K G A E G G V F E Q P Y L L D D G T S L A C A L V Y P K Q W I M L R
725E(1) E
725E(2)
725E(3) E
725E(4) E
EV13(1)
EV13(2)
EV13(3)
EV13(4)


300
J670 T N M S A T I V L P W M N S A P M D F A L R H N N W T L A V I P V C P L A G G T G N T H T Y V P I T
725E(1) C
725E(2)
725E(3) C
725E(4) C
EV13(1)
EV13(2)
EV13(3)
EV13(4)
|→ VP3
350
J670 I S I A P M C A E Y N G L R N A I T Q G V P T C L L P G S N Q F L T T D D H S S A P A F P D F S P T
725E(1)
725E(2)
725E(3)
725E(4)
EV13(1)
EV13(2)
EV13(3)
EV13(4)
400
J670 P E M H I P G Q V H S M L E I V Q I E S M M E I N N V N D A S G V E R L R V Q I S A Q S D M D Q L L
725E(1)
725E(2)
725E(3)
725E(4)
EV13(1)
EV13(2)
EV13(3)
EV13(4)
450
J670 F N I P L D I Q L E G P L R N T L L G N I S R Y Y T H W S G S L E M T F M F C G S F H T T G K L I I
725E(1)
725E(2)
725E(3)
725E(4)
EV13(1)
EV13(2)
EV13(3)
EV13(4)
500
J670 C Y T P P G G S S P T D R M Q A M L A T H V V W D F G L Q S S I T I I I P W I S G S H Y R M F N T D
725E(1)
725E(2)
725E(3)
725E(4)
EV13(1)
EV13(2)
EV13(3)
EV13(4)


550
J670 A K A I N A H V G Y V T C P H Q T N L V A P V G A A D Q C Y I V G H V A A K K D P H L R L H R D G P
725E(1)
725E(2)
725E(3)
725E(4)
EV13(1)
EV13(2)
EV13(3)
EV13(4)
|→ VP1
600
J670 D I G Q S A I L P E Q A A T T Q I G E I V K T V A N T V E S E I K A E L G V I P S L N A V E T G A T
725E(1)
725E(2)
725E(3)
725E(4)
EV13(1) V
EV13(2) V
EV13(3) V
EV13(4) V
650
J670 G N T E P E E A I G T R T V I N H H G T A E C L V E N F L G R S A L V C H R S F E Y K N H S T S T S
725E(1)
725E(2)
725E(3)
725E(4) A
EV13(1) P
EV13(2) P
EV13(3)
EV13(4)
700
J670 S I Q X N F F I W T L N T R E L V Q I R R K M E L F T Y L R F D T E I T I V P T L R L F S S S N V S
725E(1)
725E(2)
725E(3)
725E(4)
EV13(1)
EV13(2)
EV13(3)
EV13(4)
750
J670 F S G L P N L T L Q A H Y V P T G A R K P S S Q D S F E W Q S A C N P S V F F K I N D P P A R L T I
725E(1)
725E(2)
725E(3)
725E(4)
EV13(1)
EV13(2)
EV13(3)
EV13(4)


800
J670 P F M S I N S A Y A N F Y D G F A G F E K K A T V L Y G I N P A N T M G N L C L R V V N S Y Q P V Q
725E(1)
725E(2)
725E(3)
725E(4)
EV13(1)
EV13(2)
EV13(3)
EV13(4)
850
J670 Y T L T V R V Y M K P K H I K A W A P R A P R T H P Y T N I L H N N Y V G R S A A P N A P T A I V S
725E(1)
725E(2)
725E(3)
725E(4)
EV13(1)
EV13(2)
EV13(3)
EV13(4)
871
J670 D R S T I K T M P N D I N L T T A G P G Y
725E(1)
725E(2)
725E(3)
725E(4)
EV13(1)
EV13(2)
EV13(3)
EV13(4)

The amino acid substitutions identified for the mutants are listed in Table 12. All the mutants selected with mAb EV-13 had the same substitutions at amino acid position 589 (Ile → Val). In addition, mutants EV-13 (2) and (3) showed the same change at position 650 (Ser → Pro), while mutant EV-13 (1) possessed a substitution at position 649 (Thr → Ala). These alterations are all located in VP1.

No changes were common to all of the mutants selected with mAb 72-5E. However, the mutants 72-5E (1) (3) and (4) had the same substitutions at amino acid positions 173 (Ser → Cys), 223 (Val → Glu) and 264 (Ser → Cys), while mutant 72-5E (2) showed a unique change at position 143 (Asn → Thr). These differences are all situated in VP2.

TABLE 12 PREDICTED AMINO ACID DIFFERENCES IN ANTIBODY ESCAPE MUTANTS[1]

Mutant	Protein	Position[2]	Change
72 - 5E (1)	VP2	173 (104)	Ser → Cys
		223 (154)	Val → Glu
		264 (195)	Ser → Cys
72 - 5E (2)	VP2	143 (74)	Asn → Thr
72 - 5E (3)	VP2	173 (104)	Ser → Cys
		223 (154)	Val → Glu
		264 (195)	Ser → Cys
72 - 5E (4)	VP2	173 (104)	Ser → Cys
		223 (154)	Val → Glu
		264 (195)	Ser → Cys
EV - 13 (1)	VP1	589 (28)	Ile → Val
		649 (88)	Thr → Ala
EV - 13 (2)	VP1	589 (28)	Ile → Val
		650 (89)	Ser → Pro
EV - 13 (3)	VP1	589 (28)	Ile → Val
		650 (89)	Ser → Pro
EV - 13 (4)	VP1	589 (28)	Ile → Val

[1] amino acid differences listed with respect to the consensus amino acid sequence predicted for J670

[2] the amino acid position relative to amino acid 1 of the polyprotein is shown; the amino acid position relative to amino acid 1 of the VP1 or VP2 is shown in parenthesis

CHAPTER 4

DISCUSSION

The main objective of this study was the identification of neutralization associated sites of EV70. The antigenic structure of picornaviruses has been studied intensively over the past few years using a variety of methods. These include: (i) isolation and characterization of mutants that escape neutralization by mAbs; (ii) identification of hydrophilic nonconserved sequences that are probably situated at the virion surface and subject to immune pressures; (iii) analysis of reaction between mAbs and peptides believed to be located in antigenic sites; (iv) stimulation of immune responses using synthetic peptides from possible antigenic sites; and (v) identification of atomic structures that may interact with antibody, using X-ray crystallographic techniques (Minor, 1990). Among these methods, the first approach has been the most popular one. Specifically, antigenic sites of polioviruses 1, 2 and 3 (Minor *et al.*, 1986; Table 1), HRV 2 (Appleyard *et al.*, 1990; Table 3), HRV 14 (Sherry *et al.*, 1986; Table 2), mengovirus (Boege *et al.*, 1991) and HAV (Ping *et al.*, 1992) have been identified using neutralizing

mAbs to select escape mutants. Because this approach has been used widely and successfully in the field, because it is not technically difficult, and because a number of the neutralizing mAbs were available, it was the method of choice in this study for identification of neutralization associated sites of EV70.

The first series of experiments assessed the neutralizing ability of the available mAbs. The neutralization titers of the mAbs (72-5E, 73-2F, EV-13 and EV-12) were determined using the plaque reduction assay (Figure 1, Table 6). The 50% neutralization titers determined for mAbs 72-5E, 73-2F and EV-13 were high; $10^{4.1}$, $10^{4.1}$ and $10^{5.9}$, respectively. The titers for mAbs 72-5E and 73-2F were consistent with data published previously (Anderson *et al.*, 1984). One intriguing observation was that the neutralization titer of mAb EV-12 was low ($10^{2.6}$) at 25°C, but increased 19-fold if virus and antibody were incubated together at 37°C. The titer of mAb EV-12 at 37°C ($10^{3.8}$) approached the value published previously (Wiley *et al.*, 1992). The observation that increased incubation temperature improved the neutralizing ability of mAb EV-12 could be due to effects on the mAb or the virus or both. It is possible that the higher temperature alters the EV70 virion in some way to make a binding site for mAb EV-12 more accessible. There is evidence that more than one form of poliovirus particle is present in solution (Roivainen *et al.*, 1993). In addition, heating poliovirus particles appears to alter virion conformation (Fricks and Hogle, 1990), although temperatures higher than 37°C are required to effect this change. The temperature sensitivity of EV70 may be important in this regard. It is also possible that incubation at 37°C alters the conformation of the antibody molecule to make binding more efficient. To test these

possibilities, virus or mAb could be incubated separately at either 25°C or 37°C for 1 hr and cooled to room temperature before proceeding with the incubation at room temperature.

Once a working titer for each antibody was established, the next objective of this study was the isolation of antibody-escape mutants. The four neutralizing mAbs, 72-5E, 73-2F, EV-13 and EV-12 were used in attempts to isolate variants resistant to neutralization according to the method of Sherry and Rueckert (1985). Two of the mAbs, 72-5E and EV-13 gave mutants after the first round of selection and these mutants survived two additional rounds of neutralization. The other two mAbs, 73-2F and EV-12 did not yield mutants. There are several possible explanations for these observations. (1) The number of plaques screened may have been too few to identify mutants. The range of frequency of isolation of antibody-resistant mutants for other viruses is from 10^{-4} to 10^{-6} (Minor *et al.*, 1983; 1985; Sherry and Rueckert, 1985; Coelingh and Tierney, 1989; Emini *et al.*, 1983a, 1983b; Portner *et al.*, 1980). For antibodies 73-2F and EV-12, at least 10^{7} PFU were screened using various modifications of the techniques. This should have been enough PFU to identify variants that escaped neutralization, however, it remains possible that mutations at sites recognized by these two antibodies are particularly rare. If these two mAbs could be used to successfully obtain mutants, it could lead to the identification of additional neutralization sites of EV70. (2) It is possible that the sites recognized by the mAbs are key residues for virus survival. Under these circumstances, mutation at such sites would always be lethal. The inability to isolate antibody resistant mutants has been reported. A similar phenomenon was found

for poliovirus type 2 (Minor *et al.*, 1986; La Monica *et al.*, 1987). Minor *et al.* (1986) used seven mAbs to select antibody-escape mutants for the Sabin strain of type 2 poliovirus. While mutants could be isolated with five of the mAbs, the other two mAbs failed to select mutants. The same mAbs were used by La Monica *et al.* (1987) to isolate antibody-escape mutants from the Lansing strain of type 2 poliovirus, with similar results. In similar studies, Baty and Randall (1993), Nishikawa *et al.* (1983) and Coelingh and Tierney (1989) also failed to obtain mutants of simian virus 5, Newcastle disease virus and human parainfluenzavirus type 3, respectively, with mAbs that had low neutralization titers. (3) The neutralization titer of the mAbs was too low, therefore, the selection pressure used by these mAbs was not sufficient to select mutants from the parental virus. Because the titer of mAb EV-12 was lower in my hands than that published (Wiley *et al.*, 1992), this could be one of the explanations for the failure of this mAb to yield mutants. If the last two reasons are the case, additional neutralizing mAbs should be used in future experiments.

Once mutants had been obtained and plaque purified, it was confirmed that they were resistant to neutralization by the mAb used in their selection. This observation suggests that the two mAbs (72-5E and EV-13) recognize different neutralization associated determinants on EV70. mAbs 73-2F and EV-12 could have been used in the cross-neutralization tests of 72-5E and EV-13 mutants, but, unfortunately, were not. It will be of interest to find out if these mAbs recognize the same or different sites as mAbs 72-5E and EV-13. Nevertheless, this work demonstrates that at least two different neutralization-associated sites are present on EV70.

As expected, the mutants were neutralized by mAbs that were not used in their selection. Not only were they neutralized but they also appeared to be more sensitive (although the differences were small) to neutralization than wild type virus. The mutants represent a population of viruses selected for a change(s) that allows them to escape neutralization by a particular mAb. Such a change may alter other features of the virion, such as capsid conformation, which may make them more sensitive to neutralization by antibodies recognizing other epitopes.

In an effort to determine if neutralization-associated sites recognized by mAbs EV-13 and 72-5E are altered in nature, the neutralization of several wild isolates of EV70 was carried out. All of the wild isolates were neutralized by both mAbs (Figures 7 and 8) and the neutralization patterns and titers were similar to those of prototype virus. The observation for mAb 72-5E is in agreement with data of Anderson *et al.* (1984). This suggests that the neutralization associated sites identified by mAbs 72-5E and EV-13 are not altered in wild isolates of EV70. Although the numbers of mAbs and isolates were small, these results are consistent with the interpretation that there is little immunological pressure for change at the sites recognized by these two mAbs.

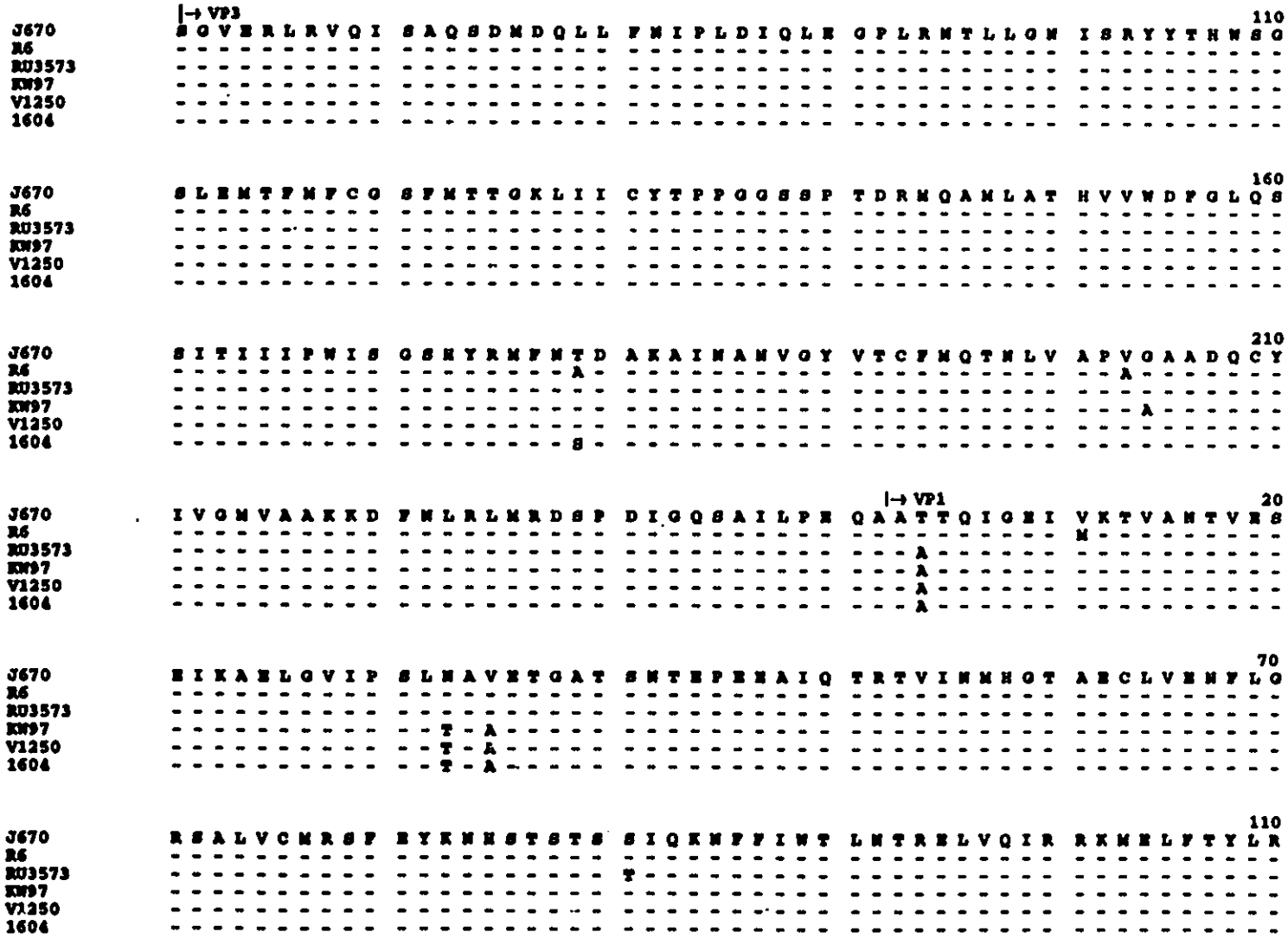
The next objective that was addressed was to identify the locations in the capsid protein of the amino acid changes that correlated with escape from neutralization. Different sites in the capsid proteins of EV70 were identified by cloning and sequence analysis of the capsid protein coding region of the EV70 mutants. As summarized in Table 12, differences were seen in all 4 of the EV-13 mutants at position 589 (Ile → Val). Two of the EV-13 mutants (EV-13 2, 3) had a substitution at position 650 (Ser →

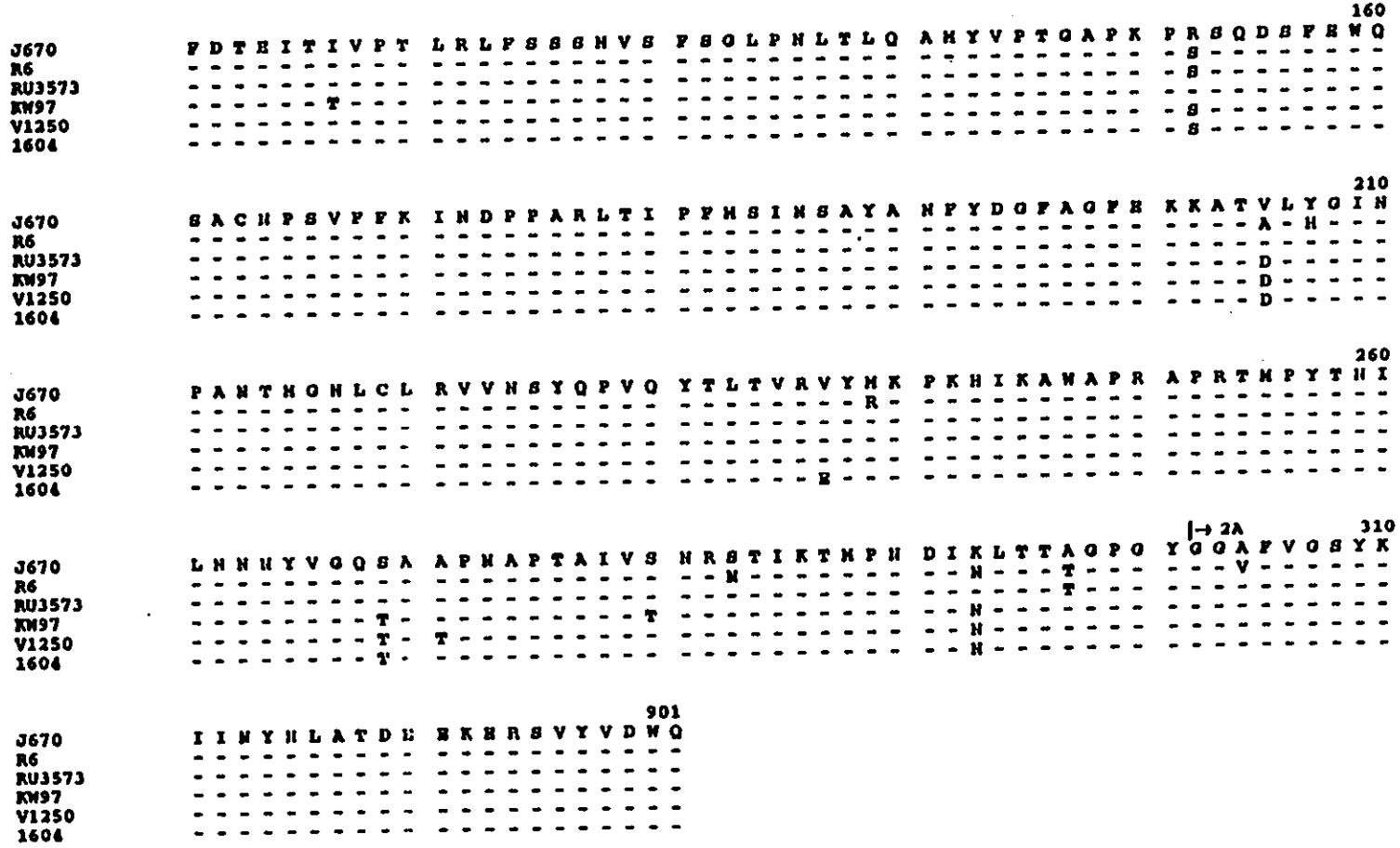
Pro) and a single mutant (EV-13 1) had a change at position 649 (Thr → Ala). All of the substitutions predicted for the EV-13 mutants were in VP1 coding sequences. Three of the 72-5E mutants (72-5E 1, 3 and 4) were predicted to have substitutions at positions 173 (Ser → Cys), 223 (Val → Glu) and 264 (Ser → Cys) in VP2, while one of the 72-5E mutants (72-5E 2) showed a unique difference from the prototype at position 143 (Asn → Thr) in VP2.

The Ile → Val substitution at position 589 (residue 28 of VP1) would appear to be the best candidate to explain escape from neutralization by mAb EV-13. This is the only change common to all of the EV-13 mutants, it was restricted to EV-13 mutants and it was not seen in any of the 72-5E mutants or wild isolates (Table 12, Figure 16). Most importantly, in one mutant (EV-13 4), this was the only substitution found and must therefore be sufficient for escape from neutralization. Amino acid 589 (28 of VP1) is located near the amino terminus of VP1. The amino terminus of picornavirus VP1 has not previously been identified as a neutralization associated site using mAbs and, in the crystal structure of several picornaviruses including poliovirus, is located near the inner surface of the capsid. However, it has been shown that the amino terminus of poliovirus VP1 is immunogenic and can lead to the production of neutralizing antibodies. Antibodies obtained by immunizing rabbits and rats with synthetic peptides derived from poliovirus VP1 residues 24-40 or 37-53 of types 1 and 3 recognize denatured VP1 and react with and neutralize whole virus (Chow and Baltimore, 1982; Hovi and Roivainen 1993; Roivainen *et al.*, 1993). In addition, peptides derived from this region of type 3 poliovirus are recognized by human sera (Roivainen *et al.*, 1991). Residues 24-40 and

Figure 16. Predicted VP1 and VP3 amino acid sequences of the prototype and wild isolates of EV70

The predicted amino acid sequences of EV70 protein VP1 and the carboxyl terminal 75% of VP3 are shown. The amino acid position in numbered on the top of each line. Amino acids of wild isolates differing from the prototype (J670) sequence are indicted below the J670 amino acid sequence. Identical amino acids are shown by dashes.


|→ VP3
110
J670 S Q V E R L R V Q I S A Q S D M D Q L L F N I P L D I Q L E G P L R M T L L Q M I S R Y Y T N W S Q
R6 -
RU3573 -
KW97 -
V1250 -
1604 -
160
J670 S L E M T F M F C G S F M T T G K L I I C Y T P P G G S S P T D R M Q A M L A T H V V N D F G L Q S
R6 -
RU3573 -
KW97 -
V1250 -
1604 -
210
J670 S I T I I I P W I S Q S H Y R M F M T D A K A I M A M V Q Y V T C F M Q T M L V A P V Q A A D Q C Y
R6 - - - - - - - - - - - - - - - - - A - A - - - - - - -
RU3573 -
KW97 - A - - -
V1250 -
1604 - - - - - - - - - - - - - - - - - S -
|→ VP1
20
J670 I V Q M V A A K K D F N L R L M R D S P D I Q Q S A I L P E Q A A T T Q I Q E I V K T V A N T V E S
R6 - M - - - - - - -
RU3573 - A - - - - - - - - - -
KW97 - A - - - - - - - - - -
V1250 - A - - - - - - - - - -
1604 - A - - - - - - - - - -
70
J670 E I K A E L G V I P S L N A V E T G A T S N T E P E N A I Q T R T V I N N H G T A E C L V E N F L Q
R6 -
RU3573 -
KW97 - - - - - - - - - - - - T - A -
V1250 - - - - - - - - - - - - T - A -
1604 - - - - - - - - - - - - T - A -
110
J670 R S A L V C M R S F E Y K N N S T S T S S I Q K N F F I N T L N T R E L V Q I R R K M E L F T Y L R
R6 -
RU3573 - T -
KW97 -
V1250 -
1604 -

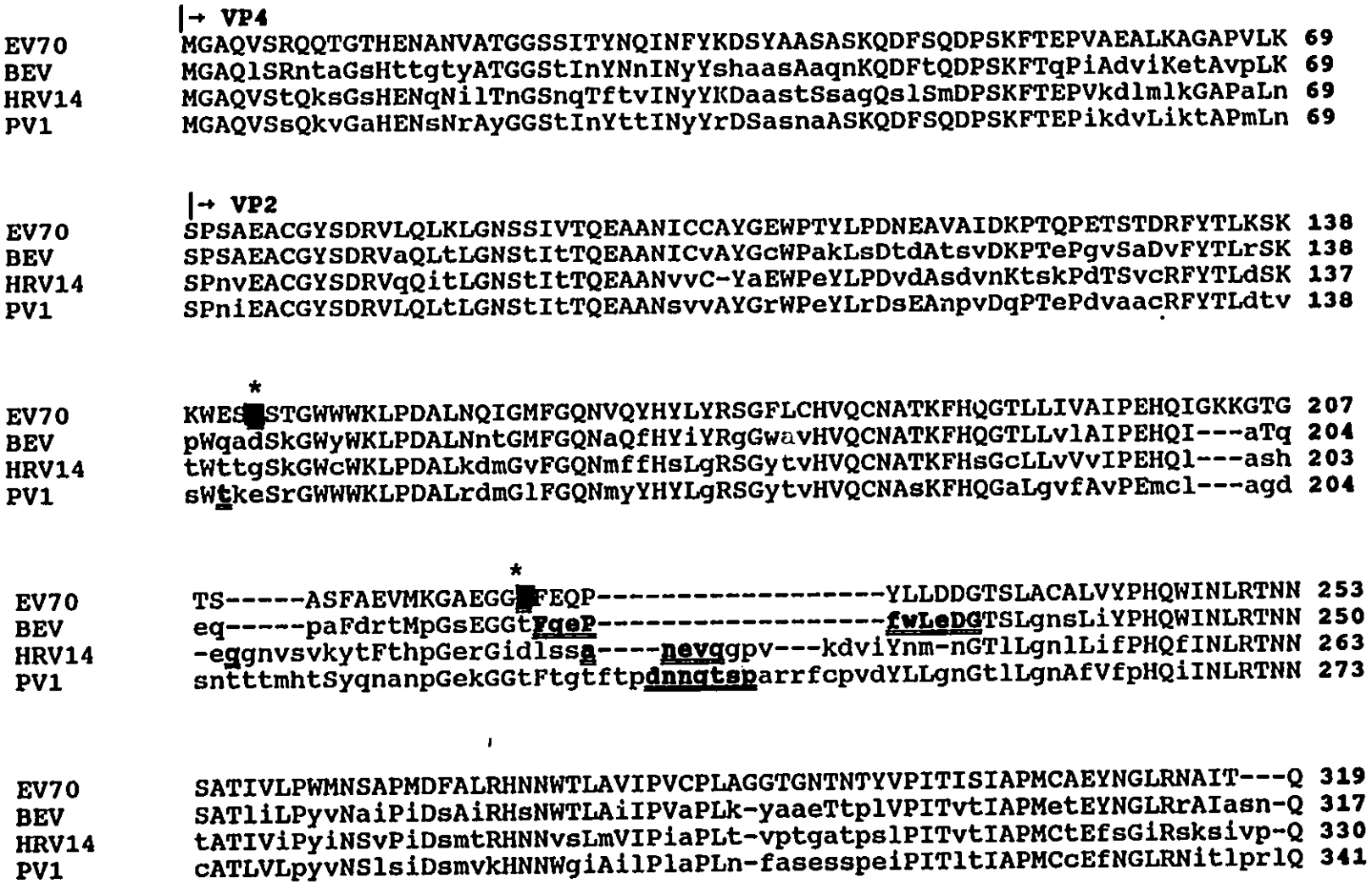

160
J670 F D T E I T I V P T L R L P S S S N V S F S Q L P N L T L Q A H Y V P T Q A P K P R S Q D S F E W Q
R6 S S
RU3573 S S
KW97 T S
V1250 S S
1604
210
J670 S A C H P S V F F K I N D P P A R L T I P Y H S I N S A Y A N F Y D Q F A Q F E K K A T V L Y Q I N
R6 A H
RU3573 D
KW97 D
V1250 D
1604 D
260
J670 P A N T H Q N L C L R V V N S Y Q P V Q Y T L T V R V Y H K P K H I K A W A P R A P R T H P Y T N I
R6 R
RU3573
KW97
V1250 E
1604
→ 2A
310
J670 L N N H Y V G Q S A A P N A P T A I V S N R S T I K T H P H D I K L T T A Q P Q Y Q Q A F V Q S Y K
R6 M N T T
RU3573 N
KW97 T T N
V1250 T T N
1604 T
901
J670 I I N Y H L A T D E E K E R S V Y V D W Q
R6
RU3573
KW97
V1250
1604

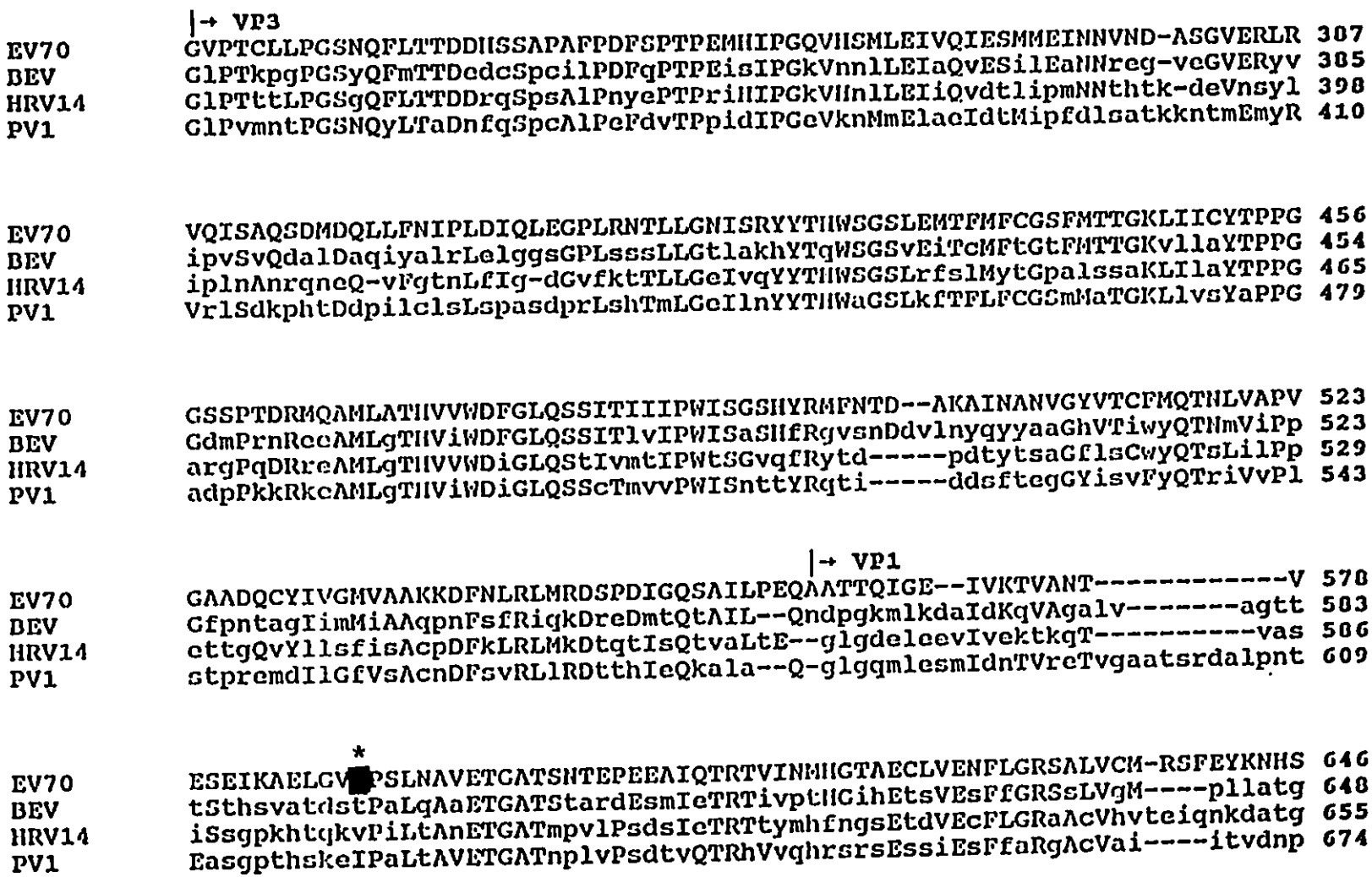
37-53 of poliovirus VP1 correspond to amino acids 17-28 and 25-41 of EV70 (Hovi and Roivainen, 1993; Figure 17). As mentioned previously, the amino terminus of VP1 is buried in the crystal structure of poliovirus but is exposed when poliovirus is heat-denatured. In addition, this region of VP1 becomes exposed on poliovirus particles that have been allowed to attach to susceptible cells and is believed to play an important role during poliovirus penetration of the plasma membrane (Fricks and Hogle, 1990). In solution, poliovirus is believed to be able to adopt two different interconvertible forms, the A form and the B form (Roivainen *et al.*, 1993). The A form is preferred at neutral pH while the B form is preferred at acidic pH (4.5). The B form also shares several properties with poliovirus particles altered by cell binding (Roivainen *et al.*, 1993). In addition, a fraction of poliovirus particles are able to interact with antibodies made to synthetic peptides derived from the amino terminus of VP1. Together, these observations suggest that polioviruses can exist in one of two conformations. One conformation likely corresponds to the crystal structure that has been described (Hogle *et al.*, 1985). The other conformation would appear to have an exposed amino-terminal VP1 domain. It has been proposed that the reversible externalization/internalization of the amino terminus of VP1 may function during poliovirus attachment and entry.

Despite its importance as an immunogenic site, the amino terminus of picornaviruses has not previously been identified as such using mAb escape mutants. The identification of EV70 escape mutants with amino acid substitutions near the amino terminus of VP1 therefore, is an unprecedented observation, and might suggest a fundamental structural difference between EV70 and polioviruses (and possibly other

Figure 17. Amino acid residues predicted to be associated with neutralization by Mabs EV-13 and 72-5E

Amino acids of the capsid proteins for four different picornaviruses, Enterovirus 70 (EV70) (Takeda *et al.*, 1989), bovine Enterovirus (BEV) (Earle *et al.*, 1988), poliovirus type 1 (PV 1) (Koch and Koch, 1985) and human rhinovirus 14 (HRV 14) (Callahan *et al.*, 1985) are presented. The alignments were done using the Pustell Sequence Analysis Programs (IBI) Version 2.04. The amino acid positions are indicated at the end of each line. The predicted cleavages between the capsid proteins marked with vertical lines and arrows. Differences with respect to EV70 are presented with small case letters below the EV70 amino acid sequence and gaps are presented by "-". The amino acid residues believed to be associated with neutralization by mAb EV-13 (in VP1) and 72-5E (in VP2) are shown in green and red respectively. The amino acids or regions predicted to correspond to the 72-5E site are shown in yellow on VP2 of the other picornavirus capsid protein sequences.


|→ VP4
EV70 MGAQVSRQQTGTHENANVATGGSSITYNQINFYKDSYAASASKQDFSQDPSKFTEPVAEALKAGAPVLK 69
BEV MGAQlSRntaGsHttgtyATGGStInYNnINyYshaasAaqnKQDFtQDPSKFTqPiAdviKetAvpLK 69
HRV14 MGAQVStQksGsHENqNilTnGSnqTftvINyYKDaastSsagQslSmDPSKFTEPVkdlmlkGAPaLn 69
PV1 MGAQVSsQkvGaHENsNrAyGGStInYttINyYrDSasnaASKQDFSQDPSKFTEPikdvLiktAPmLn 69
|→ VP2
EV70 SPSAEACGYSDRVLQLKLGNSSIVTQEAANICCAYGEWPTYLPDNEAVAIDKPTQPETSTDRFYTLKSK 138
BEV SPSAEACGYSDRVaQLtLGNStItTQEAANICvAYGcWPakLsDtdAtsvDKPTePgvSaDvFYTLrSK 138
HRV14 SPnvEACGYSDRVqQitLGNStItTQEAANvvC-YaEWPeYLPDvdAsdvnKtskPdTSvcRFYTLdSK 137
PV1 SPniEACGYSDRVLQLtLGNStItTQEAANsvvAYGrWPeYLrDsEAnpvDqPTePdvaacRFYTLdtv 138
EV70 KWES STGWWWKLPDALNQIGMFGQNVQYHYLYRSGFLCHVQCNATKFHQGTLLIVAIPEHQIGKKGTG 207
BEV pWqadSkGWyWKLPDALNntGMFGQNaQfHYiYRgGwavHVQCNATKFHQGTLLvlAIPEHQI---aTq 204
HRV14 tWttgSkGWcWKLPDALkdmGvFGQNmffHsLgRSGytvHVQCNATKFHsGcLLvVvIPEHQl---ash 203
PV1 sWtkeSrGWWWKLPDALrdmGlFGQNmyYHYLgRSGytvHVQCNAsKFHQGaLgvfAvPEmcl---agd 204
EV70 TS-----ASFAEVMKGAEGG FEQP----------YLLDDGTSLACALVYPHQWINLRTNN 253
BEV eq-----paFdrtMpGsEGGtFqeP----------fwLeDGTSLgnsLiYPHQWINLRTNN 250
HRV14 -eggnvsvkytFthpGerGidlssa----nevqgpv---kdviYnm-nGTlLgnlLifPHQfINLRTNN 263
PV1 sntttmhtSyqnanpGekGGtFtgtftpdnnqtsparrfcpvdYLLgnGtlLgnAfVfpHQiINLRTNN 273
EV70 SATIVLPWMNSAPMDFALRHNNWTLAVIPVCPLAGGTGNTNTYVPITISIAPMCAEYNGLRNAIT---Q 319
BEV SATliLPyvNaiPiDsAiRHsNWTLAiIPVaPLk-yaaeTtplVPITvtIAPMetEYNGLRrAIasn-Q 317
HRV14 tATIViPyiNSvPiDsmtRHNNvsLmVIPiaPLt-vptgatpslPITvtIAPMCtEfsGiRsksivp-Q 330
PV1 cATLVLpyvNSlsiDsmvkHNNWgiAilPlaPLn-fasesspeiPITltIAPMCcEfNGLRNitlprlQ 341


|→ VP3
EV70 GVPTCLLPGSNQFLTTDDHSSAPAFPDFSPTPEMHIPGQVHSMLEIVQIESMMEINNVND-ASGVERLR 307
BEV GlPTkpgPGSyQFmTTDedcSpcilPDFqPTPEisIPGkVnnlLEIaQvESilEaNNreg-veGVERyv 385
HRV14 GlPTttLPGSgQFLTTDDrqSpsAlPnyePTPriHIPGkVHnlLEIiQvdtlipmNNthtk-deVnsyl 398
PV1 GlPvmntPGSNQyLTaDnfqSpcAlPeFdvTPpidIPGeVknMmElaeIdtMipfdlsatkkntmEmyR 410
EV70 VQISAQSDMDQLLFNIPLDIQLEGPLRNTLLGNISRYYTHWSGSLEMTFMFCGSFMTTGKLIICYTPPG 456
BEV ipvSvQdalDaqiyalrLelggsGPLsssLLGtlakhYTqWSGSvEiTcMFtGtFMTTGKvllaYTPPG 454
HRV14 iplnAnrqneQ-vFgtnLfIg-dGvfkttLLGeIvqYYTHWSGSLrfslMytGpalssaKLIlaYTPPG 465
PV1 VrlSdkphtDdpilclsLspasdprLshTmLGeIlnYYTHWaGSLkfTFLFCGSmMaTGKLlvsYaPPG 479
EV70 GSSPTDRMQAMLATHVVWDFGLQSSITIIIPWISGSHYRMFNTD--AKAINANVGYVTCFMQTNLVAPV 523
BEV GdmPrnReeAMLgTHViWDFGLQSSITlvIPWISaSHfRgvsnDdvlnyqyyaaGhVTiwyQTNmViPp 523
HRV14 argPqDRreAMLgTHVVWDiGLQStIvmtIPWtSGvqfRytd-----pdtytsaGflsCwyQTsLilPp 529
PV1 adpPkkRkeAMLgTHViWDiGLQSScTmvvPWISnttYRqti-----ddsftegGYisvFyQTriVvPl 543
|→ VP1
EV70 GAADQCYIVGMVAAKKDFNLRLMRDSPDIGQSAILPEQAATTQIGE--IVKTVANT----------V 578
BEV GfpntagIimMiAAqpnFsfRiqkDreDmtQtAIL--QndpgkmlkdaIdKqVAgalv-------agtt 583
HRV14 ettgQvYllsfisAcpDFkLRLMkDtqtIsQtvaLtE--glgdeleevIvektkqT----------vas 586
PV1 stpremdIlGfVsAcnDFsvRLlRDtthIeQkala--Q-glgqmlesmIdnTVreTvgaatsrdalpnt 609
*
EV70 ESEIKAELGV[redacted]PSLNAVETGATSNTEPEEAIQTRTVINMHGTAECLVENFLGRSALVCM-RSFEYKNHS 646
BEV tSthsvatdstPaLqAaETGATStardEsmIeTRTivptHGihEtsVEsFfGRSsLVgM----pllatg 648
HRV14 iSsgpkhtqkvPiLtAnETGATmpvlPsdsIeTRTtymhfngsEtdVEcFLGRaAcVhvteiqnkdatg 655
PV1 EasgpthskeIPaLtAVETGATnplvPsdtvQTRhVvqhrsrsEssiEsFfaRgAcVai----itvdnp 674

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EV70    TSTSSIQKNFFIWTLNTRELVQIRRKMELFTYLRFDTEITIVPTLRLFSSSNVSFSGLPNLTLQAMYVP 715
BEV     TSith-------WridfREfVQlRaKMswFTYmRFDvEfTIiaT----SStgqnvtteqhtTyQvMYVP 706
HRV14   idnhreaKlFndWkiNlssLVQlRkKlELFTYvRFDsEyTIlaTasqpdSaNySs----NLvvQAMYVP 720
PV1     aSTtnkdKlFavWkitykdtVQlRRKlEfFTYsRFDmElTfVvTanftetnNghal---NqvyQiMYVP 740

EV70    TGARKPSSQDSFEWQSACNPSVFFKINDPPARLTIPFMSINSAYANFYDGFAGFE------KKATVLYG 778
BEV     pGApvPSnQDSFqWQSgCNPSVFadtdgPPAqfsvPFMSsanAYstvYDGyArFm------dtdpdrYG 769
HRV14   pGApnPkewDdytWQSAsNPSVFFKvgD-tsRfsvPyvglaSAYncFYDGysh--------ddAetqYG 781
PV1     pGApvPekwDdytWQtssNPSiFytygtaPARisvPyvgIsnAYshFYDGFsKvplkdqsaalgdsLYG 809

EV70    IN-PANTMGNLCLRVVNSYQPVQYTLTV--RVYMKPKHIKAWAPRAPRTMPYTNILNNNYVGRSAAPNA 844
BEV     IIlPsNflGfmyfRtledaahq-----VrfRiYaKiKHtscWiPRAPRqaPYkkryNlvfsGdSdrics 8??
HRV14   IItvlNhMGsmafRiVNehdeh--ktlVkiRVYhraKHveAWiPRAPRalPYTsIgrtNYpkntepvik 847
PV1     aa-slNdfGiLaVRVVNdhnPt--kvTskiRVYlKPkHIrvwcPRpPRavaYygpg-vdYkGdtltPls 874

EV70    PTAIVSDRSTIKTMPNDIDLTTAGPGY 871
BEV     nrA---------------sLtsy---- 840
HRV14   krkg--------------Diksy---- 856
PV1     tk----------------DLTTy---- 881
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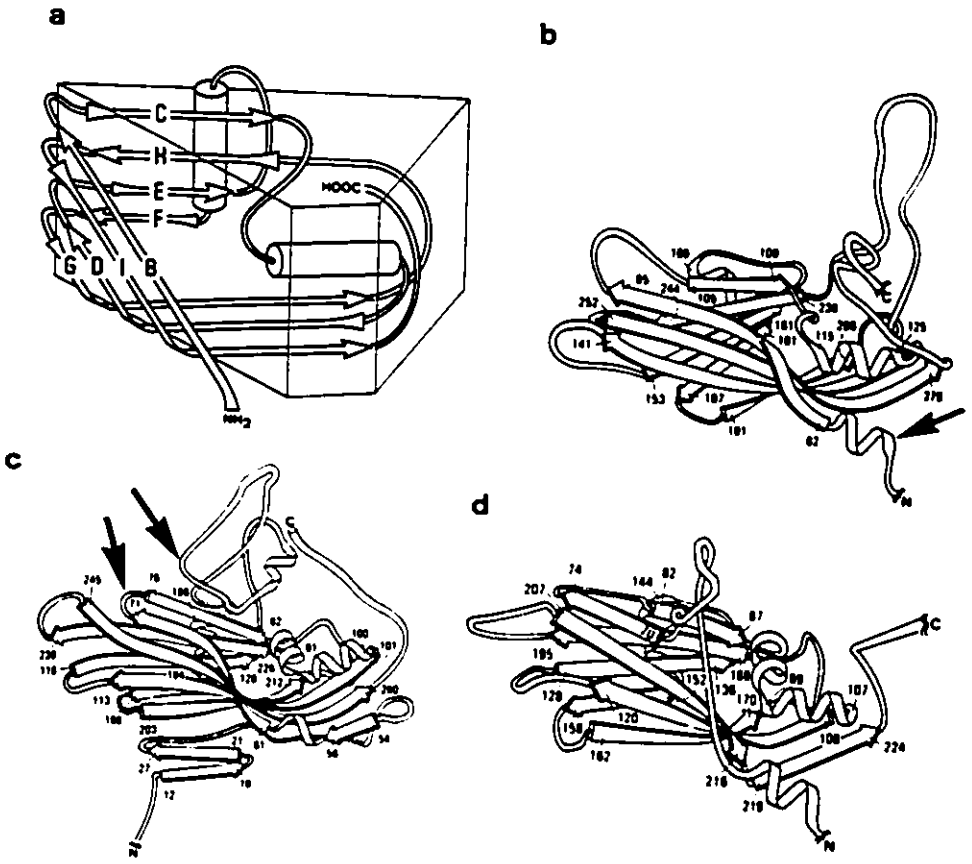
picornaviruses). The fact that EV70 is also naturally temperature sensitive may reflect such a structural difference.

The substitutions at position 649 (residue 88 of VP1) and 650 (residue 89 of VP1) were also restricted to EV-13 mutants. Residues 649 (88 of VP1) and 650 (89 of VP1) are predicted to be in the BC loop of VP1 and on the surface of EV70 virions in positions that might correspond to poliovirus antigenic site I (residues 91-102 of VP1) (Table 1), HRV 14 immunogenic site Ia (residues 91, and 95) (Table 2) and HRV 2 neutralization site A (residues 85, 86 and 92) (Table 3). One of the wild isolates, RU3573, was predicted to have a Ser → Thr substitution at position 651 (residue 90 of VP1, Figure 16). This might suggest that amino acid substitutions are permitted in this region of VP1, although it is possible that the change noted in RU3573 could be due to an error occurring during PCR amplification. More compelling, however, was the observation that the three EV-13 mutants with changes at positions 649 and 650 also had the Ile → Val substitution at position 589. This would appear to rule out these changes as responsible for escape from neutralization.

No single amino acid change in the capsid protein-coding sequences of all the 72-5E mutants was identified that could explain escape from neutralization by mAb 72-5E. The Asn → Thr substitution at position 143 (residue 74 of VP2) of mutant 72-5E (2) would appear to be responsible for the resistance of this variant to neutralization. Based on sequence alignments of picornaviral capsid proteins (Figure 17, Palmenberg, 1989) and on the structure of poliovirus VP2, amino acid 74 of the EV70 VP2 is predicted to be located in the BC loop and on the surface of EV70 virions (Figure 18). This would

Figure 18. Predicted locations of amino acid residues associated with neutralization by mAbs EV-13 and 72-5E

(a) Schematic diagrams of the wedge-shaped 8 stranded anti-parallel beta barrel cores of poliovirus VP1, VP2 and VP3. Each of the beta strands is shown by arrows and labeled alphabetically starting from the amino terminus. Helices are presented as cylinders. Diagrams of VP1, VP2 and VP3 are shown in (b), (c) and (d), respectively. The approximate location of amino acids predicted to be associated with neutralization by EV70 specific mAbs EV-13 and 72-5E are indicated with arrows on VP1 and VP2 respectively. Figure was modified from Hogle *et al.* (1989) with permission.


a
C
H
E
F
G D I B
HOOC
b
c
d

lie in a region corresponding to poliovirus antigenic site IV (IIIb) (residues 72 of poliovirus VP2) (Table 1) and the mengovirus antigenic site (residue 75 of VP2) (Boege *et al.*, 1991).

The three other mutants selected with mAb 72-5E all have identical substitutions at 3 positions; 173 (Ser → Cys, residue 104 of VP2), 223 (Val → Glu, residue 154 of VP2) and 264 (Ser → Cys, residue 195 of VP2). These changes were not found in any of the EV-13 mutants but no information is available for the wild isolates at this time. Changes at any of these three positions might account for resistance to neutralization by mAb 72-5E. Amino acid 154 of VP2 is predicted to be located in the EF loop on the surface of EV70 virions and in proximity to one another with residue 74 of VP2, while residues 104 of VP2 and 195 of VP2 are probably underneath the virion surface in the β-barrel core and may or may not have effects on conformation of surface structures. In addition, the Val → Glu substitution at position 154 in VP2 appears to be in a region that corresponds to BEV antigenic site I (152 of BEV VP2) (Table 4, Figure 17), HRV14 antigenic site II (158 of HRV14 VP2) (Table 2, Figure 17) and the mengovirus composite antigenic site (144 of mengovirus VP2) as well as HRV2 antigenic site B (Table 3). It is also located in the same loop (EF) as poliovirus antigenic site II (Table 1, Figure 17, 18). Since this change appears to align with antigenic sites of other picornaviruses, it appears to be the best candidate to explain how these three mutants escape neutralization by mAb 72-5E.

In summary, this is the first report concerning characterization of sites associated with EV70 neutralization. Specific amino acids which appear to be part of neutralization-

associated sites of EV70 were identified. It is predicted that the site recognized by mAb EV-13 includes amino acid 28 of VP1 and the site recognized by mAb 72-5E includes residues 74 and 154 of VP2. The EV-13 site would appear to consist of amino-terminal VP1 residues and is likely a linear epitope whereas the 72-5E epitope is conformational in nature. Proof that these residues are critical to antibody neutralization of EV70 will require further characterization such as site-directed mutagenesis or peptide scanning techniques. Unlike other picornaviruses, the amino terminus of VP1 was recognized as a neutralization-associated determinant using mAb selection of escape variants. This suggests that the structure of EV70 may be different from the structures of other picornaviruses, with a more exposed or accessible amino terminal VP1 domain. Verification of this possibility will require examination of EV70 structure by other methods such as X-ray crystallography.

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APPENDIX I

SEQUENCING PRIMERS

591(+)	5'- ATGGTGACAATCAGAGATTG -3'
780(+)	5'- TTGCCACTGGAGGCTCAAGC -3'
985(+)	5'- ATTAGGAAATTCCAGTATAG -3'
1200(+)	5'- TTGGTATGTTTGGTCAAAAC -3'
1500(+)	5'- GTTACCATGGATGAACAGTG -3'
1733(+)	5'- GATGACCACTCATCGGCACC -3'
1864(+)	5'- CGCTAGTGGAGTGGAGAGG -3'
2087(+)	5'- CCACCTGGTGGATCTAGCC -3'
2158(+)	5'- GCTGCAATCTAGTATCACAC -3'
2204(-)	5'- GAGACCCTGAGATCCAAGG -3'
2462(+)	5'- GAGAGTGAGATTAAGGCTG -3'
2524(-)	5'- AGCTCCTGTCTCAACAGCA -3'
2759(+)	5'- TACCTAAGGTTTGACACTG -3'
2829(-)	5'- AAGGAGACATTGCTGCTTG -3'
3068(+)	5'- CCAGCAAACACTATGGGAA -3'
3134(-)	5'- GTGTGTATTGGACTGGTTG -3'