CHARACTERIZATION OF THE GENOME OF EQUINE HERPESVIRUS 1 SUBTYPE 2

by

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TO ELEANOR,

my best friend and mother.

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SUMMARY

Equine herpesvirus type-1 (EHV-1), a member of the Alphaherpesvirinae, is a major cause of abortion and respiratory disease in horses worldwide. It is also associated with a neurological syndrome, neonatal foal disease and more rarely, coital exanthema. Collectively, these diseases represent a significant economic loss to the thoroughbred industry each year. Two antigenically and genetically distinct subtypes of EHV-1 exist. They can be unequivocally differentiated by restriction endonuclease analysis of their DNAs. Molecular epizootiological studies in America and Australia indicate that both subtypes of EHV-1 are respiratory pathogens with the potential to cause abortion, but only subtype 1 has been associated with abortion storms and the neurological form of the disease. Most of the molecular data published concern the subtype 1 virus.

At the onset of this project the genome of EHV-1 subtype 2 was totally uncharacterized. This was probably due in part to the original misconception that it was identical to EHV-1 subtype 1 and in part to the relative difficulty of growing this virus to a high titre in tissue culture. The purpose of this work was to determine whether the two subtypes of EHV-1 shared a common genome structure, to construct the first restriction endonuclease maps for EHV-1 subtype 2 and to investigate the homology between this virus and other members of the Alphaherpesvirinae by molecular hybridization and DNA sequence analysis.

Electron microscopy of EHV-1 subtype 2 DNA which had

been denatured and self-annealed indicated that a sequence of 11kbp approximately at one genome terminus is repeated in inverse orientation at one internal site. The inverted repeats were shown to be separated by a unique sequence of approximately 13kbp. The presence of repeated sequence within the EHV-1 subtype 2 genome was confirmed by hybridization studies using DNA probes isolated from virion DNA. A library of plasmid clones containing Bam HI fragments representing approximately 75% of the genome was prepared, and the clones were then used to derive Bam HI and Eco RI restriction endonuclease maps for EHV-1 subtype 2 DNA. The results show that the EHV-1 subtype 2 genome consists of two segments, L (111kbp) and S (35kbp). The S component consists of a unique sequence (U_S; 9.6kpb - 16kbp) flanked by inverted repeats (TR_S and IR_S; 9.5kbp - 12.7kbp). Published data indicate that the EHV-1 subtype 1 genome has a similar structure. However, the maps for the two subtypes are quite different. Eco RI and Bam HI cleave within the TR_S/IR_S, and so it was not possible to determine whether U_S inverts relative to the L region, as it does in subtype 1.

HSV-1 DNA fragments containing coding sequences for genes which have been shown previously to be well-conserved in the alphaherpesviruses were hybridized to EHV-1 subtype 2 DNA. Thus, the regions of the EHV-1 subtype 2 genome homologous to probes for the HSV-1 genes encoding the ribonucleotide reductase, the major capsid protein, the major DNA-binding protein and the immediate early protein V_{mw}IE175 were identified. Cloned DNA fragments of EHV-1

subtype 2 were used in comparative hybridization experiments to further determine the extent and distribution of homologous sequences in the genomes of both subtypes of EHV-1 and HSV-1. Regions of detectable homology are arranged colinearly along the genomes suggesting that the three viruses share a common gene arrangement. These results imply that it should be possible to predict the locations of most EHV-1 subtype 2 genes on the basis of our existing knowledge of HSV-1 gene location and function.

Published data indicate that the S segment is the least related region in the genomes of several members of the Alphaherpesvirinae and that the TR_S/U_S and IR_S/U_S junctions have altered in location, relative to adjacent genes, during evolution. To elucidate the nature of the genes near the EHV-1 subtype 2 TR_S/U_S junction, the DNA sequence of a 4.57kbp Bam HI fragment was determined using the Sanger chain terminating dideoxynucleotide method. The junction was located within a 100bp region by using several M13 clones in a hybridization study, indicating that TR_S/IR_S and U_S are approximately 10.9kbp and 13.1kbp in size respectively. The G+C content of the TR_S portion of the fragment is approximately 17% greater that that of the U_S portion. An 8bp sequence is tandemly repeated within the TR_S. Analysis of the sequence showed that Bam HI 1 contains two complete open reading frames and the parts of two others. The amino acid sequences of predicted EHV-1 subtype 2 proteins were compared with those coded by the S segments of VZV and HSV-1. Homologues of the four EHV-1

genes were detected in both HSV-1 and VZV. The EHV-1 genes and the TR_S/U_S junction have an arrangement intermediate between that of their HSV-1 and VZV counterparts. One of the EHV-1 subtype 2 genes apparently encodes a glycoprotein.

ABBREVIATIONS

A	adenine-containing moiety or alanine
ACV	acyclovir
Ala	alanine
Arg	arginine
Asn	asparagine
Asp	aspartate
ATP	adenosine triphosphate
BHK	baby hamster kidney cells
BHV-1	bovine herpesvirus type 1
BHV-2	bovine herpesvirus type 2
BHV-4	bovine herpesvirus type 4
BMV	bovine mammilitis virus
bp	base pairs
C	cytosine-containing moiety or cysteine
CCV	channel catfish virus
CMV	cytomegalovirus
c.p.e.	cytopathic effect
Cys	cysteine
D	aspartate
dATP	deoxyadenosine triphosphate
dCTP	deoxycytidine triphosphate
ddNTP	dideoxynucleotide triphosphate
dGTP	deoxyguanosine triphosphate
dITP	deoxyinosine triphosphate
dNTP	deoxynucleotide triphosphate
DMSO	dimethyl sulphoxide
DNase	deoxyribonuclease
E	glutamate

EBTr	bovine embryo tracheal cells
EBV	Epstein-Barr virus
EDTA	ethylenediamine tetra-acetic acid
EHV-1	equine herpesvirus type 1
EHV-2	equine herpesvirus type 2
EHV-3	equine herpesvirus type 3
F	phenylalanine
G	guanine-containing moiety or glycine
G+C content	moles per cent deoxyguanosine plus deoxycytidine moieties
Gln	glutamine
Glu	glutamate
Gly	glycine
GPCMV	guinea pig cytomegalovirus
H	histidine
HCMV	human cytomegalovirus
His	histidine
HSV	herpes simplex virus
HSV-1	herpes simplex virus type 1
HSV-2	herpes simplex virus type 2
HVPan	herpesvirus pan
HVPapio	herpesvirus papio
HVS	herpesvirus saimiri
I	isoleucine
IE	immediate early
Ile	isoleucine
K	lysine
kb	kilobases
KB	human carcinoma cells

kbp	kilobase pairs
L	leucine
Leu	leucine
Lys	lysine
M	methionine
MCP	major capsid protein
MDBP	major DNA-binding protein
MDV	Marek's disease virus
Met	methionine
min	minutes
m.o.i.	multiplicity of infection
mRNA	messenger RNA
N	asparagine
n	nucleotides
NBL-6	equine dermal cell line
NP40	Nonidet P40
oz	ounces
P	proline
PAA	phosphonoacetic acid
Phe	phenylalanine
PK(15)	pig kidney cell line
Pro	proline
PRV	pseudorabies virus
Q	glutamine
R	purine or arginine
RNase	ribonuclease
r.p.m.	revolutions per minute
RK13	rabbit kidney cell line
S	serine

SDS	sodium dodecyl sulphate
sec	seconds
Ser	serine
SHELUT	SV40-transformed sheep lung fibroblasts
SV40	simian virus 40
T	thymidine-containing moiety or threonine
TK	thymidine kinase
Thr	threonine
Trp	tryptophan
ts	temperature-sensitive
Tyr	tyrosine
UV	ultraviolet
V	valine
Val	valine
vol/vol	volume/volume
VZV	varicella-zoster virus
wt/vol	weight/volume
W	tryptophane
Y	tyrosine

Abbreviations for media and solutions are given in the Materials

CHAPTER 1

INTRODUCTION

THE FAMILY HERPESVIRIDAE

General Description

Herpesviruses are highly disseminated throughout the world. Approximately eighty members of the family Herpesviridae are currently recognized (Roizman, 1982), and these have been isolated from a wide range of hosts including fish (e.g. channel catfish virus, Wolf and Darlington, 1971), reptiles (e.g. green iguana virus, Zeigel and Clark, 1972), birds (e.g. pigeon herpesvirus 1; Cornwell et al., 1970) and many mammals including monkeys, apes and man (Nahmias, 1972; Barahona et al., 1974). There are five human herpesviruses, herpes simplex virus type 1 (HSV-1), herpes simplex virus type 2 (HSV-2), varicella-zoster virus (VZV), Epstein-Barr virus (EBV) and human cytomegalovirus (HCMV). Herpesviruses cause several economically important diseases in domestic animals. For example, pseudorabies virus (PRV) causes Aujeszky's disease in swine, bovine herpesvirus 1 (BHV-1) is the aetiological agent of infectious bovine rhinotracheitis and equine herpesvirus 1 (EHV-1) is a major cause of abortion and respiratory disease in horses.

Clinical manifestations of herpesvirus infection range from inapparent infection, to localized disease and to fatal systemic illness. Some herpesviruses are associated with neoplastic disease in their natural host. For example, Lucké virus causes renal adenocarcinoma in the frog (Gravell, 1971), Mareks disease virus (MDV) induces

transformation of lymphoblastoid cells in chickens (Churchill and Biggs, 1968) and EBV is clearly involved in Burkitt's lymphoma in humans (Epstein et al., 1964; Klein, 1979). Herpesviruses are able to persist in a latent form after primary infection, and then may reactivate to cause additional, and often recurrent, episodes of disease during the lifetime of the host.

Classification

Members of the family Herpesviridae have been classified on the basis of morphology and cytopathology (Matthews, 1979, 1982). The herpesvirion comprises four major structural elements: the core, capsid, tegument and envelope. The nucleocapsid consists of an internal DNA-containing core surrounded by an icosahedral capsid. The amorphous material arranged around the nucleocapsid is known as the tegument, and the entire structure is surrounded by a lipid envelope containing glycoproteins (Wildy et al., 1960; Roizman and Furlong, 1974).

Despite these morphological similarities herpesviruses show an immense diversity of biological properties and do not lend themselves readily to subclassification. However, attempts have been made to subdivide the family Herpesviridae on the basis of biological properties (Barahona et al., 1974; Honess and Watson, 1977; Roizman et al., 1981) and, to a limited degree on the basis of genome structure (Roizman et al., 1981; Roizman, 1982).

Subclassification of Herpesviruses on the Basis of Biological Properties

Subclassification on the basis of host range, cytopathology, duration of reproductive cycle and characteristics of latent infection has led to the definition of three subfamilies: the Alphaherpesvirinae, Betaherpesvirinae and Gammaherpesvirinae (Roizman et al., 1981; Roizman, 1982; Honess, 1984).

The Alphaherpesvirinae have a variable host range in vitro. Their reproductive cycle is relatively short and they spread rapidly in tissue culture with mass destruction of infected cells. Clinically, these viruses can cause serious generalized disease in the newborn or immunocompromised adult. The more typical syndrome is a mild infection of the skin, genitalia, or respiratory tract. Latency, usually in the neurons of the ganglia, is a common sequela to primary infection (Wildy et al., 1982). Members of this group include HSV-1, HSV-2, VZV, PRV, EHV-1 and bovine herpesvirus type 2 (BHV-2).

The Betaherpesvirinae or cytomegaloviruses are species-specific in vivo and in vitro (Wright, 1973). Even in these cells they have a relatively long reproductive cycle. Infected cells often become enlarged (cytomegalia) and persistently infected cell cultures are readily established. In normal adults infection is usually subclinical but generalized disease may occur in neonates or immunocompromised adults. Transplacental transmission of many of these viruses may result in congenital

malformations. Sites of persistence include secretory glands, lymphoreticular cells, kidneys and other tissues. Members of the Betaherpesvirinae include human cytomegalovirus (HCMV) and equine herpesvirus 2 (EHV-2).

The Gammaherpesvirinae are specific either for B lymphocytes (e.g. EBV, belonging to the γ_1 subgroup) or T lymphocytes (e.g. Herpesvirus saimiri (HVS), belonging to the γ_2 subgroup). Several of these viruses cause lymphoproliferative diseases. All members of this subfamily are able to replicate in lymphoblastoid cells in vitro and some are able to grow in epithelial cells and fibroblasts. Latent virus is commonly detected in host lymphoid tissue.

Subclassification of Herpesviruses on the Basis of Genome Structure

Despite the generally convincing concordance of biological characteristics displayed by members of each subfamily, it must be noted that this is a subjective classification based on an assessment of a multitude of complex properties. In some cases the assignment of a virus to a particular group is equivocal. Bovine herpesvirus 4 (BHV-4) has a long replicative cycle, is enveloped primarily by budding on smooth cytoplasmic membranes of Golgi body elements and gives rise to the formation of cytoplasmic inclusions. On this basis it was classified as a cytomegalovirus (Storz et al., 1984). However, the BHV-4 genome is 144kbp in size and has polyrepetitive DNA segments at both termini. This bears closer resemblance to the genome of the lymphotropic virus

HVS, a member of the Gammaherpesvirinae, than to the cytomegaloviruses (Ehlers et al., 1985). Furthermore, the recently identified thymidine kinase induced by BHV-4 (Kit et al., 1985) provides another similarity between HVS and BHV-4 absent from HCMV. Such anomalies have stimulated subclassification by criteria other than biological properties.

Subclassification on the basis of genome structure depends on the arrangement within the genome of reiterated DNA sequences of at least 100bp (Roizman, 1982). Although the majority of herpesviruses can be classified according to biological properties, only a minority have been characterized sufficiently to group them by this criterion. The human pathogen, HSV-1, is the type species of the Herpesviridae (Fenner, 1976), and was also the first herpesvirus whose genome structure was analysed in detail. Thus, an account of the types of herpesvirus genome structure will be preceded by a description of the HSV-1 genome, with occasional reference to HSV-2.

Structure of Herpes Simplex Virus DNA

The genome of HSV-1, like that of all herpesviruses studied to date consists of a linear, double-stranded DNA molecule (Ben-Porat and Kaplan, 1962; Russell and Crawford, 1963; Becker et al., 1968). The molecular weight of HSV DNA is approximately 1×10^8 as determined by electron microscopy, sedimentation velocity and restriction endonuclease analyses (Becker et al., 1968; Kieff et al., 1971; Wilkie, 1973; Grafstrom et al., 1974; Wadsworth et

al., 1975; Clements et al., 1976; Wilkie, 1976; Skare and Summers, 1977). The genome has a nucleotide composition of 67% G+C (Russell and Crawford, 1963; Kieff et al., 1971). HSV DNA sediments homogeneously on a neutral sucrose gradient but heterogeneously on an alkaline gradient (Kieff et al., 1971). This alkali sensitivity of the DNA has been ascribed by some to single strand interruptions (Frenkel and Roizman, 1972a; Wilkie 1973; Ecker and Hyman, 1981) and by others to covalently bound ribonucleotides (Hirsch and Vonka, 1974; Muller et al., 1979).

The outstanding paper in the elucidation of the structural organisation of the HSV-1 genome was undoubtedly that of Sheldrick and Berthelot (1974). Using electron microscopy, they demonstrated that single strands of HSV-1 DNA could fold back on themselves to form dumb-bell shaped structures (C2) consisting of two single-stranded circles joined by an extensive and highly homologous double-stranded region. A diagrammatic representation of a C2 molecule is shown in Figure 1.1a. For this structure to arise from a linear precursor, two sets of inverted repetitions must be present in the genome. The presence of "tails" in the duplex region of several C2 molecules and the scrutiny of molecules consisting of a single circle and duplex suggested that the genome termini resided near the middle of the duplex. Annealing of HSV-1 DNA treated with exonuclease III resulted in circular duplex molecules, indicating the presence of a directly, repeated sequence at the genome termini. These three important observations, as

Figure 1.1

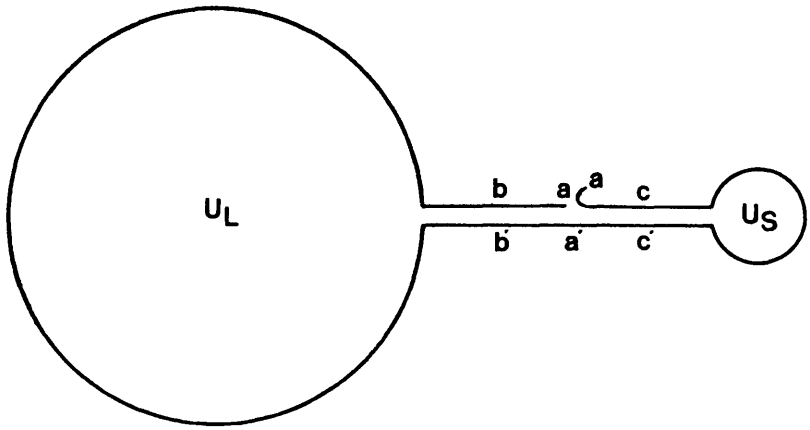
The Genome Structure of HSV-1

a. The dumbell shaped structure (C2) observed by electron microscopy of self-annealed HSV DNA Sheldrick and Berthelot, 1974). TR_L = b + a, IR_L = b' + a', TR_S = c + a, IR_S = c' + a'.

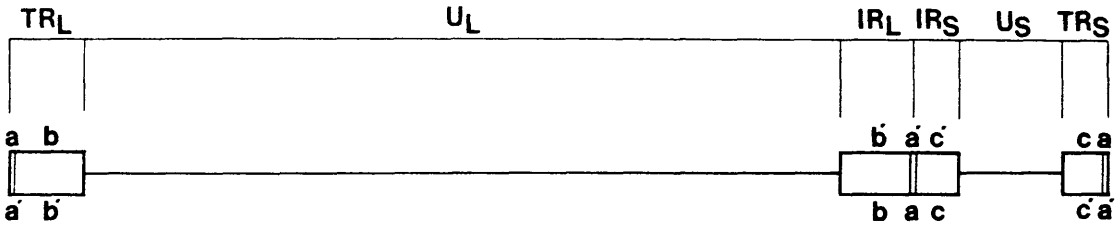
b. Structural arrangement of the HSV-1 genome comprising two unique sequences U_L and U_S bounded by inverted repetitions TR_L, IR_L, TR_S, and IR_S. Sequence a represents the terminal repetition.

c. The four permutations of sequence arrangement of HSV-1 DNA. The four arrangements have been designated P, I_L, I_S and I_{SL} (Roizman et al., 1979) depending on the orientation of the U_L and U_S. Restriction maps of HSV DNA are conventionally displayed in the P arrangement. Both U_L and U_S are found in two orientations which occur with equal frequency. As a consequence of this inversion a restriction enzyme which cleaves only in the U_L and U_S will generate four quarter molar fragments (a+b, c+b, a+d and c+d), and the genome termini will form half molar fragments.

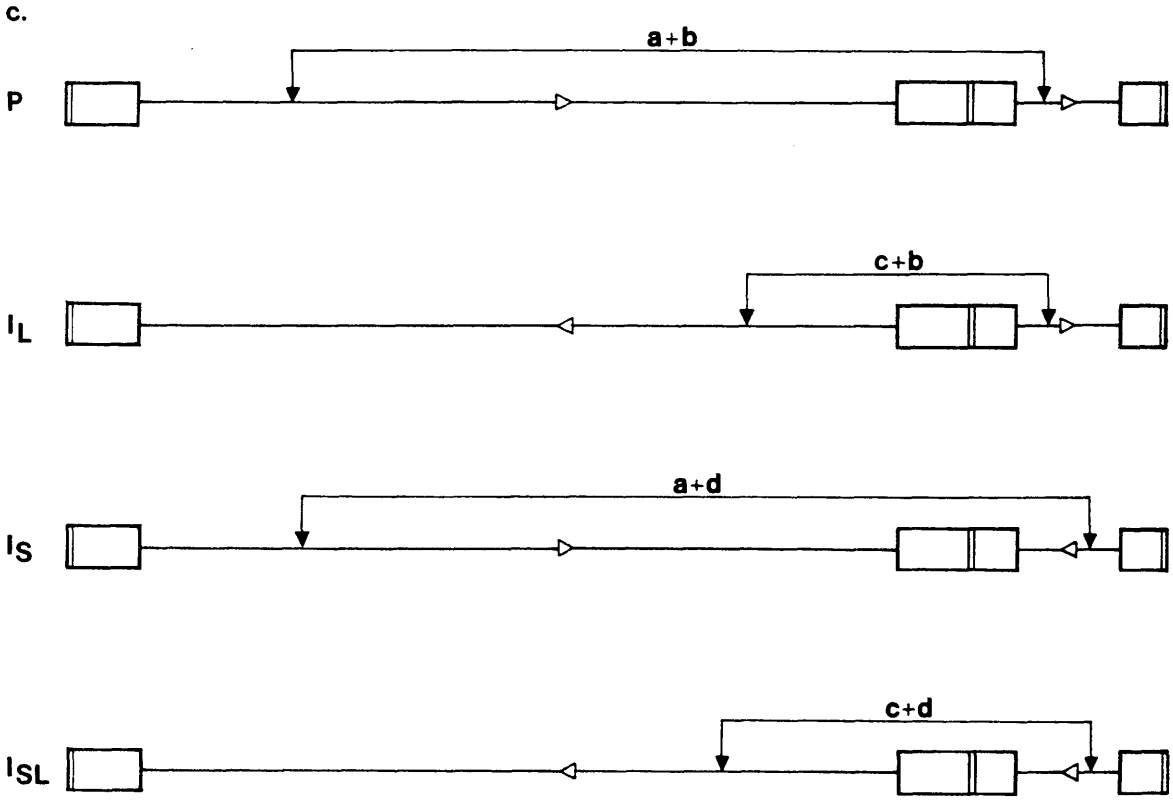
a.


U_L
b a a c
b' a' c'
U_S

b.


TR_L
U_L
IR_L
IR_S
U_S
TR_S
a b
a' b'
b' a' c'
b a c
c a
c' a'

c.


a+b
P
c+b
I_L
a+d
I_S
c+d
I_{SL}

well as others, were used to propose the following model for the genome structure of HSV. In summary, the DNA molecule consists of a long unique sequence (U_L) bracketed by inverted repeat sequences (IR_L and TR_L), linked to a short unique sequence (U_S) flanked by a second pair of inverted repeat sequences (IR_S and TR_S). This arrangement is shown in Figure 1.1b. Consideration of this model suggested that inter- or intra-molecular recombination could result in inversion of the L (TR_L + U_L + IR_L) and S (TR_S + U_S + IR_S) components to generate four isomeric forms of virion DNA (Sheldrick and Berthelot, 1974). This possibility was confirmed by partial denaturation mapping (Delius and Clements, 1976). Restriction endonuclease analysis confirmed the genome structure and demonstrated that the four isomers are of equal abundance in virion DNA (Hayward et al., 1975; Clements et al., 1976; Wilkie and Cortini, 1976). Gel electrophoresis of HSV-1 DNA digested completely with certain restriction endonucleases resulted in the visualisation of 0.5M and 0.25M fragments. The size of the fragments generated by an enzyme which cleaves only in the unique sequences depends on the orientation of those sequences. Figure 1.1c illustrates four 0.25M fragments spanning the L-S junction a+b, c+b, a+d and c+d. As a consequence of inversion these are generated by digestion with a hypothetical enzyme which cleaves in the U_S and the U_L. Similarly four 0.5M fragments are generated from the termini. The four genome arrangements of HSV have been designated P (prototype), I_L (L inverted), I_S (S inverted) and I_{SL} (L and S inverted) (Roizman et al., 1979).

Sheldrick and Berthelot, (1974) proposed that TR_L/IR_L and TR_S/IR_S were identical, but further electron microscopic analysis showed that the two sets of inverted repeats differ in size and in partial denaturation profile (Wadsworth et al., 1975; Delius and Clements, 1976). This was confirmed by the observation that certain restriction endonucleases cleave only within one set of inverted repeats (Hayward et al., 1975; Clements et al., 1976; Wilkie and Cortini, 1976). TR_S/IR_S are subdefined as a'c' and ca in Figure 1.1b and TR_L/IR_L as ab and b'a' (Wadsworth et al., 1975). The a sequence is present as a direct repeat at the genome termini and in inverse orientation at the L-S junction. A variable number of a sequences is present at the L-S joint and L terminus (Wilkie 1976; Wilkie et al., 1977; Wagner and Summers, 1978). The size of the HSV-1 a sequence was estimated to be 400 to 600bp by electron microscopy (Grafstrom et al., 1975; Wadsworth et al., 1976; Kudler and Hyman 1979) and 265bp by restriction endonuclease mapping (Wagner and Summers, 1978). Davison and Wilkie (1981) determined the nucleotide sequence of the a sequence of HSV-1 and HSV-2 and found they had approximate sizes of 400bp and 250bp, respectively. However, heterogeneity of size is characteristic of the HSV-1 a sequence and appears to be due to variability in the copy number of a tandemly reiterated G+C-rich sequence within the a sequence (Wagner and Summers, 1978; Davison and Wilkie, 1981). The a sequence has been reported to play a major role in the inversion of the L and S components (Mocarski and Roizman, 1982) and in

encapsidation of DNA (Stow et al., 1983).

HSV-2 has a G+C content of 69% (Goodheart et al., 1968; Kieff et al., 1971, Halliburton, 1972), and approximately 50% of its genome is homologous to HSV-1 DNA (Kieff et al., 1972). The HSV-2 genome structure is the same as that of HSV-1 (Hayward et al., 1975; Skare et al., 1975; Cortini and Wilkie, 1978) and the two viruses have a colinear gene arrangement (Esparaza et al., 1976, Davison and Wilkie, 1983). The substantial differences between the HSV-1 and HSV-2 restriction endonuclease maps is of diagnostic value in determining the subtype of an HSV isolate (Lonsdale, 1979).

i) Group A

This genome type has a single direct repeat at each terminus. The group is exemplified by the channel catfish herpesvirus (CCV), the genome of which is illustrated in Figure 1.2 (Chousterman et al., 1979).

ii) Group B

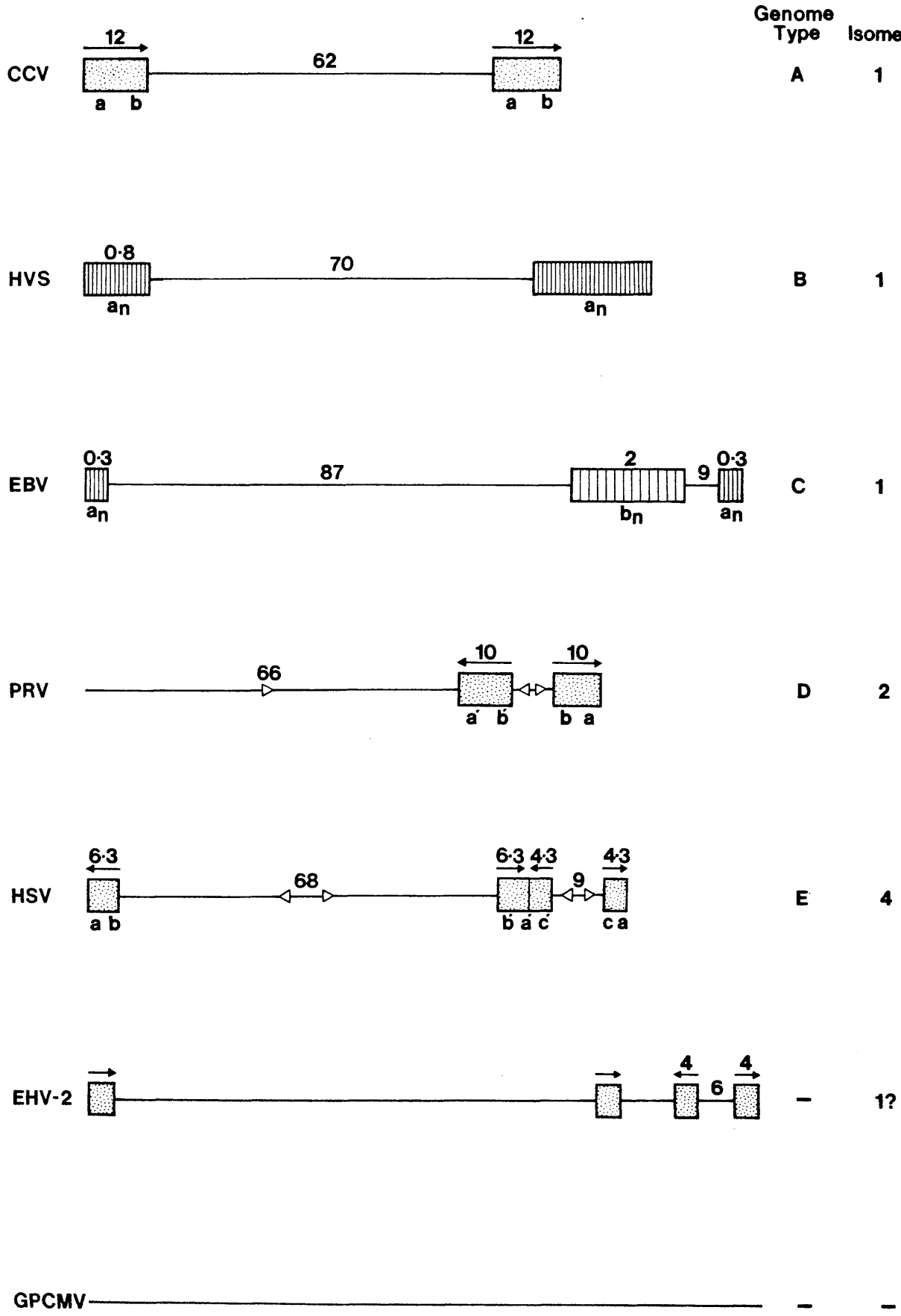
As in Group A, group B genomes are characterized by terminal redundancy. The two groups differ in that redundant regions in the latter are composed of multiple reiterations of one set of sequences (Figure 1.2). Structural analysis of the genomes of three oncogenic herpesviruses of nonhuman primates, HVS, herpesvirus ateles and herpesvirus aotus type 2 has shown that they belong to this group (Bornkamm et al., 1976; Fleckenstein et al., 1978; Fuchs et al., 1985). In each case the genome consists of a unique A+T-rich 110kbp sequence flanked by

Figure 1.2

Sequence Arrangements in Herpesvirus DNAs

Herpesviruses can be sub-classified into different groups on the basis of their genome structure. The groups A, B, C, D and E are represented by chanel catfish herpesvirus (CCV), herpesvirus saimiri (HVS), Epstein-Barr virus (EBV), pseudorabies virus (PRV) and herpes simplex virus (HSV) respectively. While most of the herpesvirus DNAs studied to date fall into one of these groups this is not true of a number of the cytomegaloviruses such as guinea pig cytomegalovirus (GPCMV) and equine cytomegalovirus (EHV-2).

In this schematic diagram the horizontal lines represent unique sequences. Where appropriate the observed orientations of the U_L and U_S are indicated by arrowheads. Repeat sequences larger than 1kbp in length, other than terminal reiterations, are shown as dot-filled rectangles. The arrows above these rectangles indicate the orientation of the repeat sequences. The vertical lines and letters a_n and b_n signify multiple tandem repeat sequences. The letters a, b, c have also been used to denote the orientation of repeat sequences and their complements a', b', c'. The use of these letters does not imply homology between different herpesviruses. Numbers above the lines represent molecular weight (x 10^6). The numbers above the vertical lines refer to a unit length of the reiterated sequence.


Genome Type
Isomer
CCV
12
62
12
a b
a b
A
1
HVS
0·8
70
an
an
B
1
EBV
0·3
87
2
9
0·3
an
bn
an
C
1
PRV
66
10
10
a' b'
b a
D
2
HSV
6·3
68
6·3
4·3
9
4·3
a b
b a c
c a
E
4
EHV-2
4
4
6
–
1?
GPCMV
–
–

repetitive G+C-rich sequences. Restriction endonuclease cleavage patterns of the terminal repeats of HSV and herpesvirus ateles indicate that these regions are composed of identical tandemly repeated sequences of approximately 1.4kbp and 1.5kbp, respectively (Bornkamm et al., 1976; Fleckenstein et al., 1978; Fleckenstein and Desrosiers, 1982). The reiterations of herpesvirus aotus are more variable. Analyses with restriction enzymes indicates the existence of two types of unit, one 2.1kbp and one 2.3kbp in size, which share at least 1.3kbp of identical DNA sequence. The repeat units are arranged in tandem and appear to be random in order (Fuchs et al., 1985). HVS, herpesvirus ateles and herpesvirus aotus type 2 all show genomic size heterogeneity due to variability in the number of reiterated H-units (Bornkamm et al., 1976; Fleckenstein et al., 1978; Fuchs et al., 1985). Isopycnic centrifugation of intact DNA demonstrated that a population of any of these three viruses contains a minority of defective genomes comprising reiterations of the terminal repeat unit. Their lack of infectivity corresponds to their low genetic complexity (Bornkamm et al 1976; Fleckenstein et al., 1978; Fuchs et al., 1985).

Bovine cytomegalovirus is the first non-primate herpes virus reported to possess a genome structure similar to those of the viruses discussed above (Ehlers et al., 1985). Tandemly repeated DNA sequences are present at both genome termini and amount to 20 to 25% of the 144kbp genome.

iii) Group C

This group is exemplified by EBV, which has tandemly

reiterated sequences in direct orientation at the genomic termini and a different set of internal tandem reiterations (Figure 1.2). Three primate β-lymphotropic viruses, EBV, herpesvirus papio (HVPapio) and herpesvirus pan (HVPan), exhibit this type of genome structure (Dambaugh et al., 1980; Heller and Kieff, 1981; Heller et al., 1981, 1982a). Of the three, the genome of EBV is the best characterized. The DNA is approximately 180kbp in size and consists of two regions of unique sequences U_L and U_S separated by a direct internal repeat region, IR1. As in group B, both ends of the DNA contain a variable number of direct, tandem repeats (TR). The TR is 500bp and there are usually 4 - 8 copies per terminus. The internal reiteration, IR1, is 3,071bp in size and variable in copy number (Given and Kieff, 1979; Cheung and Kieff, 1982). Other features of the EBV genome include two partially homologous sequences designated DL and DR, near the left and right ends of U_L (Raab-Traub et al., 1980). D_L contains 11 to 14 repeats of a 124bp sequence designated IR2. DR contains approximately 30 reiterations of a related 103bp sequence designated IR4 (Dambaugh and Kieff, 1982). Another repeat sequence, IR3, composed of only three nucleotide triplet elements is located between IR2 and IR4 (Heller et al., 1982b). The determination of the complete DNA sequence of the B95-8 strain of EBV revealed the existence of other repetitive sequences scattered throughout the genome (Baer et al., 1984).

HVPapio and HVPan DNA are approximately 40% homologous to EBV DNA (Falk et al., 1976; Gerber et al., 1976) and

the size and organization of their DNA is similar to that of EBV (Heller and Kieff, 1981; Heller et al., 1982a). There is extensive homology among the IRs of the three viruses and the duplicated DL and DR regions are conserved. No homology is detected, however, among the TRs (Heller and Kieff, 1981; Heller et al., 1982a).

iv) Group D

The genomes of group D herpesviruses such as PRV, EHV-1 subtype 1, EHV-3 and BHV-1 consist of two regions, L and S. The unique sequence of S (U_S) is bracketed by inverted repeat sequences (IR_S and TR_S). The S component inverts relative to the L component which consists of a unique sequence in a fixed orientation. The genome structure of PRV is shown in Figure 1.2. Electron microscopic examination of self-annealed single-stranded PRV DNA (Stevely, 1977; Ben-Porat et al., 1979), EHV-1 subtype 1 DNA (Ruyechan et al., 1982), EHV-3 DNA (Atherton et al., 1982) and VZV DNA (Ecker and Hyman, 1982) revealed structures consisting of single-stranded loops joined to a double-stranded region which terminates in a long single-stranded tail. This is consistent with the repetition of DNA sequences at one terminus in inverse orientation within the genome. Evidence for the existence of two isomeric forms of these virus genomes comes from the existence of four 0.5M restriction digest fragments and the presence of three terminal fragments, two in 0.5M amounts, in some restriction digests. The S region inverts and yields two terminal fragments, whereas the L region produces only one

terminal fragment as it is in a fixed orientation (Dumas et al., 1981; Henry et al., 1981; Whalley et al., 1981; Atherton et al., 1982; Sullivan et al., 1984). Restriction endonuclease maps were derived by enzyme and Southern hybridization analyses for the genome structure of EHV-1, subtype 1 (Whalley et al., 1981; Henry et al., 1981), EHV-3 (Sullivan et al., 1984), PRV (Powell, 1979), VZV (Dumas et al., 1981) and BHV-1 (Mayfield et al., 1983). Interestingly, unlike any other PRV strains that have been studied, the Norden vaccine strain has a Group E genome structure similar to that of HSV. Both the U_S and U_L regions of the genome invert giving rise to four isomeric forms. This is most likely due to the fact that a DNA sequence found normally only at the end of U_L is also present in the inverted form next to the internal IR_S of the Norden genome (Lomniczi et al., 1984). DNA sequence analysis of the L-S joint and genome termini of VZV revealed that U_L is flanked by an inverted repeat of 88.5bp, and hybridization studies indicated that 5% of VZV DNA molecules have an inverted L component (Davison, 1984). Thus, whereas the VZV genome could be regarded as a member of Group E described below, the size of the VZV IR_L/TR_L is less than the minimum 100 nucleotides considered for classification purposes (Roizman, 1982).

v) Group E

This group is exemplified by the HSV genome, the structure of which has been discussed in some detail above. Electron microscopic studies, partial denaturation mapping (Kilpatrick and Huang, 1977) and restriction endonuclease

digestion and Southern hybridization analyses of cloned fragments of HCMV DNA (Spector et al., 1982) showed that it has a similar structure to HSV DNA. BHV-2 was the first non-human herpesvirus to be shown to be closely related to HSV. The antigenic relationship of these viruses was demonstrated by complement fixation, immunofluorescence and immunodiffusion tests while DNA-DNA hybridization analyses showed that they share about 14% sequence homology (Sterz et al., 1973/74). These findings stimulated interest in BHV-2 DNA. Analysis of the genome structure demonstrated that, although smaller, BHV-2 DNA has a similar sequence arrangement to HSV (Buchman and Roizman 1978a,b). Group E also includes MDV (Fukuchi et al., 1984) and herpesvirus aotus types 1 and 3 (Ebeling et al., 1983a).

Other Genome Structures

The genome structures of a number of cytomegaloviruses are not readily classified into any of the groups discussed above. The genomes of murine CMV (Mercer et al., 1983; Ebeling et al., 1983b), guinea-pig CMV (Gao and Isom, 1984) and rat CMV (Meijer et al., 1986) consist of a long unique sequence without invertible components or extensive repeated sequences (Figure 1.2). Preliminary data on the genome of EHV-2 DNA indicates that it contains inverted repeats at one terminus. At the other terminus there is a sequence which is directly repeated within the genome (Figure 1.2). There is no evidence to suggest that the EHV-2 genome exists in more than one isomeric form (O'Callaghan et al., 1984).

THE PATHOGENESIS OF EQUINE HERPESVIRUSES

Introduction

Three antigenically distinct types of equine herpesvirus have long been recognized. These are equine herpesvirus type 1 (EHV-1), also known as equine abortion virus or equine rhinopneumonitis virus, equine herpesvirus type 2 (EHV-2) or equine cytomegalovirus (ECMV) and equine herpesvirus type 3 (EHV-3) or equine coital exanthema virus. There is now conclusive evidence that viruses currently classified as EHV-1 consist of two genetically and antigenically distinct subtypes. It has been suggested that one of these subtypes be renamed equine herpesvirus type 4 (EHV-4) (Studdert et al., 1981), and it seems probable that this change in nomenclature will be implemented by the Herpesvirus Study Group of the International Committee for the Nomenclature of Viruses. The equine herpesviruses have recently been the subject of a number of comprehensive review articles (O'Callaghan et al., 1978; O'Callaghan et al., 1983; Campbell and Studdert, 1983; Allen and Bryans, 1986). Viruses classified at present as EHV-1 cause considerable financial loss to the horse industry each year. They are a major cause of respiratory disease and abortion in horse populations throughout the world and are also associated with neonatal foal disease, neurological disease and coital exanthema (Kemen, 1975; O'Callaghan et al., 1983). EHV-2, an antigenically heterogeneous group of viruses (Plummer et al., 1969, 1973; Erasmus, 1970; Mumford and Thompson, 1978) is widely disseminated throughout the horse

population. The virus has been isolated from a variety of equine tissues (Kono and Kobayash, 1964; Kemeny and Pearson, 1970; Studdert, 1974) but has not yet been implicated conclusively in any disease syndrome (Studdert, 1974; Gleeson and Studdert, 1977; Burrell, 1985). Horses appear to be infected early in life, become persistent carriers and shed virus constantly (Studdert, 1974). EHV-3 is the aetiological agent of a progenital disease known as equine coital exanthema. Systemic signs are uncommon. Lesions, which appear initially as papules and progress rapidly to pustules and ulcers, are usually confined to the penile and vulvar mucosa (Bryans and Allen, 1973; Studdert, 1974) but may occur around the lips and nose (Krogsrud and Onstad, 1971). Breeding must cease for the duration of the disease.

Abortion Caused by EHV-1

Equine abortion of putative viral aetiology was first recorded in Kentucky in 1932, and the transmissibility of the filterable agent and pathological lesions of the aborted foetus were described soon afterwards (Dimock and Edwards, 1932, 1933, 1936; Dimock, 1940). In 1941, Manninger and Csontos reported that pregnant mares inoculated with filtered material from aborted foetuses suffered a mild upper respiratory tract disease which was transmitted to in-contact animals. Their conclusion, that abortion is a sequel to respiratory tract infection, was confirmed by numerous other studies (Salyi, 1942; Manninger, 1949; Hansen and Holst, 1950). In Kentucky,

two viruses isolated from the respiratory tract, Army 183 (Jones et al., 1948) and the Grayson I strain were shown to induce similar pathological lesions to the Kentucky-A strain of equine abortion virus (Dimock and Edwards, 1933) on inoculation of Syrian hamsters (Doll et al., 1953) or equine foetuses (Doll and Kinter, 1954). In 1957, Doll et al. suggested that the agent previously described as equine abortion virus be regarded primarily as a respiratory virus of horses and be designated as equine rhinopneumonitis virus. Later electron miscroscopy identified the causal agent of equine rhinopneumonitis as a herpesvirus, and since that investigation it has been known as equine herpesvirus type 1 (Plummer and Waterson, 1963).

EHV-1 is a major cause of both sporadic and epizootic abortion in horse populations throughout the world. In Kentucky, EHV-1 was shown to be responsible for an annual loss of 0.4 to 2.6% of foetuses (Bryans, 1981), and in the absence of equine arteritis virus (Mumford, 1985a), EHV-1 is virtually the only known cause of epidemic abortion in mares in these islands at present. It is widely accepted that abortion occurs subsequent to a respiratory tract infection which may be subclinical or so mild as to go unnoticed by the mare's handlers. The incubation period is extremely variable under natural conditions, and experimental inoculation of pregnant mares by the intranasal, subcutaneous, intravenous or intramuscular route resulted in an incubation period varying from 14 to 120 days (Doll and Bryans, 1962). A transient viraemia occurring after infection with abortigenic EHV-1 has been

Figure 1.3

EHV-1 Abortion

Necropsy of an aborted foetus showing some of the classical lesions of EHV-1 infection;

(a) splenomegaly with prominent lymph follicles, excess pleural fluid and jaundice.

(b) necrotic foci in the liver.

The photographs were a kind gift of Dr. C. Whitwell, The Equine Research Centre, Newmarket.

EHV-1 Abortion

Figure 1·3a

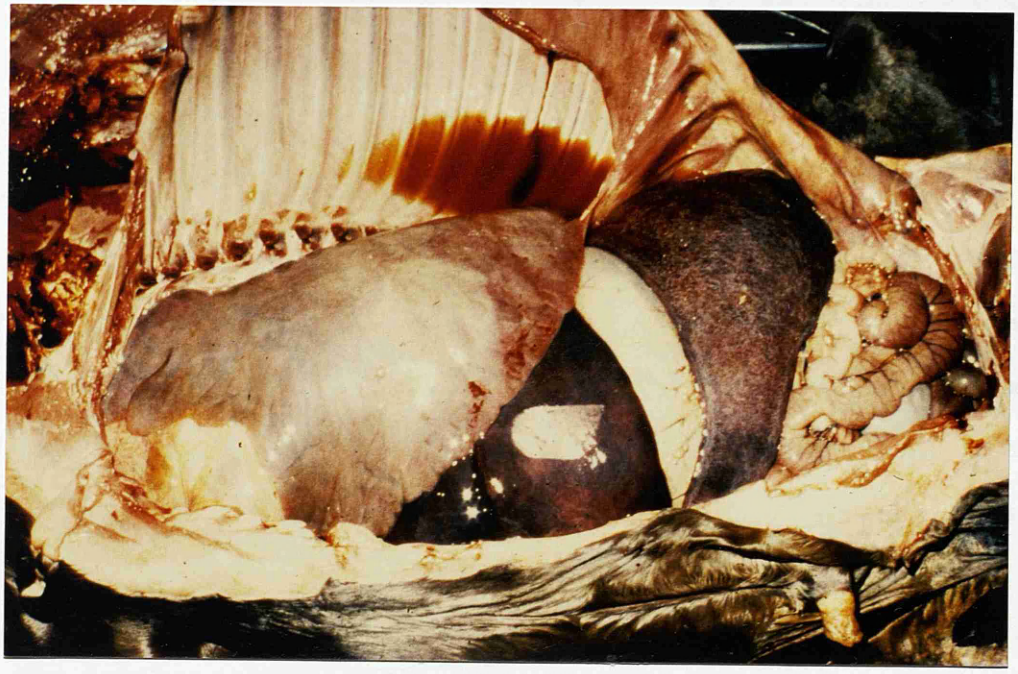
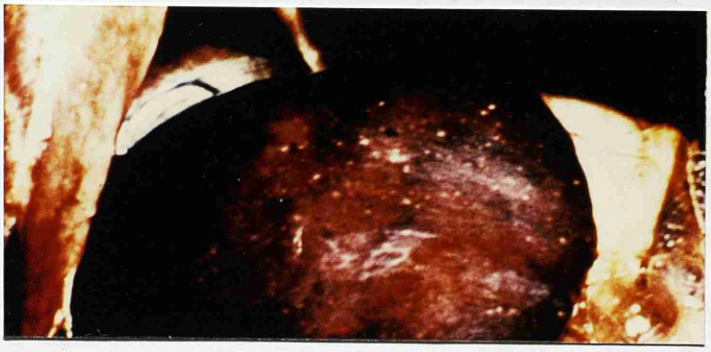


Figure 1·3b



well documented (Bryans, 1969; Bryans and Prickett, 1969; Burrows and Goodridge, 1973; Gleeson and Coggins, 1980), but infectious virus has not been detected in circulating leukocytes beyond the first 24 days after infection (Bryans, 1969). The period between natural respiratory infection and expulsion of the foetus ranges from 14 to 90 days (Doll and Bryans, 1962), and the location of the virus for much of this period is unknown. An extra-foetal location is suggested as direct inoculation into the foetus or chorioallantoic sac results in abortion within 9 days.

The majority of abortions occur between the 8th and 11th months of gestation (Doll and Bryans, 1963b). The mare usually aborts without premonitory signs, the foetal membranes are not retained, and the subsequent fertility of the mare is unaffected (Dimock, 1940). The pathological lesions depend on the age of the foetus (Prickett, 1970) and probably the degree of development of the foetal immune system. Foetuses aborted prior to the sixth month of gestation are severely autolyzed, and foetuses aborted between the seventh month and full term present the classical lesions described originally by Dimock et al., (1942, 1947). The most prominent gross lesions are jaundice, petechiation of mucous membranes, necrotic foci in the liver, splenomegaly with prominent lymph follicles, excess pleural fluid and marked oedema of the lungs. Some of these lesions are shown in Figure 1.3. The principal microscopic lesions are focal necrosis of spleen and liver, bronchiolitis and pneumonitis. Foetuses aborted during the 6th or 7th months of gestation tend to show a mixture of

general autolysis and local tissue reaction.

The cellular and humoral immune responses, to EHV-1 are poorly understood. Mares with high levels of virus neutralizing antibody are susceptible to either natural or experimental reinfection of the respiratory mucosa, and may abort (Doll, 1961; Doll and Bryans, 1963b; Bryans, 1969). Bryans (1969) suggested that the reinfecting virus escapes neutralization by becoming situated intracellularly in leukocytes. However, the same study in Kentucky showed that mares with a serum virus neutralizing antibody titre of 2.0 or higher withstood challenge with EHV-1 by the natural route. This immunity to infection was not maintained when virus was inoculated parenterally. The ability of EHV-1 to induce abortion in the presence of a high serum neutralizing antibody titre suggests that cell mediated immunity may play an important role in protection. Gerber et al. (1977) demonstrated that the cell-mediated immune response of mares in the latter stages of pregnancy was suppressed. However, although this is the stage in gestation when 95% of EHV-1 abortions occur, Dutta and Campbell (1977) found no significant difference in the stimulating index of lymphocytes from mares which suffered EHV-1 abortion and those which foaled normally. While the immune response elicited by abortion is more protective than that stimulated by EHV-1 respiratory disease it tends to be variable (Doll, 1961). Most mares do not abort as a result of EHV-1 infection more than once in their lifetime but animals have been known to abort in consecutive pregnancies (Doll and Bryans, 1962) or after an interval of

2 years or longer (Doll, 1961).

Respiratory Disease Caused by EHV-1

Several European studies which suggested that equine abortion virus was a pneumotropic virus (Manninger and Csontos, 1941; Manninger, 1949; Hansen and Holst, 1950) prompted Doll et al. (1954) to investigate the pathogenesis of EHV-1 in young horses. On experimental inoculation of weanling horses, the virus caused mild respiratory catarrh, fever and leukopenia. Serological surveys indicated that EHV-1 was the aetiological agent of an annual epizootic rhinitis in young horses in Kentucky. EHV-1 has since been shown to be the major cause of acute upper respiratory tract disease in young horses worldwide (Studdert, 1974). Clinical disease is most prevalent in horses under 2 years of age, and infection of perinatal foals may lead to a fatal bronchopneumonia (Bryans, 1969). The common clinical manifestations of EHV-1 respiratory infection in a susceptible animal are anorexia, fever, serous nasal discharge (Figure 1.4), coughing, enlarged intermandibular and parapharyngeal lymph nodes and congestion of the conjunctivae (Doll et al., 1954; Coggins, 1979). Coughing is a less prominent feature of the disease than with influenza while the nasal discharge tends to be more copious (Powell, 1975). While recovery is usually uneventful the disease is sometimes complicated by secondary bacterial infection (Figure, 1.4) often with streptococci of Lancefield group C (Studdert, 1974).

Once maternal antibodies have waned, usually at 3-4 months of age (Bagust et al., 1972), the immune response

Figure 1.4

EHV-1 Respiratory Disease

a. Horse with serous nasal discharge typical of acute virus infection of the upper respiratory tract.

b. Purulent discharge indicative of secondary bacterial infection.

Photographs were a kind gift of Dr. D. Onions, Glasgow Veterinary School.

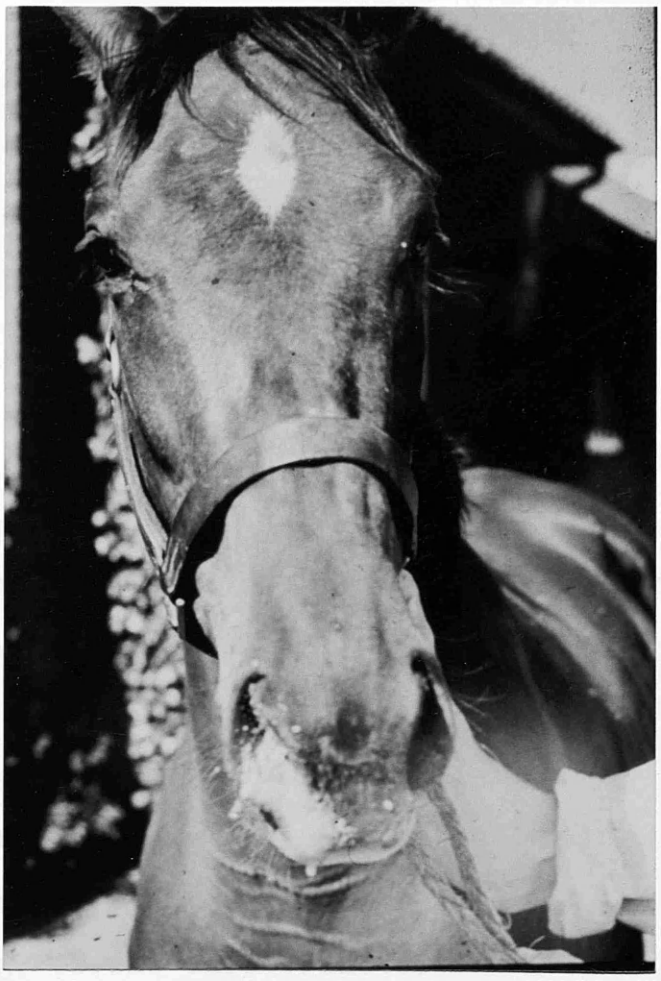


Figure 1·4a

Figure 1·4b

elicited by respiratory infection with EHV-1 is short-lived and horses may be reinfected every 3 or 4 months (Doll, 1961; Doll and Bryans, 1963b). Consecutive infections do appear to induce some measure of protection, however, as clinical signs of the disease are seldom seen in the older animal. It is thought that mild and subclinical infections and the resulting inappetance may play a role in the "poor performance" syndrome in racehorses (Mumford and Rossdale, 1980; Strickland, 1986; Powell, 1986). The virus is highly contagious, and when a susceptible population is exposed disease spread may be very rapid. Such a situation occurs commonly at weaning when young stressed animals are often grouped together in close proximity for the first time (Doll and Bryans, 1963b). In young horses in training, serial infections of different animals can cause a yard to lose an entire racing season. In 30 training stables in 6 areas of England surveyed from 1971-1976, only 2 were completely free of respiratory disease for 12 months. EHV-1 was involved in approximately 45% of outbreaks of respiratory disease investigated in this survey (Powell et al., 1978).

EHV-1 Neurological Syndrome

The first definitive account of EHV-1 neurological disease was by Saxegaard (1966), who isolated the virus from the tissues of two adult male horses which had shown serious nervous symptoms. Since then this syndrome has been recorded worldwide in horses of all ages and both sexes (Dinter and Klingeborne, 1976; Collins, 1972;

Greenwood and Simpson, 1980; Bartra et al., 1982) and the disease has been induced experimentally (Jackson et al., 1977; Mumford and Edington, 1980). Three years ago an outbreak of EHV-1 neurological disease among the Lippizaner horses in Piber, Austria received international media coverage. The syndrome has been associated both with abortion and outbreaks of respiratory disease. The clinical signs vary with the severity of the case, and may include inco-ordination, ataxia, weakness, incontinence and posterior paralysis. Severe cases become laterally recumbent and either die or necessitate euthanasia. The prognosis for non-recumbent animals is usually favourable and although a period of months may be required for a complete return to normal, recovered horses have won races (Little and Thorsen, 1976).

Gross lesions tend to be minimal at necropsy, while the histopathological changes vary with the severity of the disease. A vasculitis of the smaller arteries and veins of the central nervous system with associated malacia is common to all cases. The vasculitis is characterized by necrosis of the endothelium, often with concomitant thrombosis. The cellular response to endothelial necrosis is manifested as a perivascular cuffing of inflammatory cells; initially neutrophils and later, lymphocytes (Jackson et al., 1977). Other lesions range from minimal gliosis and neuronal degeneration associated with foci of malacia in the brain and spinal cord (Jackson and Kendrick, 1971) to severe disseminated meningoencephalitis (Charlton et al., 1976). While virus has been recovered from a

variety of organs at necropsy, including the brain and spinal cord (Saxegaard, 1966; Mumford and Edington, 1980), efforts to isolate virus are not always successful (Platt et al., 1980).

Unlike many other herpesviruses, such as HSV, BHV-1 and PRV, EHV-1 does not appear to be neurotropic (Jackson et al., 1977). There is no evidence that the virus infects neuronal cells and the neurological signs evident in the affected animal appear to be a consequence of ischaemia, malacia and inflammation of nervous tissue subsequent to a disruption in the cerebrospinal blood supply. EHV-1 antigen has been demonstrated in the vascular endothelial cells (Patel et al., 1982; Edington et al., 1986) thus supporting the observation made by Dinter and Klingeborn (1976) that EHV-1 paralysis may be an immune complex mediated disease. Experimental EHV-1 infection of ponies resulted in a hypocomplementemia, a characteristic of several virus diseases where immune complexes play a role in the pathogenesis (Snyder et al., 1981). The suggestion that the pathogenesis of the neurological syndrome was immunologically based prompted Greenwood and Simson (1980) to attempt corticosteroid therapy on affected cases in Newmarket. Treatment was unfortunately initiated too late in the outbreak to assess its efficacy. Recently HSV-1 IgM immune complexes were detected by immunofluorescence in the brain of a human patient with herpes encephalitis. Histopathologically, perivascular cuffing composed mostly of T lymphocytes were prominent in various parts of the brain. It was suggested that the immune complexes played a

role in the development of the pathology by attracting these lymphocytes through the activation of a complement pathway (Hayashi et al., 1986).

Neonatal Foal Disease Caused by EHV-1

Outbreaks of neonatal foal disease associated with EHV-1 have been described in Kentucky (Bryans et al., 1977) and in Australia (Dixon et al., 1978; Hartley and Dixon, 1979). Bryans et al., (1977) isolated EHV-1 from three of nine foals which died in the second week of life. Most of these foals were normal at birth but succumbed after a few days to respiratory distress, weakness and diarrhoea. An interstitial pneumonitis and hypoplasia of the spleen and thymus was observed at necropsy. The authors suggested that this depletion and/or degeneration of lymphocytes in spleen and thymus caused by EHV-1 may have increased the susceptibility of the foals to lethal bacterial infections. Dixon et al. (1978) reported the occurrence of primary uncomplicated perinatal EHV-1 infection. A number of foals were unable to stand or suckle without assistance and died with varying degrees of respiratory distress within 24 hours of birth. Other foals were normal at birth and no clinical signs were observed until 18 to 24 hours later, when severe signs of respiratory distress commenced. At necropsy the lungs were enlarged, firm and plum purple in colour. Splenomegaly with prominent lymphoid follicles was apparent, but some of the classical lesions associated with EHV-1 abortion, such as jaundice, focal liver lesions and excess pleural fluids, were absent. EHV-1 was isolated from all foals examined.

Coital Exanthema Caused by EHV-1

EHV-1 is the aetiological agent of a form of coital exanthema similar to, but less severe than, that caused by EHV-3. This appears to be a relatively rare manifestation of EHV-1 infection and is of little economic importance (Bryans and Allen, 1973: O'Callaghan et al., 1983).

The Differentiation of Two Subtypes of EHV-1

For more than a quarter of a century evidence has accumulated to indicate the existence of two distinct subtypes of EHV-1 differing in antigenicity, tissue culture characteristics, epizootiology, pathogenicity and molecular biology. EHV-1 subtype 1 is commonly referred to as the abortigenic subtype while subtype 2 is known as the respiratory subtype. However, EHV-1 subtype 1 and subtype 2 are not necessarily equivalent to foetal and respiratory isolates. Both subtypes have the potential to cause respiratory disease and abortion.

All isolates of EHV-1 are regarded as belonging to the same serotype as they are neutralized to some extent by convalescent sera or by antisera prepared in laboratory animals (Burrows and Goodridge, 1973). However, as early as 1959, Shimizu et al. reported that the virus could be divided into two subtypes on the basis of cross-neutralization tests. This was subsequently confirmed by other workers (Matumoto et al., 1965; Mayr et al., 1965; Borgen and Ludwig, 1974). Burrows and Goodridge (1973), in a study of 17 isolates of foetal or respiratory origin, demonstrated far greater antigenic differences between the

two groups than between different isolates recovered from the same site. Polyspecific serum has been used to differentiate strains of each subtype in plaque reduction and kinetic neutralization tests and in enzyme-linked immunosorbent assays (Turtinen, 1983). The latter technique measures both neutralizing and non-neutralizing antibodies and was found to be somewhat less sensitive than the other techniques in discriminating between the two subtypes. Yeargan et al. (1985) produced EHV-1 monoclonal antibodies, several of which demonstrate subtype specificity. These specific monoclonal reagents allow subtype identification within 3 hours after the isolation of virus in tissue culture.

Serological surveys have indicated a worldwide dissemination of EHV-1 (Matumoto et al., 1965). However, while the respiratory form of the disease is common in all horse populations EHV-1 abortion has a far higher incidence in the United States and parts of Europe than it does in Australia, South Africa, Japan and Great Britain (Platt 1973; Powell 1975; Burrows and Goodridge, 1979; Coggins, 1979; Mumford and Rossdale, 1980). EHV-1 abortion in Britain and Ireland tended, for many years, to be sporadic and associated with imported animals (Farrelly, 1966; Burrows, 1968). The relative rarity of EHV-1 abortion in Ireland, where rhinopneumonitis occurs annually in young stock, led Farrelly to suggest, as early as 1966, that there were two strains of EHV-1: an aborting strain which could cross the placenta and a non-aborting strain which failed to pass this barrier. EHV-1 has been isolated, in

Australia, from horses suffering from respiratory disease since 1962 (Duxbury and Oxer, 1968; Bagust and Pascoe, 1968; Studdert et al., 1970), but no case of EHV-1 abortion was recorded until 1977, when it is thought that an abortigenic subtype 1 virus was introduced into the country for the first time (Sabine, 1980; Sabine et al., 1983). Retrospective analysis of the sole EHV-1 isolate obtained from an aborted foetus prior to 1977 (Peet et al., 1978) showed it to be a respiratory subtype or subtype 2 isolate (Sabine et al., 1983), thus supporting the view that the previously endemic respiratory subtype occasionally caused abortion.

The geographical variation in the clinical manifestations of EHV-1, and the apparent existence of strains of virus differing in virulence, instigated a study of the growth characteristics of EHV-1 isolates by Burrows and Goodridge (1973). In general, foetal isolates grew more quickly in cell culture, had a wider host cell range and were more cytopathic than respiratory isolates. Comparisons were made both in cell monolayers and organ cultures of susceptible tissues. Studdert and Blackney (1979) compared the plaque size and host cell range of several Australian foetal and respiratory isolates. Their results supported those of Burrows and Goodridge (1973), and indicated that bovine embryo tracheal (EBTr) cells are useful for differentiating the two subtypes. All but one of 11 foetal isolates grew on EBTr cells, but none of the 7 respiratory isolates tested grew in this cell line. Respiratory isolates also produced larger plaques than

foetal isolates. Growth characteristics of the two groups of isolates *in vitro* correlated well with those *in vivo*. After inoculation into partly immune horses, viruses recovered from aborted foetuses were more infectious, grew better in the nasopharynx, were more likely to be found in the buffy coat of the blood, were excreted in greater quantities and elicited a better neutralizing antibody response than the respiratory isolates (Burrows and Goodridge, 1973, 1975). Patel *et al*. (1982) showed by immunofluorescence studies in experimentally infected foals that a subtype 2 respiratory isolate was confined to the respiratory tract epithelium, macrophages and regional lymph nodes. Both paresis and foetal subtype 1 isolates also replicated in the epithelium of the small intestine, but only the paresis isolate infected blood vessel endothelial cells. Only subtype 1 isolates initiated a viraemia. Further studies in mice demonstrated that subtype 1 isolates, unlike subtype 2 isolates, infected the brain following intracerebral inoculation (Patel and Edington, 1983).

Sabine *et al*. (1981) first demonstrated conclusively that there were two subtypes of EHV-1 by restriction endonuclease analysis of virus DNA. Virus isolates within each subtype have similar restriction endonuclease profiles which are entirely different from those of viruses of the other subtype. While anomalies frequently arose in all the differentiation studies discussed above, the electrophoretic pattern of restriction digest fragments provides a definitive way of classifying a viral isolate

into either subtype. It has been suggested that viruses with a respiratory subtype or subtype 2 electropherotype be renamed EHV-4 (Studdert et al., 1981). Both subtypes are respiratory pathogens, but only viruses which have a subtype 1 electropherotype are commonly associated with abortion. The use of restriction endonuclease analysis in epizootiological studies of EHV-1 in Kentucky (Allen et al., 1983a) and Australia (Studdert, 1983) indicate that subtype 2 virus, like subtype 1, causes epizootics of respiratory disease in young horses, but is rarely the cause of abortion and does not appear to be associated with abortion storms or the neurological syndrome. It was thought, initially, that both subtypes of EHV-1 were similar to those of HSV in that no two epidemiologically unrelated viruses had identical restriction endonuclease fingerprints (Studdert et al., 1981). However, on examination of a larger number of field isolates this proved not to be the case. The abortigenic subtype, or subtype 1, of EHV-1 demonstrates remarkable genetic homogeneity for a herpesvirus (Allen et al., 1985). Two electropherotypes were responsible for greater than 90% of 176 epizootically unrelated outbreaks of abortion, over a 24 year period in Kentucky, excluding those which were vaccine-related (Allen et al., 1983a). In contrast, DNA fingerprints of the respiratory subtype show greater heterogeneity than those of the abortigenic (Allen et al., 1983a; Allen and Bryans, 1986).

Kinetic analysis of DNA-DNA reassociation suggests that the two subtypes of EHV-1 share only 17% of their

genome nucleotide sequence (Allen and Turtinen, 1982). Thus, they are more distantly related than HSV-1 and HSV-2, which demonstrate a genetic homology of 50% (Kieff et al., 1972).

Latency of EHV-1

One of the major problems associated with the majority of infections by herpesvirus including HSV (Baringer and Swoveland, 1973; Stevens, 1975; Wildy et al., 1982), BHV-1 (Snowden, 1964, 1965; Pastoret et al., 1984), PRV (Gutekunst, 1979; Beran et al., 1980) and feline herpesvirus 1 (FHV-1) (Herrman et al., 1984) is their ability to establish latency. Latent virus may reactivate, usually under stress, at any time during the life of the host, and not only cause clinical disease but also serve as a potential source of infection for the animal's cohorts. Much circumstantial evidence for the persistence of EHV-1 and its reactivation following a variety of stress situations has been accumulated. Erasmus (1966) isolated EHV-1 from groups of horses after inoculation with an attenuated African horse sickness virus vaccine while Burrows and Goodridge (1984) noted increases in EHV-1 complement fixing antibody following influenza vaccination. In a self-contained pony herd at the Animal Virus Research Institute in Pirbright, virus was recovered from ponies shortly after castration, rehousing, weaning of offspring and in the terminal stages of grass sickness. No clinical abnormalities were detected in these animals but a number of ponies developed four-fold or greater increases in EHV-1

neutralizing antibody (Burrows and Goodridge, 1978, 1979). Edington et al., (1985) recently presented the first experimental evidence that EHV-1 can exist in the latent form and be reactivated following immunosuppression with dexamethasone and prednisolone. Three months after experimental infection EHV-1 was recovered from six ponies in a group of eight within two weeks of corticosteroid treatment. While five of these ponies were viraemic only three shed virus in nasal secretions arguing in favour of reactivation rather than secondary reinfection. No clinical signs of respiratory disease were detected after virus reactivation and two animals failed to show a significant increase in complement fixing antibody suggesting that reactivation may go undiagnosed at both the clinical and laboratory level (Mumford, 1985b).

The sensory ganglia have been shown to be a site of latent infection for HSV in man (Bastian et al., 1972; Baringer and Swoveland, 1973; Baringer, 1974), in mice (Stevens and Cook, 1971; Cook and Stevens, 1973, 1976) and in rabbits (Stevens et al., 1972), for BHV-1 in cattle (Homan and Easterday, 1980) for PRV in pigs (Beran et al., 1980) and FHV-1 in cats (Gaskell et al., 1985) by isolation of infectious virus in explant and co-cultivation studies. Attempts to demonstrate EHV-1 in the trigeminal ganglia and other tissues using these techniques have, with one exception, failed (Burrows and Goodridge, 1984; Allen and Bryans, 1986). The site of EHV-1 latency remains to be identified. In the acutely infected animal EHV-1 subtype 1 can be isolated from leukocytes for two to three weeks

after infection by cocultivation onto permissive cell monolayers (Bryans, 1969). EHV-1 can only be isolated from viable undisrupted leukocytes indicating that the virus may be present in a noninfective or subvirion form (Gleeson and Coggins, 1980; Scott et al., 1983). It has been suggested that this cell-associated viraemia represents a latent infection of the white blood cells by EHV-1 (Gleeson and Coggins, 1980).

THE MOLECULAR BIOLOGY OF EHV-1 SUBTYPE 1

The Structure of EHV-1

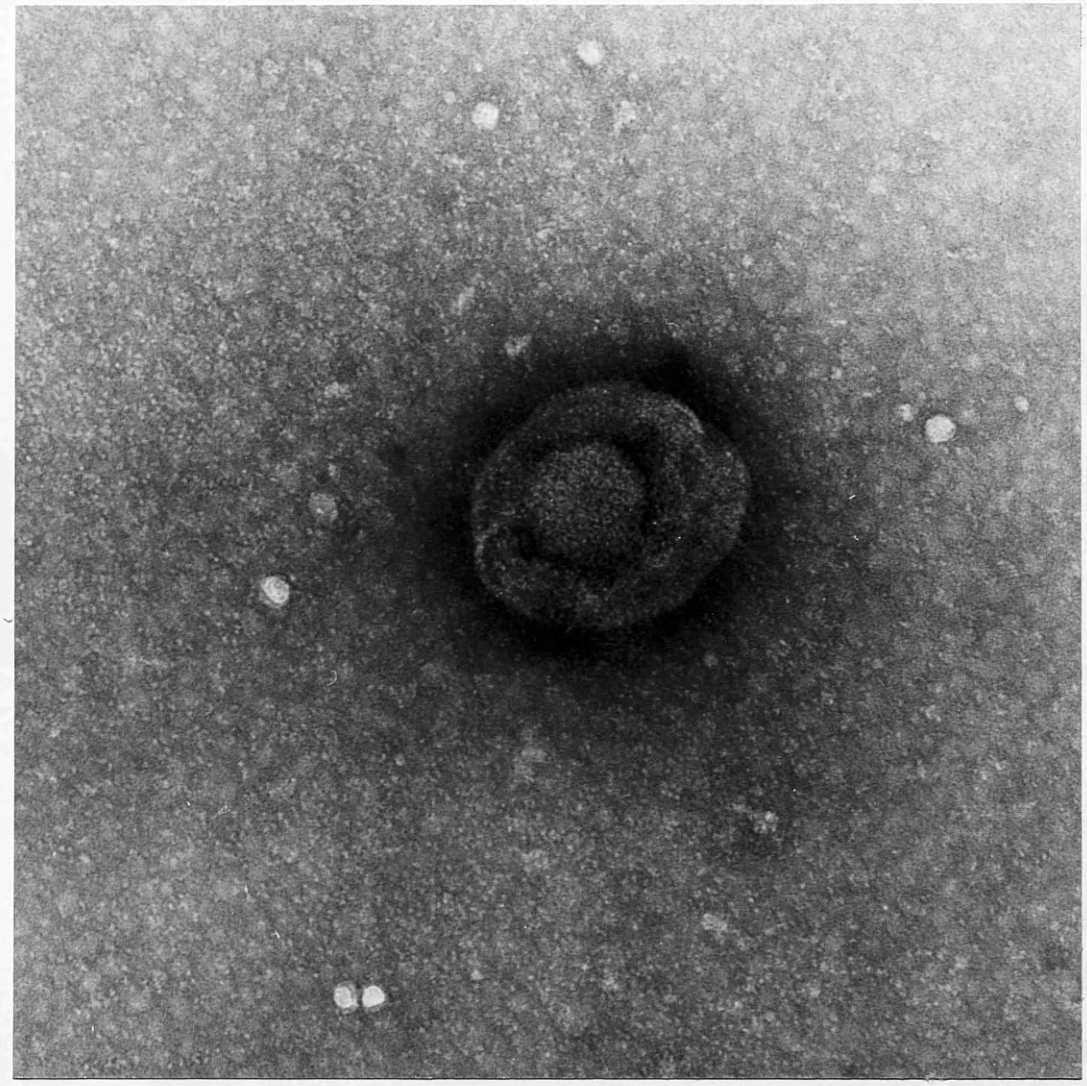
The two subtypes of EHV-1 are morphologically indistinguishable from each other and similar to all members of the Herpesvirinae. Virions are approximately 150 - 170nm in size and are composed of four major subvirion components: the envelope, tegument, capsid and core (Plummer and Waterson, 1963; Abodeely et al., 1970; Darlington and Moss, 1968; O'Callaghan and Randall, 1976). An electron micrograph of an EHV-1 subtype 2 virion is shown in Figure 1.5.

The lipoprotein envelope is a triple-layered membranous structure that surrounds the nucleocapsid. It is derived from the nuclear membrane of the infected cell (Darlington and James, 1966; Darlington and Moss, 1968; O'Callaghan and Randall, 1976; O'Callaghan et al., 1978). Virus envelope glycoproteins play an important role in the adsorption and penetration of virus into cells, cell fusion and spread of virus from cell to cell and egress of progeny virus (Manservigi et al., 1977; Sarmiento et al., 1979; Noble et al., 1983; Buckmaster et al., 1984). Thus, they are important determinants of viral pathogenicity. They elicit both a humoral and a cell-mediated immune response in the host (Powell et al., 1974; Papp-Vid and Derbyshire, 1978, 1979; Norrild et al., 1979; Bishop et al., 1983, 1984). Their identification and characterization is thus of primary importance to the elucidation of viral pathogenesis and prophylaxsis of disease. Six major and

Figure 1.5

Electron micrograph of an EHV-1 subtype 2 virion .

Magnification = 145,000X



six minor glycoproteins have been identified in the envelope of both subtypes of EHV-1 (Turtinen and Allen, 1982; Turtinen, 1983; Allen and Bryans, 1986). The six major glycoproteins are between 240,000 and 40,000 in molecular weight, and are designated VGP 2, 10, 13, 14, 18 and 22a based on the original nomenclature of O'Callaghan and Randall, (1976). Equivalent species are found in both subtypes (Turtinen, 1983; Allen and Bryans, 1986).

The amorphous area between the envelope and the nucleocapsid is known as the tegument (Roizman and Furlong, 1974). Variation in size of this region is thought to be responsible for the differences in overall virion size noted among EHV-1 populations (O'Callaghan et al., 1983). Several EHV-1 proteins have been tentatively assigned to this region (O'Callaghan et al., 1983; Turtinen, 1983; Allen and Bryans, 1986). However, little is known about the detailed composition of the EHV-1 tegument.

EHV-1 capsids are approximately 100nm in diameter (Abodeely et al., 1970) and a comparative electron microscopy study by Vernon et al. (1974) demonstrated that there were no significant structural differences between HSV-1 and EHV-1 capsids. The HSV-1 capsid is composed of 162 morphological subunits called capsomeres, arranged to form an icosadeltahedron which exhibits 2-, 3- and 5- fold symmetry (Wildy et al., 1960). The fully assembled EHV-1 nucleocapsid is made up of six major proteins, one of which a phosphoprotein with a molecular weight of 148,000 constitutes approximately 68% of total capsid protein

(Perdue et al., 1974, 1975, 1976; O'Callaghan et al., 1977, 1978).

The core contains the EHV-1 DNA molecule and associated proteins and is located centrally within the capsid. It is an electron dense toroidal structure occasionally containing a less dense centre (Perdue et al., 1975; O'Callaghan and Randall, 1976). This centre consists of a bar structure around which the DNA is spooled or wound in strands with a regular spacing (Furlong et al., 1972; O'Callaghan and Randall 1976; O'Callaghan et al., 1978, 1983).

The Genome of EHV-1, Subtype 1

Early studies which found that EHV-1 inclusions stained Feulgen-positive indicated that the virus contained DNA (Randall and Bracken, 1957; Gentry and Randall, 1960). Further evidence for this was obtained from the autoradiographic study of hepatic cell nuclei isolated from EHV-1 infected hamsters labelled with tritiated thymidine (Gentry et al., 1962). Chemical analysis of virus purified from the plasma of infected hamsters indicated that the DNA has a G+C content of approximately 56% (Darlington and Randall, 1963). A detailed study by Soehner et al. (1965) of the buoyant density and melting temperature of EHV-1 DNA showed that the G+C content is 57%. Like HSV-1 DNA (Frenkel and Roizman, 1972a) EHV-1 DNA fragments upon treatment with alkali (O'Callaghan et al., 1983).

A combination of techniques such as electron microscopy, restriction endonuclease digestion, end

labelling and Southern blot hybridization have been used to demonstrate that EHV-1 subtype 1 has a genome structure characteristic of a Group D herpesvirus (Figure 1.2). Thus, it comprises two covalently linked segments designated L (long) and S (short). The S segment consists of a unique region U_S bracketed by inverted repeat sequences (TR_S, IR_S). Electron microscopic studies showed that when single stranded EHV-1 DNA is allowed to self-anneal the inverted repeat sequences (TR_S, IR_S) hybridize to form a double-stranded region which is contiguous at one end with a single stranded loop (U_S) and at the other with a long single stranded tail (L) (Ruyechan *et al.*, 1982). The presence in virion DNA of two equimolar isomers of the EHV-1 genome differing in the orientation of U_S is indicated by the existence in certain restriction endonuclease digests of three terminal fragments and 0.5M fragments which share significant homology (Henry *et al.*, 1981; Whalley *et al.*, 1981).

Henry *et al.* (1981) investigated the genome structure of the Kentucky A strain of EHV-1 subtype 1. Their findings indicated that the L and S segments have molecular weights of 71.6×10^6 and 20.4×10^6, respectively. The average molecular weight of U_S on electron microscopic examination was found to be 6.4×10^6 (Ruyechan *et al.*, 1982). Whalley *et al.* (1981) analysed the genome structure of an Australian isolate of EHV-1 subtype 1 (isolate HVS 25), and reported molecular weights of 74×10^6 and 26×10^6 for the L and S segments, respectively. The molecular weight of U_S was estimated as at least 7.9×10^6. The DNA

Figure 1.6

The Genome Structure of EHV-1, Subtype 1

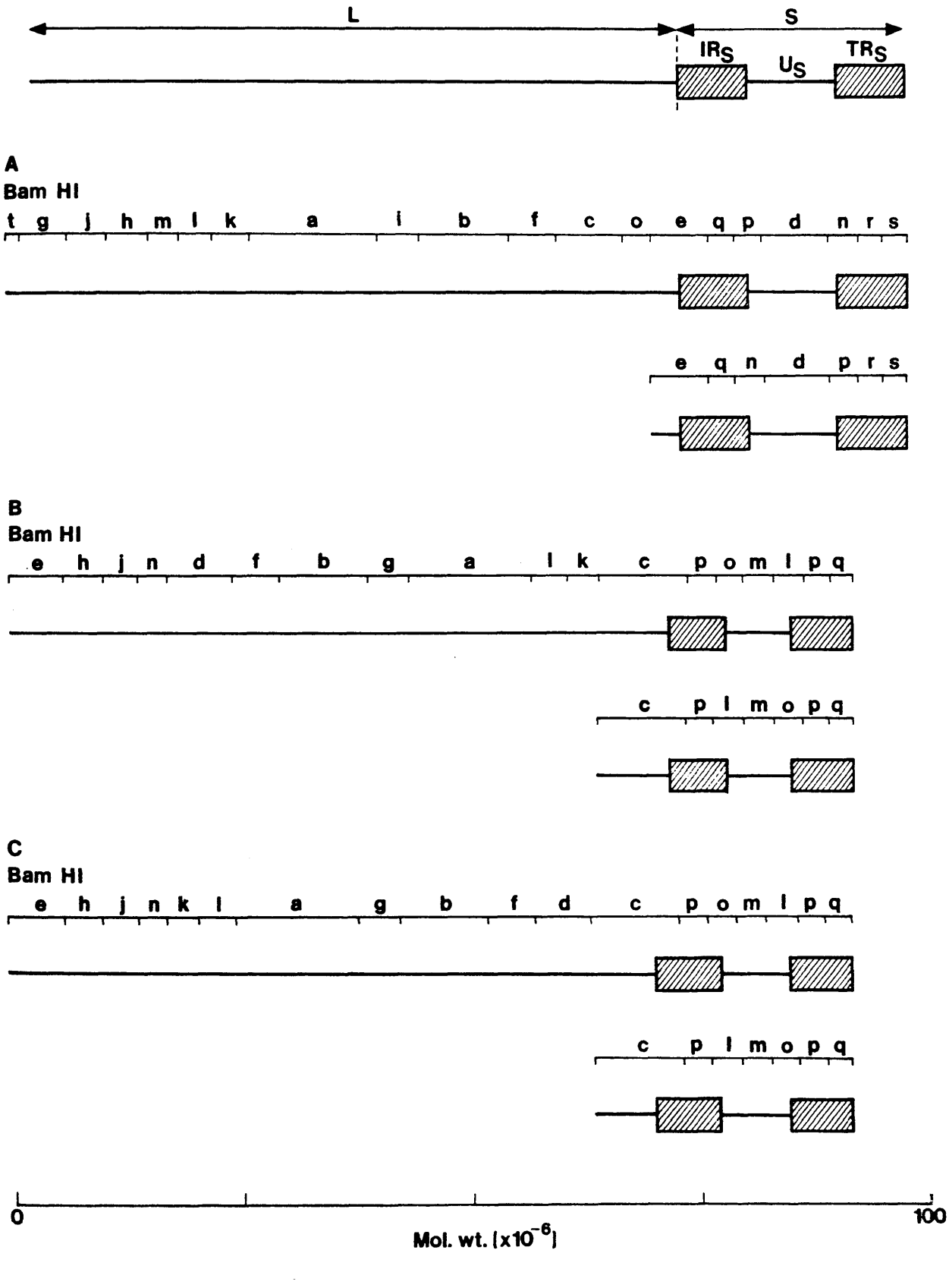
A. The Bam HI cleavage map of isolate HVS-25 of EHV-1, subtype 1, derived by Whalley et al., (1981).

B. The Bam HI cleavage map of the Kentucky A strain of EHV-1, subtype 1, derived by Henry et al., (1981).

C. The revised Bam HI cleavage map of the Kentucky A strain (Baumann et al., 1986),

The two hatched boxes represent the repeat regions. Both orientations of the S region are shown.

EHV-1 DNA Structure



was not examined by electron microscopy. The latter values are used for comparative analysis in this thesis.

Eco RI, Bgl II, Xba I and Bam HI cleavage maps were derived for the Kentucky A strain by Henry et al. (1981), and Eco RI, Bgl II and Bam HI maps of isolate HVS-25 were derived by Whalley et al. (1981). The original maps for the Kentucky A strain (Henry et al., 1981) suggested a difference in the arrangement of fragments of the U_L of these two viruses. However, a later modification of the Kentucky A maps consisting of the inversion of DNA sequences between 0.2 and 0.7 map units, showed that the Kentucky strain has an arrangement of restriction endonuclease sites similar to HVS-25 (Baumann et al., 1986). This is illustrated in Figure 1.6.

Lytic Infection With EHV-1.

The replicative cycle of EHV-1 subtype 1 has been studied in mouse fibroblast (L-M) cells and in the young Syrian hamster. The latter was used for 20 years for propagation of EHV-1. In this animal EHV-1 causes a consistently reproducible lethal hepatitis and virus is released into the blood in large amounts. All animals die approximately 12 hours post inoculation, and more than 95% of the hepatic parenchymal cells show a typical intranuclear inclusion (Bracken and Randall, 1957; Gentry and Randall, 1960; Gentry et al., 1960; O'Callaghan et al., 1972). The maximum virus titer is obtained from monolayers of L-M cells 18-24 hours postinfection, and 95% of the virus is released from the cell (O'Callaghan et al., 1968a, 1978, 1983).

Initial Stages of Infection

Herpesviruses attach to specific unidentified receptors on the cell surface. The mechanism of virus entry into the cell has not been fully elucidated, but it is generally accepted that it is mediated by fusion of the virus envelope with the cell membrane (Morgan et al., 1968). This is supported by evidence that envelope glycoproteins play a role in adsorption and penetration of HSV-1 (Sarmiento et al., 1979; Little et al., 1981) and that HSV-1 envelope Fc receptors could be detected in the cell surface membrane immediately after penetration and in the absence of viral gene expression (Para et al., 1980). However, virus entry by fusion has not been definitively established and several early electron microscopic studies indicate that HSV is taken up by phagocytosis (Holmes and Watson, 1963; Hummeler et al., 1969; Dales and Silverberg, 1969). Abodeely et al. (1970), studied the entry of EHV-1 subtype 1 particles into L-M cells by electron microscopy. The virus entered the cell by viropexis within 5 min postinfection. The particles were engulfed by pseudopodia phagocytized and remained in intracytoplasmic vacuoles for up to two hours postinfection. The mechanisms by which parental virus DNA is uncoated and translocated into the nucleus is unknown. However, release of virus DNA into the nucleoplasm is obviously dependent on a viral function as viral capsids accumulate at the nuclear pores of cells infected at the nonpermissive temperature with the HSV-1 temperature-

sensitive mutant tsB7. These capsids do not release DNA for at least 6 hours postinfection, unless the cells are shifted to the permissive temperature, in which case DNA release occurs within 30 min (Batterson et al., 1983).

Effect of Virus Infection on Host Macromolecular Synthesis

Shutoff of host macromolecular metabolism is a recognised feature of herpesvirus infections (Roizman and Roane, 1964; Sydiskis and Roizman, 1966; Gergely et al., 1971; Rakusanova et al., 1971), but the mechanisms involved are poorly understood. EHV-1 infection causes significant alterations in host nucleic acid and protein synthesis. Inhibition of host DNA synthesis commences early in infection of L-M cells and is eventually suppressed by 95% (Randall and Walker, 1963; O'Callaghan et al., 1968a). Also, pulse labelling with radioactive uridine demonstrated a dramatic inhibition of cellular RNA synthesis in L-M cells (O'Callaghan et al., 1968b) and in human carcinoma cells (KB) (Lawrence, 1971) commencing early after infection with EHV-1 subtype 1. O'Callaghan et al. (1968a) measured protein synthesis in EHV-1 infected L-M cells by incorporation of [^{14}C]leucine into the protein fraction. Cell replication ceased a few hours after infection, but total protein synthesis continued at a linear rate. The authors suggested that EHV-1 infected cells undergo a smooth transition from the synthesis of host protein to viral protein. However, Lawrence (1971) using pulse labelling with [^{14}C]valine in KB cells found the rate of total protein synthesis decreased between 5 and 6 hours after infection and decreased progressively for the

remainder of the infectious cycle. Recent studies on the regulation of EHV-1 polypeptides using SDS-polyacrylamide gel electrophoresis indicated that a gradual but progressive decline in cellular protein synthesis occurs during the course of infection (Caughman et al., 1985).

Replication of Viral DNA

The kinetics of viral DNA synthesis in EHV-1-infected L-M cells were examined by measuring the incorporation of labelled thymidine and deoxycytidine (O'Callaghan et al., 1968a,b). EHV-1 DNA synthesis began at 4-6 hours postinfection, increased rapidly and reached a peak at 12-14 hours. The rate of EHV-1 DNA synthesis decreased after 14 hours postinfection and by 16-18 hours was reduced to less than one-third of the maximal rate. Studies of EHV-1 infected Syrian hamsters demonstrated a similar virus growth curve. A short latent period was followed by a sharp increase in virus titre. DNA synthesis began at 3-4 hours postinfection and continued to increase until 8 hours postinfection, after which synthesis decreased until the animals died at 12-14 hours postinfection (O'Callaghan et al., 1972).

Electron microscopic studies, pulse labelling experiments and restriction endonuclease analysis of replicating DNA suggest that herpesvirus replication involves circularization of the linear input DNA and the formation of head-to-tail concatamers probably by a rolling circle mechanism (Jean and Ben-Porat, 1976; Ben-Porat et al., 1976; Jacob and Roizman, 1977; Jacob et al., 1979;

Ben-Porat and Rixon, 1979). Sequences essential for HSV-1 amplification in permissive cells have been analysed in detail. One origin of replication is present twice in the genome, in IR_S/UR_S (Stow, 1982; Mocarski and Roizman 1982; Stow and McMonagle, 1983), and the second is located in the centre of U_L between the genes encoding the major DNA-binding protein and DNA polymerase (Locker et al., 1982; Spaete and Frenkel, 1982; Weller et al., 1985). No such information is available on the locations of the EHV-1 origins of replication.

Transcription

No EHV-1 RNA is synthesised in the presence of α-amanitin, a potent inhibitor of RNA polymerase II (Kemp et al., 1975). EHV-1 transcription was first investigated by Huang et al. (1971) who used DNA-RNA hybridization to demonstrate that virus-specific RNA synthesis commenced at 2-4 hours postinfection and continued until 12-14 hours postinfection. Hybridization competition experiments indicated that the virus-specific RNA species transcribed early in infection were a subset of RNA transcribed at late times. Temporal regulation of RNA transcription has been described in detail in other herpesviruses particularly HSV-1 (Rakusanova et al., 1971; Frenkel and Roizman, 1972b; Wagner, 1972; Swanstrom and Wagner, 1974).

The transcription of the HSV genome is sequentially ordered, and three major groups of viral mRNA immediate-early (IE) or α, early (E) or β and late (L) or γ have been identified (Frenkel and Roizman, 1972b; Swanstrom and

Wagner, 1974; Kozak and Roizman 1974; Clements et al., 1977). The synthesis of HSV IE mRNA occurs in the presence of protein synthesis inhibitors (Kozak and Roizman, 1974). The gene for a virion polypeptide synthesised late in infection which specifically activates transcription from IE genes has been mapped (Campbell et al., 1984) and its DNA sequence determined (Dalrymple et al., 1986). IE gene products are required for the transcription of both E and L genes (Watson and Clements, 1978; Preston 1979, 1981; Everett, 1984; O'Hare and Hayward, 1985). Analysis of the effects of metabolic inhibitors on EHV-1 polypeptide synthesis indicates that viral gene expression is temporally regulated in a manner similar to that of HSV (Caughman et al., 1985).

Protein Synthesis

Honess and Roizman (1974, 1975) used amino-acid analogues and inhibitors of protein synthesis to demonstrate the existence of three classes of HSV polypeptides α, β and γ. These correlate in general to the three temporal classes of viral transcripts, IE, E and L, discussed above. The synthesis of α polypeptides, the earliest polypeptides made, is maximal between 3 and 4 hours postinfection and requires no prior protein synthesis. The function of one or more α polypeptides is required for synthesis of β polypeptides, which is maximal at between 5 and 7 hours postinfection. The β polypeptides inhibit α polypeptide synthesis. One or more β polypeptides is required for the onset of synthesis of the γ

group which in turn inhibit the production of β polypeptides (Honess and Roizman, 1974, 1975; Kozak and Roizman, 1974; Roizman et al., 1974). Analysis of polypeptides produced by HSV-1 ts mutants suggests that this initial account of the cascade synthesis of HSV polypeptides was an oversimplification (Marsden et al., 1976). Although the IE viral polypeptide V_{mw}IE 175 and possibly V_{mw}IE 110 mediate the synthesis of non-IE viral polypeptides (Watson and Clements, 1978, 1980; Preston 1979, 1981; Everett, 1984; O'Hare and Hayward, 1985), the requirement does not appear to be absolute (Pereira et al., 1977; Locker et al., 1982). Two groups of β polypeptides which differ in the kinetics of their synthesis have been identified (Pereira et al., 1977). This is also true of the γ group which is subdivided into two subgroups γ_1 and γ_2. γ_1 polypeptides such as glycoprotein B are made in the absence of DNA synthesis, but in substantially lesser amounts than in a normal productive infection. γ_2 proteins have an absolute requirement for DNA synthesis (Conley et al., 1981; Wolf and Roizman, 1978).

Caughman et al. (1985) demonstrated that EHV-1 polypeptide synthesis is a coordinately regulated and sequentially ordered process similar to that of HSV-1. Four IE, 17 E and 12 L polypeptides were identified. The kinetics of synthesis of at least 3 of the E polypeptides appear to be linked to viral DNA replication.

Virus Associated Enzymes

Several changes in enzymatic activity are associated with infection by EHV-1 subtype 1. A virus coded thymidine kinase (TK) has been identified and characterized (Allen et al., 1978b, 1979; McGowan et al., 1979). The existence of this enzyme was first suggested by the inhibition of EHV-1 replication in both L-M cells and in the Syrian hamster by arabinosylthymine (ara-T), a thymidine analogue which is selectively phosphorylated by herpesvirus TKs (Aswell et al., 1977). Increased TK activity was observed in the presence of sufficient TTP to inhibit host cell TK after infection of four different cell types with EHV-1. This was also observed in liver homogenates from EHV-1-infected hamsters (Allen et al., 1978b). Neutralization of the novel TK activity occurred on preincubation of the cells with antiserum against EHV-1-infected cells (Allen et al., 1978b). Moreover, biochemical transformation of TK- negative cells to the TK-positive phenotype with EHV-1 subtype 1 was demonstrated (Allen et al., 1978b). The purified EHV-1-induced TK was shown to be more closely related to that of PRV than to that of HSV by its lack of ability to phosphorylate deoxycytidine. However, EHV-1 TK resembled the HSV-coded enzyme in its resistance to feedback inhibition by TTP and dCTP and its ability to use a variety of nucleoside triphosphates as phosphate donors (Allen et al., 1979). Purified TK, from EHV-1 transformed TK-negative mouse cells, was identical to the TK purified from these cells on lytic infection with EHV-1 with respect to its phosphate donor specificity, molecular weight,

electrophoretic mobility, substrate specificity and antigenicity (McGowan et al., 1979).

EHV-1 DNA polymerase has been purified from the nuclei of infected hamster livers and characterized (Allen et al., 1977a). The main features which distinguish it from the host enzyme are immunological specificity, high salt requirement for maximal activity and sensitivity to phosphonoacetic acid (Kemp et al., 1975; Allen et al., 1977a). EHV-1 DNA polymerase shows no immunological cross reactivity with, and is far more sensitive to phosphonoacetic acid, than the enzyme coded by HSV-1 (Honess and Watson, 1977; Allen et al., 1977a). HSV-1 ribonucleotide reductase is composed of two non identical virus-coded subunits (Langelier and Buttin, 1981; Cohen et al., 1985; Frame et al., 1985; Bachetti et al., 1986). The resistance of EHV-1 replication to hydroxyurea and TTP concentrations sufficent to completely block host cellular DNA synthesis suggests that EHV-1 also specifies ribonucleotide reductase (Cohen et al., 1975 Allen et al., 1978a). Significant alterations in ribonucleotide reductase activity in EHV-1 infected cells have also been observed (Cohen et al., 1977).

A protein kinase was identified by Randall et al. (1972) in the virions of EHV-1. There is also some evidence for the existence of an EHV-1-induced deoxyribonuclease although the results of much of this work are confused by the contamination of virus stocks with Mycoplasma hominis (Stock and Gentry., 1969).

Assembly of EHV-1 Virions.

Capsids are assembled and the DNA packaged in the nucleus of the infected cell. Three major species of EHV-1 nucleocapsid have been isolated and characterized (Perdue et al., 1974, 1975, 1976; O'Callaghan and Randall, 1976; O'Callaghan et al., 1977, 1978). They are classified on the basis of their densities as Light (L), Intermediate (I) and Heavy (H) (Perdue et al., 1975). It was deduced from pulse chase experiments that the I capsids are precursors of the H capsids and that the L capsids are defective by-products of virus assembly (Perdue et al., 1976). Only the mature, core-containing H capsids are enveloped (O'Callaghan and Randall, 1976; O'Callaghan et al., 1977). Envelopment occurs at the nuclear membrane either by budding or invagination of the nuclear membrane (Darlington and Moss, 1968; O'Callaghan and Randall, 1976; O'Callaghan et al., 1977).

Oncogenic Transformation by EHV-1

EHV-1 subtype 1, EHV-2 and EHV-3 have been shown to have oncogenic potential in tissue culture and in laboratory animals. Transformed and tumour cell lines have been established (Robinson et al., 1980a,b; Dauenhauer et al., 1982; Staczek et al., 1984). EHV-1 subtype 1 preparations enriched for defective interfering (DI) particles, or UV-irradiated virus, can oncogenically transform permissive primary hamster cells (Robinson et al., 1980a,b,c). Infection with the former results in the coestablishment of oncogenically transformed and persistently infected cell populations (Robinson et al.,

1980c; Dauenhauer et al., 1982). Infection of nonpermissive, primary BALB/c mouse embryo cell cultures with infectious EHV-1 or transfection of these cells with EHV-1 DNA also resulted in oncogenic transformation (O'Callaghan et al., 1983). EHV-1 transformed and tumour cells of all three systems contain integrated EHV-1 DNA sequences, express viral antigens and are oncogenic in laboratory animals (Robinson and O'Callaghan 1983; O'Callaghan et al., 1983).

There is no evidence that any of the equine herpesviruses are associated with oncogenesis in the horse.

CHAPTER 2

MATERIALS AND METHODS

MATERIALS

Cells

Rabbit kidney cells, (RK13), and pig kidney, [PK(15)], both established cell lines, were obtained from Flow Laboratories, Irvine, Scotland. Equine dermal cells (NBL-6), a finite cell line, were also purchased from Flow Laboratories. An SV40-transformed cell line derived from sheep lung fibroblasts (SHELUT) was a generous gift of Dr. J. Macnab, Institute of Virology, Glasgow, U.K. (Macnab, 1972).

Viruses

Several primary EHV-1 isolates were kindly supplied by Dr. J. Mumford, The Animal Health Trust, Newmarket, U.K. These included the two viruses used throughout these studies: Vo1939 which was isolated from an aborted foetus, and 1942, an isolate from a young horse suffering from upper respiratory tract disease.

Tissue Culture Media and Solutions

PK(15), RK13 and NBL-6 cells were grown in Earle's Minimum Essential Medium (Flow) supplemented with 0.2% sodium bicarbonate, 1% non-essential amino acids, 1% glutamine, 100 units/ml penicillin, 100mg/ml streptomycin and 10% foetal calf serum.

SHELUT cells were grown in Glasgow's Modified BHK Medium (Flow) (Busby *et al.*, 1964) supplemented with 0.2% sodium bicarbonate, 1% glutamine, 100 units/ml penicillin,

100mg/ml streptomycin and 10% newborn calf serum.

Other Solutions Used for Tissue Culture

Agar	3.8% sea plaque agar.
Giemsa strain	1.5% (vol/vol) suspension of Giemsa in glycerol, heated to 56^{o} for 90-120 min and diluted with an equal volume of methanol.
Phosphate-buffered Saline (PBS)	170mM NaCl; 3.4mM KCl; 1mM NaH_2PO_4; 2mM KH_2PO_4; 0.7mM $CaCl_2$; 0.5mM $MgCl_2$; in distilled water, pH 7.2.
PBS/Cidex	50% (vol/vol) PBS plus 50% (vol/vol) Cidex.
Trypsin	0.25% wt/vol Difco trypsin in 140mM NaCl; 30mM KCl; 0.28mM Na_2HPO_4; 1mg per ml dextrose; 0.0015% wt/vol phenol red; 25mM Tris-HCl, pH7.4; 100 units/ml penicillin and 100 ug/ml streptomycin.
Versene	6mM EDTA in PBS containing 0.0015% wt/vol phenol red.
Trypsin/Versene	1 volume of trypsin solution plus 4 volumes of versene solution.

Tissue Culture Ware

Plastic roller bottles, flasks and petri dishes were purchased from Flow Laboratories, Irvine, Scotland.

Chemicals and Enzymes

Unless otherwise stated, all chemicals were of Analar grade and obtained from B.D.H. Chemicals Ltd., Poole, Dorset, U.K.

Agarose (type II), bovine serum albumin (5% solution (wt/vol) in 0.7g/litre NaCl), cytochrome c, deoxyribonuclease I, ethidium bromide, lysozyme, polyvinylpyrollidone, ribonuclease A and yeast RNA were purchased from the Sigma London Chemical Co. Ltd., Kingston-upon-Thames, Surrey, U.K.

TEMED (N,N,N',N'-tetra-ethylenediamine) was purchased from Bio-Rad Laboratories Inc., Richmond, California, U.S.A.

Formamide was purchased from, Fluorochem, Glossop, Derbyshire, U.K.

Parlodion was purchased from Mallinkcrodt, Rochester, New York, U.S.A.

Dextran sulphate, Ficoll 400 and Percoll were purchased from Pharmacia Fine Chemicals, Uppsala, Sweden.

Sea plaque agarose was purchased from FMC Corporation, Marine Colloids Division, Rockland, U.S.A.

Dimethyl sulphoxide (DMSO) was purchased from Aldrich Chemical Co. Ltd., Gillingham, Dorset, U.K.

Ampicillin was purchased from Beecham Research Laboratories, Brentford, Middlesex, U.K.

Radiochemicals, [α-^{32}P] -dCTP, -dGTP, -dATP and -TTP (3000Ci/mmole) were purchased from Amersham International, Bucks. U.K.

Caesium chloride, calf intestinal phosphatase, T4 DNA ligase, E. coli DNA polymerase I and Klenow DNA polymerase were purchased from the Boehringer Corporation (London) Ltd., Lewes, East Sussex, U.K.

Bam HI, Eco RI and other restriction endonucleases were purchased either from Boehringer Corporation (London) Ltd., Lewes, East Sussex or from Bethesda Research Laboratories (BRL), Inc., Cambridge, U.K.

Wacker reagent was purchased from Wacker Chemical Company, Munich, West Germany.

DNA

Calf thymus DNA was obtained from Sigma (London) Chemical

Company, Kingston-upon-Thames, London, U.K.

ϕX174 RFI DNA was purchased from New England Biolabs, Beverly, Massachussetts, U.S.A.

pUC9 DNA and M13mp18 RF DNA were purchased from Bethesda Research Laboratories, Inc., Cambridge, U.K.

HSV-1 DNA was generously supplied by Dr. N. Stow.

Kpn I fragments of HSV-1 DNA cloned in pAT153 were a kind gift of Dr. A.J. Davison.

Miscellaneous Materials

Nitrocellulose membrane filters - Schleicher and Schull, Dassel, West Germany.

X-Omat and XS1 film - Kodak Ltd., London.

Fix (Amfix) - May and Baker Ltd., Dagenham, Essex, U.K.

Filter papers - Whatman, Ltd., Maidstone, Kent, U.K.

Membrane Filters (0.4μm and 0.22μm pore size) - Millipore (U.K.) Ltd., London.

All deoxynucleoside and dideoxynucleoside triphosphates were purchased from Pharmacia, P L Biochemicals, Hounslow, Middlesex, U.K.

Commonly Used Solutions and Buffers

E buffer 0.036M Trizma; 0.036M NaH_2PO_4; 0.001M EDTA, pH 7.8.

TBE buffer 0.089M Trizma; 0.089M boric acid; 0.0025M EDTA.

TE 0.01M Tris-HCl pH 8.0; 0.001M EDTA.

NTE 0.01M Tris-HCl, pH 7.4; 0.1M NaCl; 0.001M EDTA.

SSC 0.15M NaCl; 0.015M sodium citrate, pH 7.5.

TM buffer 0.1M Tris-HCl, pH 8.0; 0.1M $MgCl_2$.

METHODS

Growth of Cells

RK13, PK(15), NBL-6 and SHELUT cells were grown in slowly rotating 80 oz bottles in 100ml of the appropriate medium at 37°C in an atmosphere of 5% CO_2. When the monolayers were confluent, the medium was decanted, the cells washed twice with trypsin-versene and rotated until they detached from the bottle surface. Roller bottles were usually seeded at approximately 3×10^7 cells/ bottle and 50mm Petri dishes at 1×10^6 cells/dish in 4ml of medium.

Stocks of cells were stored by freezing at-140°C in the appropriate medium containing either 20% glycerol or 10% dimethyl sulphoxide.

Production of Virus Stocks

Roller bottles of slightly sub-confluent monolayers were infected with virus at a m.o.i. of 0.003. Virus was added in 20ml of medium per bottle and allowed to adsorb for 60 min at 37°C. At the end of the adsorption period, 30ml of medium were added to each bottle, and the infected cells were incubated at 31°C for 3-6 days. When the cell monolayer showed 100% c.p.e., the cells were shaken into the medium and pelleted at 1,500 r.p.m. for 5 min at 4°C in an MSE Coolspin centrifuge. The clarified supernatant was centrifuged at 12,000 r.p.m. for 2 hours in a Sorvall GSA rotor and the resultant virus pellet resuspended in the appropriate medium. Virus stock was stored in sterile vials at -70°C.

Virus Titration

Sonicated virus stock was diluted serially at tenfold dilutions in the appropriate medium. Drained confluent monolayers of cells in 50mm Petri dishes were inoculated with 100µl of virus suspension at the required dilution. Each dilution was assayed in duplicate. Virus was adsorbed for 60 min at 37°C, the plates being agitated periodically to prevent dehydration of the cell sheet. After adsorption the medium was aspirated, and the monolayers overlaid with 4ml of medium containing 0.75% (wt/vol) sea plaque agarose. The plates were then incubated at 37°C until plaques were clearly visible (5-7 days). The monolayers were fixed for at least 6 hours with Cidex/PBS, stained with Giemsa, and the plaques counted.

Plaque Purification of Virus

Virus was titrated on the appropriate cell monolayers, and plaques allowed to develop under an agarose overlay. The plates were then overlaid with agarose containing 0.016% (wt/vol) neutral red chloride, and well separated virus plaques were picked with a Pasteur pipette using a Wild M7A stereoscopic dissecting microscope. The infected cells were transferred to 1ml of medium and sonicated. Cell monolayers inoculated with tenfold dilutions of this virus were incubated at 37°C until plaques developed which were suitable for picking. The virus was plaque purified three times in this way before 50% of the progeny of a plaque were used to inoculate a 50mm plate of cells to prepare a primary virus stock. The stock was used to inoculate several flasks of cells. Virus harvested from

the infected monolayers in the flasks was titrated and used to infect 80 oz roller bottles for production of working stocks of virus.

Preparation of EHV-1 DNA

The required number of roller bottles of confluent cells were infected with the appropriate subtype of EHV-1 at a m.o.i. of 0.003 as described above. They were incubated at 31°C until extensive c.p.e. was evident and the majority of cells had detached from the bottle surface (2-6 days, depending on the virus subtype used). The infected cell medium was centrifuged at 5,000 r.p.m. for 5 min to pellet the cells, and the supernatent was centrifuged at 12,000 r.p.m. for 2 hours in a Sorvall GSA 6 X 200ml rotor. The pellet was resuspended in 5ml PBS, sonicated and centrifuged at 11,000 r.p.m. in a Sorvall SS34 rotor for 5 min to spin down cellular debris. Virus was then pelleted by centrifugation at 18,000 r.p.m. in a Sorvall SS34 rotor for 1 hour, resuspended in 0.5ml 5mM Tris, 0.1% bovine serum albumin, sonicated and layered onto a Percoll gradient. The Percoll gradient solution was prepared by the method of Pertoft described in the manufacturer's instructions for the purification of HSV. Centrifugation was for 30 min at 40,000 r.p.m. in a Beckman Ti50 rotor at 4°C. The virus banded about one third the way down the self-generated gradient. The fraction containing the virus was collected in a 2ml syringe, by inserting the needle through the side of the tube below the visible band, placed on a 3ml 60% sucrose cushion and

centrifuged at 35,000 r.p.m. for 1 hour in a Beckman AH650 rotor. The Percoll sedimented into the sucrose layer, and the virus remained in the upper iso-osmotic solution. The virus was diluted in 0.1M Tris then pelleted at 35,000 r.p.m. in a Beckman AH650 for 1 hour. After resuspension in a small volume of NTE (0.5 - 5ml), virus DNA was extracted.

The virus suspension was sonicated in an ultrasonic bath and 20% (wt/vol) SDS added to a final concentration of 1%. An equal volume of NTE-equilibrated phenol was added and the tube agitated gently on a rock-and-roller for 10 min. The mixture was centrifuged at 2,000 r.p.m. at 6^{o}C for 10 min, in an MSE Coolspin centrifuge. The upper aqueous phase was removed and re-extracted with two further volumes of NTE-equilibrated phenol. Finally the aqueous phase was extracted with chloroform to remove all traces of phenol. The DNA was precipitated by adding 0.1 volumes of 3M NaAc pH 7.0 and 2.5 volumes of ethanol and leaving on dry ice for at least 30 min. The DNA was pelleted, washed with 70% ethanol, drained well and resuspended in a small volume of 0.1 x SSC. The DNA concentration was estimated approximately by visual comparison of samples with known quantities of DNA on ethidium bromide stained agarose mini-gels under UV illumination (365nm). EHV-1 DNA was stored at -20^{o}C.

EHV-1 subtype 2 DNA was prepared, in some instances, by an alternative method which did not involve use of Percoll gradients. A virus pellet was obtained as described above except that, it was resuspended in 10ml NTE and briefly sonicated instead of loading onto a Percoll

gradient. Contaminating cellular DNA was degraded by adding DNase at 10µg/ml and incubating at 37°C for 1 hour. SDS was added to a final concentration of 2%, and the preparation was extracted approximately 3 times with NTE-equilibrated phenol until a clear interphase was obtained. A chloroform extraction was followed by ethanol precipitation of the DNA as described above. The DNA was pelleted, washed with 70% ethanol, resuspended in 10ml of 100mM NaCl and 10µg/ml RNase and left overnight at room temperature. Further purification was achieved by treatment with 1mg/ml proteinase K for 2 hours at 31°C. The DNA was extracted once with phenol:chloroform (1:1 vol/vol), once with chloroform, ethanol precipitated, drained well and resuspended in 0.1 X SSC.

Electron Microscopy of EHV-1 Subtype 2 DNA

Intramolecular annealing studies were performed essentially by the method of Ruyechan et al. (1982). 1ug of EHV-1 subtype 2 DNA in 10µl 0.1M SSC was mixed with 40µl of deionised formamide and denatured by heating at 60°C for 10 min. The formamide concentration was reduced from 80% to 66% by the addition of 0.2M Tris-HCl, 0.02M EDTA pH 8.5, and the single-stranded DNA allowed to self-anneal at 25°C for 2 hours. The sample was diluted to a final concentration of 50% formamide, 100mM Tris-HCl (pH 8.5), 10mM EDTA and 100µg/ml cytochrome c. Both single-stranded and double-stranded circles of ϕX174 RF DNA were added as markers. For visualising by electron microscopy, 10µl of this mixture was spread onto 20% formamide, 10mM Tris-HCl (pH 8.5), 1mM EDTA and picked up on a parlodion-coated 400

mesh copper grid. The preparation was stained for 10 sec in 10μg/ml uranyl acetate in 90% ethanol, rinsed in 90% ethanol for 5 sec and rotary shadowed at 70°C with platinum/palladium. The grid was stabilised by shadowing with carbon and examined at 90kv in a Jeol 100S electron microscope. All photographs were taken and the length of EHV-1 and ϕX174 DNA was measured using a Summagraphics ID digitizing tablet controlled by a PDP-11 minicomputer (Rixon et al., 1984a). The sizes of the single-stranded loops and double-stranded stems of the self-annealed EHV-1 DNA, which corresponded to the U_S and TR_S/IR_S regions respectively, were calculated by the ratio of their lengths with respect to the marker DNA lengths. Histograms of the measurements were drawn.

Restriction Endonuclease Digestion of DNA

All restriction digests were carried out in 20mM Tris-HCl (pH 7.5), 20mM $MgCl_2$, 100mM NaCl, 6mM 2-mercaptoethanol and 0.02% wt/vol sterile bovine serum albumin. Sufficient enzyme was added to produce a limit digest of the purified DNA, and the mixture was incubated at 37°C for a minimum of 3 hours. Reactions were terminated by the addition of 2% vol/vol of dye Ficoll, which contained 10% wt/vol Ficoll 400, 0.1M EDTA and 0.1% wt/vol bromophenol blue in 5 x E buffer.

Agarose Gel Electrophoresis

DNA fragments resulting from restriction endonuclease cleavage were fractionated by electrophoresis. Horizontal gels were used with agarose concentrations of 0.5-1.0% as

indicated in the appropriate figure legends. The agarose was dissolved in E buffer by boiling in a Tappan microwave oven. The fluorescent dye ethidium bromide (0.5µg/ml) was added (Sharp et al., 1973) and the agarose allowed to cool to about 50°C before pouring onto a glass plate. A comb was fixed in the molten agar and removed when the gel had set to leave appropriate-sized loading wells. The gel was submerged in E buffer containing 0.5µg/ml ethidium bromide, and the DNA samples with added dye-Ficoll introduced into the wells. Electrophoresis was for 15-20 hours at 2V/cm at room temperature or until the samples had migrated the required distance in the gel. Gels were photographed under long (365nm) or short (254nm) wavelength UV irradiation using Polaroid type 667 or type 665 film.

Isolation of Restriction Fragments

A technique similar to that described by Weislander (1979) was used for the recovery of DNA from low melting temperature agarose. Restriction fragments were electrophoresed on a 0.7% low-melting-point agarose gel in 1 x TBE in the presence of 0.5µg/ml ethidium bromide. Electrophoresis was carried out at 2V/cm for 36 hours at room temperature. The fragments were visualised under long (365nm) wavelength UV irradiation and excised from the gel. To minimise cross-contamination a clean scalpel blade was used for each fragment. Gel slices were melted in about 3 volumes of TE by heating at 65°C for 10 min. Except where the DNA was to be used in recombination experiments 20µg of yeast tRNA was added to aid DNA precipitation. The melted gel slice was extracted with two volumes of TE-equilibrated

phenol at 65°C, mixed and centrifuged for 2 min in a mini-centrifuge. The aqueous phase was recovered and re-extracted twice with TE-equilibrated phenol at 65°C. The preparation was extracted once with ether and the DNA precipitated with 1/10 volume of 3M NaAc and 2.5 volumes of ethanol on dry ice. The DNA was pelleted by centrifugation and dried in a lyophiliser.

Nick Translation of DNA

In vitro labelling of DNA with [α-^{32}P] deoxyribonucleoside triphosphates was done essentially as described by Ribgy et al. (1977). Nick translation reactions were set up on ice and typically contained 0.5µg of DNA in a volume of 20µl of the following solution:- 50mM Tris-HCl pH 7.5, 5mM $MgCl_2$, 1mM dithiothreitol, 0.05% wt/vol bovine serum albumin, 1µl each of unlabelled 2mM dATP, dTTP, and dGTP and 1µl α-^{32}P dCTP (10µCi). The reaction mixture was incubated at 15°C for 90 min after addition of DNase to 10^{-4} mg/ml and 1 unit of DNA polymerase I. After incubation the mixture was made up to 50µl with distilled water and the labelled DNA precipitated twice with 1/10 volume 3M NaAc and 0.75 volume isopropanol on dry ice for a minimum of 30 min. The radioactivity of the DNA was monitored using a portable beta counter.

Radiolabelling of M13 Probes

Approximately 0.5µg of M13 clone DNA was annealed for 30 min at 37°C to approximately 4ng of hybridization probe primer in a final volume of 10ul of 15mM Tris, 15mM $MgCl_2$ pH 8.5. The reaction mixture was then incubated at 37°C

for 15 min in the presence of 1µl of each of 0.5mM dGTP, dCTP, dTTP, 1ul α-^{32}P dATP (10µCi) and 1µl of Klenow DNA polymerase. At the end of this period the reaction was terminated by the addition of 1µl 0.25M EDTA and the DNA precipitated twice with isopropanol, as described above.

Transfer of DNA to Nitrocellulose

The transfer of DNA fragments from agarose gels to nitrocellulose membranes was accomplished by the technique of Southern (1975). The DNA was digested with the appropriate restriction enzyme and electrophoresed in a single wide-slot 0.5% agarose gel. After electrophoresis the DNA was denatured by soaking the gel for 45 min in gel soak I (0.2M NaOH, 1M NaCl) and then neutralized by soaking for 45 min in gel soak II (1M Tris-HCl pH 8, 1M NaCl). The gel was transferred to a glass plate wrapped in 2 sheets of Whatman 3MM paper moistened with 10 x SSC. The plate was supported in a tray of 2 litres of 10 x SSC. Care was taken to ensure that there were no air bubbles between the 3MM paper and either the plate or the gel. A sheet of nitrocellulose paper was floated on the surface of the 10 x SSC until it was soaked from beneath, then it was applied to the gel surface. All air bubbles between the gel and the filter were removed. Two moistened sheets of 3MM paper cut to the same size as the filter were placed on top of the nitrocellulose followed by a weighted stack of paper towels. DNA fragments transferred from the gel to the nitrocellulose overnight, maintaining their original spatial arrangement. The filter was dried at room

temperature and baked in a vacuum oven at 80°C for 2 hours.

Hybridization

Radioactively labelled probes were used to detect homologous sequences in the single-stranded DNA immobilized on the nitrocellulose. The stringency of the hybridization conditions was varied by altering the temperature or the formamide concentration (McConaughy et al., 1969; Hutton, 1977). Hybridizations were performed at 37°C in 30% vol/vol formamide or at 42°C in 50% vol/vol formamide. The hybridization of M13 cloned DNA to virus DNA was at 68°C in the absence of formamide. The nitrocellulose filter was cut into 5mm strips and placed in individual 15ml plastic tubes with tightly fitting metal caps. The strips were incubated overnight in 5ml of the appropriate hybridization solution at 37°C or 42°C in a shaking water bath. Hybridization solutions routinely used consisted of 3 x SSC, 5 x Denhardt's solution (Denhardt, 1966; 0.02% wt/vol Ficoll 400, 0.02% wt/vol polyvinylpyrrolidone, 0.02% wt/vol bovine serum albumin) and 100µg/ml denatured salmon sperm DNA in 50% or 30% vol/vol formamide. Hybridization solution for M13 probes consisted fo 20mM Tris pH 7.5, 3 x SSC, 5 x Denhardt's solution, 100µg/ml denatured salmon sperm DNA and 0.5% SDS.

Nick translated probe DNA was denatured by heating at 100°C for 2-3 min in 80% vol/vol formamide. The tubes containing the preincubated nitrocellulose strips were drained and the denatured probe added in 5ml of fresh hybridization solution. Hybridization at 42°C in 50% vol/vol formamide were incubated in a shaking water bath

overnight. The filters were then transferred to a plastic sandwich box and washed in 3 x SSC, 0.2% SDS, 10mM $NaPO_4$ pH 7.5 at 50°C for at least three hours. The buffer was changed every hour. Hybridization in 30% vol/vol formamide at 37°C was allowed to proceed for 2-3 days and the strips were then washed at 45°C. Hybridization of M13 probes was overnight at 68°C. Washing was for three hours in 2 x SSC, 0.5% SDS, changing the washing solution every hour. After washing, the strips were dried in air and baked in a vacuum oven at 80°C for 15 min. They were then applied to a glass plate using double-sided sellotape and autoradiographed.

Autoradiography

Nitrocellulose strips hybridized at 42° in 50% vol/vol formamide were autoradiographed at room temperature with Kodak X-Omat S film. Those hybridized at 37°C in 30% vol/vol formamide were autoradiographed at -70°C with preflashed Kodak X-Omat film and a Dupont Cronex phosphotungstate screen.

Cloning of EHV-1 Subtype 2 DNA

Ligation of EHV DNA into Plasmids

EHV-1 subtype 2 DNA fragments were ligated into the vector pUC9, a plasmid which includes the ampicillin-resistance gene from pBR322 and the polylinker region from M13mp9 (Vieira and Messing, 1982). 5µg of EHV-1 subtype 2 DNA and 5µg pUC9 DNA were separately digested with Bam HI. 22 units of calf intestinal phosphatase were also added to the pUC9 digestion to remove terminal 5'- phosphate groups.

Complete digestion was verified by gel electrophoresis of aliquots of the reactions and then the DNA was extracted twice with an equal volume of phenol:chloroform (1:1) and ethanol-precipitated. Ligation was performed essentially by the method of Tanaka and Weisblum (1975). Approximately 0.1µg of Bam HI digested pUC9 and 1µg of Bam HI-digested EHV-1 DNA were mixed in 50mM Tris-HCl pH 7.5, 8mM $MgCl_2$, 10mM dithiothreitol, 1mM ATP in a final volume of 40µl. 2 units of T4 DNA ligase (0.5µl) were then added. Two control reactions were also set up, one identical to the ligation mixture above but containing no EHV-1 DNA, the second containing neither EHV-1 DNA nor T4 DNA ligase. The three reactions were incubated at 4°C for 16 hours. The extent of ligation was ascertained by comparing gel electrophoretic patterns of aliquots of the reaction mixtures with undigested pUC9 and Bam HI-digested EHV-1 subtype 2 DNA.

Transfection of Bacteria with Recombinant Plasmids

Calcium-shocked E. coli DHI (Hanahan, 1983) were transformed with recombinant DNA essentially as described by Cohen et al. (1972). The host was grown at 37°C in 100ml of L-broth (0.17M NaCl, 10g/l Difco bactotryptone, 5g/l yeast extract) to an optical density of 0.6 at 630nm in a Gallenkamp orbital incubator. The cells were harvested by centrifugation at 8,000 r.p.m. for 2 min at 4°C, washed by resuspending gently in 50ml of ice-cold 10mM $CaCl_2$ and centrifuged again at 8,000 r.p.m. for 2 min at 4°. The pellet was gently resuspended in 40ml of ice-cold

75mM $CaCl_2$ and left on ice for 20 min. The cells were harvested as before and resuspended in 1ml 75mM $CaCl_2$ and kept on ice. The three ligation mixtures and an additional control of 5×10^{-4}µg undigested pUC9 were added to 0.2ml aliquots of competent cells, and after a further 60 min on ice were incubated for 2 min at 42°. Each sample was then added to 3ml L-broth and incubated at 37°C for 60 min in a Gallenkamp orbital shaker.

Aliquots of 0.1ml were spread on 90mm L-broth agar plates containing 100µg/ml ampicillin, and the plates were incubated at 37°C overnight. Transformation efficiency was usually around 10^6/colonies/µg. All procedures involving growth of live bacteria subsequent to transformation were done under conditions of good microbiological practice, as advised by the Genetic Manipulation Advisory Group and the local safety committee.

Analysis of Recombinant DNA Clones

Bacterial colonies were picked using sterile cocktail sticks and incubated overnight in 2ml of L-broth containing 100µg/ml ampicillin at 37°C in an orbital shaker. The recombinant plasmids were harvested from minicultures by the method of Holmes and Quigley (1981). 0.5ml of the overnight cultures was centrifuged for 2 min in an Eppendorf centrifuge and resuspended in 50ul of 8% sucrose, 0.5% Triton X-100, 50mM EDTA, 10mM Tris-HCl pH 8.0. 5µl of a freshly-prepared solution of lysozyme (10mg/ml) was added and the mixture placed in a boiling water bath for 40 sec. The preparation was immediately centrifuged in an Eppendorf

centrifuge for 10 min. The supernatant was removed into a clean 1.5ml tube and the DNA precipitated by adding 1/10 volume 3M NaAC pH 7.5 and an equal volume isopropanol and left at -20°C for 1 hour. The DNA was pelleted, washed in 70% ethanol, dried and resuspended in distilled water. Insert sizes were ascertained by gel electrophoresis of Bam HI cleavage products. Those colonies chosen to form the clone library were colony purified once, and bacterial stocks were stored at -20°C in 1% Difco Bactopeptone, 40% glycerol. Stocks of plasmid DNA were prepared from these bacteria.

Large-scale Isolation of Plasmid DNA

A bacterial colony or 20µl from the stock was picked with a sterile loop and used to inoculate 5ml of 2TY broth (10g Difco bactotryptone, 10g yeast extract, 5g/l NaCl) containing 100µg/ml of ampicillin. The starter culture was incubated at 37°C for 24 hours and was then added to 200ml of 2TY broth not containing ampicillin. The culture was incubated at 37°C overnight in a Gallenkamp orbital incubator. The cells were harvested by centrifugation at 8,000 r.p.m. for 5 min in a GSA rotor. They were resuspended in 5ml ice cold 25% sucrose, 50mM Tris pH 8 and kept on ice. 2.5ml of a freshly-made solution of lysozyme (5mg/ml) were added and the cells left on ice for 30 min. At the end of this period 2.5ml of ice-cold 0.2M EDTA were added and the bacteria left on ice for 20 min, when 2ml of cold 1.5% NP40 were added. After a further 10 min on ice the cells were centrifuged at 18,000 r.p.m. for 30 min in an SS34 rotor to pellet bacterial debris. The supernatant

was extracted twice with phenol:chloroform (1:1) after the addition of SDS to a final concentration of 1%. 20µl of 100mg/ml RNase A was added and the preparation left for 30 min at 37°C. After another phenol:chloroform (1:1) extraction, the DNA was precipitated overnight at room temperature by adding 1/10 volume 3M NaAc pH 7 and 0.5 volume isopropanol. A DNA pellet was obtained by centrifugation at 2,200 r.p.m. for 15 min. The plasmid DNA was then purified by equilibrium centrifugation in a caesium chloride-ethidium bromide gradient. 63.5g of caesium chloride were added to 4.25ml ethidium bromide (10mg/ml) and made up to a final volume of 85ml with distilled water. The DNA pellet was resuspended by vortexing with 4.7ml of this mixture, and the resuspended DNA was centrifuged in a TV865 rotor at 40,000 r.p.m. for 16 hours at 15°C. The DNA formed two bands, the lower of which consisted of closed circular plasmid DNA. A hypodermic needle was inserted into the side of the tube and the lower band of DNA collected. The ethidium bromide was removed by extracting several times with an equal volume of caesium chloride saturated isopropanol, and the DNA was dialysed overnight against 0.1 x SSC. The DNA was ethanol precipitated, pelleted, drained well and resuspended. Finally, the DNA concentration was estimated by spectrophotometry.

The Preparation of Single-stranded Template DNA for the Determination of DNA Sequences

DNA was sequenced using the M13-dideoxynucleotide chain

terminator method (Sanger et al., 1977, 1980). Single-stranded template DNA was prepared as follows.

Approximately 50µg of a plasmid containing the Bam HI 1 fragment of EHV-1 subtype 2 were digested with Bam HI and electrophoresed on a 1% low melting temperature agarose gel. Approximately 10µg of Bam HI 1 were purified by phenol extraction of the appropriate gel slice. The DNA was resuspended in 50µl of ligase buffer (500mM Tris-HCl pH 7.5, 100mM $MgCl_2$, 100mM dithiothreitol) to which 10 units of ligase were added and incubated overnight at 15°C. The sample was then diluted to 0.5ml in NTE, sonicated at 0°C for 7 x 3 sec with 1 min intervals and ethanol precipitated. The ends of the sheared fragments were repaired by resuspending the DNA in a 20µl solution of 67mM Tris-HCl pH 7.9, 6.7mM $MgCl_2$, 5mM dithiothreitol, 0.2mM each of dATP, dGTP, dCTP and TTP and 10 units of T4 polymerase. The reaction was incubated for 2 - 3 hours at 15°C. The repaired DNA was extracted twice with an equal volume of NTE-equilibrated phenol and precipitated by adding 2.5 volumes of ethanol on dry ice. The centrifuged pellet was washed in 70% ethanol, dried, resuspended in 8% PEG and 0.41M NaCl, and left overnight on ice. The DNA was pelleted and washed in 70% ethanol. It was then resuspended in 50µl NTE and precipitated by the addition of an equal volume of isopropanol on dry ice. The pellet was washed with 70% ethanol and dissolved in 20µl of distilled water. Gel electrophoresis showed that sheared fragments were sized 400-1,500bp. 5µg of the processed EHV-1 DNA fragments were ligated as described above into 1µg of

M13mp18 DNA which had been cleaved with Sma I and treated simultaneously with calf intestinal phosphatase. 100 units of T4 DNA ligase were used to ligate the DNA in a volume of 20µl. Competent cells for transfection by the ligated DNA were prepared as follows. A colony of E. coli K12 strain JM101 (Messing, 1979) was inoculated into 100ml of 2YT broth and incubated until the optical density at a wavelength of 630nm was 0.3. The cells were harvested by centrifugation at 8,000 r.p.m. for 2 min at 8°C and resuspended in 50ml of ice-cold 50mM $CaCl_2$. After 30 min on ice the cells were again pelleted by brief centrifugation at 8,000 r.p.m. The pellet was resuspended in 5ml of ice-cold 50mM $CaCl_2$ and kept on ice. Appropriate controls were included in the transfection. 0.2ml aliquots of calcium-shocked cells were added to tubes containing the ligation reactions, mixed well and left on ice for 40 - 60 min. The cells were then incubated at 42°C for 2 min. E. coli JM101 cells in exponential phase (0.2ml), isopropyl-β-D-thio-galactopyranoside (25ul of 25mg/ml in water) and 5-bromo-4-chloro-3-indoyl-β-galactosidase (25ul of 25mg/ml in dimethylformamide) were dispensed into 3ml aliquots of H-top agar (10g bactotryptone, 8g NaCl and 8g agar per litre) at 42°C and the transformed cells added. The mixture was swirled before plating out on L-broth-agar plates (7.5g/l bacto-agar in L-broth). The plates were inverted and incubated overnight at 37°C. Colourless plaques were picked with sterile cocktail sticks into 1.5ml aliquots of a 1/100 dilution of an overnight JM101 culture in 2YT broth, and incubated at 37°C for six hours in an

T A B L E 1.1

	dGTP mix	dATP mix	TTP mix	dCTP mix
0.5mM dTTP	200	200	10	200
0.5mM dCTP	200	200	200	10
0.5mM dGTP	10	200	200	200
50mM Tris-HCl pH 8 1mM Na_2EDTA	50	50	50	50

	0.2mM ddGTP	0.7mM ddATP	0.3mM ddTTP	0.7mM ddCTP
5mM ddGTP	40	-	-	-
5mM ddATP	-	14	-	-
5mM ddTTP	-	-	60	-
5mM ddCTP	-	-	-	14
water	960	986	940	986

	dNTPmix	ddNTP mix	Water	
dGTP/ddGTP mix	400	150	250	
dATP/ddATP mix	400	200	200	
dTTP/ddTTP mix	400	400	-	
dCTP/ddCTP mix	400	200	200	

orbital incubator. The bacteria were pelleted by microfuging for five min. Each supernatent was decanted into a fresh tube, and the phage precipitated by the addition of 150ul 20% polyethylene glycol 6000 in 2.5M NaCl. The preparations were mixed well and left overnight at 4°C. The phage was pelleted by microfuging for 5 min, resuspended in 100µl TE and extracted with 50µl of TE-equilibrated phenol. Phage DNA was ethanol precipitated, washed with 70% ethanol and lyophilised. The lyophilised pellets of single-stranded DNA were redissolved in 30µl of distilled water and stored at -20°C. Of the 319 templates prepared in this way the DNA sequences of 305 were determined and those of 186 formed the database.

DNA Sequencing Reactions

Eighteen templates were sequenced and electrophoresed on a single gel. 9µl of annealing mix (1µl TM buffer, 1µl pentadecamer primer, 7µl distilled water) were aliquoted into 18 1.5ml tubes. 1µl of template was added to each of the tubes, which were whirlimixed and then incubated at 37°C for 30 min. Meanwhile, 40µl dATP mix (23.3µl 0.5mM dATP, 976.6µl water) were added to 8µl (80µCi) [^{32}P]dATP (PB.10204; Amersham) and whirlimixed briefly. dNTP/ddNTP mixes were made as shown in Table 1.1.

Approximately 80 units of the Klenow fragment of E. coli DNA polymerase I were added to 50µl 0.1 x TM, whirlimixed briefly, and then 2µl were added to each annealed template. The templates were centrifuged briefly and 2µl of each template was dispensed into the bottom of four wells of a 96-well round-bottomed Nunc microtitre

plate. 2µl aliquots of the appropriate [^{32}P]dATP/dNTP/ddNTP mix were aliquoted onto the side of the appropriate wells. The lid of the microtitre plate was replaced and the drops combined by tapping the plate sharply against the edge of the bench. The plate was then whirlimixed well, and incubated at 31°C for 15 min. At the end of this period, 40 units of the Klenow fragment of DNA polymerase I were added to 150µl dNTP chase mix (dNTPs at 0.25mM), and 2µl aliquots were added to the side of each well and mixed as described above. The reactions were incubated at 31°C for 30 min and electrophoresed either immediately or as after storing overnight at -20°C

Preparation of DNA Sequencing Gels.

Two 45 x 45cm glass plates, one notched, were cleaned thoroughly. The notched plate was then scrubbed with 5ml of ethanol, then coated with 5ml of B.D.H. Repelcote and scrubbed again with 5ml of ethanol. The plain glass plate was scrubbed with 5ml ethanol and coated with Wacker solution (5ml ethanol, 0.15ml 10% acetic acid, 25µl Wacker) and then scrubbed vigorously three times with 5ml ethanol. The two plates were then assembled, treated sides inwards, with Sylglass tape after applying 0.6mm Plasticard spacers. The gel mixes were prepared. The basic solutions are 10 x TBE, 40% acrylamide (380g/l acrylamide, 20g/l N,N'-methylenebis-acrylamide, deionized) and 575g/l deionized urea. Top gel mix for each gel was made by combining 80ml urea with 5ml 10 x TBE and 15ml of 40% acrylamide solution. Bottom gel mix consists of 460g/l urea, 250ml/l 10 x TBE,

150ml/l 40% acrylamide, 50g/l sucrose, 50mg/l bromophenol blue.

Two foldback clips were fixed to each edge of the gel sandwich before pouring the gel. 120µl of 25% (wt/vol) ammonium persulphate were added to 100ml top gel mix, and 28µl to 14ml bottom gel mix. The same volumes of TEMED were then added to the solutions. 12ml of polymerizing top gel mix were taken up immediately into a 25ml pipette, followed by 12ml of polymerizing bottom gel mix. Four or five air bubbles were allowed to pass up the pipette, and the gradient was poured down one side into the gel sandwich, which was positioned vertically. A 60ml syringe was then used to quickly pour 40ml polymerizing top gel mix across the width of the gel, followed by 30ml down the other side of the sandwich. All trapped air bubbles were tapped out vigourously with the heel of the hand, the gel was supported at an angle of 10 - 15^{o} from the horizontal and two 40-tooth 0.6mm Plasticard combs were placed into the top of the gel and clipped into position with foldback clips. When the gel had set completely (about 10 min), the clips and the tape at the bottom of the sandwich were removed, and the sandwich was placed in a gel kit. Three litres of 1 x TBE was divided between the top and bottom buffer tanks. The combs were removed carefully and the wells washed out with buffer from the top tank. 2µl of 1g/l bromophenol blue, 1g/l xylene cyanol FF, 10mM Na_2EDTA in deionized formamide were added to the sequencing reactions and the DNA denatured by heating in a boiling bath for 1 min. The lanes in the first half of the gel

were washed out again and loaded with 3µl of the samples using a 10µl Hamilton syringe. When the first half of the gel was loaded, the second half was washed out and loaded. Eighteen templates were electrophoresed on 72 lanes in each gel at 60 watts for 2.5 to 3 hours, until about 15 min had elapsed after the bromophenol blue dye had electrophoresed from the bottom of the gel. The radioactive tank buffer was syphoned out, the gel removed from the kit, the remaining Sylglas tape removed and the glass plates separated. The plain glass plate, to which the gel was covalently attached, was soaked in 10% acetic acid for 30 min with occasional shaking. The gel was rinsed with water and baked in a circulating hot air oven at 130°C for at least 2 hours. The dried gel was allowed to cool and exposed to a sheet of 35 x 43cm Kodak XS1 (1596) film for 24 - 48 hours.

Sequencing Gel Artefacts

Problems associated with secondary structure, which give rise to "compressed" regions in the sequencing ladder were solved by substituting dGTP and ddGTP by dITP and ddITP in the sequencing reactions. The 0.5mM dGTP in the dNTP mixes was replaced by 2.0mM dITP and the 0.2mM ddGTP was replaced by 1mM ddITP. The chase mix was as described above.

Interpretation and Analysis of Sequencing Data

DNA sequence data were manipulated and analysed in a DEC PDP -11/44 computer running under the R5X11M system. The programs described by Staden (1977, 1978, 1979, 1980,

1982) were used to screen gel readings for vector pUC9 sequences and Bam HI sites, to construct the database and to analyse the resulting completed sequence. Codon usage was examined by the program of Staden and McLachlan (1982). Amino acid sequences were obtained using the translation program of Taylor (1986). Analysis of predicted amino acid sequences was carried out using a matrix comparison program (Pustell and Kafatos, 1982), an alignment program (Taylor, 1984) and a hydrophobicity program (Kyte and Doolittle, 1982).

CHAPTER 3

STUDIES ON THE STRUCTURE OF THE EHV-1 SUBTYPE 2 GENOME

RESULTS

Introduction

It has recently been shown that viruses currently classified as equine herpesvirus type 1 (EHV-1) represent two distinct herpesviruses: subtype 1, primarily an abortigenic virus, and subtype 2, primarily associated with upper respiratory tract disease. Almost all available molecular data concern the former, but at the commencement of this work nothing was known about the genome structure of the latter. Thus, the main objectives of the first part of this project were to characterize the genome of the respiratory subtype of EHV-1 in order to determine whether the two subtypes share a common genome structure, and to construct the first restriction endonuclease maps of the virus DNA.

Virus Growth and DNA Preparation

The abortigenic subtype of EHV-1 grows on a wide variety of animal cell lines. Bagust (1971) listed 17 primary cultures and 14 continuous cell line cultures which support virus growth. Plummer and Waterson (1963) recorded the ready propagation of EHV-1 in rabbit kidney cells. The virus grows to a moderately high titre in cell culture. For example, in the work described here, stocks of EHV-1 subtype 1 strain Vo1939 with titres of 10^8 p.f.u./ml and particle/p.f.u. ratios of approximately 10^2 were prepared on RK13 cells.

Subtype 2 of EHV-1 grows to a lower titre in cell culture and shows a greater host cell specificity than the

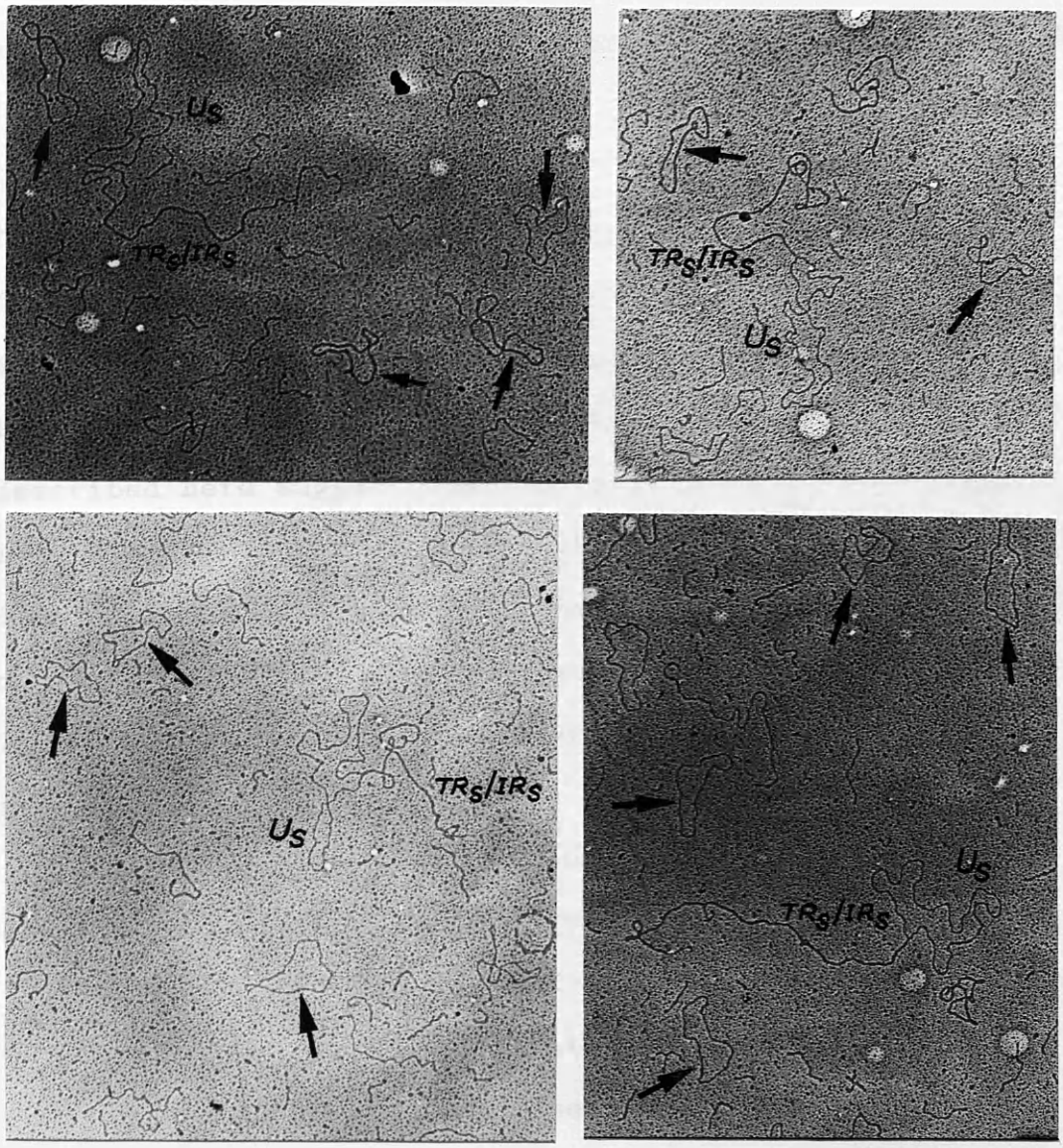
abortigenic virus (Burrows and Goodridge, 1973; Studdert and Blackney, 1979). The subtype 2 virus used in this work was plaque-purified on NBL-6 cells, the only equine cell line commercially available in the U.K. One disadvantage of this cell line is that on some occasions monolayers failed to attain confluency. Subtype 2 isolates have been grown on pig kidney cells (Burrows and Goodridge, 1973; Studdert and Blackney, 1979), so initial attempts were made to plaque-purify the virus on PK(15), an established cell line. These cells were more reliable in their growth properties than NBL-6 cells, but plaques were smaller and virus yields were lower. Therefore, NBL-6 cells were used for plaque purification and preparation of working stocks of respiratory isolate 1942. Titres of up to 10^6 p.f.u./ml with particle/p.f.u. ratios of 1-5 x 10^3 were obtained. Virus growth in NBL-6 cells yielded negligible amounts of DNA, but an average yield of 1-2μg of virus DNA per roller bottle was obtained when the virus was cultured in an SV40-transformed sheep cell line (SHELUT).

Electron Microscopy of EHV-1 DNA

EHV-1 subtype 2 DNA was denatured and the single strands allowed to self-anneal as described in the Methods. The aim of this approach was to identify inverted repeat sequences in the EHV-1 subtype 2 genome. Figure 3.1 shows electron micrographs of EHV-1 self-annealed single strands. Each consists of a single-stranded loop joined to a double-stranded region contiguous with a long single strand. The structures are interpreted as the result of annealing of a

FIGURE 3.1

Electron micrographs of annealed single-stranded EHV-1 subtype 2 DNA molecules. The molecules show a single-stranded loop (U_S) with a double-stranded stem (TR_S/IR_S) contiguous with a long single-stranded tail. Single-stranded ϕX174 DNA molecules are indicated by arrows.


Us
TRs/IRs
TRs/IRs
Us
TRs/IRs
Us
Us
TRs/IRs

terminal sequence to an internal inverted complementary sequence to form a double-stranded region. The single-stranded loop connecting these sequences and the long single-stranded tail, thus represent unique sequences. The results were similar to those obtained in electron microscopic studies of annealed single strands from PRV DNA (Stevely, 1977; Ben-Porat et al.,1979), EHV-1 subtype 1 (Ruyechan et al., 1982) and EHV-3 (Atherton et al., 1982). The genome structure of these viruses consists of an L (long) segment of unique sequence linked to an S (short) segment comprising an unique sequence (U_S) flanked by inverted repeat sequences (TR_S/IR_S). Therefore, the data described here suggest that the genome of EHV-1 subtype 2 is similar to those of EHV-1 subtype 1, EHV-3 and PRV. The loop in the electron micrographs would represent U_S and the double-stranded region would result from the annealing of TR_S and IR_S. The long single-stranded tail would represent L.

No "dumbell-shaped" molecules (two single-stranded circles joined by a duplex region), indicative of an HSV-type genome structure (group E), were observed, and there was no evidence to suggest that sequences from the L terminus were also present elsewhere in the genome in inverted orientation. However, it was concluded from the variable length of the single-stranded tails that no full length EHV-1 DNA molecules were observed. This was probably owing to the presence of nicks or gaps within the DNA (O'Callaghan et al., 1983). Thus, the presence of additional inverted repeats in the L segment is not ruled

FIGURE 3.2a

Histogram showing the length distribution of molecules of single-stranded ϕX174 DNA.

Total number of molecules: 394

Mean length: 5,385.51n (actual length = 5,386n)

Standard deviation: 436.29n

FIGURE 3.2b

Histogram showing the length distribution of single stranded loops of self-annealed EHV-1 subtype 2 DNA. The peak at around 5,500 nucleotides (indicated by the arrow) probably represents the juxtapositioning of ϕX174 molecules with EHV-1 DNA. Values between 12,000 and 14,500 nucleotides which constitute the second major peak were used to estimate the mean size of U_S.

Sample size: 27

Mean length: 13,304.58n

Standard deviation: 511.30n

Figure 3·2a

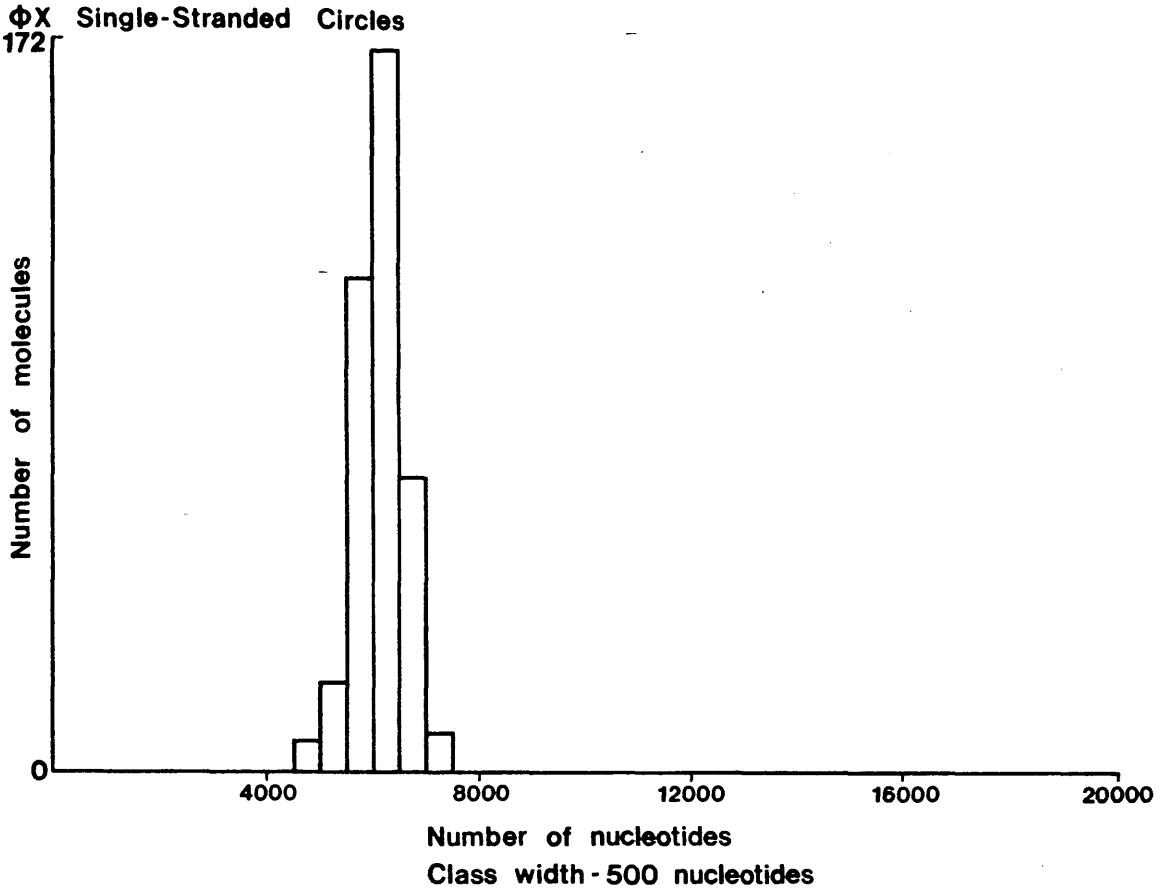
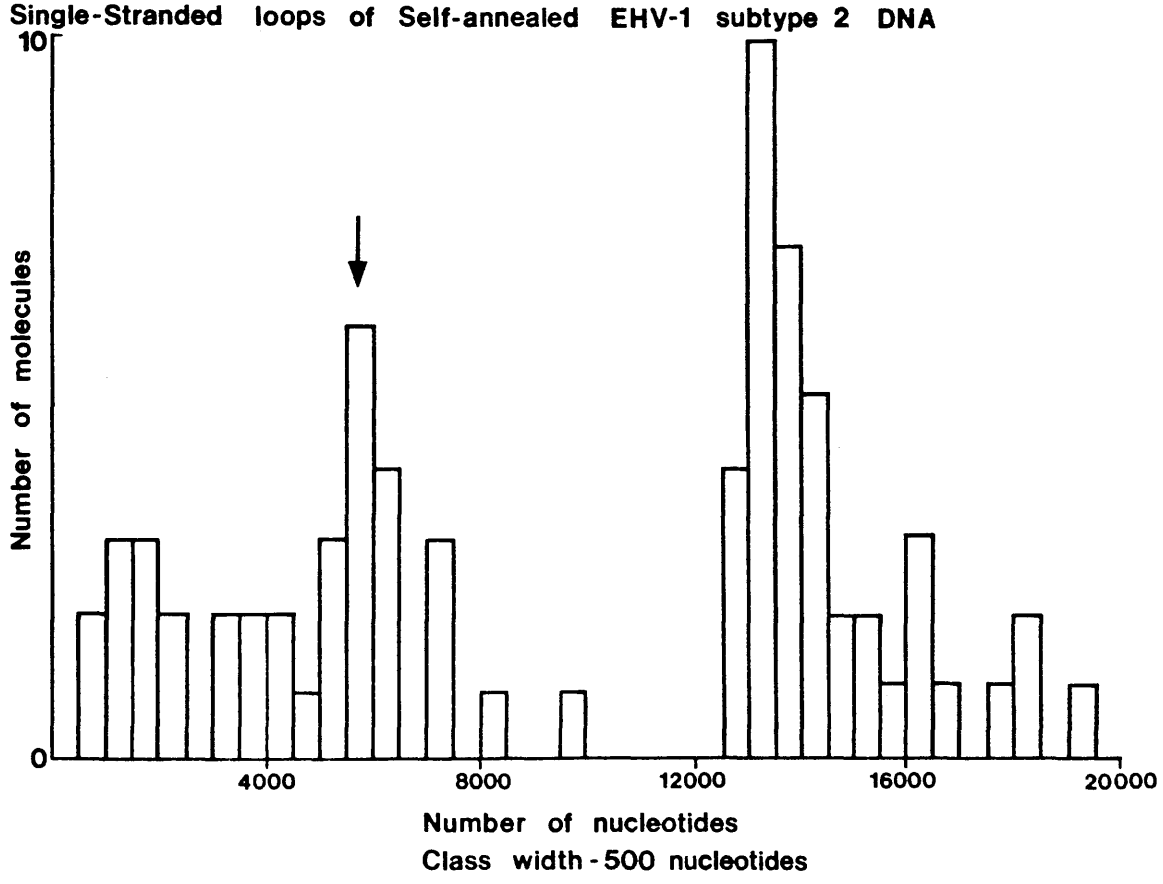


Figure 3·2b



out by the electron microscopic data.

The lengths of single-stranded loops in self-annealed molecules were measured using a Summagraphics digitizing tablet. The size of U_S was calculated from the ratio of these lengths to those of single-stranded ϕX174 DNA. Histograms of the measurements of ϕX174 and EHV-1 subtype 2 DNA are shown in Figures 3.2a and 3.2b, respectively. The EHV-1 single-stranded loops are represented by a much wider range of values than the ϕX174 circles. The reason for this is unknown, but it is possible that the ends of the single-stranded L regions of some EHV-1 DNA molecules may have folded back to give apparent loops. The larger loops may be due to partial dissociation of double-stranded stems. The first peak at 5,500n for the measurement of EHV-1 loops (see arrow in Figure 3.2b) coincides with the peak value in measurements of single-stranded ϕX174 circles (Figure 3.2a) and is probably an artefact due to juxtapositioning of ϕX174 molecules with EHV-1 DNA. To calculate the size of U_S of EHV-1 subtype 2, only the values representing the main peak, between 12,000 and 14,500n were considered. The mean value of the 27 samples in this range was 13,304.38n. The size of U_S was estimated at 13,306n, a result derived by dividing the product of the known length of single-stranded ϕX174 (5,386n; Sanger et al., 1978) and the appropriate mean sample value of the EHV-1 loops (13,304.38n) by the mean value of the measurements of the ϕX174 circles (5,385.51n).

Measurement of the double-stranded stem structures characteristic of the inverted repeat sequences was

problematic as the boundary of the duplex was difficult to distinguish, but the largest measurements gave an estimated length of 8,896bp by comparison with measurements of ϕX174 RF DNA.

Restriction Enzyme Patterns of EHV-1 DNA

It has been recognized for some time that EHV-1 isolates from the respiratory tract tend to differ from those from aborted foetuses on the grounds of epidemiology (Miller, 1966), pathogenicity (Burrows and Goodridge, 1973) and growth characteristics *in vitro* (Burrows and Goodridge, 1973; Studdert and Blackney, 1979). More recently, it has been shown both with Australian and American isolates that the two virus subtypes can be clearly differentiated by restriction endonuclease analysis (Sabine *et al.*, 1981; Studdert *et al.*, 1981; Turtinen *et al.*, 1982). Samples of purified DNA from the two British isolates used in this study were digested with restriction enzymes *Bam* HI and *Eco* RI and separated by agarose gel electrophoresis. Figure 3.3a shows that the respiratory isolate (1942) is clearly distinguishable from the abortigenic isolate (Vo1939). The restriction endonuclease analysis of the two British isolates correlates well with the published profiles of the two subtypes (Allen *et al.*, 1983a; Studdert, 1983). The electropherotype common to the majority of isolates from cases of EHV-1-associated abortion in the central area of Kentucky from 1960 to 1981 was designated 1P. This strain now appears to have been largely replaced by one with a different electropherotype designated 1B (Allen *et al.*, 1985). Vo1939 has the restriction profile of a 1P isolate.

FIGURE 3.3

Restriction endonuclease analysis of EHV-1 DNA

(a) Horizontal 0.7% agarose gel showing products of EHV-1 subtype 1 and subtype 2 DNA digested with restriction endonucleases.

The molecular weights of the EHV-1 subtype 2 DNA fragments were calibrated from the *Hind* III digest of λ DNA shown in tracks 1 and 6.

Figure 3·3a

Restriction Endonuclease Analysis of EHV-1 DNA

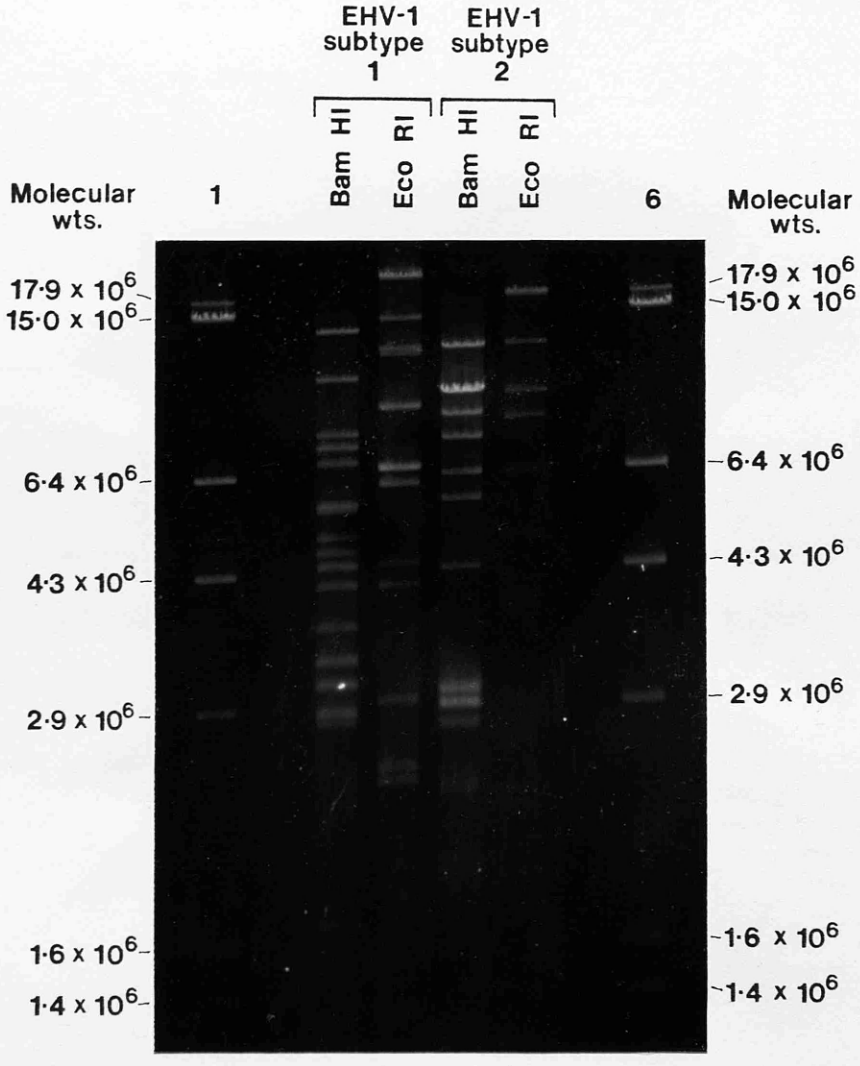


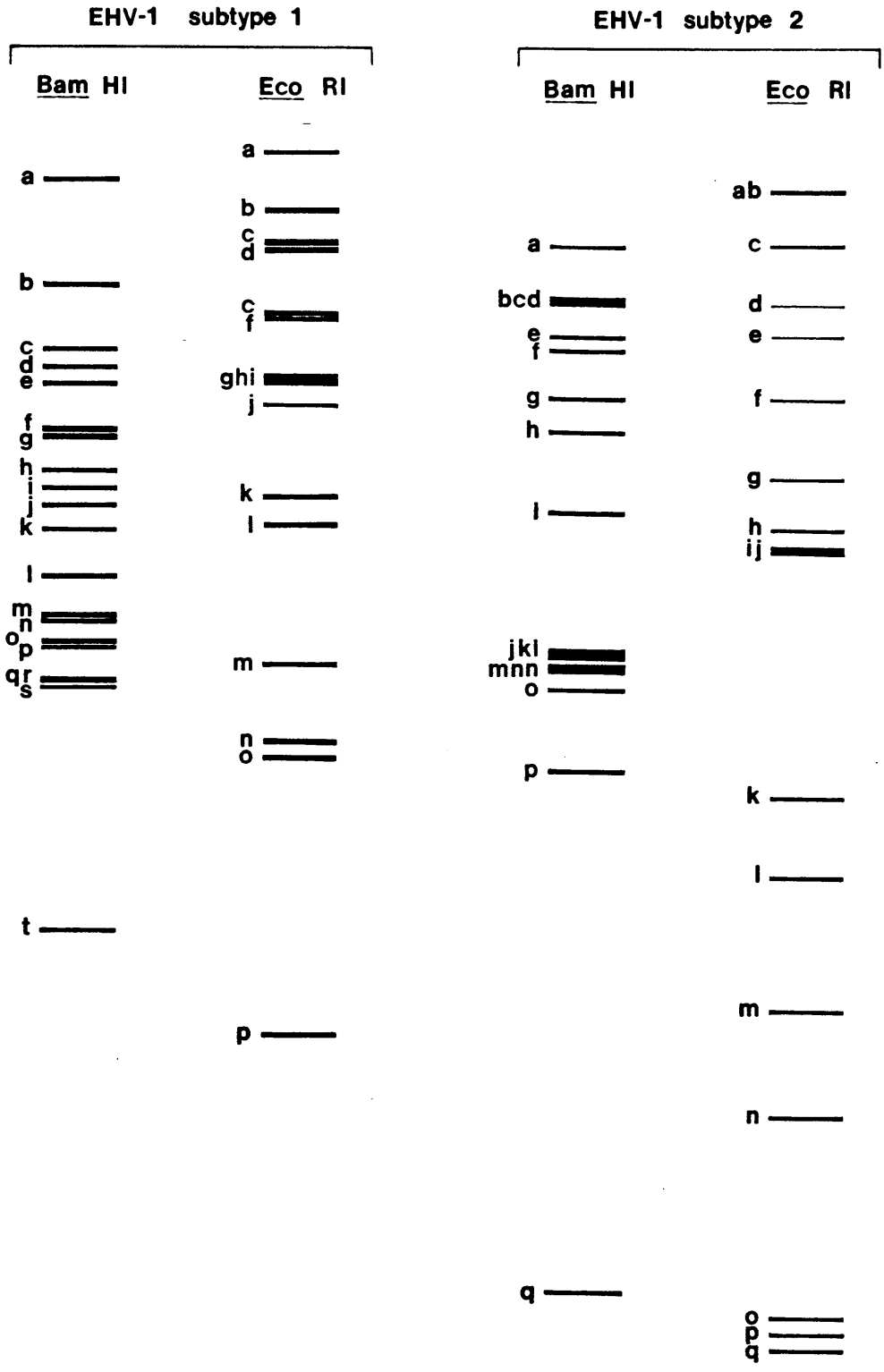
FIGURE 3.3 (continued)

Restriction endonuclease analysis of EHV-1 DNA

(b) A diagrammatic representation of Bam HI and Eco RI digests of the DNA of both subtypes of EHV-1, showing the assignment of letters to bands.

Figure 3·3b

Restriction Endonuclease Analysis of EHV-1 DNA



T A B L E 3.1

SIZES (kbp) OF EHV-1 SUBTYPE 2 DNA FRAGMENTS

Bam HI			Eco RI		
Fragments	Estimated Size	Estimated Molarity	Fragments	Estimated Size	Estimated Molarity
a	18.5	1	ab	27.5	2
bcd	14.1	3	c	18.5	1
e	12.7	1	d	14.1	1
f	11.0	1	e	12.0	1
g	9.5	1	f	9.5	1
h	8.4	1	g	7.3	1
i	6.4	1	h	6.3	1
jkl	4.6	3	ij	6.0	2
mnn	4.5	3	k	3.4	1
o	4.2	1	l	2.9	1
p	3.6	1	m	2.2	1

Molecular Weights of Restriction Digest Fragments of EHV-1 Subtype 2 DNA

The molecular weights of restriction endonuclease fragments of EHV-1 subtype 1 DNA have been published (Whalley et al., 1981; Henry et al., 1981). Figure 3.3a shows that digestion of EHV-1 subtype 2 DNA with restriction endonucleases Bam HI and Eco RI yielded 11 visible bands in each case, when electrophoresed in a 0.7% agarose gel. The molecular weights of fragments were determined by comparison with a Hind III digest of phage λ DNA (Maniatis et al., 1975). The distance migrated by each DNA fragment was plotted against the logarithm of the molecular weight, and the calibration curve was then used to estimate the molecular weights of EHV-1 restriction fragments. Bam HI digestion generated fragments ranging from 18.5kbp to 3.6kbp in size, and Eco RI fragments ranged from 27.5kbp to 2.2kbp. Fragments of both digests were assigned letters alphabetically in descending order of molecular weight (Figure 3.3b, Table 3.1). The relative molarities of the bands of EHV-1 subtype 2 DNA visualized in agarose gels were estimated from densitometric traces (Table 3.1) and these values were supported by hybridization studies using EHV-1 subtype 2 DNA fragments isolated from virion DNA or cloned in a plasmid vector. The relative molarities of the bands were taken into account when estimating the size of the genome. The sum of the sizes of individual restriction fragments gives the approximate size of the entire EHV-1 subtype 2 genome as 144kbp.

FIGURE 3.4

Autoradiograph showing the results of hybridizing [^{32}P]-labelled Bam HI fragments of EHV-1 subtype 2 DNA to a Bam HI digest of the same DNA.

The radiolabelled fragments were hybridized for approximately 12 hours to unlabelled DNA immobilized on nitrocellulose at 42^{o}C in hybridization solution containing 50% formamide. The nitrocellulose strips were then washed at 50^{o}C for three hours and autoradiographed.

A summary of the results of this experiment is shown in Table 3.2.

EHV-1 subtype 2 Bam HI

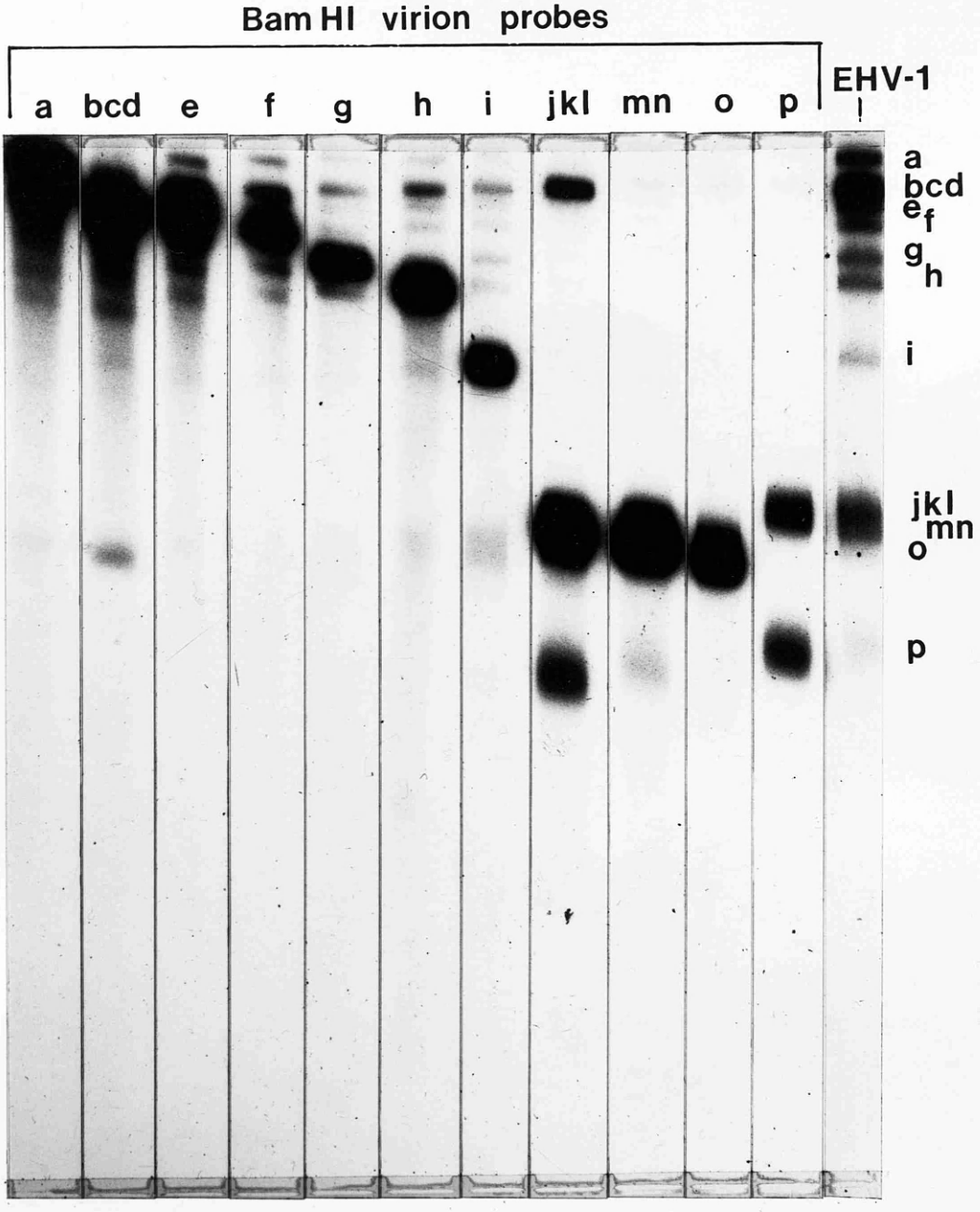


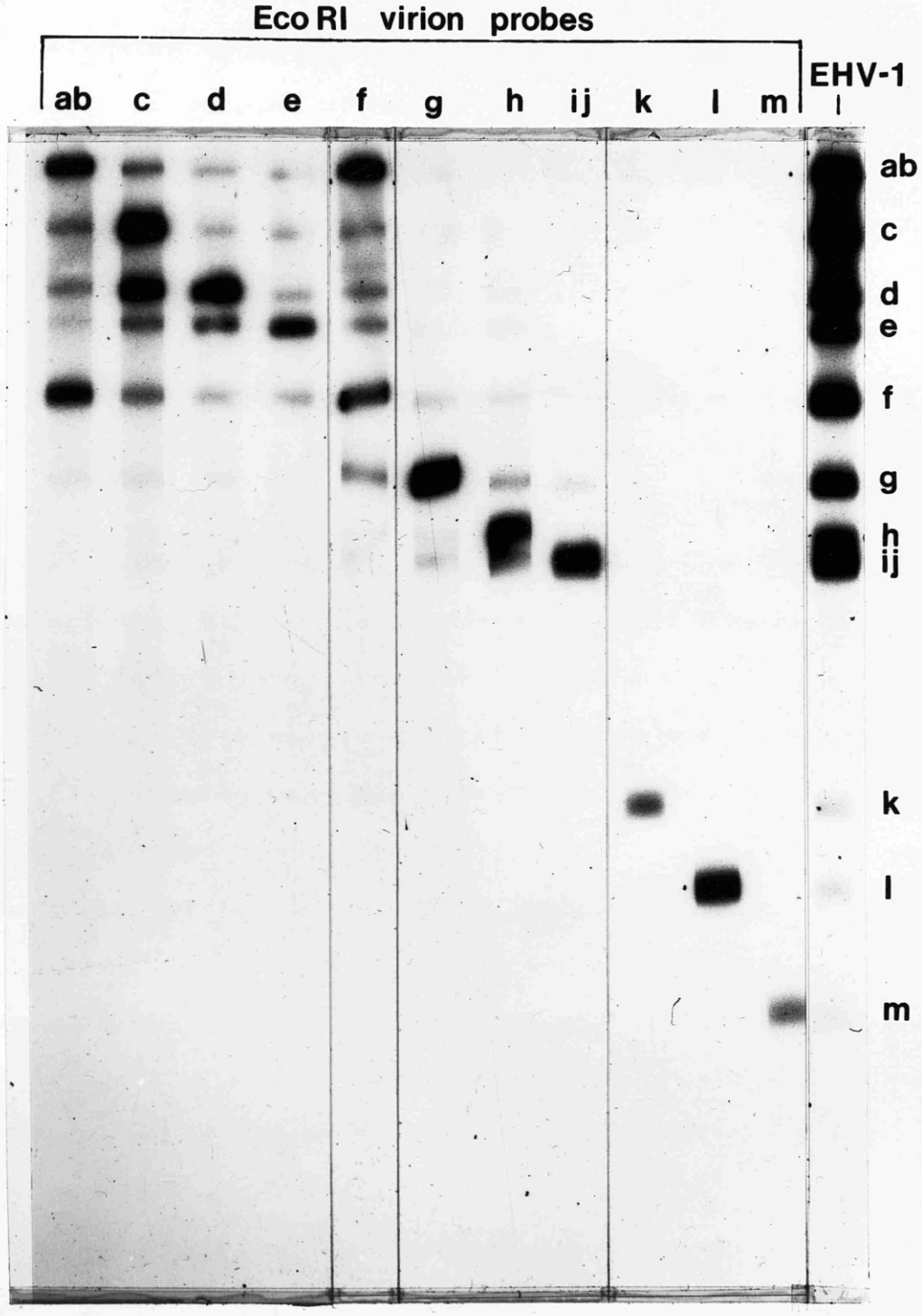
FIGURE 3.5

Autoradiograph showing the results of hybridizing [^{32}P]-labelled Eco RI fragments of EHV-1 subtype 2 DNA to an Eco RI digest of the same DNA.

Conditions of hybridization and washing were identical to those referred to in Figure 3.4.

A summary of the results is shown in Table 3.3.

EHV-1 subtype 2 EcoRI



Hybridization Studies of EHV-1 Subtype 2 DNA Using Probes Isolated from Virion DNA

Sequence homology between DNA fragments may be investigated by hybridization studies using the blotting technique developed by Southern (1975). Figure 3.4 shows an autoradiograph of the results of hybridizing [^{32}P]-labelled Bam HI fragments of EHV-1 subtype 2 DNA to a Bam HI digest of EHV-1 subtype 2 DNA. This experiment was designed to verify the presence of the repeated DNA sequences detected by electron microscopy. A Bam HI digest of EHV-1 subtype 2 DNA was electrophoresed in a 0.5% agarose gel and transferred onto nitrocellulose. A second Bam HI digest of subtype 2 DNA was electrophoresed in a 1% low melting temperature agarose gel. The fragments were excised individually, purified and nick translated. The nitrocellulose blot was cut into strips and radiolabelled Bam HI fragments used as probes. The results are shown in Figure 3.4 and summarized in Table 3.2. As expected, each DNA probe hybridized to the equivalent fragment in the Bam HI digest. Each probe fragment was unavoidably contaminated with fragments which migrated above and below it in the low melting temperature gel. For example, in the first track of Figure 3.4, hybridization of probe a to band bcd was due to the contamination of probe a with bcd. The hybridization resulting from contaminating fragments was taken into account when formulating Tables 3.2 and 3.3. However, the hybridization of probe bcd to jkl, of probe jkl to bcd and to p, and probe p to jkl cannot be explained on the basis of contamination. These results indicate the

FIGURE 3.6

Autoradiograph showing the results of hybridizing [^{32}P]-labelled Bam HI fragments of EHV-1 subtype 2 DNA to an Eco RI digest of the same DNA. Conditions for hybridization and washing were identical to those referred to in Figure 3.4. A summary of the results is shown in Table 3.2.

The hybridization of probe Bam HI jkl to Eco RI p and of probe Bam HI bcd to Eco RI q was clearer on a longer autoradiographic exposure.

EHV-1 subtype 2 Eco RI

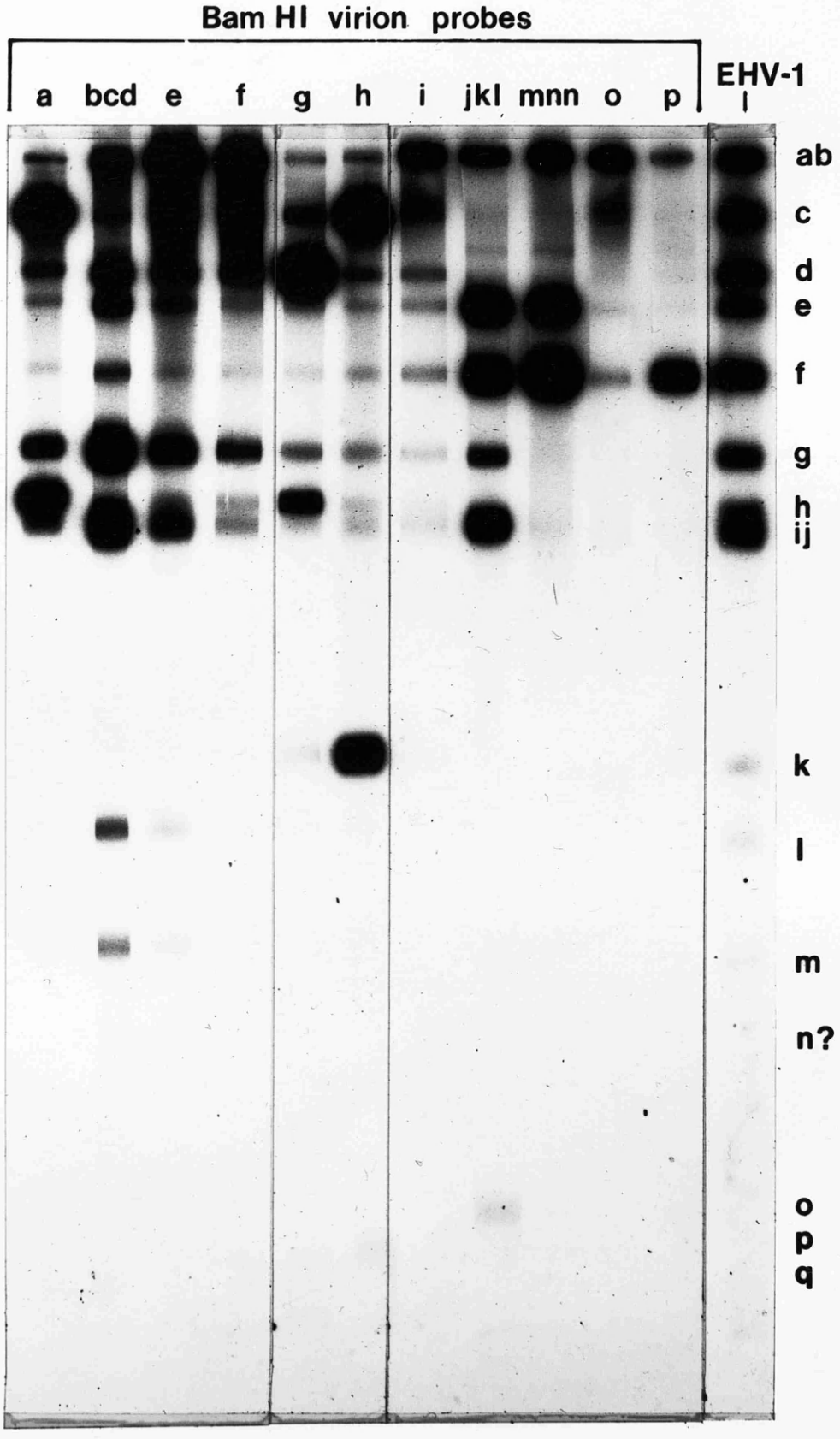
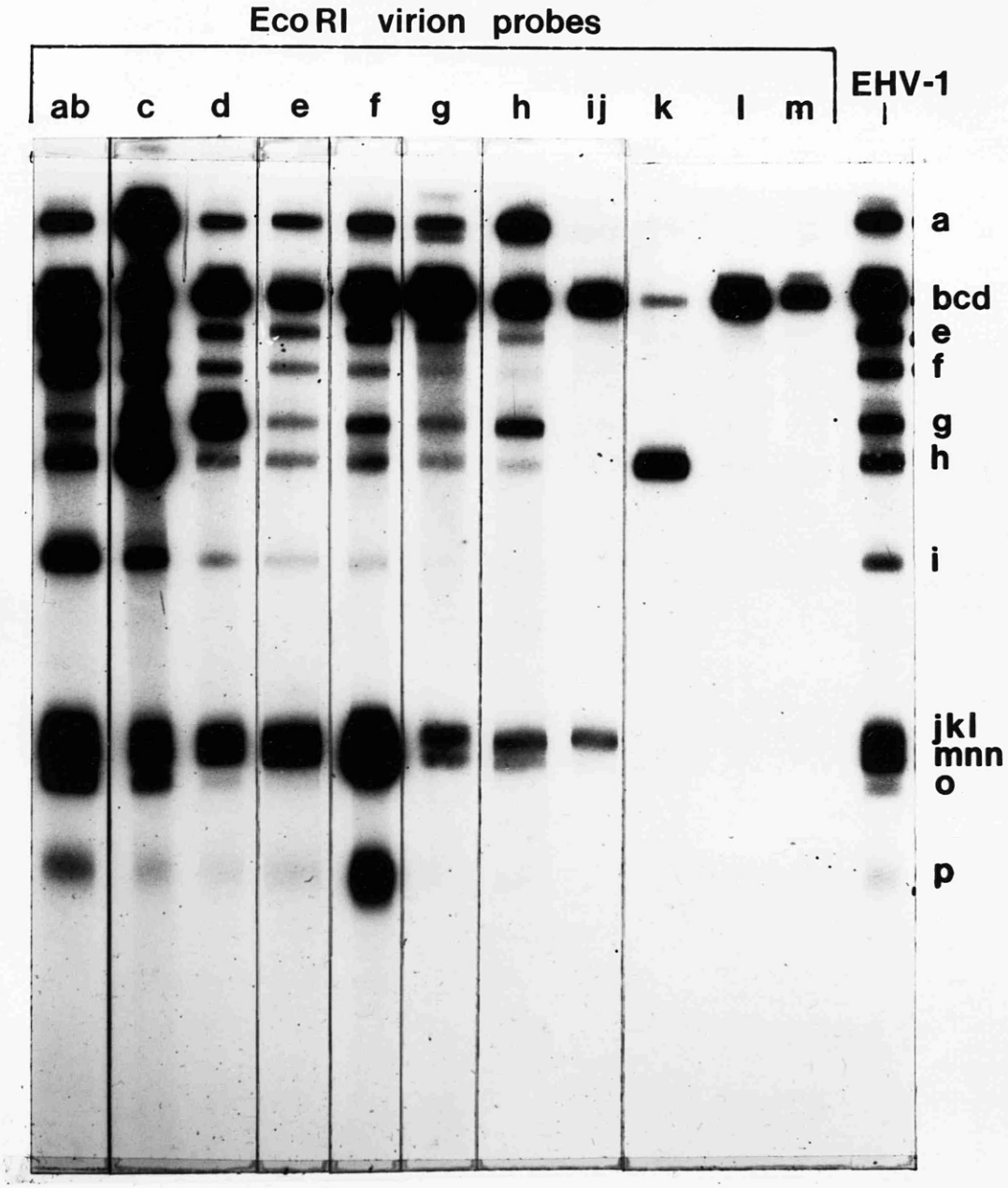


FIGURE 3.7

Autoradiograph showing the results of hybridizing [^{32}P]-labelled Eco RI fragments of EHV-1 subtype 2 DNA to a Bam HI digest of the same DNA.
Conditions for hybridization and washing were identical to those referred to in Figure 3.4. A summary of these results is shown in Table 3.3.

EHV-1 subtype 2 Bam HI



T A B L E 3.2

Results of Hybridizing-Nick-Translated Bam HI Fragments prepared from EHV-1 Subtype 2 Virion DNA, to Bam HI or Eco RI Fragments of EHV-1 Subtype 2 DNA.

Bam HI Probe	Hybridizing Bam HI Fragment	Hybridizing Eco RI Fragment
a	a	c,h
bcd	bcd,jkl	ab,d,e,f,g,ij,l,m,n,q
e	e	ab
f	f	ab
g	g	d,h
h	h	c,k,p
i	i	ab
jkl	jkl,bcd,p	ab,e,f,g,ij,o,p
mnn	mnn	ab,e,f
o	o	ab
p	jkl,p	ab,f

T A B L E 3.3

Results of Hybridizing Nick-Translated Eco RI Fragments prepared from EHV-1 Subtype 2 Virion DNA to Eco RI or Bam HI Fragments of EHV-1 Subtype 2 DNA.

Eco RI Probe	Hybridizing Eco RI Fragments	Hybridizing Bam HI Fragments
ab	ab,f	bcd,e,f,i,jkl,mnn,o,p
c	c	a,h
d	d	bcd,g
e	e	bcd,jkl,mnn
f	f,ab	bcd,jkl,mnn,p
g	g,ij	bcd,jkl
h	h	a,g
ij	ij,g	bcd,jkl
k	k	h
l	l	bcd
m	m	bcd

presence of repeated DNA sequences within the EHV-1 subtype 2 genome.

Figure 3.5 shows an autoradiograph of the hybridization of [^{32}P]-labelled Eco RI fragments isolated from virion DNA to an Eco RI digest of EHV-1 subtype 2 DNA. These data are summarized in Table 3.3. Again, each fragment hybridized to its equivalent, and the existence of repeated sequences is demonstrated by the hybridization of probe ab to f and probe f to ab. There is also some indication that probe ij hybridized to g and that probe g hybridized to ij.

Figure 3.6 shows an autoradiograph of the hybridization of Bam HI probes prepared from virion DNA to an Eco RI digest of EHV-1 subtype 2 DNA, and Figure 3.7 shows the hybridization of Eco RI probes prepared from virion DNA to a Bam HI digest. These data are summarized in Tables 3.2 and 3.3. Both Eco RI ab and f hybridized to, among other fragments, Bam HI bcd, jkl and p. Similarly, in addition to other fragments, Bam HI bcd, jkl and p hybridized to Eco RI ab and f. These results are consistent with the presence of portions of these fragments in repeated regions of the genome.

In addition to identifying fragments from the repeated regions, it was possible from these data to determine the relative locations of several fragments in other regions of the EHV-1 subtype 2 genome. For example, Eco RI h hybridized to Bam HI a and g, implying that these two Bam HI fragments are adjacent to each other in the genome. In turn, Bam HI a hybridized to Eco RI c and h implying that

Eco RI c and h are beside each other in the genome. However, the presence of bands containing more than one fragment (e.g. Bam HI bcd and jkl), and the fact that the majority of these gave very complex hybridization patterns owing to the presence of component fragments in repeated regions, made it impossible to derive complete restriction endonuclease maps from these data alone. Fragment contamination also gave rise to major problems in the interpretation of the results.

Cloning of the EHV-1 Subtype 2 Genome and Use of Clones in Mapping Studies

Bam HI fragments of EHV-1 subtype 2 DNA were cloned into plasmid vector pUC9 and propagated in E. coli K12 strain JM83 (Vieira and Messing, 1982). Figure 3.8 demonstrates that most of the clones contained small inserts and that clones containing larger inserts were rare. Of the 112 cloned EHV-1 subtype 2 DNA fragments analysed, none was larger than 10kbp. Additional clones were prepared and propagated in E. coli K12 strain DH1 (Hanahan, 1983). 117 clones with inserts were analysed. Most of the larger Bam HI fragments of EHV-1 subtype 2 were cloned successfully. A library of 13 clones which represents approximately 75% of the genome was compiled and a stock of each plasmid DNA was prepared. Clones of Bam HI k,p,d and e were not obtained. The hybridization and restriction endonuclease double digest data discussed below suggested that clone 4 may contain Bam HI k and Bam HI b with the loss of a Bam HI site. However, the structure of

FIGURE 3.8

Electrophoretic analysis of plasmids containing EHV-1 subtype 2 Bam HI inserts. Approximately 1ug of each plasmid was digested to completion with Bam HI and electrophoresed on a horizontal 1% agarose gel. The centre track shows standard EHV-1 subtype 2 DNA digested with Bam HI.

Figure 3·8

Electrophoretic analysis of plasmids containing EHV-1 subtype 2 Bam HI fragments

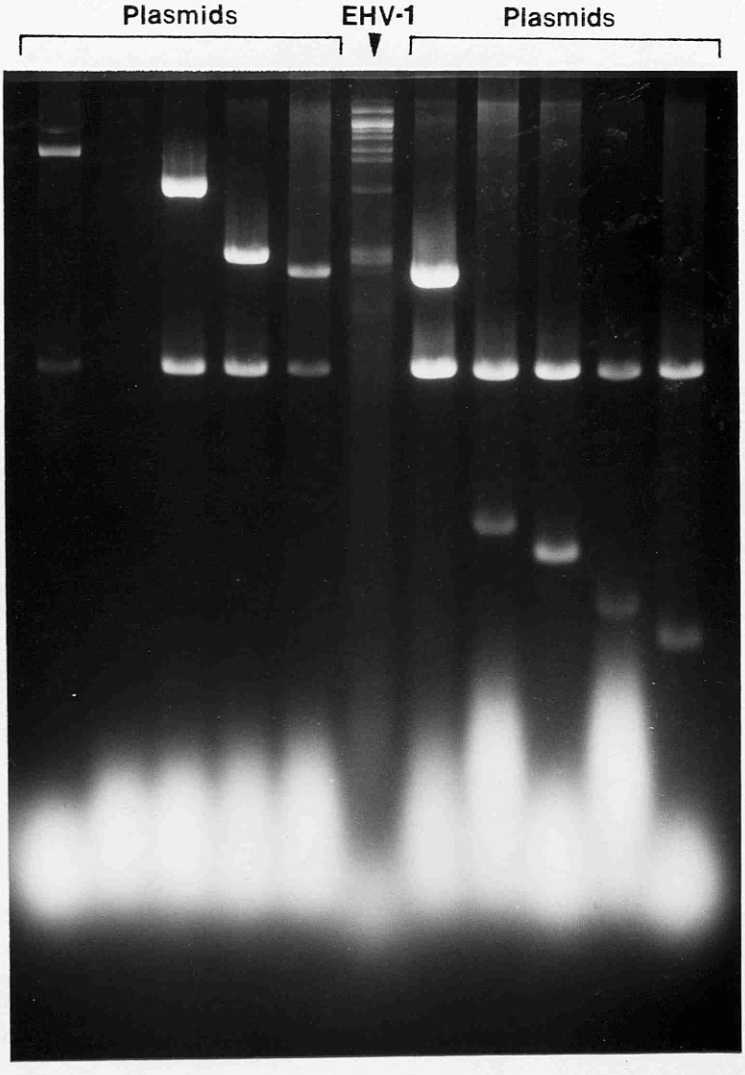


FIGURE 3.9

Autoradiograph showing the results of hybridizing [^{32}P]-labelled Bam HI cloned fragments of EHV-1 subtype 2 DNA to a Bam HI digest of the same DNA. Hybridization and washing conditions were identical to those referred to in Figure 3.4. The results are listed in Table 3.4. The faint band common to all tracks was thought to be a contaminant, perhaps pUC9, which may have been present in the dye Ficoll.

EHV-1 subtype 2 Bam HI

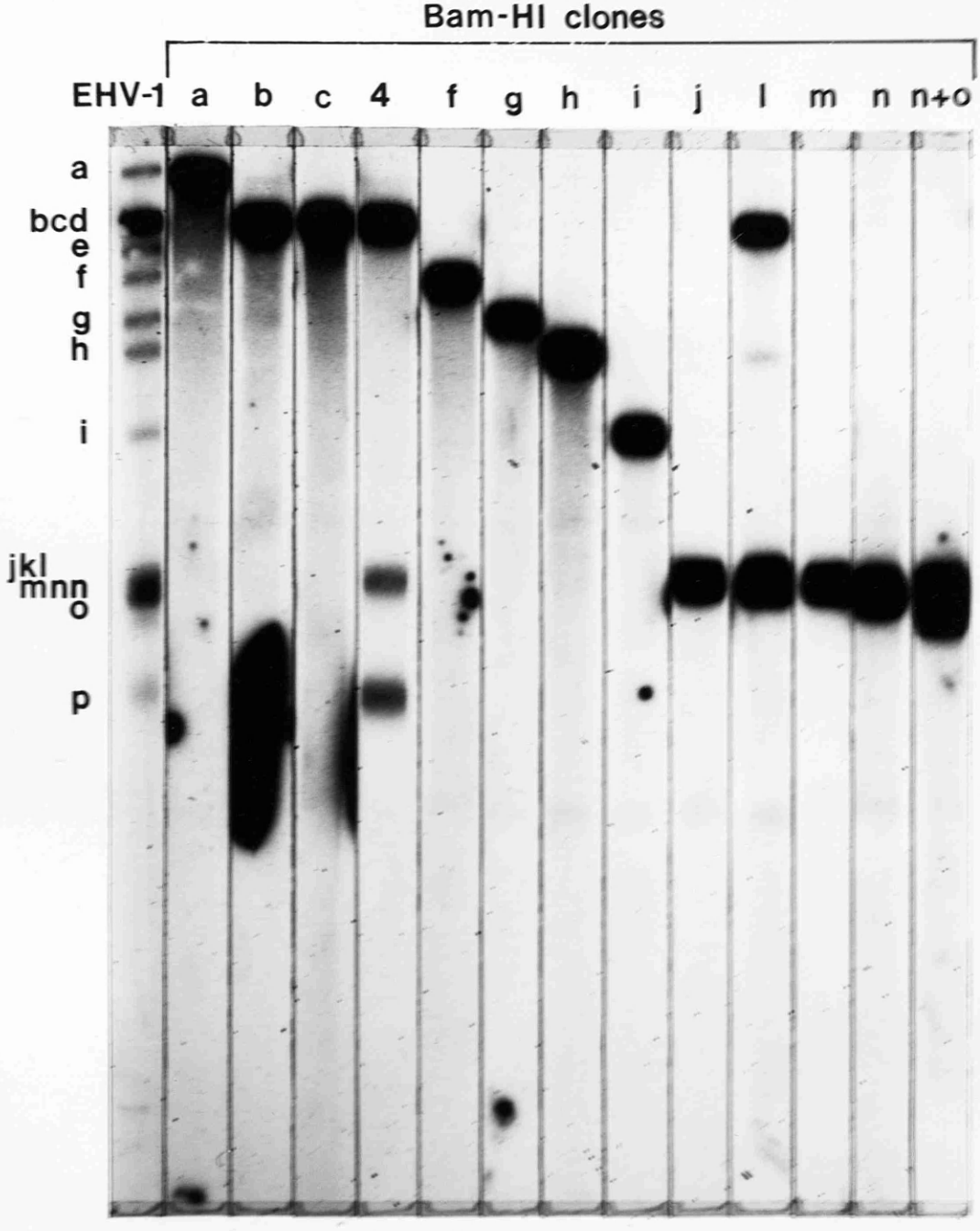


FIGURE 3.10

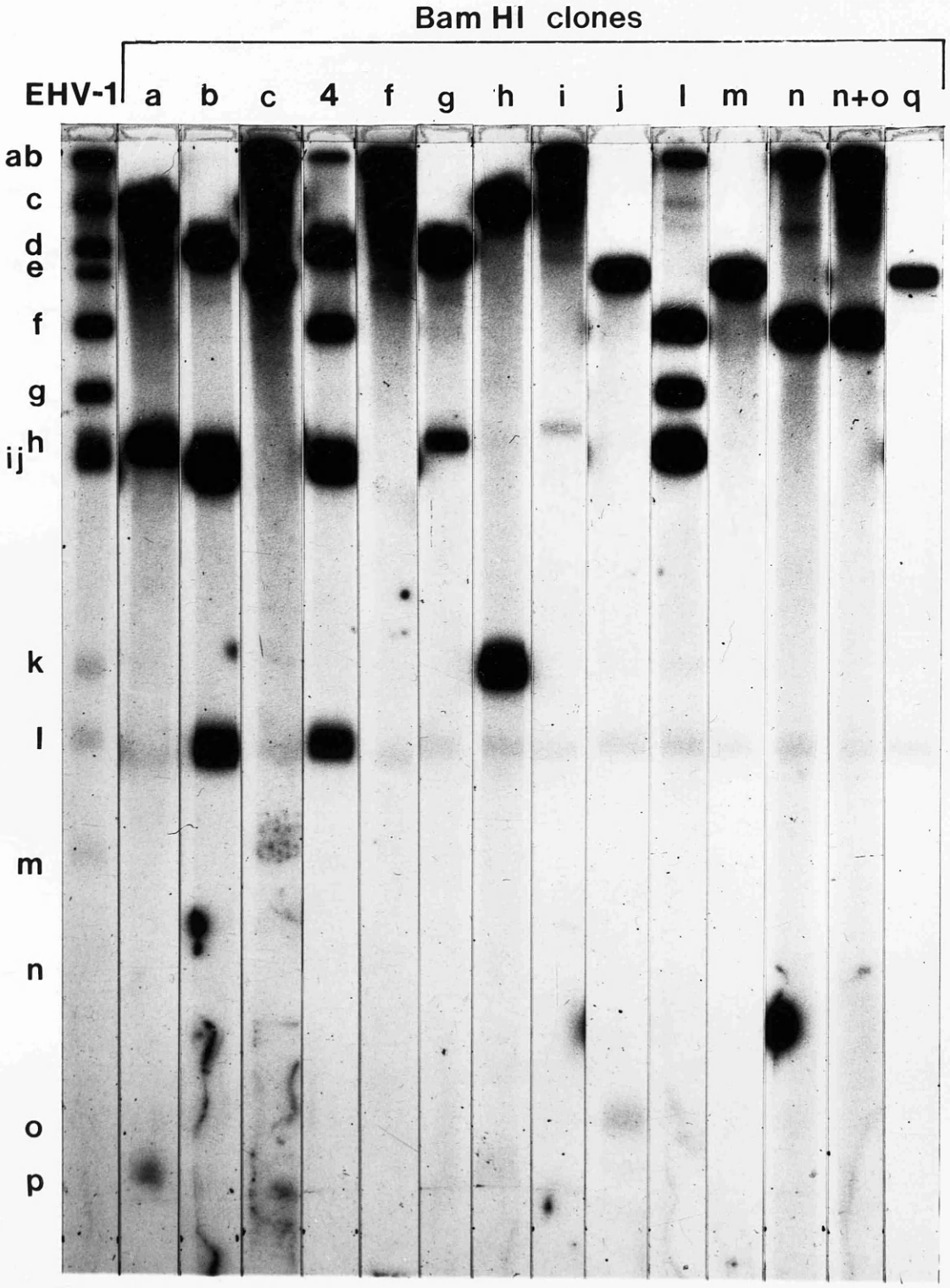
Autoradiograph showing the results of hybridizing [^{32}P]-labelled Bam HI cloned fragments of EHV-1 subtype 2 DNA to an Eco RI digest of the same DNA. Hybridization and washing conditions were identical to those referred to in Figure 3.4.

The results are listed in Table 3.4.

The hybridization of cloned Bam HI h and j to Eco RI p was clearer on a longer autoradiographic exposure.

The faint band migrating below Eco RI 1, in all tracks may represent contaminating pUC9 in the dye Ficoll.

EHV-1 subtype 2 Eco RI



T A B L E 3.4

HYBRIDIZATION DATA FOR BAM HI CLONES

Bam HI Cloned Probes	Hybridizing Bam HI Bands	Hybridizing Eco RI Bands
a	a	c,h
b	bcd	d,ij,l
c	bcd	ab,e
4	bcd,jkl,p	ab,d,f,ij,l
f	f	ab
g	g	d,h
h	h	p,k,c
i	i	ab
j	jkl	e,o,p
l	bcd,jkl	ab,f,g,ij
m	mnn	e
n	mnn	ab,f
n+o	mnn,o	ab,f
q	-	e

this insert was not clearly ascertained. A second clone used in most hybridization experiments contains Bam HI n and o. Clones containing Bam HI n and o separately were also obtained.

The clones were used in two ways to map the genome of EHV-1 subtype 2. Firstly, they were used in hybridization studies analogous to those described above in order to determine the order of restriction fragments in the genome. Secondly, the products of digestion by two restriction endonucleases were analysed in order to elucidate the locations of Bam HI and Eco RI cleavage sites in the genome.

Hybridization Studies Using Cloned DNA

The use of cloned DNA fragments in hybridization experiments eliminated problems due to fragment contamination evident in the autoradiographs shown in Figures 3.4 - 3.7, and allowed fragments in multiple bands (e.g. Bam HI bcd and jkl) to be hybridized separately. 0.5ug of each plasmid DNA was nick translated and hybridized to Bam HI and Eco RI digests of EHV-1 subtype 2 DNA. The results are shown in Figures 3.9 and 3.10 and summarized in Table 3.4.

Restriction Endonuclease Double Digest Experiments

The plasmids were used in restriction endonuclease double digest experiments in order to determine the locations of Eco RI sites in each cloned Bam HI fragment. Figure 3.11 shows selected results; the data for all digestions are summarized in Table 3.5. The sum of the

FIGURE 3.11

Cleavage of EHV-1 subtype 2 plasmids with Bam HI and Eco RI.

The DNA was digested and electrophoresed on a 0.7% agarose gel. Hind III digests of λ DNA were used as size markers (tracks M).

a. Fragments produced by cleavage with Bam HI alone.

b. Fragments produced by cleavage with Bam and Eco RI.

c. Fragments produced by cleavage with Eco RI alone.

Figure 3·11

Restriction Endonuclease analysis of
EHV-1 subtype 2 plasmids

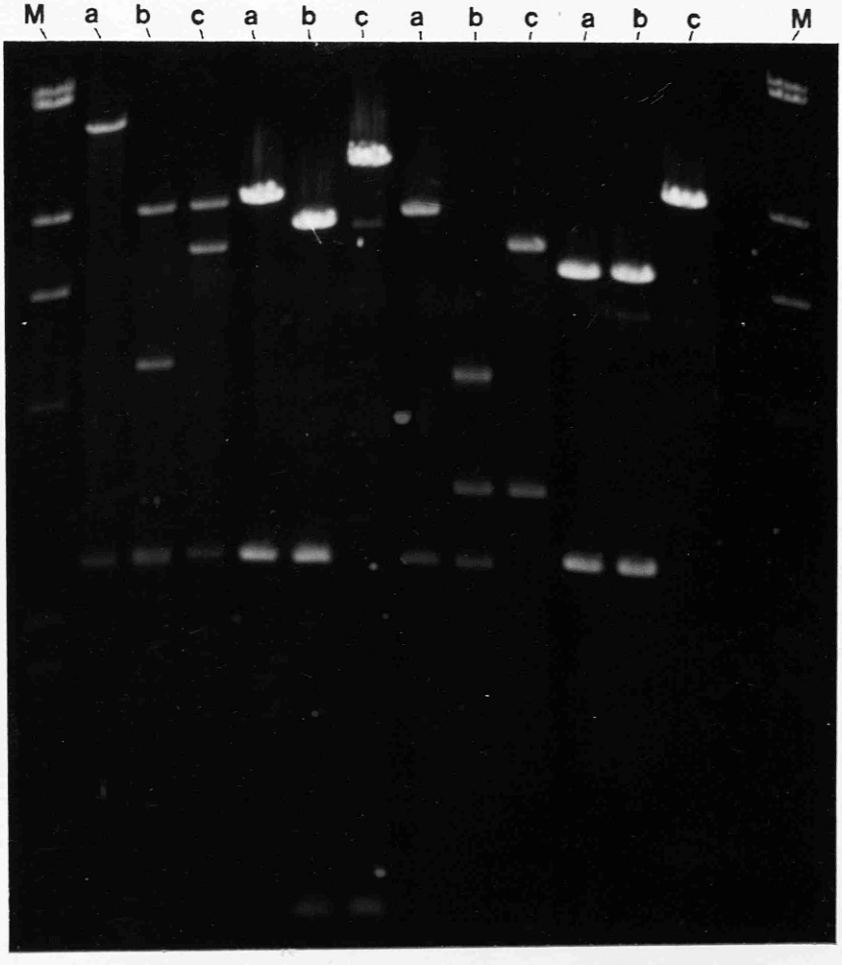


FIGURE 3.11 (continued)

M. Hind III digest of λ DNA.

a. Fragments produced by cleavage with Bam HI alone.

b. Fragments produced by cleavage with Bam HI and Eco RI.

c. Fragments produced by cleavage with Eco RI alone.

Figure 3·11 cont.

Restriction Endonuclease analysis of
EHV-1 subtype 2 plasmids

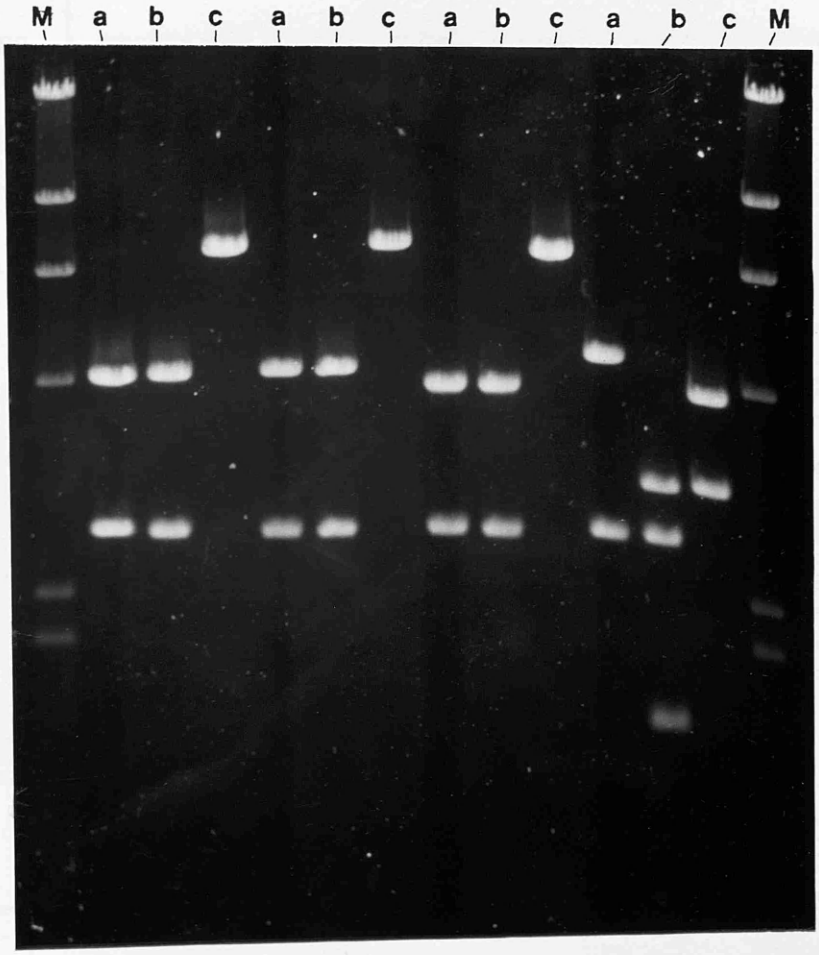


TABLE 3.5

Sizes (kbp) of Fragments generated by digesting cloned EHV-1 Subtype 2, DNA Fragments with Bam HI and Eco RI

CLONES EHV-1 DNA FRAGMENTS	PRODUCTS of Bam HI DIGESTION	PRODUCTS of Bam HI + Eco RI DIGESTION	PRODUCTS of Eco RI DIGESTION
a	18.5, 2.7	13.7, 4.8, 2.7	13.7, 7.5
b	14.1, 2.7	6.3, 5.0, 2.8, 2.7	6.3, 7.8, 2.7
c	14.1, 2.7	10.0, 3.1, 2.7, 1.0	12.6, 3.2, 1.0
4	18.7, 2.7	9.8, 5.0, 2.8, 2.7	9.8, 7.8, 2.7
f	11.0, 2.7	11.0, 2.7	13.7
g	9.5, 2.7	8.0, 2.7, 1.5	1.5, 10.7
h	8.4, 2.7	4.7, 3.3, 2.7	7.4, 3.3
i	6.4, 2.7	6.4, 2.7	9.1
j	4.6, 2.7	3.4, 2.7, 1.0	7.3
l	4.6, 2.7	3.0, 2.7, 1.6	4.3, 3.0
m	4.5, 2.7	4.5, 2.7	7.2
n	4.5, 2.7	4.5, 2.7	7.2
o	4.2, 2.7	4.2, 2.7	6.9
q	1.0, 2.7	1.0, 2.7	3.7

pUC9 = 2.7 kbp in size.

molecular weights of EHV-1 DNA fragments resulting from digestion with Bam HI plus Eco RI agreed well with the molecular weight of each intact fragment.

Derivation of the Restriction Endonuclease Map for the S Region of the EHV-1 Subtype 2 Genome

The hybridization of Bam HI virion DNA fragments to a Bam HI digest of EHV-1 subtype 2 DNA (Figure 3.4) showed that, in addition to hybridizing to themselves, Bam HI bcd hybridized to jkl, Bam HI jkl hybridized to bcd and to p, and Bam HI p hybridized to jkl. These data indicate the presence of portions of the fragments in these bands in repeated DNA sequences. The use of cloned DNA as hybridization probes helped to identify the individual fragments mapping in the repeats. Cloned Bam HI l hybridized to Bam HI jkl and bcd, but neither cloned Bam HI b nor cloned Bam HI c hybridized to Bam HI jkl (Figure 3.9). This implies that Bam HI l hybridizes only to Bam HI d, which was not cloned. Bam HI p from virion DNA hybridized to Bam HI jkl (Figure 3.4) and, since neither cloned Bam HI l nor cloned Bam HI j hybridized to p, Bam HI k, which was not cloned, must hybridize to p (Figure 3.9).

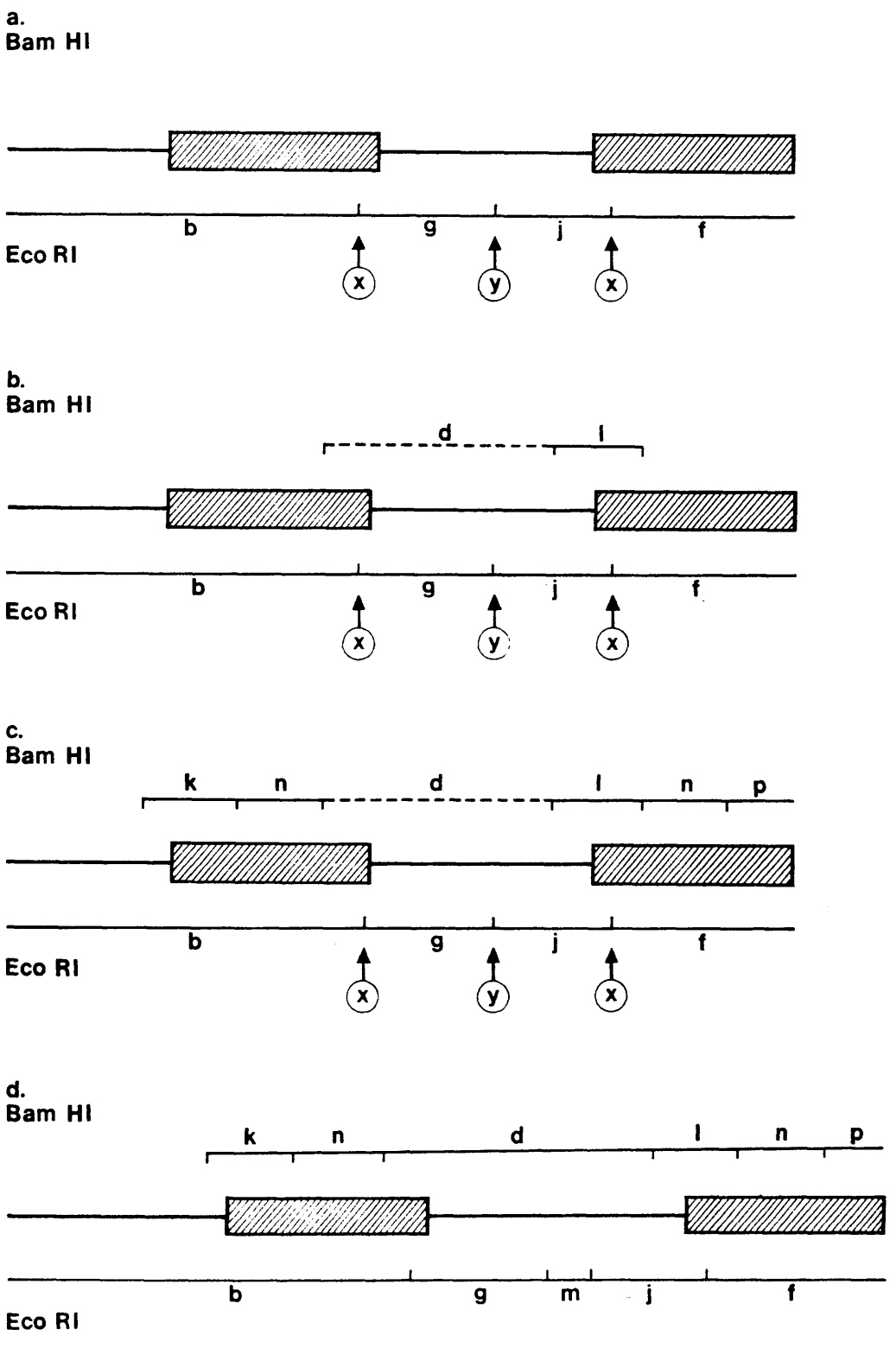
The hybridization of Eco RI virion DNA probes to an Eco RI digest of EHV-1 subtype 2 DNA (Figure 3.5) indicated the presence of portions of Eco RI f and one or both of Eco RI ab in the repeated regions, as Eco RI f and ab hybridized to themselves and to each other. Similarly, the results in Figure 3.5 suggest that Eco RI g and either or both of ij hybridized to each other. However, the weak hybridization between Eco RI g and ij indicates that

FIGURE 3.12

A diagrammatic representation of the derivation of the Bam HI and Eco RI linkage maps for the S segment of the EHV-1 subtype 2 genome. The linkage maps are shown above and below the representative genome, in which inverted repeats are denoted by hatched rectangles. Fragments, not drawn to scale are represented by broken lines. The letters x and y denote possible additional Eco RI fragments at the locations indicated by the arrows.

Figure 3·12

Derivation of the Bam HI and Eco RI Linkage Maps for the S region of the EHV-1 subtype 2 genome



hybridizing fragments share only a short sequence. These data are interpreted as follows. Firstly, Eco RI b and f, which show a high degree of homology originate from the S terminus and L-S joint. Eco RI f is the smaller of the fragments and therefore represents the S terminal fragment, and Eco RI b contains the L-S joint. Secondly, Eco RI g and j contain the TR_S/U_S and IR_S/U_S junctions. The proposed arrangement of Eco RI fragments in S is illustrated in Figure 3.12a, but does not exclude the presence of additional Eco RI fragments between b and g, g and j, and j and f. Hybridization of cloned Bam HI 1 to Eco RI ab, f, g and ij (Figure 3.10) shows that this fragment spans the junction between Eco RI b and g or j and f. The stronger hybridization to Eco RI ij than to g indicates that Bam HI 1 shares more sequence with the former, and therefore that Bam HI 1 spans the junction between Eco RI j and f. This arrangement is illustrated in Figure 3.12b. Restriction endonuclease analysis showed that Bam HI 1 is cleaved by Eco RI to give fragments of 3kbp and 1.6kbp (Table 3.5). The stronger hybridization of Bam HI 1 to Eco RI ij than to Eco RI f (Figure 3.10) suggests that the Eco RI site is nearer the right end of Bam HI 1. This found proof in the DNA sequence of Bam HI 1 discussed in Chapter 5. The Bam HI site which divides 1 and n is located approximately 8kbp from the S terminus, and an equivalent Bam HI site is located in IR_S. The genome location of Bam HI d, which hybridized to Bam HI 1 (Figure 3.9), is shown in Figure 3.12b.

Eco RI f hybridized to Bam HI bands bcd, jkl, mnn and

p (Figure 3.7). Cloned Bam HI n hybridized to Eco RI ab and f, and Bam HI mnn, verifying the location of this fragment completely within TR_S/IR_S (Figures 3.9 and 3.10). Bam HI n was not cleaved by Eco RI (Table 3.5), and is unlikely to be the S terminal fragment because it was cloned. Eco RI f also hybridized to Bam HI p, which hybridized to itself and to Bam HI k (Figures 3.4 and 3.7). Neither Bam HI p nor Bam HI k were cloned, but, on the basis of size, it is proposed p is at the S terminus and k at the L-S joint. The data are consistent with the location of Bam HI n between l and p, as shown in Figure 3.12c. The sum of the sizes of Bam HI p, n and the portion of l within Eco RI f corresponds to the size of Eco RI f, implying that all large Bam HI fragments in TR_S/IR_S have been accounted for. However, the sum of the sizes of Bam HI d and l is 18.7kbp, whereas the sum of the sizes of Eco RI j and Eco RI g plus twice the size of the portion of Bam HI l to the right of the Eco RI site is only 16.5kbp. The difference between these sizes implies that there is a 2.2kbp fragment between Eco RI g and j. Bam HI bcd from virion DNA hybridized to Eco RI ab, d, e, f, g, ij, l, m and n (Figure 3.6). Cloned Bam HI b hybridized to Eco RI d, ij and l while clone Bam HI c hybridized to Eco RI ab and e (Figure 3.10). Thus Eco RI m and n are candidates for the small fragment between Eco RI g and j. Eco RI n is anomalous in being present in reduced molarity on the control filter in Figure 3.6, and is possibly related to another fragment such as Eco RI m. Eco RI m has a size of 2.2kbp, and is therefore the best candidate for the small

fragment between Eco RI g and j. The complete Bam HI and Eco RI restriction endonuclease maps for the S segment of the genome of EHV-1 subtype 2 is shown in Figure 3.12d.

Bam HI k, which contains the L-S junction, was not cloned. Cloned Bam HI b and cloned 4 both hybridized to Bam HI bcd (Figure 3.9) and to Eco RI d, ij and l (Figure 3.10). Clone 4 also hybridized to Bam HI jkl and p (Figure 3.9) and to Eco RI ab and f (Figure 3.10). This suggests that clone 4 contains sequences from Bam HI b and Bam HI k.

Derivation of the Restriction Endonuclease Map for the L Segment of the EHV-1 Subtype 2 Genome

Eco RI b contains the L-S joint and a portion of this fragment makes up the major part of IR_S (Figure 3.12d). The hybridization data concerning Eco RI ab were analysed in an attempt to determine the relationship of Eco RI b to other fragments in U_L and to ascertain which Bam HI fragments hybridized to Eco RI a and not to b. Probe Eco RI ab hybridized to Bam HI bcd, e, f, i, jkl, mnn, o and p (Figure 3.7). Bam HI d, k, l, n and p hybridized to a portion of Eco RI b in IR_S. Cloned Bam HI fragments b, j and m did not hybridize to Eco RI ab (Figure 3.10). Thus, Bam HI c, e, f, i and o overlap Eco RI a or the portion of b in U_L. The hybridization patterns of these Bam HI fragments were studied to elucidate the location of Eco RI a. Cloned Bam HI c hybridized to Eco RI ab and e (Figure 3.10) and produced fragments of 10kbp, 1.0kbp and 3.1kbp on digestion with Eco RI and Bam HI (Table 3.5). These data imply that the left end of Eco RI b or one end of Eco RI a

FIGURE 3.13

A diagrammatic representation of the derivation of the Bam and Eco RI linkage maps for a portion of the U_L of the EHV-1 subtype 2 genome. The linkage maps are shown above and below the representative genome, in which inverted repeats are denoted by hatched rectangles. Fragments not drawn to scale are represented by broken lines.

Figure 3·13

Derivation of the Bam HI and Eco RI Linkage Maps for the U_L of the EHV-1 subtype 2 Genome

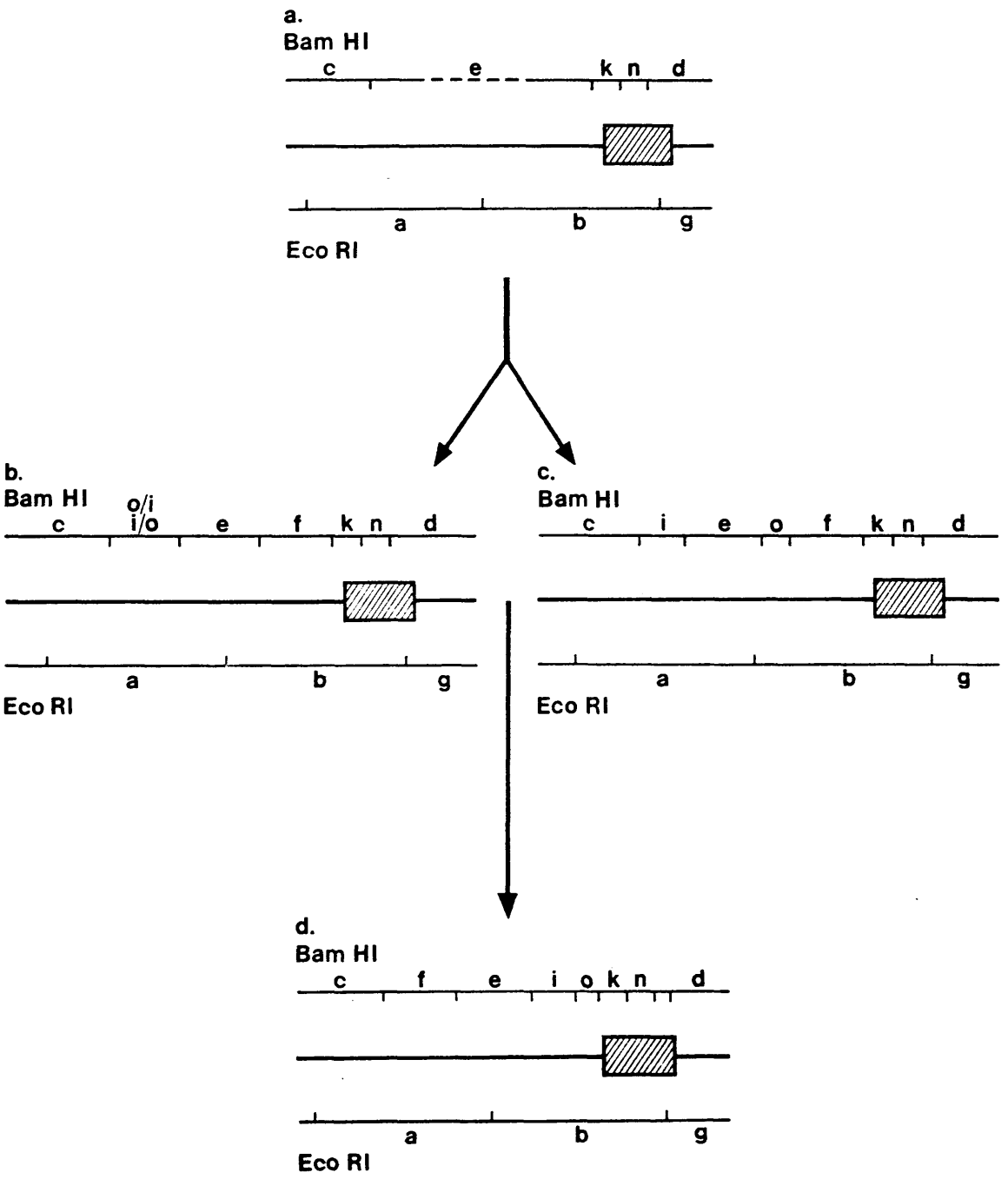
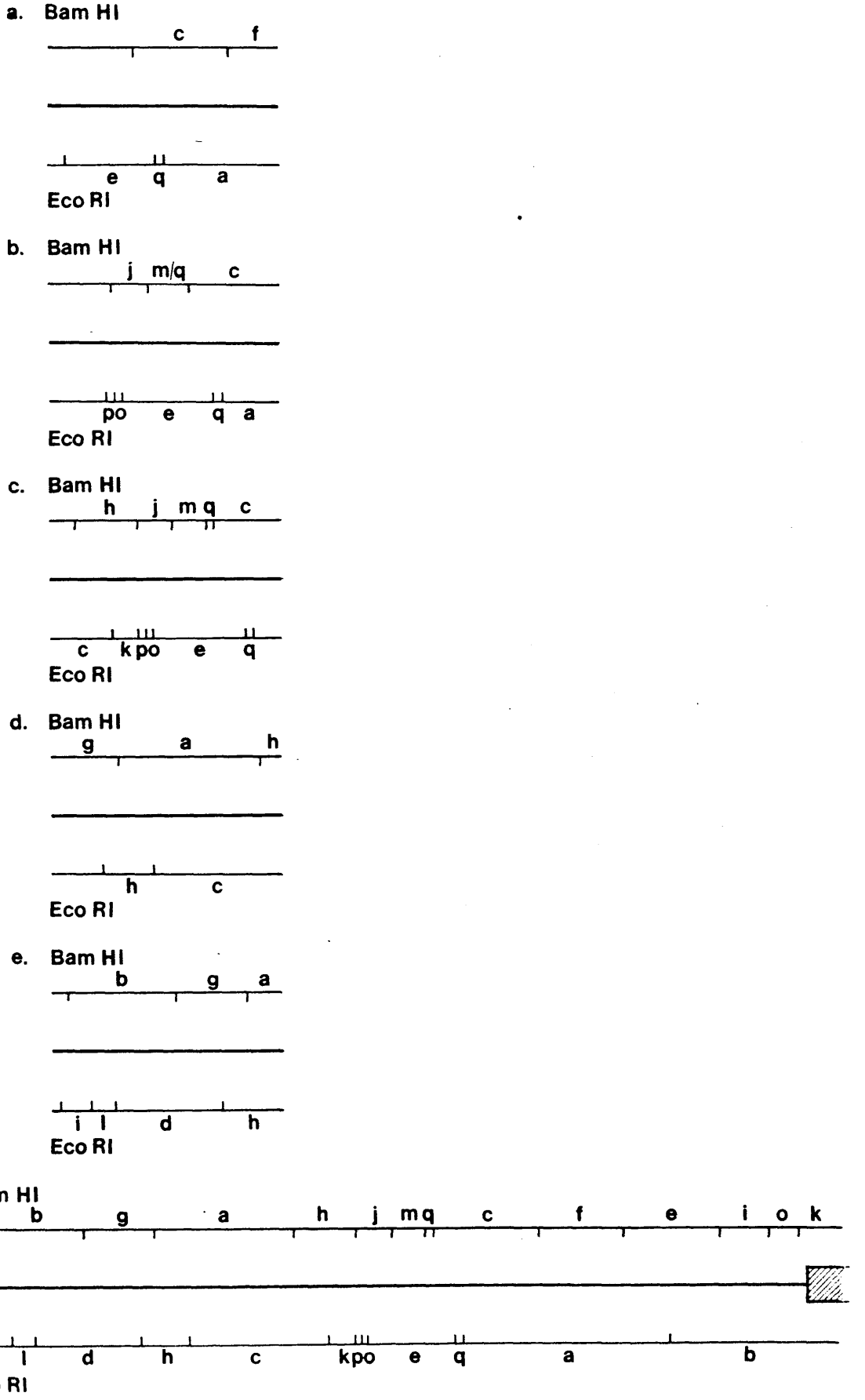


FIGURE 3.14

A diagrammatic representation of the derivation of the Bam HI and Eco RI linkage maps for a portion of the U_L of EHV-1 subtype 2 genome. The linkage maps are shown above and below the representative genome.

Figure 3·14

Derivation of the Bam HI and Eco RI Linkage Maps for the U_L of the EHV-1 subtype 2 Genome



contained within Bam HI c and located between Eco RI e and a was identified as Eco RI q on the basis of hybridization of Bam HI bcd to an Eco RI digest of EHV-1 subtype 2 DNA (Figure 3.14a). Probe Eco RI e hybridized to Bam HI bcd, jkl and mnn (Figure 3.7). Cloned Bam HI q and m hybridized only to Eco RI e, and cloned Bam HI j hybridized to Eco RI e and to two smaller fragments, Eco RI o and p (Figure 3.10). Therefore, Bam HI fragments m and q, which were not cleaved by Eco RI (Table 3.5), are located between Bam HI j and c. It was not possible to determine the order of Bam HI m and q from these data (Figure 3.14b). They are shown in the order m-q in the final map of U_L in Figure 3.14.

Cloned Bam HI j and h hybridized to Eco RI p but only Bam HI j hybridized to Eco RI o, thus implying that Eco RI o is contained completely within Bam HI j and maps adjacent to Eco RI e (Figures 3.10 and 3.14b). Digestion of cloned Bam HI j with Bam HI and Eco RI resulted in fragments of 3.4kbp and 1kbp (Table 3.5), which represent a portion of Eco RI e and the whole of Eco RI o, respectively. Eco RI p contains the Bam HI site between Bam HI j and h, but the small portion of this fragment within Bam HI j was not detected by restriction endonuclease digestion (Figure 3.14c).

Cloned Bam HI h hybridized to Eco RI p, k and c (Figure 3.10). Eco RI k hybridized only to Bam HI h (Figure 3.7) and one of the fragments resulting from digestion of cloned Bam HI h with Bam HI and Eco RI is equivalent in size to Eco RI k (Table 3.5). This indicates that Eco RI k is contained wholly within Bam HI h, and is

thus located between Eco RI p and Eco RI c (Figure 3.14c). Hybridization of Eco RI c to a Bam HI digest of EHV-1 subtype 2 DNA (Figure 3.7) showed that Bam HI a and h are adjacent (Figure 3.14d). Cloned Bam HI a hybridized to Eco RI c and h (Figure 3.10), thus locating Eco RI h next to Eco RI c, and Eco RI h hybridized to Bam HI g and a (Figure 3.7), indicating that Bam HI g is next to Bam HI a (Figure 3.14d). These fragment locations were confirmed by double digestion of cloned Bam HI a and g (Table 3.5).

Eco RI d hybridized to Bam HI bcd and g (Figure 3.7). As Bam HI c and d were mapped previously, Bam HI b must be adjacent to Bam HI g (Figure 3.14e). Cloned Bam HI b hybridized to Eco RI d, ij and l (Figure 3.10) and on digestion with Bam HI and Eco RI yielded products of 6.3kbp, 5kbp and 2.8kbp. The 6.3kbp product plus the 8kbp product of cloned Bam HI g is equivalent in size to Eco RI d. The 2.8kbp product represents the whole of l, and the remaining 5kbp is equivalent to the portion of Eco RI i which overlaps Bam HI b. Bam HI b was cloned and therefore is unlikely to be the fragment at the L terminus. The restriction endonuclease digestion results suggest that the 1kbp of Eco RI i which does not map in Bam HI b is present in a small Bam HI fragment at the L terminus. The complete Bam HI and Eco RI restriction endonuclease maps of the EHV-1 subtype 2 genome are shown in Figure 3.15.

FIGURE 3.15

Bam HI and Eco RI maps of EHV-1 subtype 2 DNA. The location of the cleavage sites are shown above and below the representative genome. The inverted repeat sequences are represented by hatched rectangles.

Figure 3·15
Restriction Endonuclease Maps of the EHV-1 subtype 2 Genome

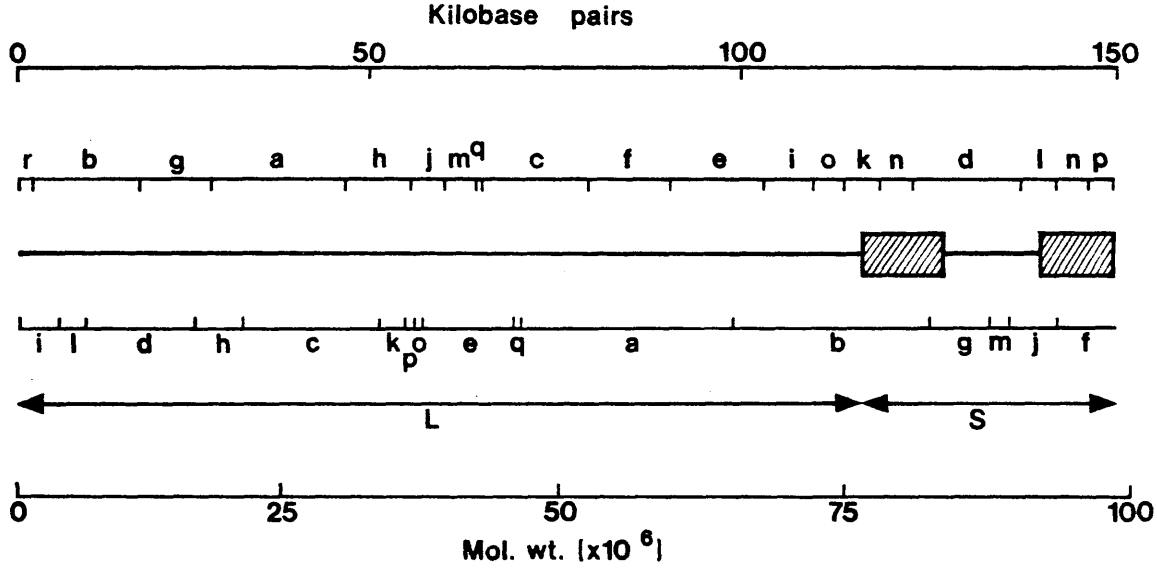


Figure 3.16

Restriction endonuclease maps of EHV-1 subtype 1 and subtype 2 DNA. The locations of the Bam HI and Eco RI cleavage sites are shown above the representative genomes. The inverted repeat sequences are represented by hatched rectangles.

DISCUSSION

The analysis of EHV-1 subtype 2 DNA is consistent with a model for the genome structure similar to that for EHV-1 subtype 1. However, the comparison of restriction endonuclease maps in Figure 3.16 shows that the two subtypes are quite different in the arrangement of restriction sites.

The size of the EHV-1 subtype 2 genome was estimated at approximately 144kbp (93.5 x 10^6 daltons) from the sums of the sizes of Bam HI and Eco RI fragments. However, hybridization studies and double digestion of cloned DNA fragments revealed the existence of several additional small fragments, allowing the original estimate to be revised to 146kbp (95 x 10^6 daltons). The genome consists of two segments: L (111kbp) and S (35kbp). The S segment comprises a unique sequence (U_S) flanked by inverted repeat sequences (TR_S/IR_S). From estimates of fragment size, TR_S/IR_S are larger than 9.5kbp (Eco RI f) and less than 12.7kbp (Bam HI 1+n+p). The electron microscopy data indicate a size of 13.3kbp for U_S, suggesting that the TR_S/IR_S are 10.8kbp in size, a value which falls within this range. The genome of EHV-1 subtype 1 is 153kbp (100 x 10^6 daltons) in size (Whalley et al., 1981) . The S segment of EHV-1 subtype 1 (40kbp) is approximately 5kbp larger than that of EHV-1 subtype 2, and the estimated size of IR_S/TR_S is between 8.6kbp and 13.4kbp, implying that U_S has a minimum size of 12.1kbp.

The hybridization data do not indicate the presence of an inverted repeat flanking U_L of EHV-1 subtype 2.

However, a very small repeat, such as that present in the VZV genome (Davison, 1984), cannot be ruled out at present. Similarly, no indication of a terminal redundancy akin to the HSV-1 a sequence was found.

Since both Eco RI and Bam HI cleave within the inverted repeat sequences of the subtype 2 genome it is not known whether U_S inverts relative to a fixed orientation of L, as occurs in subtype 1.

CHAPTER 4

STUDIES ON THE LOCATION OF HOMOLOGOUS SEQUENCES IN THE GENOMES OF THE TWO SUBTYPES OF EHV-1 AND HSV-1

RESULTS

Introduction

At the onset of this project none of the genes of EHV-1 subtype 2 had been mapped. The object of this section was to use comparative hybridization to study the extent and distribution of homologous sequences between the genomes of the two subtypes of EHV-1 and between the genomes of EHV-1 subtype 2 and HSV-1. Since the development of methods for immobilizing DNA on nitrocellulose filters (Nygaard and Hall, 1964) and for detecting bound nucleic acid with radiolabelled probes (Denhardt, 1966; Gillespie and Spiegelman, 1965), hybridization procedures have been used widely to determine relationships between nucleic acids. Moreover, the Southern blot technique allows DNA sequences fractionated by agarose gel electrophoresis to be transfered to nitrocellulose prior to detection by hybridization (Southern, 1975). Southern hybridization was employed by Davison and Wilkie (1983) and Ben-Porat et al. (1983) to identify the more highly conserved regions in the genomes of several members of the Alphaherpesvirinae. Davison and Wilkie (1983) used cloned DNA probes to investigate homologous sequences in the genomes of HSV-1, HSV-2, EHV-1 subtype 1, VZV and PRV. Conserved sequences include those which in HSV-1 code for the major DNA-binding protein, the major capsid protein, the DNA polymerase, the immediate-early protein V_{mw}IE 175 and one or both of the early proteins V_{mw}136 and V_{mw}38, which are the two subunits of the active HSV-1 ribonucleotide reductase (Bacchetti et al., 1984; Preston

FIGURE 4.1

Hybridization of cloned Kpn I fragments of HSV-1 to a Bam HI digest of EHV-1 subtype 2 DNA. Hybridization was carried out in 30% formamide at 37°C for approximately 50 hours, after which the nitrocellulose strips were washed at 45°C.

EHV-1 subtype 2 BamHI

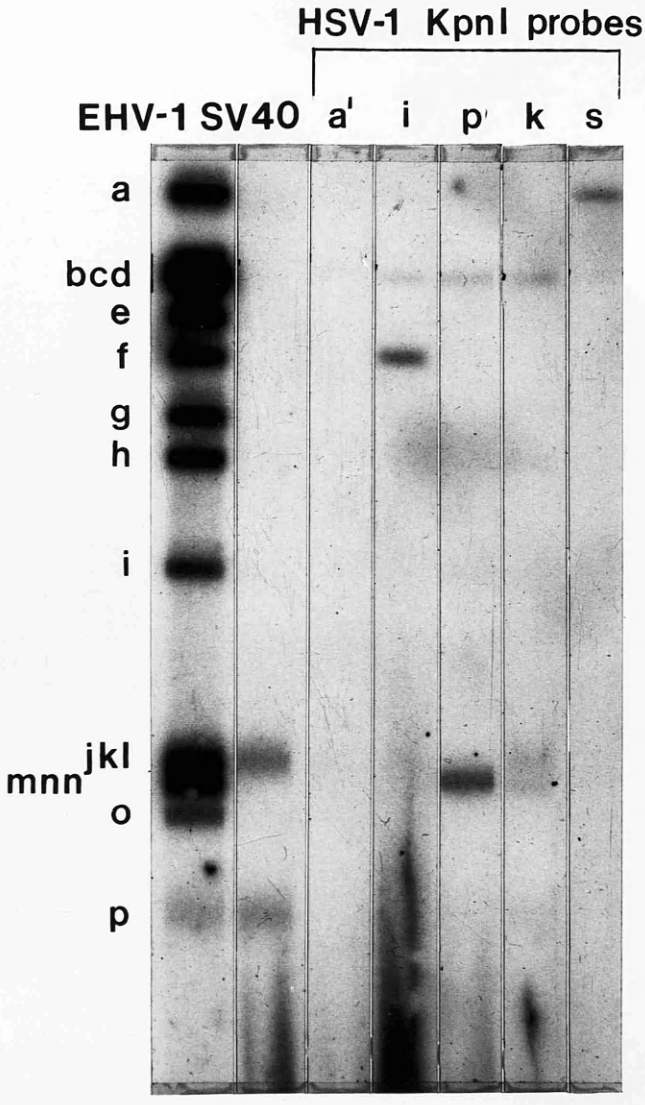
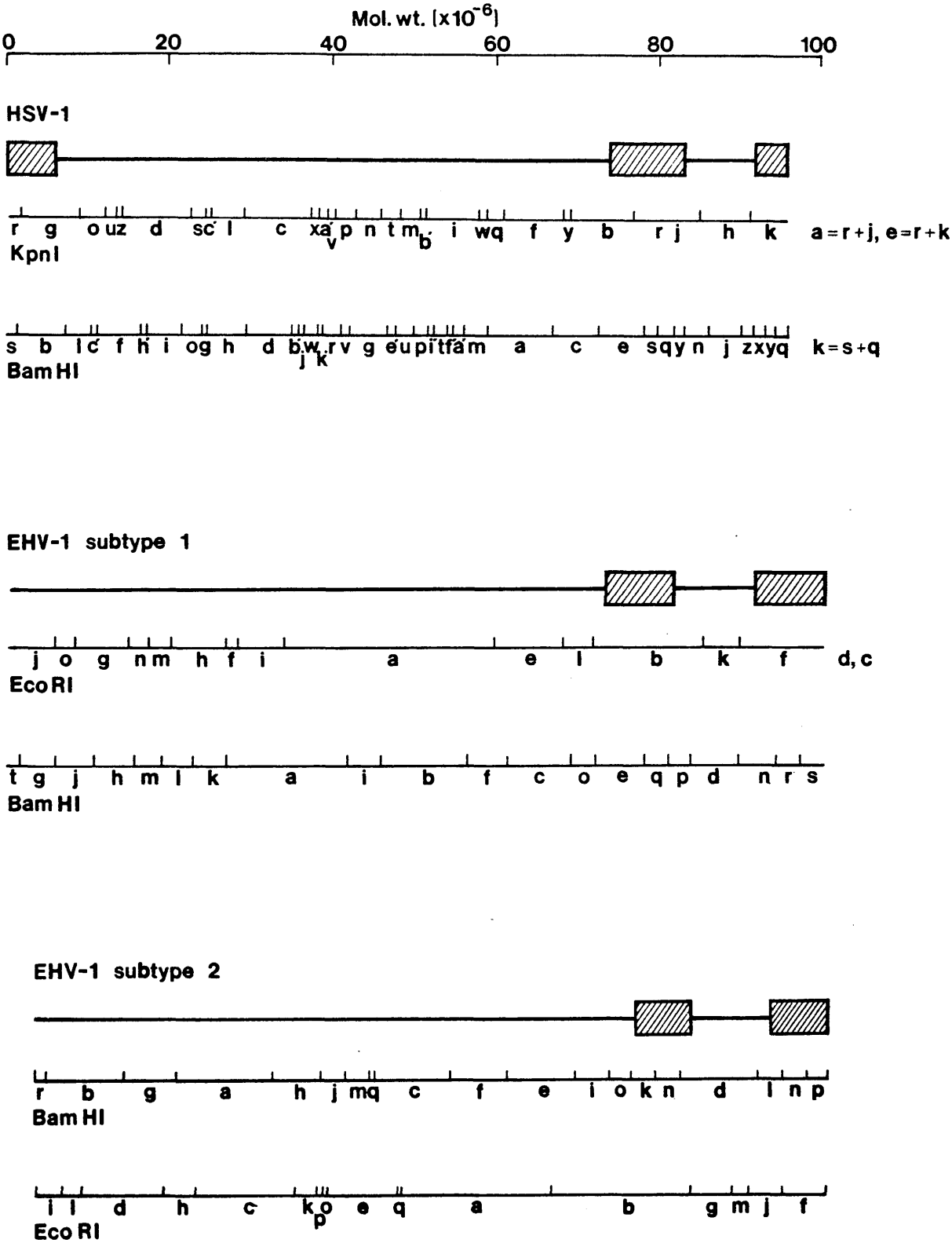


FIGURE 4.2

Restriction endonuclease maps of HSV-1 and EHV-1 subtypes 1 and 2. The maps are shown below each representative genome and those for HSV-1 are in the I_L arrangement. The inverted repeat sequences are represented by cross-hatched boxes. The HSV-1 Bam HI map is from Davison and Wilkie (1981), the HSV-1 Kpn I map is from Preston et al. (1978), the EHV-1 subtype 1 maps are from Whalley et al. (1981) and the EHV-1 subtype 2 maps have been discussed in Chapter 3.

Figure 4·2

Restriction endonuclease maps of the DNA of HSV-1 and EHV-1 subtypes 1 & 2



et al., 1984; Frame et al., 1985). Davison and Wilkie (1983) concluded that HSV-1 probes which contain sequences from better conserved genes could be used to locate counterparts of these genes in other members of the Alphaherpesvirinae.

The Location of Highly Conserved Genes on the EHV-1 Subtype 2 Genome

Figure 4.1 shows the result of hybridizing five nick-translated HSV-1 probes to a Bam HI digest of EHV-1 subtype 2 DNA immobilized on nitrocellulose. Hybridization was carried out under conditions of low stringency, as EHV-1 subtype 2 and HSV-1 are only distantly related. The probes comprised Kpn I fragments of HSV-1 (Figure 4.2) cloned in pAT153, and each contains a conserved region identified by Davison and Wilkie (1983). Kpn I a' (1.1kbp) is completely contained within the protein-coding sequence of the DNA polymerase gene (Quinn and McGeoch, 1985); Kpn I i (9.7kbp) contains the entire coding sequences of three genes, including the major capsid protein gene, and portions of two other genes (Davison and Scott, 1986b); Kpn I p (3.5kbp) contains the majority of the coding region for the major DNA binding protein (Quinn and McGeoch, 1985) and one other gene of unknown function; Kpn I k (7.4kbp) encodes V_{mw}IE 175, V_{mw}IE 12 and portions of a DNA-binding protein and a tegument phosphoprotein (Murchie and McGeoch, 1982; McGeoch et al., 1985; Dalziel and Marsden, 1984; Frame et al., 1986b); Kpn I s (3.4kbp) contains portions of the genes encoding the two subunits of ribonucleotide

TABLE 4.1

Hybridizing of Plasmids Containing HSV-1 DNA Fragments to EHV-1 Subtype 2 DNA Blotted Onto Nitrocellulose.

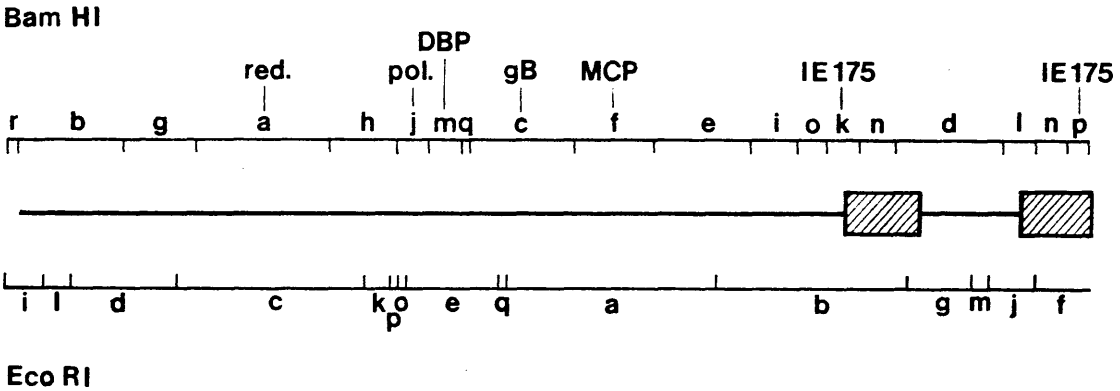
Probe DNA HSV-1 *Kpn* I	*Bam* HI Fragments of EHV-1 Subtype 2 to which Probe Hybridized
a'	-
i	f
p	mnn
k	jkl,mnn,p
s	a
SV40 DNA	jkl,p

FIGURE 4.3

Location of highly conserved genes on the EHV-1 subtype 2 genome. The Bam HI and Eco RI maps are shown above and below the representative genome. The inverted repeat sequences are represented by cross-hatched boxes. The predicted location of the gene coding for the ribonucleotide reductase (red), the DNA polymerase (pol), the DNA-binding protein (DBP), glycoprotein B (gB), the major capsid protein V_{mw}175 (IE175) are indicated by vertical lines.

Figure 4·3

Location of highly conserved genes on the EHV-1 subtype 2 genome



reductase (Draper et al., 1982; McLauchlan and Clements, 1983). The hybridization results are listed in Table 4.1 and the approximate positions of several highly conserved genes on the EHV-1 subtype 2 genome are indicated in Figure 4.3.

No hybridization of HSV Kpn I a' to EHV-1 subtype 2 DNA was detected. The predicted amino acid sequence of the HSV-1 and EBV DNA polymerases share considerable homology, reflected in the DNA sequences, but the most highly conserved region (amino acid residues 880 to 896 in the HSV-1 DNA polymerase) is coded by Kpn I x (Quinn and McGeoch, 1985). It is possible that a probe from elsewhere in the HSV-1 DNA polymerase gene would have hybridized to EHV-1 subtype 2 DNA. SV40 DNA was also used as a probe in the experiment in order to eliminate the remote possibility that EHV-1 subtype 2 DNA prepared from SHELUT cells, a line generated by SV40 transformation of sheep cells, contained SV40 DNA. Under conditions of low stringency the SV40 probe hybridized to EHV-1 subtype 2 Bam HI jkl and p. This hybridization is probably non-specific and indicative of the higher G+C content of the TR_S/IR_S, as discussed in Chapter 5. However, it suggests that care must be taken in the interpretation of the results of hybridization of other probes to these fragments.

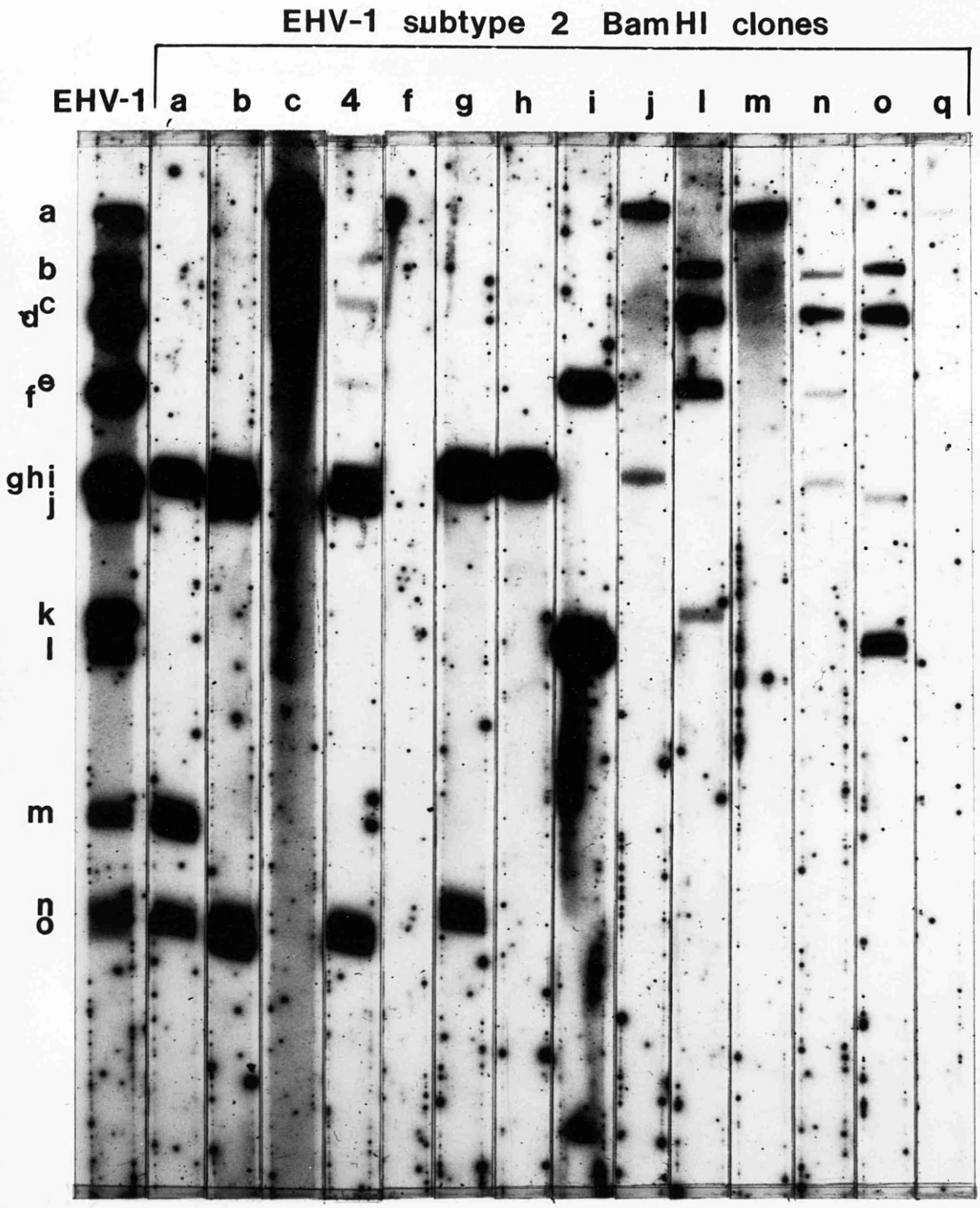
Regions of Homology in the Genomes of the Two Subtypes of EHV-1

The two subtypes of EHV-1 cross-neutralize and share multiple antigens (Allen and Bryans, 1986). Kinetic analysis of re-association experiments indicate that the

FIGURE 4.4

Hybridization of cloned Bam HI fragments of EHV-1 subtype 2 DNA to an Eco RI digest of EHV-1 subtype 1 DNA. Hybridization was carried out in 30% formamide at 37°C for approximately 24 hours, after which the nitrocellulose strips were washed at 45°C. The results of this experiment are listed in Table 4.2.

EHV-1 subtype 1 EcoRI



T A B L E 4.2

Homologous DNA Fragments of EHV-1 Subtypes 1 and 2

PROBE	HYBRIDIZATION TO BLOTS	
Bam HI Clones EHV-1 Subtype 2	Eco RI EHV-1 Subtype 1	Bam HI EHV-1 Subtype 1
a	ghi,n,m,p	a,m,l,k
b	ghi,j,o	g,j,t
c	a	b,i
4	b,c,d,f,ghi,j,o	g,e,j,n,t
f	a	b,f
g	ghi,n	h,j
h	ghi	a
i	e,l	c,o
j	a,ghi	a
l	b,c,d,f,k	d,n,p
m	a	a
n	b,c,d,f,ghi	qrs
o	b,d,l,j	e
q	a	a

FIGURE 4.5

Summary of the regions of homology between the genomes of the two subtypes of EHV-1. Representative genome structures define the axes, and regions of homology are shown by stippling. Only regions of homology identified by the hybridization of EHV-1 subtype 2 fragments to both a Bam HI and Eco RI digest of EHV-1 subtype 1 are represented. The map locations of several EHV-1 subtype 2 Bam HI fragments are ambiguous. Homology between these fragments and EHV-1 subtype 1 DNA is indicated by open boxes. The uncloned EHV-1 subtype 2 fragments k, p, d and e are indicated by a line below the genome. No data are available on the homology between these regions of the genome and EHV-1 subtype 1 DNA.

Figure 4·5

Homology between the Genomes of the two subtypes of EHV-1

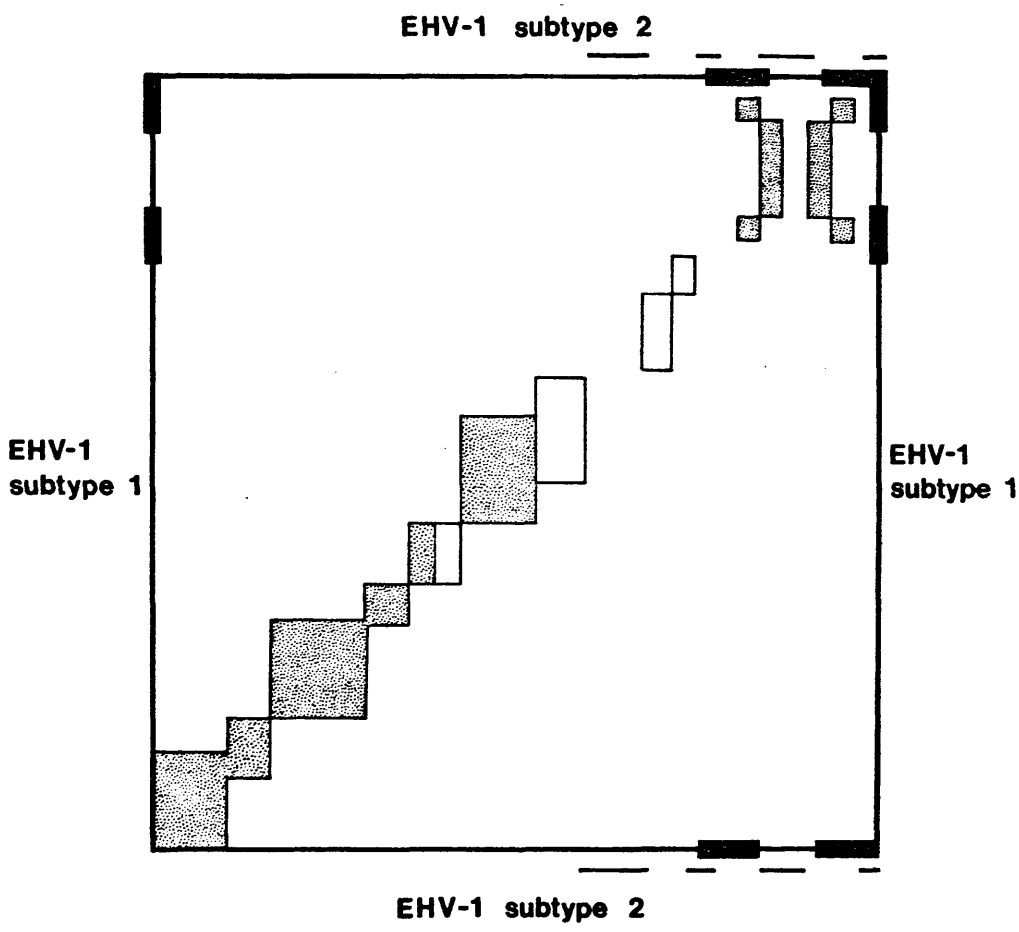


FIGURE 4.6

Hybridization of cloned Bam HI fragments of EHV-1 subtype 2 DNA to a Kpn I digest of HSV-1 DNA. Hybridization was carried out in 30% formamide at 37°C for approximately 50 hours, after which the nitrocellulose strips were washed at 45°C. The results of this experiment are listed in Table 4.3.

HSV-1 Kpn 1

EHV-1 Bam HI clones

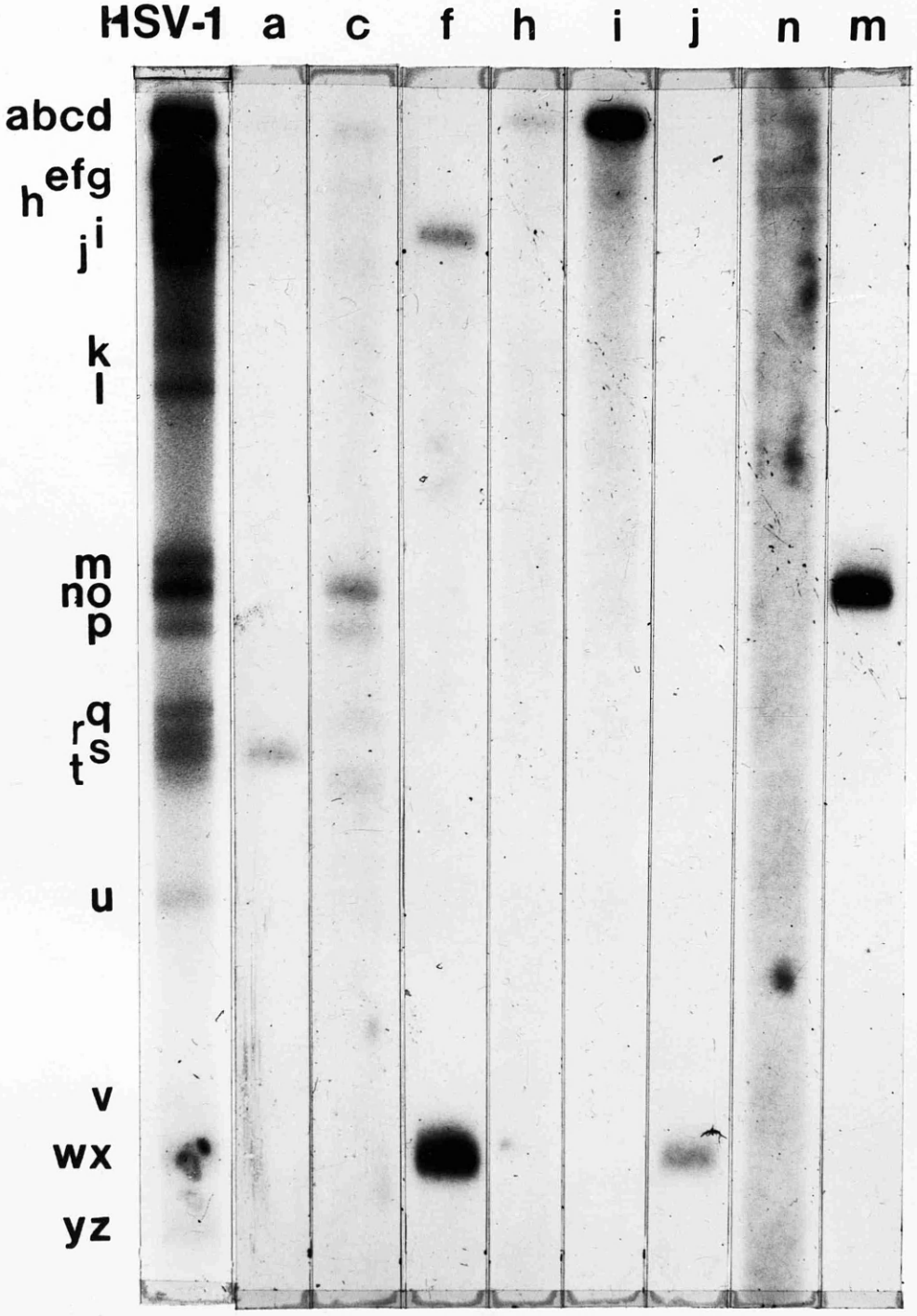


FIGURE 4.7

Hybridization of cloned Bam HI fragments of EHV-1 subtype 2 DNA to a Bam HI digest of HSV-1 DNA. Hybridization was in 30% formamide at 37°C for approximately 50 hours, after which the nitrocellulose strips were washed at 45°C.

The results of this experiment are listed in Table 4.3.

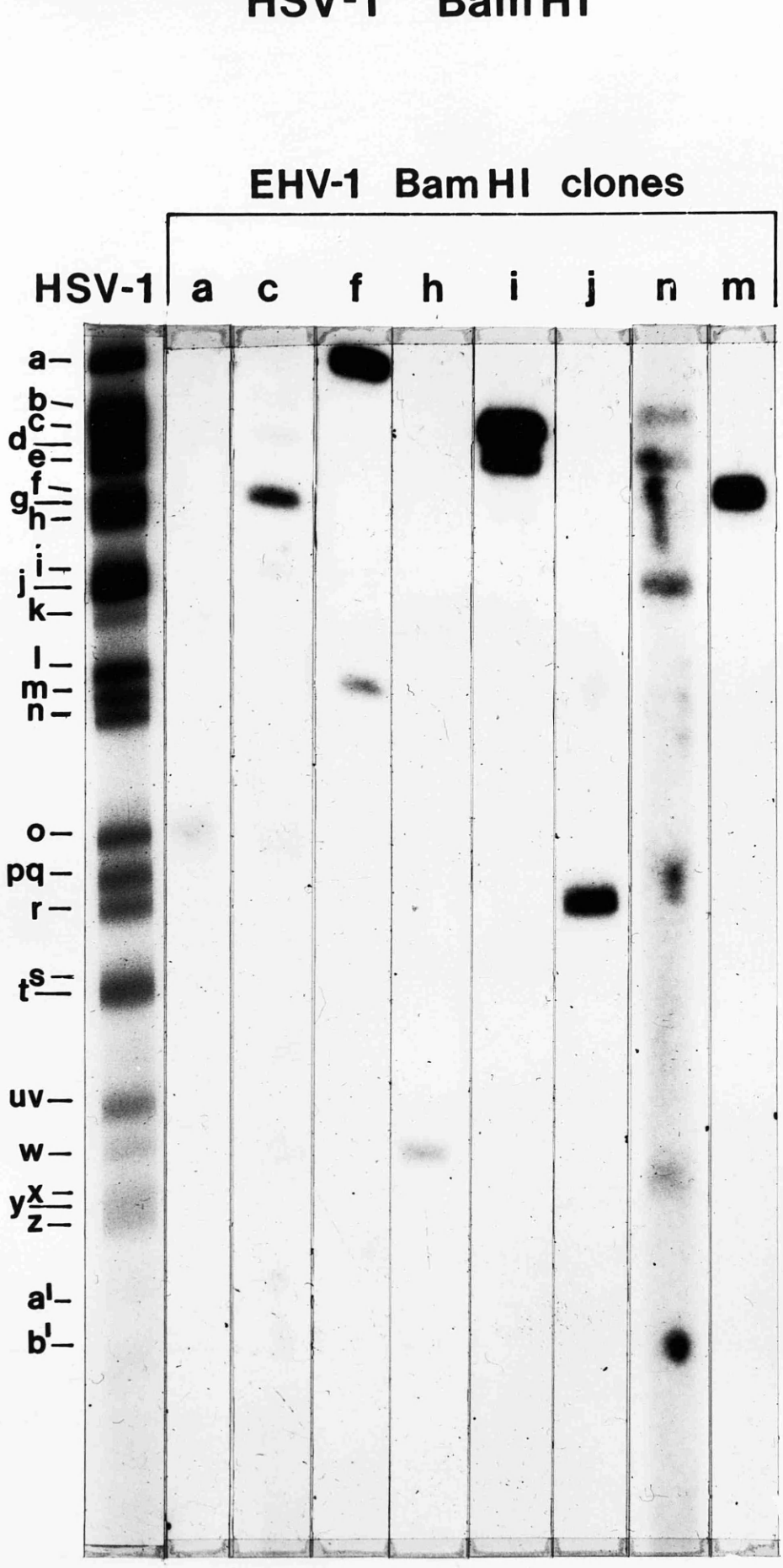

HSV-1 Bam HI
EHV-1 Bam HI clones
HSV-1
a
c
f
h
i
j
n
m
a
b
c
d
e
f
g
h
i
j
k
l
m
n
o
pq
r
s
t
uv
w
x
y
z
a'
b'

TABLE 4.3

Homologous DNA Fragments of HSV-1 and EHV-1 Subtype 2

PROBE DNA	FRAGMENTS TO WHICH CLONES HYBRIDIZED	
Bam HI Clones EHV-1 Subtype 2	Bam HI HSV-1	Kpn I HSV-1
a	o	s
b	N	N
c	g	no,p,t
4	N	N
f	a,m	wx,i
g	N	N
h	w	abcd
i	c,e	abcd
j	r	wx
l	N	N
m	g	p
n	b,e,j	abcd,efg,h
o	N	N
q	N	N

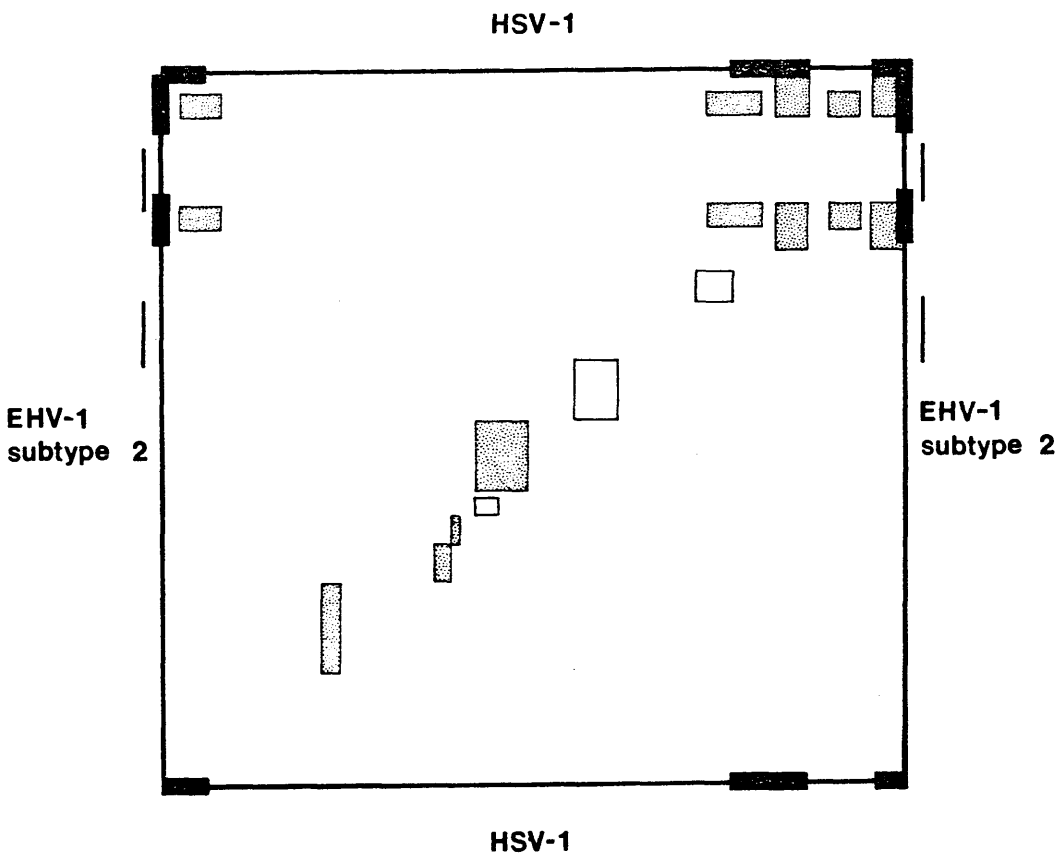
N = No significant hybridization.

FIGURE 4.8

Summary of homology between the genomes of EHV-1 subtype 2 and HSV-1. These regions were identified by the hybridization of cloned HSV-1 Kpn I fragments to EHV-1 DNA (Figure 4.1) and by hybridization of cloned EHV-1 subtype 2 Bam HI fragments to HSV-1 DNA (Figures 4.6 and 4.7). Representative genome structures define the axes, and regions of homology are shown by stippling. The HSV-1 genome is illustrated in the I_L orientation. EHV-1 subtype 2 Bam HI fragments k, p, d and e were not cloned. HSV-1 cloned Kpn I k hybridized to the former two fragments amongst others (Figure 4.1). No homology data are available for EHV-1 subtype 2 Bam HI d and e. These fragments are indicated by a line at the side of the EHV-1 genome. Hybridization of regions of the EHV-1 subtype 2 genome which were not unambiguously mapped are indicated by open boxes.

Figure 4·8

Regions of Homology between the Genomes of HSV-1 and EHV-1 subtype 2



(1983) demonstrated that HSV-1 DNA probes hybridized to limited areas of the EHV-1 subtype 1 genome and that homologous regions in the EHV-1 genome are colinear with the I_L arrangement (Figure 1.1) of the HSV genome. As the genomes of the two subtypes of EHV-1 appear to be colinear (Figure 4.5), it is likely that the genome of EHV-1 subtype 2, like that of subtype 1 is also colinear with HSV. To test this hypothesis, cloned fragments of EHV-1 subtype 2 DNA were hybridized to restriction endonuclease digests of HSV-1 DNA. These experiments also served to verify the results of hybridizing HSV-1 probes containing sequences from highly conserved genes to EHV-1 subtype 2 DNA. Representative autoradiographs showing the results of hybridizing cloned Bam HI fragments of EHV-1 subtype 2 DNA to Kpn I and Bam HI digests of HSV-1 DNA are shown in Figures 4.6 and 4.7, respectively. The results are listed in Table 4.3 and summarized graphically in Figure 4.8. Hybridization of EHV-1 subtype 2 clones to HSV-1 DNA was weaker than that observed between the two subtypes of EHV-1 under identical conditions of low stringency. Also, under these conditions, homology was confined to certain regions of the genomes. Bam HI b, g,l, o and q of EHV-1 subtype 2 demonstrated no significant hybridization to HSV-1 DNA. Homologous regions correspond well to those containing the highly conserved HSV-1 genes identified by Davison and Wilkie (1983). Bam HI f of EHV-1 subtype 2, which hybridized to the HSV-1 probe containing the major capsid protein gene (Figure 4.1), hybridized in these experiments to HSV-1 Kpn I w and i and Bam HI a and m. The gene

encoding the HSV-1 major capsid protein is located in these fragments. Bam HI a of EHV-1 subtype 2 hybridized to HSV-1 Bam HI o and Kpn I s, and EHV-1 Bam HI m hybridized to HSV-1 Kpn I p and Bam HI g. These results substantiate the hybridization of HSV-1 probes for the ribonucleotide reductase and major DNA binding protein genes to EHV-1 subtype 2 Bam HI a and m, respectively (Figure 4.1). EHV-1 subtype 2 Bam HI j hybridized to HSV-1 Bam HI r and Kpn I x, which contain sequences from the DNA polymerase gene. The functions of proteins encoded by HSV-1 DNA sequences which hybridized strongly to Bam HI i of EHV-1 subtype 2 are unknown. However, proteins coded by DNA sequences in this region are highly homologous to the equivalent VZV protein (D.J. McGeoch and A.J. Davison, personal communication). EHV-1 subtype 2 Bam HI h hybridized to HSV-1 sequences adjacent to those encoding the DNA polymerase, and Bam HI c hybridized to the region of the HSV-1 genome which encodes the major DNA binding protein and glycoprotein gB. In summary, Figure 4.8 shows that homologous regions in the EHV-1 subtype 2 genome are colinear with the I_L or I_{SL} genome arrangement of HSV-1.

DISCUSSION

Re-association kinetic and thermal denaturation analysis of the equine herpesvirus genomes indicated that the EHV-1 subtype 1 and EHV-3 genomes share 7 - 10% sequence homology, whereas EHV-2 is less closely related to either virus, sharing 2 - 3% homology with EHV-1 subtype 1 and 1 - 2% homology with EHV-3 (Allen et al., 1977b; Staczek et al., 1983). In cross-hybridization studies the two subtypes of EHV-1 showed 17% homology (Allen and Turtinen 1982), and similar experiments indicated that HSV-1 and HSV-2 share up to 50% of their genome nucleotide sequence (Kieff et al., 1972; Ludwig et al., 1972; Sugino and Kingsbury, 1976). Antigenic cross-reactivity and overlap in disease potential suggests that the relationship between the subtypes of EHV-1 may be somewhat analogous to that between HSV-1 and HSV-2 (Turtinen, 1983). Wilkie et al. (1979) demonstrated that almost all regions of HSV-1 and HSV-2 were homologous to some extent, areas of least homology including TR_L/IR_L and most of the S segment. The close relationship between and colinearity of the HSV-1 and HSV-2 genomes was verified by Davison and Wilkie (1983). In 50% formamide at 37% every Kpn I fragment of the HSV-1 genome hybridized to filters containing HSV-2 DNA fragments. The colinear distribution of homologous regions complemented earlier genetic data indicating colinearity between the genomes of HSV-1 and HSV-2 (Chartrand et al., 1979; Easton and Clements, 1980; Halliburton et al., 1980). In the experiments described above, all cloned EHV-

1 subtype 2 fragments hybridized to EHV-1 subtype 1 DNA at colinear positions. This suggests that, like HSV-1 and HSV-2, the two subtypes of EHV-1 share a common gene arrangement. These findings also support the notion that the latter evolved from a common ancestor, the subtype 2 virus having lost the ability, except on rare occasions, to cross the placenta (Turtinen, 1983). Southern blot hybridization analyses also indicated a highly conserved evolutionary relationship between EHV-1 and EHV-3 which does not appear to be shared to the same degree by EHV-2 (O'Callaghan et al., 1984; Baumann et al., 1986). Initial studies by O'Callaghan et al. (1984) revealed that, although probes from the U_L region of the EHV-1 subtype 1 genome hybridized only to sequences present in the EHV-3 U_L, many homologous regions were non-colinear in distribution. It was suggested that sequences in EHV-1 subtype 1 U_L from 0.20 - 0.64 map units are inverted relative to the homologous sequences in EHV-3 U_L. This conclusion was later refuted by the subsequent revision of the EHV-1 subtype 1 restriction endonuclease maps derived by this group, and the EHV-1 and EHV-3 genomes were found to hybridize to each other colinearly (Baumann et al., 1986). The hybridization conditions used by Baumann et al. (1986) permitted up to 23% base pair mismatch. Under these conditions no homology between EHV-1 U_S and EHV-3 U_S was detected. This is consistent with the findings of Davison and Wilkie (1983), who concluded that U_S is the most heterologous region in several genomes of the Alphaherpesvirinae. In this study, homology between EHV-1

subtype 1 U_S and subtype 2 U_S could not be ascertained because Bam HI d, which contains the major portion of EHV-1 subtype 2 U_S, was not cloned.

HSV-1 is by far the best characterized member of the Herpesviridae. In contrast very little is known about the genetic organisation of the equine herpesviruses. Under hybridization conditions which permitted detection of DNA sequences that displayed approximately 25% or less base pair mismatch, EHV-1 subtype 2 cloned fragments hybridized to colinear HSV-1 DNA sequences (Figures 4.6 and 4.7). This is illustrated graphically in Figure 4.8. The observation that regions of homology between EHV-1 subtype 2 and HSV-1 are dispersed throughout the genomes in a colinear manner is consistent with the colinearity between the EHV-1 subtype 1 and HSV-1 genomes (Davison and Wilkie, 1983) and the colinearity between the two subtypes of EHV-1 discussed above. The molecular hybridization studies of Davison and Wilkie (1983) indicated that HSV-1, HSV-2, EHV-1 subtype 1, VZV and PRV have a similar gene arrangement. These authors proposed that genetic colinearity in these viruses is not limited to highly conserved genes but extends to those for which no hybridization was detected. Thus, they suggested that the locations of many genes, in HSV-2, EHV-1 subtype 1, VZV and PRV, including those for which no homology was detected, could be predicted on the basis of existing knowledge of HSV-1 gene location. This has proven to be correct for VZV. VZV is the first member of the Alphaherpesvirinae whose complete DNA sequence has been determined (Davison and Scott, 1986a). On comparison

of the amino acid sequences of predicted VZV proteins with those available for HSV-1, the genetic arrangement of the two viruses was found to be very similar (Davison and Scott, 1986a). In the context of the results discussed above this implies that the locations of most EHV-1 subtype 2 genes may be predicted on a similar basis. Thus, the large body of existing knowledge on the molecular genetics of HSV-1 can be applied to a virus whose gene layout is currently unstudied.

Several proteins are highly conserved amongst the Alphaherpesvirinae. These include two essential virus specific enzymes, the DNA polymerase and ribonucleotide reductase, and the major DNA-binding protein, the major capsid protein, glycoprotein B (gB) and an immediate-early protein, V_{mw}IE 175. The latter protein plays an essential role in the transition from immediate-early gene expression to delayed-early and late gene expression (Watson and Clements, 1978, 1980; Preston, 1979, 1981; Dixon and Schaffer, 1980). The major DNA-binding protein has been shown to be essential in HSV replication (Powell et al., 1981; Conley et al., 1981) but the exact function of this protein is at present unclear. In immunodiffusion tests the major DNA-binding proteins, and to a lesser extent the major capsid proteins of HSV-1, HSV-2, BMV, PRV and EHV-1 subtype 1, demonstrate cross-reactive antigenicity (Yeo et al., 1981). gB, one of the major glycoproteins specified by HSV is involved in fusion of the virion envelope to the cellular membrane and plays an important role in stimulating the hosts immune response (Manservigi et al.,

1977; Sarmiento et al., 1979; Spear, 1984; Yasukawa and Zarling, 1985). The antigenic relatedness of HSV-1 gB and HSV-2 gB to their EHV-1 subtype 1 and BMV counterpart has been demonstrated by agar gel immunodiffusion and by Western blot analysis (Snowden et al., 1985; Snowden and Halliburton, 1985). Tryptic peptide analysis was employed to illustrate the structural similarities between the cross-reacting DNA-binding proteins of HSV-1, HSV-2, BMV, PRV and EHV-1 subtype 1 (Littler et al., 1981) and the gBs of HSV-1, HSV-2, BMV and EHV-1 subtype 1 (Snowden et al., 1985).

Davison and Wilkie (1983) noted that virion DNA probes from EHV-1, PRV and VZV hybridized to a subset of HSV sequences. These homologous regions contain several genes of unknown function, as well as those encoding the major DNA-binding protein, the major capsid protein, the DNA polymerase, the ribonucleotide reductase and V_{mw}IE 175. Moreover, conservation of these genes in EHV-1 subtype 2 is evident from the results discussed above. Davison and Wilkie (1983) proposed that HSV-1 probes containing detectably conserved genes could be used to locate counterparts of these genes in heterologous genomes. The approximate locations of the EHV-1 subtype 2 genes encoding the major DNA binding protein, the major capsid protein, V_{mw}IE 175 and ribonucleotide reductase were determined using the appropriate HSV-1 probes. In addition hybridization of cloned fragments of EHV-1 subtype 2 DNA to HSV-1 DNA allowed the location of genes encoding the EHV-1 subtype 2 DNA polymerase and gB counterparts to be

determined. The usefulness of hybridization assays to locate highly conserved genes has recently been confirmed by the derivation of the complete DNA sequence of VZV (Davison and Scott, 1986a). The locations of the VZV genes encoding the homologues of HSV-1 DNA polymerase, the major DNA-binding protein, the major capsid protein, V_{mw}IE 175 and the ribonucleotide reductase corresponded to those predicted from the results of the hybridization studies of Davison and Wilkie (1983). The HSV-1 DNA polymerase (Quinn and McGeoch, 1985), the major capsid protein (Davison and Scott, 1986b), the major DNA-binding protein (Quinn and McGeoch, 1985), gB (Bzik et al., 1984), V_{mw}IE 175 (McGeoch et al., 1986a) and the small (McLauchlan and Clements, 1983) and large (Y. Nikas and J.B. Clements, personal communication) subunits of the HSV ribonucleotide reductase are among the proteins whose predicted amino acid sequences are strongly homologous to proteins predicted from the VZV sequence (Davison and Scott, 1986a). Moreover, the conservation of proteins is not limited to the Alphaherpesvirinae. Comparisons of the amino acid sequences of predicted HSV and EBV proteins have demonstrated that the two subunits of the ribonucleotide reductase (Gibson et al., 1984), the DNA polymerase (Quinn and McGeoch, 1985; Gibbs et al., 1985), the major DNA-binding protein (Quinn and McGeoch, 1985), glycoproteins gB (Pellet et al., 1985) and gH (McGeoch and Davison, 1986), the exonuclease (McGeoch et al., 1986b), the major capsid protein (Davison and Scott, 1986b) and three genes of unknown function (Costa et al., 1985; McGeoch et al.,

1986b) are conserved. VZV counterparts for these and other EBV genes have been identified by sequence comparison (Davison and Taylor, 1986). Of these proteins the ribonucleotide reductase and the DNA polymerase appear to be the most highly conserved whereas the homology between the VZV DNA-binding protein and its EBV counterpart is weak (Davison and Taylor, 1986). Interestingly, no EBV homologue for V_{mw}IE 175 or its VZV counterpart has been detected (McGeoch et al., 1986a; Davison and Taylor, 1986).

The hybridization data published to date and presented here constitute compelling evidence for the divergence of the Alphaherpesvirinae from an ancestral herpesvirus. Molecular hybridization studies demonstrated the genetic colinearity of HSV-1, HSV-2, EHV-1 subtype 1 and VZV (Davison and Wilkie, 1983) and of EHV-1 subtype 1 and EHV-3 (Baumann et al., 1986) and EHV-1 subtype 2 (see above). The PRV genome is colinear with HSV with the exception of a region comprising about half of U_L which is relatively inverted (Davison and Wilkie, 1983; Ben-Porat et al., 1983). This inversion is probably indicative of a rearrangement which occured during the evolution of PRV from the progenitor virus.

Comparisons of VZV and HSV-1 DNA sequence data revealed relationships between genes which had not been detected by DNA-DNA hybridization and confirmed that these two members of the Alphaherpesvirinae have a similar gene arrangement (Davison and Scott, 1986a). DNA sequence analysis of the S segment of HSV-1 (McGeoch et al., 1985, 1986a) and VZV (Davison, 1983; Davison and Scott, 1985) and comparison of

the amino acid sequences of predicted proteins demonstrated that the seven unique VZV genes in this region all have counterparts amongst the 13 unique genes of the HSV-1 S region (Davison and McGeoch, 1986). Thus, although these regions of the two genomes are among the most heterologous (Davison and Wilkie, 1983) and differ significantly in gene layout, they are clearly related. An evolutionary model has been proposed for their descent from the S segment of an ancestral virus by a series of recombinational events (Davison and McGeoch, 1986).

Evolutionary comparisons have not been confined to members of a single subfamily. While VZV and HSV-1 are more closely related than is VZV to EBV the counterparts of 29 of the 67 unique VZV genes have been found in EBV by sequence comparison (Davison and Taylor, 1986). Moreover, certain VZV and EBV genes are similar in size, location and orientation, and hydrophobicity profile, yet they share no detectable homology (Davison and Taylor, 1986). Whether or not these similarities are considered, the large number of genes conserved in VZV and EBV is strong evidence that these two viruses as representatives of the Alphaherpesvirinae and Gammaherpesvirinae respectively diverged from an ancestral herpesvirus. All conserved genes are also located in U_L and their quasi-colinear layout suggests that large-scale sequence rearrangements have occured in the evolution of one or both of these viruses from the progenitor. In conclusion, there is substantial evidence to suggest that the alpha- and gamma-herpesviruses evolved from an ancestral virus.

CHAPTER 5

THE DNA SEQUENCE ANALYSIS OF AN EHV-1 SUBTYPE 2 FRAGMENT CONTAINING THE JUNCTION BETWEEN U_S AND TR_S

INTRODUCTION

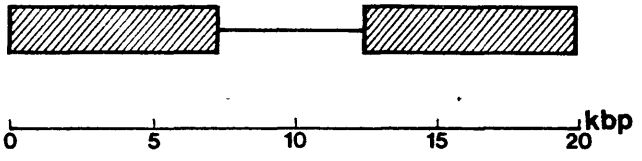
The genetic relatedness of the Alphaherpesvirinae has been demonstrated by molecular hybridization (Davison and Wilkie, 1983; Ben-Porat et al., 1983) and by DNA sequence analysis (Davison and McGeoch, 1986; Robbins et al., 1986; McGeoch and Davison, 1986; Davison and Scott, 1986a,b). The S segment is the most heterologous component of the Alphaherpesvirinae genomes (Davison and Wilkie, 1983; Davison and McGeoch, 1986). The DNA sequences of the S segment of HSV-1 (Murchie and McGeoch, 1982; McGeoch et al., 1985, 1986a) and VZV (Davison 1983; Davison and Scott, 1985) have been determined and the predicted encoded proteins compared (Davison and McGeoch, 1986). The HSV-1 U_S and TR_S/IR_S are larger than those of VZV (Figure 5.1) and six of the genes in HSV-1 S have no VZV homologues (Davison and McGeoch, 1986). Each gene in the VZV S segment has an HSV-1 counterpart (Davison and McGeoch, 1986). It is clear from the restriction endonuclease maps of the genome of EHV-1 subtype 2 (Figure 3.15) that the S segment (32kbp) is larger than the S segment of HSV (26kbp) or VZV (20kbp) (Figure 5.1). This suggests that the S segment of the EHV-1 subtype 2 may contain more genes than the S segments of HSV-1 and VZV. The cloned fragment containing the junction between the U_S and TR_S of the EHV-1, subtype 2 genome showed no homology to HSV-1 DNA in comparative hybridization experiments. The DNA sequence of this fragment was determined to elucidate the nature of the genes near the U_S - TR_S junction. These data also allowed

FIGURE 5.1

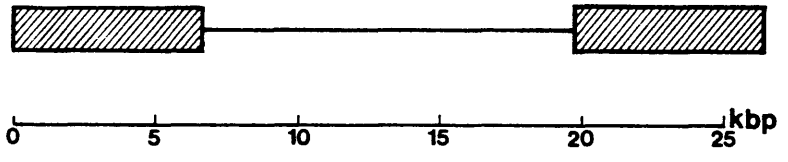
A diagrammatic representation of the S segments of VZV, HSV-1 and EHV-1 subtype 1 and 2. The inverted repeat sequences are represented by cross-hatched boxes.

S Regions of VZV, HSV-1 and EHV-1 subtypes 1 and 2

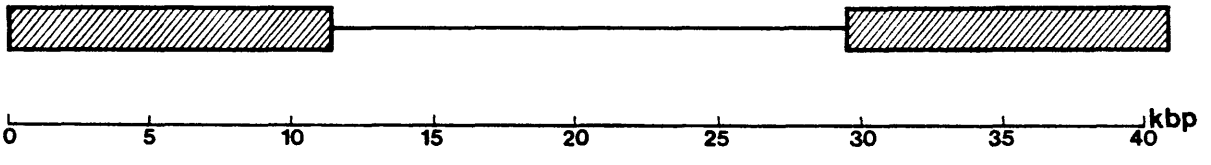
VZV



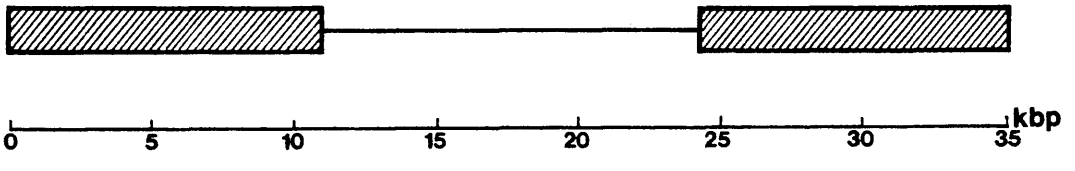
HSV-1



EHV-1 subtype 1



EHV-1 subtype 2



a more accurate estimation of the exact location of the junction than that afforded by the restriction endonuclease maps.

RESULTS AND DISCUSSION

DNA Sequence Analysis

EHV-1 subtype 2 Bam HI 1 is a 4.57kbp fragment which contains the junction between U_S and TR_S. Random fragments of Bam HI 1 isolated from a plasmid were generated by sonication and cloned into bacteriophage M13mp8. Single-stranded DNA was isolated from the M13 clones and sequenced using the Sanger dideoxynucleotide chain terminating method. The data were compiled with the aid of a computer, as described in Materials and Methods. An autoradiograph of one of the gels used to sequence Bam HI 1 is shown in Figure 5.2 and the sequence database is shown in Figure 5.3. 98% of the fragment was sequenced on both strands. The DNA sequence of EHV-1 subtype 2 Bam HI 1 is shown in Figure 5.4. The sequence was analysed for potential protein-coding regions by considering the locations of open reading frames (ORFs), initiator ATG triplets (Kozak, 1981, 1983) and elements associated with polyadenylation (Proudfoot and Brownlee, 1976). Codon usage within the sequence was examined using the program of Staden and McLachlan (1982). The analysis revealed the presence of two complete ATG-initiated ORFs and parts of two additional ORFs likely to encode proteins. The locations of three copies of the element AATAAA, a sequence usually present (as AAUAAA) close upstream of the 3' termini of eukaryotic

FIGURE 5.2

Autoradiograph of one of the 6% polyacrylamide-urea gels used to sequence EHV-1 subtype 2 Bam HI 1.

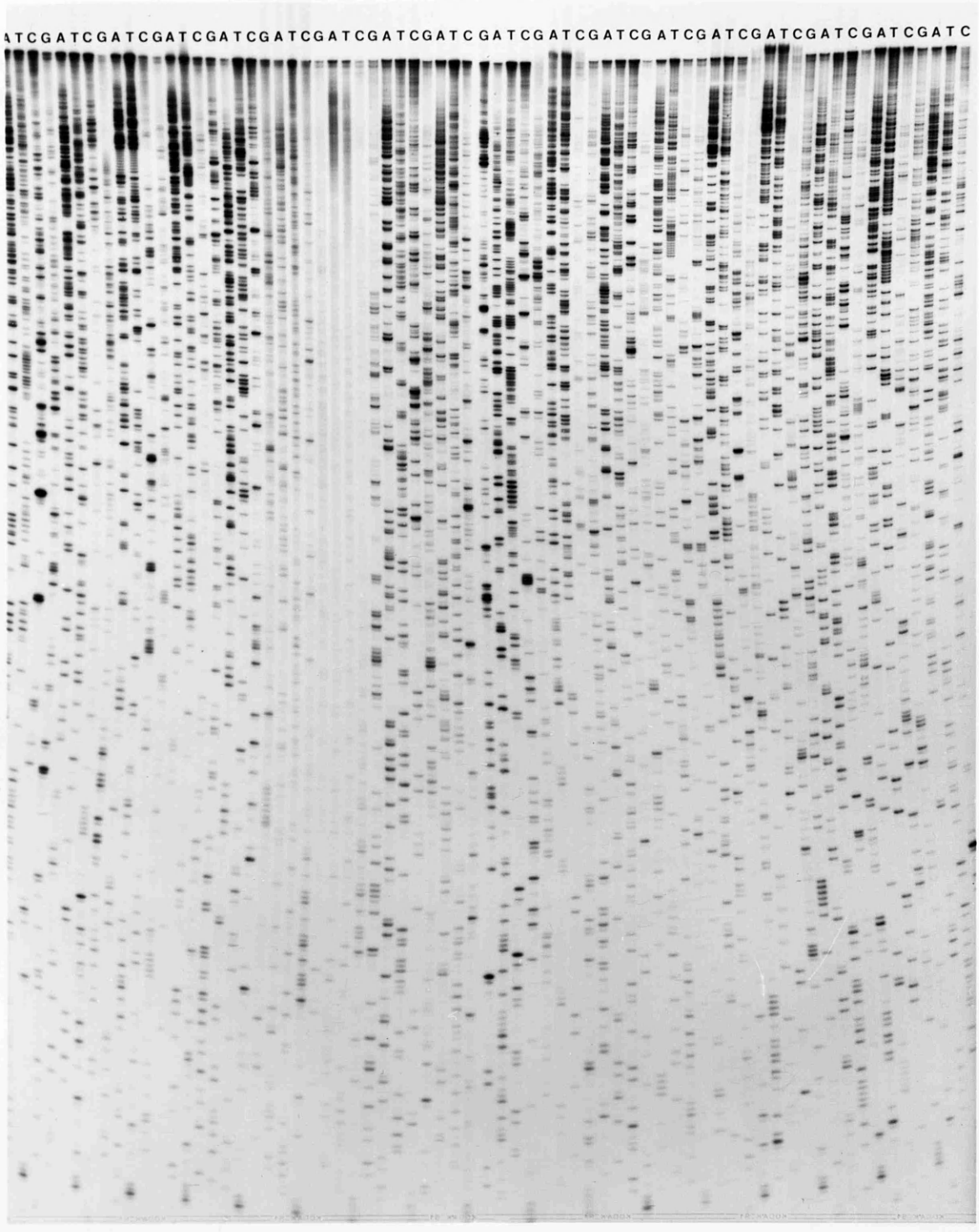

ATCGATCGATCGATCGATCGATCGATCGATCGATCGATCGATCGATCGATCGATCGATCGATCGATC

FIGURE 5.3

Database of the DNA sequence of M13 subclones of Bam HI 1 of EHV-1 subtype 2. The numbers on the left represent the individual M13 clones the DNA sequences of which are shown as the rightward 5'-3' strand. Those clones assigned a negative value were sequenced on the other strand. The consensus sequence is unnumbered. The total database contains approximately 40,000 nucleotides and each nucleotide was sequenced, on average, 8.6 times. The asterisks represent padding characters inserted by the computer program to produce an alignment. After the database was compiled initially all errors were removed by careful reading of the best sequence for each strand at each nucleotide location.

```
          10        20        30        40        50        60        70        80        90       100       110       120
-28  GGATCCACATGGCCTTCTCGGTGCCATAGTACTTTGTTGGGCGATAGGCCACATTTTATCCAACCGGCACCAAACAGGGTAGACTTGTTATTCAAAGATATACCCGA*ATCACGACCGGG
-72  GGATCCACATGGCCT*CTC
 73  GGATCCACATC*******CGGTGCCATAGTACTTTGTTGGGCGATAGGCCACATTTTATCCAACCGGCACCAAACAGGGTAGACTTGTTATTCAAAGATATACCCGA*TCAGCGACCGGG
102  GGATCCACATGGCCTTCTCGG**CCATAGTACTTTGTTGGGCGATAGGCCACATTTTATCCAACCGGCACCAAACAGGGTAGACTTGTTATTCAAAGATATACCCGAATCAGCGACCGGG
-140 GGATCCACATGGCCTTCTCGGTGCCATAGTACTTTGTTGGGC
146  GGATCCACATGGCCTTCTCGGTGCCATAGTACTTTGTTGGGCGATAGGCCACATTTTATCCAACCGGCACCAAACAGGGTAGACTTGTTATTCAAAGATATACCCGAATCAGCGACCGGG
120                                                                                                                   GGG
     GGATCCACATGGCCTTCTCGGTGCCATAGTACTTTGTTGGGCGATAGGCCACATTTTATCCAACCGGCACCAAACAGGGTAGACTTGTTATTCAAAGATATACCCGAATCAGCGACCGGG

          130       140       150       160       170       180       190       200       210       220       230       240
-28  TTGTATGTGTTTGTGTTATTGTACAACGGACATCCGGAGGCGTGGACGTATACGTTGCTTTCTACAGCAAATCACTTTATGAACGTGCTTACGGACCGAACACGCCCACGGCTAGGAGAG
 73  TTCTATGTGTTTGTGTTATTGTACAACGGACATCCGGAGGCGTGGACGTATACGTTGC*TTCTCAAGCAATATTTTAGACTGTT
102  TTGTATGTGTTTGTGTTATTGTACAACGGACATCCGGAGGCGTGGACGTATACGTTGCTTTCTACAGCAAATCACTTTATG*ACGTGCTTACGGACCGAACACGCCCAGGCCTAGGAGAG
146  TTGTATGTGTTTGTGTTATTGTACAC
120  TTGTATGTGTTTGTGTTATTGTACAACGGACATCCGGAGGCGTGGACGTATACGTTGCTTTCTACAGCAAATCACTTTATGAACGTGCTTACGGACCGAACACGCCCACGGCTAGGAGAG
 10                                                            CTACAGCAAATCACTTTATGAACGTGCTTACGGACCGAACACGCCCACGGCTAGGAGAG
-107                                                           TACAGCAAATCACTTTATGAACGTGCTTACGGACCGAACACGCCCACGGCTAGGAGAG
 -35                                                                  AACGTGCTTACGGACCGAACACGCCCACGGCTAGGAAGA
  78                                                                      TTACGGACCGAACACGCCCACGGCTAGGAGAG
 142                                                                                                 CTAGGAGAG
     TTGTATGTGTTTGTGTTATTGTACAACGGACATCCGGAGGCGTGGACGTATACGTTGCTTTCTACAGCAAATCACTTTATGAACGTGCTTACGGACCGAACACGCCCACGGCTAGGAGAG

          250       260       270       280       290       300       310       320       330       340       350       360
-28  C
102  CACTTT
120  CACTTTTATA*GGACCACGGGCACACGCTTTTCACTC*TCATCCATCTGA
 10  CACTTTTATACGGACCACGGGCACCAGCTTTTCACTCCTCATCCATCTGAGGCAACAACTCAAGAGTTGGGAGCTTGGACCAGACACTACCTCGCTTTTTTGTTGATCATAATCTGCACC
-107 CACTTTTATACGGACCACGGGCACCAGCTTTTCACTCCTCATCCATCTGAGGCAACAACTCAAGAGTTGGGAGCTTGGACCAGACACTACCTCGCTTTTTTGTTGATCATAATCTGCACC
 -35 CAC*TTTATACGGACCACGGGCACCACTT*TTCACTCCTCATCCATCTGAGGCAACAACTCAAGAGTTGGGAGCTTGGACCAGACACTACCTCGCTTTTTTGTTGATCATAATCTGCACC
  78 CACTTTTATACGGACCACGGGCACCAGCTTCTCACTCCTCATCCATCTGAGGCAACAACTCAAGAGTTGGGAGCT*GGACCAGACATA**CTCGCTTTTG***TGATCATATCTCGACT
 142 CACTTTTATACGGACCACGGGCACCAGCTTTTCACTCCTCATCCATCTGAGGCAACAACTCAAGAGTTGGAGCTTGGACCAGACA*TACCTCGC**TTTTGTTGATCATA*TCTGCAC*
  -2                                 TGTTCACTCCTCATCCATCTGAGGCAACAACTCAAGAGTTGG*AGCTTGGACCAGACACTACCTCGCTTTTT*GTTGATCATAATCTGCACC
 -26                                       ATCCATCT*AGGCAACAACTCAAGAGTTGGGAGCTTGGACCAGACACTACCTCGC*TTTTTGTTGATCATAATCTGCACC
  32                                                                  CTTGGACCAGACACTACCTCGCTTTTTTGTTGATCATAATCTGCACC
     CACTTTTATACGGACCACGGGCACCAGCTTTTCACTCCTCATCCATCTGAGGCAACAACTCAAGAGTTGGGAGCTTGGACCAGACACTACCTCGCTTTTTTGTTGATCATAATCTGCACC

          370       380       390       400       410       420       430       440       450       460       470       480
 10  TGTGCCGCGCTGCTAATTGCCTTGGTGGTGTGGGGCTGCATTCTATACATCCGAAGCAACCGCAAGCCGTATGAAGTACTAAACC**TTT
-107 TGTGCCGCGCTGCTAATTGCCTTGGTGGTGTGGGGCTGCATTCTATACATCCGAA*CAACCGCAAGCCGTATGAAGTACTAAACCCGTTT
 -35 TGTGCCGCGCTGCTAATTGCCTTGGTGGTGTGGGGCTGCATTCTATACATCCGAAGCAACCGCAAGCCGTATGAAGTACTAAACCCGTTTGAAACGGTTTA
 142 TGT
  -2 TGTGCCGCGCTGCTAATTGCCTTGGTGGTGTGGGGCTGCATTCTATACATCCGAAGCAACCGCAAGCCGTATGAAGTACTAAACCCGTTTGAAACGGTTTACACAAGCGTTCCCAGCAAC
 -26 TGTGCCGCGCTGCTAATTGCCTTGGTGGTGTGGGGCTGCATTCTATACATCCGAAGCAACCGCAAGCCGTATGAAGTACTAAACCCGTTTGAAACGGTTTACACAAGCGTTCCCAGCAAC
  32 TGTGCCGCGCTGCTAATTGCCTTGGTGGTGTGGGGCTGCATTCTATACATCCGAAGCAACCGCAAGCCGTATGAAGTACTAAACCCGTTTGAAACGGTTTACACAAGCGTTCCCAGCAAC
  -7           TGCTAATTGCCTTGGTGGTGTGGGGCTGCATTCTATACATCCGAAGCAACCGCAAGCCGTATGAAGTACT*G*TTGAAACGGTTTACACAA*CGTTCCCAGCAAC
 -56                                GGGGCTGCA*TCTATACATCCGAAGCAACCGCAAGCCGTATGAAGTACTAAA*CCGTTTGAAACGGTTTACACAAGCGTTCCCAGCAAC
 -71                                                  AGATCCGA**CAACCG**AGCCGTATGAAGTACTACCA**GTTT*AAACGGTTTACAC*AGCGTTCCCAGCAAC
  63                                                                                 CCGTTTGAAACGGTTTACACAAGCGTTCCCAGCAAC
     TGTGCCGCGCTGCTAATTGCCTTGGTGGTGTGGGGCTGCATTCTATACATCCGAAGCAACCGCAAGCCGTATGAAGTACTAAACCCGTTTGAAACGGTTTACACAAGCGTTCCCAGCAAC

          490       500       510       520       530       540       550       560       570       580       590       600
  -2 GATCCAACCGACGAAGTCTTGGTATTTGAGCGTCTGGCTTCAGACTCCGACGACTCCTTCGACTCAAGTTCAGACGA
 -26 GATCCAACCGACGAAGTCTTGGTATTTGA*CGTCTGGCTTCAGACTCCGACGACTCCTTCGACTCAAGTTCAGACGAAGAATTGGAACTACCACAACCTCCACCAGCCGCACAACTTCAG
  32 GATCCAACCGACGAAGTCTTGGTATTTGAGCGTCTGGCTT*AGACTCCGACGACTCCTTCGACTCAAGTT*AGACGAAGAATTGGAACTACCACAACCTCCACCAGCCGCACAACTTCAG
  -7 GATCCAACCGACGAA*TCTTGGTATTTGA*CGTCTGGCTTCAGACTCCGACGACTCCTTCGACTCAAGTTCAGACGAAGAATTGGAACTACCACAACCTCCACCAGCCGCACAACTTCAG
 -56 GATCCAACCGACGAAGTCTTGGTATTTGAGCGTCTGGCTTCAGACTCCGACGACTCCTTCGACTCAAGTTCAGACGAAGAATTGGAACTACCACAACCTCCACCAGCCGCACAACTTCAG
 -71 GATCCAAC*GACGAA*TCTTGGTATTTGAGCGTCTGGCTTCAGACTCCGACGACTCCTTCGACTCAA
  63 GATCCAACCGACGAAGTCTTGGTATTTGAGCGTCTGGCTTCAGACTCCGACGACTCCTTCGACTCAAGTTCAGACGAAGAATTGGAACTACCACAACCTCCACCAGCCGCACAACTTCAG
     GATCCAACCGACGAAGTCTTGGTATTTGAGCGTCTGGCTTCAGACTCCGACGACTCCTTCGACTCAAGTTCAGACGAAGAATTGGAACTACCACAACCTCCACCAGCCGCACAACTTCAG

          610       620       630       640       650       660       670       680       690       700       710       720
-26 CCGTATAGTTCACTAGAAAGTGCAGA
 32 CCGTATAGTTCACTAGAAAGTGCAGAC
 -7 CCGTATAGTTCACTAGAAAGTGCAGACGCGTCGAGAGGCCGGTCGGGTTTCAAG
-56 CCGTATAGTTCACTAG
 63 CCGTATAGTTCACTAGAAAGTGCAGACGCGTCGAGAGGCCGGTCGGGTTTCAAGGTCTGGTTCCGCGATACACCAGAGGCGTCTCCGGAGCCGCTTCATAGACCAA*CCCACCCGTCGGA
 22      TAGTTCACTAGAAAGTGCAGACGCGTCGAGAGGCCGGTCGGGTTTCAAGGTCTGGTTCCGCGATACACCAGAGGCGTCTCCGGAGCCGCTTCATAGACCAACCCCACCCGTCGGA
 58            ACTAGAAAGTGCAGACGCGTCGAGAGGCCGGTCGGGTTTCAAGGTCTGGTTCCGCGATACACCAGAGGCGTCTCCGGAGCCGCTTCATAGACCAACCCCACCCGTCGGA
-91                                  GAAGAGGCCGGTCGGGTTTCAAGGTCTGGTTCCGCGATACACCAGAGGCGTCTCCGGAGCCGCTTCATAGACCAACCC*A*CCGTCGGA
-30                                    AGAGGCCGGTC*GGTTTCAAGGTCTGGTTCCGCGATACACCAGAGGCGTCTCCGGAGC*GCTTCATAGACCAACCCCACCCGT*GGA
138                                                                                                 TCCGGAGCCGCTTCATAGACCAACCCCACCCGTCGGA
     CCGTATAGTTCACTAGAAAGTGCAGACGCGTCGAGAGGCCGGTCGGGTTTCAAGGTCTGGTTCCGCGATACACCAGAGGCGTCTCCGGAGCCGCTTCATAGACCAACCCCACCCGTCGGA

          730       740       750       760       770       780       790       800       810       820       830       840
 63 CCGGACTACAGCAAGG
 22 CCGGACTACAGCAAGGTCGCGTCAAAGCTCAGGTCTATCCTAAAATGAATTT*AACAACAAAGATACCGCTTGCGCAGGAAATGTGTGCTATGCTGAAGGACTACGCAATCGTAAGAGTA
 58 CCGGACTACAGCAAGGTCGCGTCAAAGCTCAGGTCTATCCTAAAATGAATTTCAACAACAAAGATACCGCTTGCGCAGGAAATGTGTGCTATGCTGAAGGACTACGCAATCGTAAGAGTA
-91 CCGGACTACAGCAAGGTCGCGTCAAAGCTCAGGTCTATCCTAAAATGAATTTCAACAACAAAGATACCG*TTGCGCAGGAAATGTGTGCTATGCTGAAGGACTACGCAATCGTAAGAGTA
-30 CCGGACTACAGCAAGGTCGCGTCAAAGCTCAGGTCTATCCTAAAATGAATTTCAACAACAAAGATACCG*TTGCGCAGGAAATGTGTGCTATGCTGAAGGACTACGCAATCGTAAGAGTA
138 CCGGACTACAGCAAGGTCGCGTCAAAGCTCAGGTCTATCCTAAAATGAATTTCAACAACAAAGATACCGCTTGCG*AGGAAATGTGTGCTATGCTGAAGGACTACGCAATCGTAAGAGTA
-90            AAGGTCGCGTCAGAGCTCAGGTCTATCCTAAAATGAATTTCAACAACAAAGATACCG*TTGCGCAGGAAATGTGTGCTATGCTGAAGGACTACGCAATCGTAAGAGTA
-114                                                                 TACGTTGCGCAGGAAATGTGTGCTATGCTGAAGGACTA*GCAATCGTAAGAGTA
-79                                                                                                      AAGAGTA
     CCGGACTACAGCAAGGTCGCGTCAAAGCTCAGGTCTATCCTAAAATGAATTTCAACAACAAAGATACCGCTTGCGCAGGAAATGTGTGCTATGCTGAAGGACTACGCAATCGTAAGAGTA

          850       860       870       880       890       900       910       920       930       940       950       960
 22 GTCCGGTTCGAAACAGCACCTTCGAAGAGTATCTCGA*TCACTTAATAATTACGACCGCCG*TTGAGAGCTGACTCAACTTCAGATTCGGACTCTGG*TGTAAA**CCCTCTGA*GAGCA
 58 GTCCGGTTCGAAACAGCACCTTCGAAGAGTATCTCGACTCACTTAATA*TTACG*CCGCCGTTTAG
-91 GTCCGGTTCGAAACAGCACCTTCGAAGAGTATCTCGACTCACTTAATAATTACGACCGCCGTTTGAGAGCT
-30 GTCCGGTTCGAAACAGCACCTTCGAAGAGTATCTCGACTCACTTAATAATTACGACCGCCGTTTGAGAGCTGACTCAACTTCAGATTCGGACTCTGGGTGTAAACCCCCTCTGAAGACGA
138 GTCCGGTTCGAACACA***CTTCGAGA
-90 GTCCGGTTCGAAACAGCACCTTCGAAGAGTATCTCGACTCACTTAATAATTACGACCGCCGTTTGAGAGCTGACTCAACTTCAGATTCGGACTCTGG*TGTAAACCCCCTCTGAAGACGA
-114 GTCCGGTTCGAAACAGCACCTTCGAAGAGTATCTCGACT*ACTTAATAATTACGACCGCCGTTTGAGAGCTGACT*AACTTCAGATTCGGACTCTGGGTGTAAACCCCCTCTGAAGACGA
-79 GTCCG**TCGAAACAACACCTTCG*AGAGTATCTCGACT*ACTTAATAATTACGAC*GCCG*TTGAGAGCTGACTC*ACTTCAGATTCGGACTCTGG*TGTAAACCCCCTCTGAAGACGA
-80          GAAACAGGACCT*CG*AGAGTATCTCGACTTACTTAATAATTACGACGGCCGTTTGAGAGCTGACTGAACTTCAGATTCGGACTCTGGGTGTAAACCCCCTCTGAAGACGA
  4                                                                 GCCGTTTGAGAGCTGACTCAACTTCAGATTCGGACTCTGGGTGTAAACCCCCTCTGAAGACGA
 24                                                                                                                  AAGACGA
151                                                                                                                  CGA
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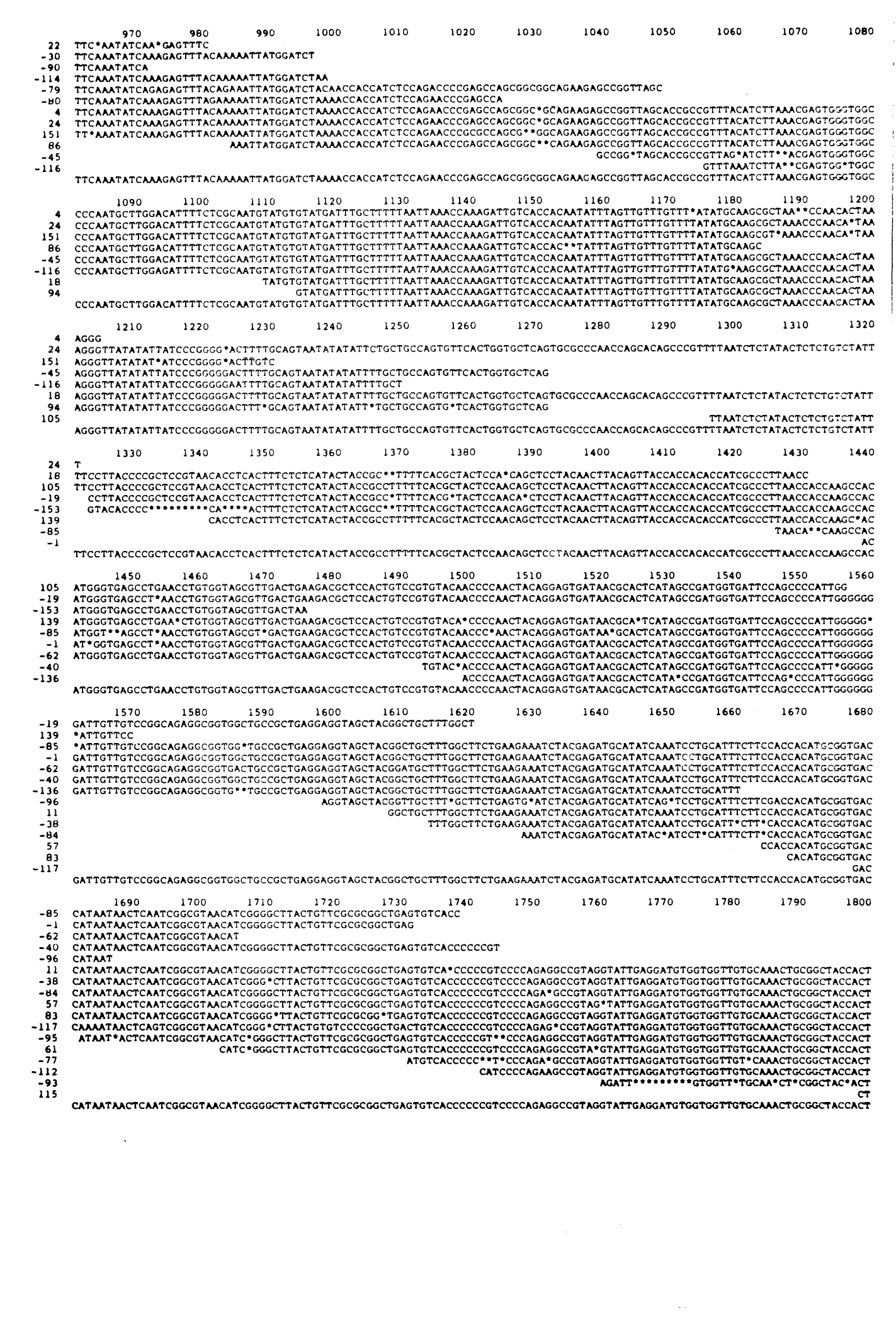

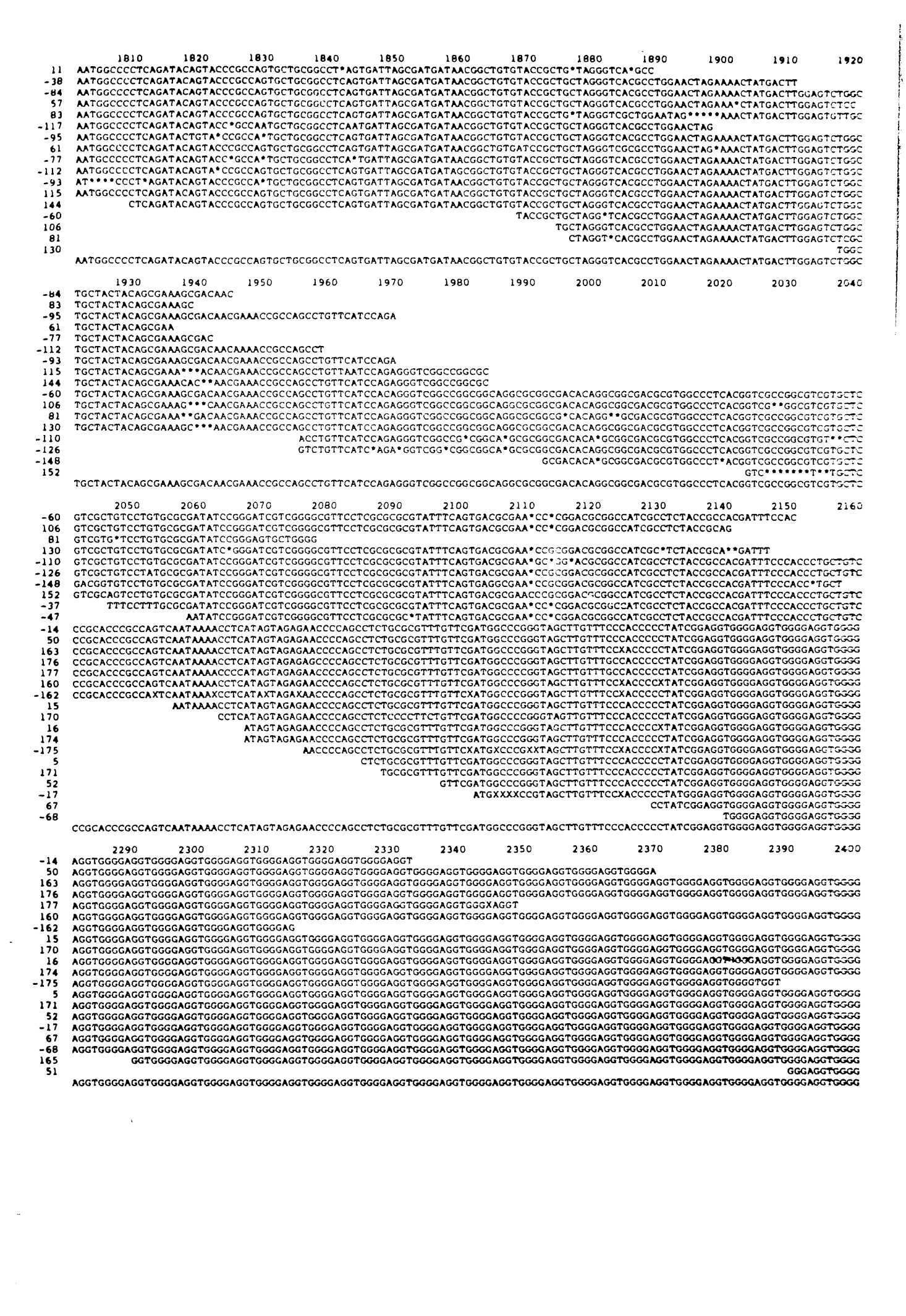

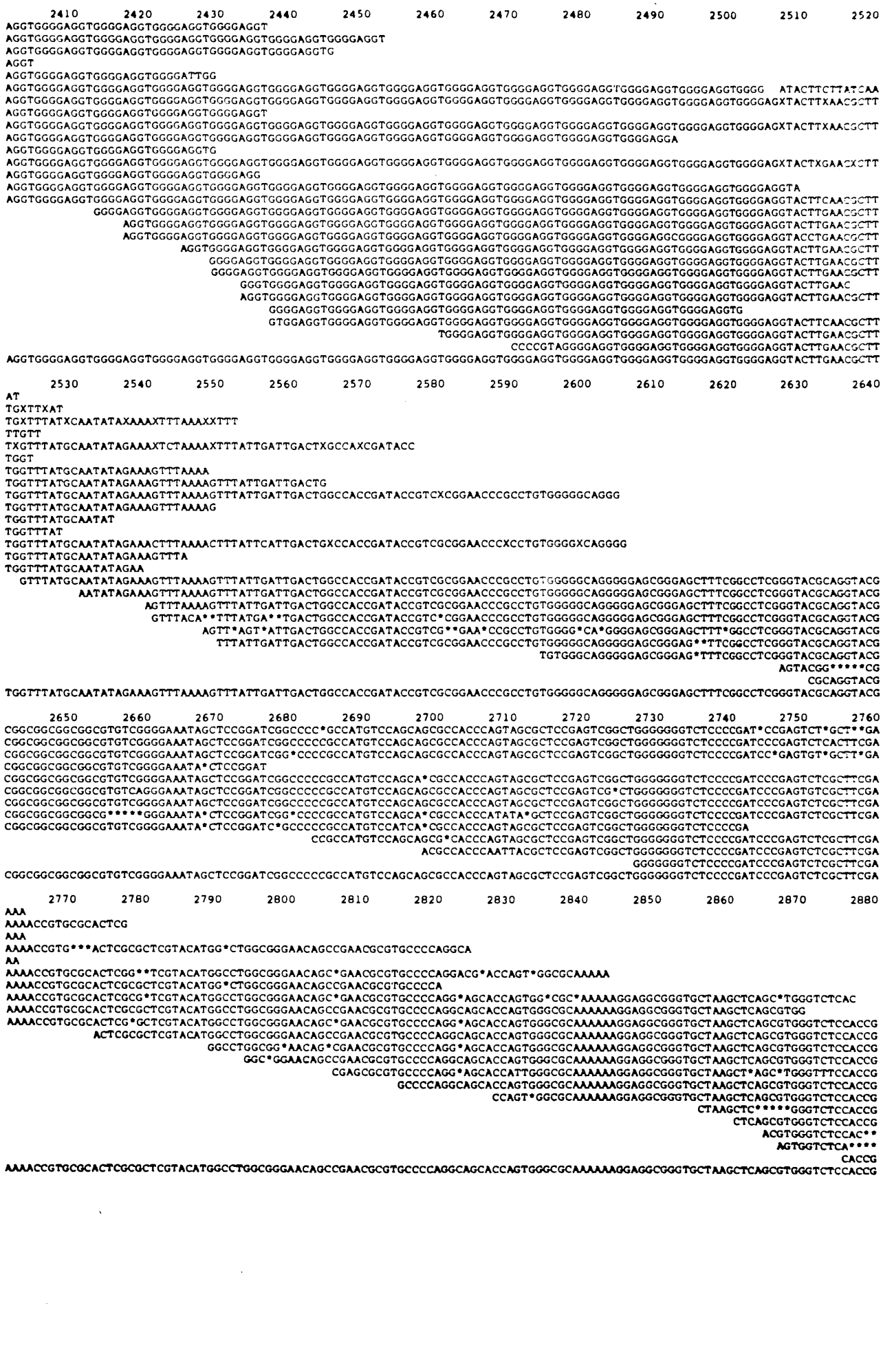

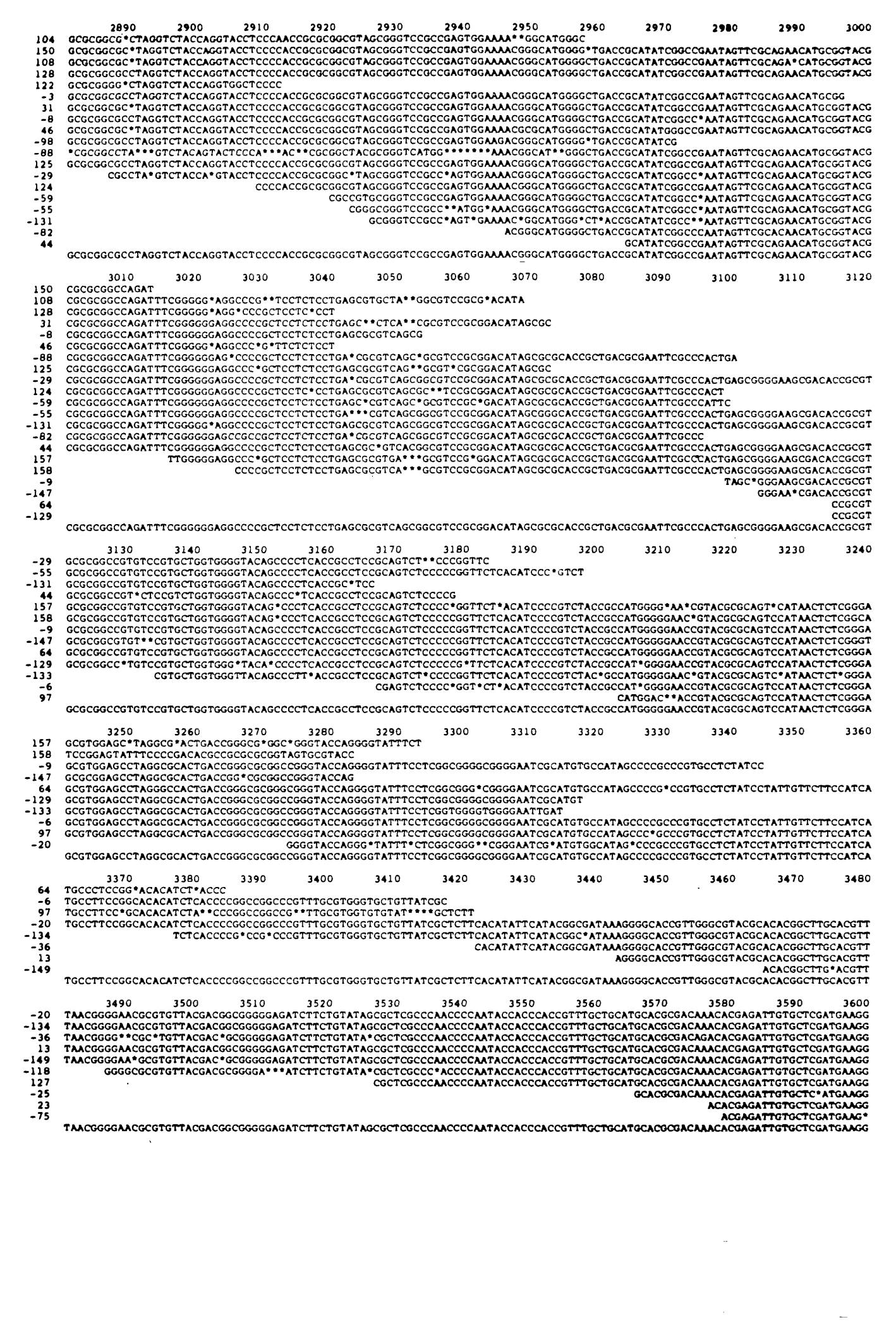

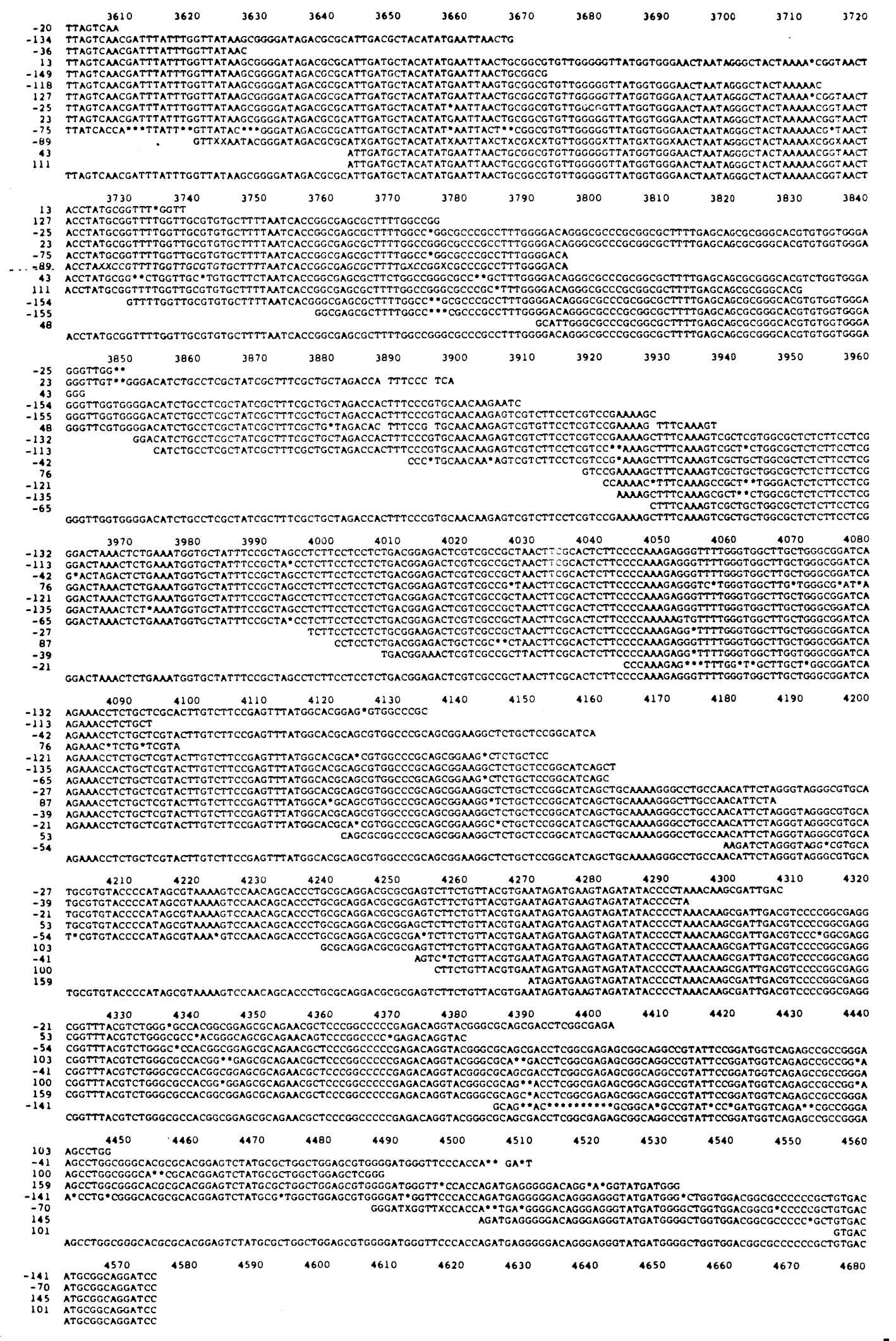

FIGURE 5.4

The DNA sequence of Bam HI 1 of EHV-1 subtype 2 shown as the rightward 5' - 3' strand. The amino acid sequences of predicted encoded proteins are shown in single letter amino acid code above (rightward encoded) or below (leftward encoded) the corresponding DNA sequence. Polyadenylation associated sequences AATAAA and ATTAAA are indicated by asterisks. Reiterated sequences are marked ><.

```
1   G  S  T  W  P  S  R  C  H  S  T  L  L  G  D  R  P  H  F  I  Q  P  A  P  N  R  V  D  L  L    30
    GGATCCACATGGCCTTCTCGGTGCCATAGTACTTTGITGGGCGATAGGCCACATTTTATCCAACCGGCACCAAACAGGGTAGACTTGTTA    90

    F  K  D  I  P  E  S  A  T  G  L  Y  V  F  V  L  L  Y  N  G  H  P  E  A  W  T  Y  T  L  L    60
    TTCAAAGATATACCCGAATCAGCGACCGGGTTGTATGTGTTTGTGTTATTGTACAACGGACATCCGGAGGCGTGGACGTATACGTTGCTT   180

    S  T  A  N  H  F  M  N  V  L  T  D  R  T  R  P  R  L  G  E  H  F  Y  T  D  H  G  H  Q  L    90
    TCTACAGCAAATCACTTTATGAACGTGCTTACGGACCGAACACGCCCACGGCTAGGAGAGCACTTTTATACGGACCACGGGCACCAGCTT   270

    F  T  P  H  P  S  E  A  T  T  Q  E  L  G  A  W  T  R  H  Y  L  A  F  L  L  I  I  I  C  T   120
    TTCACTCCTCATCCATCTGAGGCAACAACTCAAGAGTTGGGAGCTTGGACCAGACACTACCTCGCTTTTTTGTTGATCATAATCTGCACC   360

    C  A  A  L  L  I  A  L  V  V  W  G  C  I  L  Y  I  R  S  N  R  K  P  Y  E  V  L  N  P  F   150
    TGTGCCGCGCTGCTAATTGCCTTGGTGGTGTGGGGCTGCATTCTATACATCCGAAGCAACCGCAAGCCGTATGAAGTACTAAACCCGTTT   450

    E  T  V  Y  T  S  V  P  S  N  D  P  T  D  E  V  L  V  F  E  R  L  A  S  D  S  D  D  S  F   180
    GAAACGGTTTACACAAGCGTTCCCAGCAACGATCCAACCGACGAAGTCTTGGTATTTGAGCGTCTGGCTTCAGACTCCGACGACTCCTTC   540

    D  S  S  S  D  E  E  L  E  L  P  Q  P  P  P  A  A  Q  L  Q  P  Y  S  S  L  E  S  A  D  A   210
    GACTCAAGTTCAGACGAAGAATTGGAACTACCACAACCTCCACCAGCCGCACAACTTCAGCCGTATAGTTCACTAGAAAGTGCAGACGCG   630

    S  R  G  R  S  G  F  K  V  W  F  R  D  T  P  E  A  S  P  E  P  L  H  R  P  T  P  P  V  G   240
    TCGAGAGGCCGGTCGGGTTTCAAGGTCTGGTTCCGCGATACACCAGAGGCGTCTCCGGAGCCGCTTCATAGACCAACCCCACCCGTCGGA   720

    P  D  Y  S  K  V  A  S  K  L  R  S  I  L  K  -                                             255
    CCGGACTACAGCAAGGTCGCGTCAAAGCTCAGGTCTATCCTAAAATGAATTTCAACAACAAAGATACCGCTTGCGCAGGAAATGTGTGCT   810

    ATGCTGAAGGACTACGCAATCGTAAGAGTAGTCCGGTTCGAAACAGCACCTTCGAAGAGTATCTCGACTCACTTAATAATTACGACCGCC   900

    GTTTGAGAGCTGACTCAACTTCAGATTCGGACTCTGGGTGTAAACCCCCTCTGAAGACGATTCAAATATCAAAGAGTTTACAAAAATTAT   990

    GGATCTAAAACCACCATCTCCAGAACCCGAGCCAGCGGCGGCAGAAGAGCCGGTTAGCACCGCCGTTTACATCTTAAACGAGTGGGTGGC  1080

                                                      ******
    CCCAATGCTTGGACATTTTCTCGCAATGTATGTGTATGATTTGCTTTTTAATTAAACCAAAGATTGTCACCACAATATTTAGTTGTTTGT  1170

    TTTATATGCAAGCGCTAAACCCAACACTAAAGGGTTATATATTATCCCGGGGGACTTTTGCAGTAATATATATTTTGCTGCCAGTGTTCA  1260

    CTGGTGCTCAGTGCGCCCAACCAGCACAGCCCGTTTTAATCTCTATACTCTCTGTCTATTTTCCTTACCCCGCTCCGTAACACCTCACTT  1350

    TCTCTCATACTACCGCCTTTTTCACGCTACTCCAACAGCTCCTACAACTTACAGTTACCACCACACCATCGCCCTTAACCACCAAGCCAC  1440

2   M  G  E  P  E  P  V  V  A  L  T  E  D  A  P  L  S  V  Y  N  P  N  Y  R  S  D  N  A  L  I    30
    ATGGGTGAGCCTGAACCTGTGGTAGCGTTGACTGAAGACGCTCCACTGTCCGTGTACAACCCCAACTACAGGAGTGATAACGCACTCATA  1530

    A  D  G  D  S  S  P  I  G  G  D  C  C  P  A  E  A  V  A  A  A  E  E  V  A  T  A  A  L  A    60
    GCCGATGGTGATTCCAGCCCCATTGGGGGGGATTGTTGTCCGGCAGAGGCGGTGGCTGCCGCTGAGGAGGTAGCTACGGCTGCTTTGGCT  1620

    S  E  E  I  Y  E  M  H  I  K  S  C  I  S  S  T  T  C  G  D  H  N  N  S  I  G  V  T  S  G    90
    TCTGAAGAAATCTACGAGATGCATATCAAATCCTGCATTTCTTCCACCACATGCGGTGACCATAATAACTCAATCGGCGTAACATCGGGG  1710

    L  T  V  R  A  A  E  C  H  P  P  S  P  E  A  V  G  I  E  D  V  V  V  V  Q  T  A  A  T  T   120
    CTTACTGTTCGCGCGGCTGAGTGTCACCCCCCGTCCCCAGAGGCCGTAGGTATTGAGGATGTGGTGGTTGTGCAAACTGCGGCTACCACT  1800
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 N  G  P  S  D  T  V  P  A  S  A  A  A  S  V  I  S  D  D  N  G  C  V  P  L  L  G  S  R  L  150
AATGGCCCCTCAGATACAGTACCCGCCAGTGCTGCGGCCTCAGTGATTAGCGATGATAACGGCTGTGTACCGCTGCTAGGGTCACGCCTG 1890

 E  L  E  N  Y  D  L  E  S  G  C  Y  Y  S  E  S  D  N  E  T  A  S  L  F  I  Q  R  V  G  R  180
GAACTAGAAAACTATGACTTGGAGTCTGGCTGCTACTACAGCGAAAGCGACAACGAAACCGCCAGCCTGTTCATCCAGAGGGTCGGCCGG 1980

 R  Q  A  R  R  H  R  R  R  R  V  A  L  T  V  A  G  V  V  L  V  A  V  L  C  A  I  S  G  I  210
CGGCAGGCGCGGCGACACAGGCGGCGACGCGTGGCCCTCACGGTCGCCGGCGTCGTGCTCGTCGCTGTCCTGTGCGCGATATCCGGGATC 2070

 V  G  A  F  L  A  R  V  F  Q  -                                                          220
GTCGGGGCGTTCCTCGCGCGCGTATTTCAGTGACGCGAACCCGCGGACGCGGCCATCGCCTCTACCGCCACGATTTCCCACCCTGCTGTC 2160

                  ******
CCGCACCCGCCAGTCAATAAAACCTCATAGTAGAGAACCCCAGCCTCTGCGCGTTTGTTCGATGGCCCGGGTAGCTTGTTTCCCACCCCC 2250

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 ><    ><    ><    ><    ><    ><    ><    ><    ><    ><    ><    >
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                                  ******
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3                   -  F  N  I  S  Q  S  A  V  S  V  T  A  S  G  A  Q  P  P  L  P  L  P  238

GGAGCTTTCGGCCTCGGGTACGCAGGTACGCGGCGGCGGCGGCGTGTCGGGGAAATAGCTCCGGATCGGCCCCCGCCATGTCCAGCAGCG 2700
  L  K  R  G  R  T  R  L  Y  A  A  A  A  A  A  H  R  P  F  L  E  P  D  A  G  A  M  D  L  L  A  208

CCACCCAGTAGCGCTCCGAGTCGGCTGGGGGGGTCTCCCCGATCCCGAGTCTCGCTTCGAAAAACCGTGCGCACTCGCGCTCGTACATGG 2790
  V  W  Y  R  E  S  D  A  P  P  T  E  G  I  G  L  R  A  E  F  F  R  A  C  E  R  E  Y  M  A  178

CCTGGCGGGAACAGCCGAACGCGTGCCCCAGGCAGCACCAGTGGGCGCAAAAAAGGAGGCGGGTGCTAAGCTCAGCGTGGGTCTCCACCG 2880
  Q  R  S  C  G  F  A  H  G  L  C  C  W  H  A  C  F  L  L  R  T  S  L  E  A  H  T  E  V  P  148

GCGCGGCGCCTAGGTCTACCAGGTACCTCCCCACCGCGCGGCGTAGCGGGTCCGCCGAGTGGAAAACGGGCATGGGGCTGACCGCATATC 2970
  A  A  G  L  D  V  L  Y  R  G  V  A  R  R  L  P  D  A  S  H  F  V  P  M  P  S  V  A  Y  R  118

GGCCGAATAGTTCGCAGAACATGCGGTACGCGCGCGGCCAGATTTCGGGGGGAGGCCCCGCTCCTCTCCTGAGCGCGTCAGCGGCGTCCG 3060
  G  F  L  E  C  F  M  R  Y  A  R  P  W  I  E  P  P  P  G  A  G  R  R  L  A  D  A  A  D  A  88

CGGACATAGCGCGCACCGCTGACGCGAATTCGCCCACTGAGCGGGGAAGCGACACCGCGTGCGCGGCCGTGTCCGTGCTGGTGGGGTACA 3150
  S  M  A  R  V  A  S  A  F  E  G  V  S  R  P  L  S  V  A  H  A  A  T  D  T  S  T  P  Y  L  58

GCCCCTCACCGCCTCCGCAGTCTCCCCCGGTTCTCACATCCCCGTCTACCGCCATGGGGGAACCGTACGCGCAGTCCATAACTCTCGGGA 3240
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                                                                ******
GAACTAATAGGGCTACTAAAAACGGTAACTACCTATGCGGTTTTGGTTGCGTGTGCTTTTAATCACCGGCGAGCGCTTTTGGCCGGGCGC 3780
4                                                                 - R R A S K A P R   266

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S E S S S W K G T C C S D D E E D S F S E F D S S A S E E E   206

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L S Q E P M L Q L L A Q W C E L T P R A H T Y G M A Y F D L   116

AGCACCCTGCGCAGGACGCGCGAGTCTTCTGTTACGTGAATAGATGAAGTAGATATACCCCTAAACAAGCGATTGACGTCCCCGGCGAGG 4320
L V R R L V R S D E T V H I S S T S I G R F L R N V D G A L    86

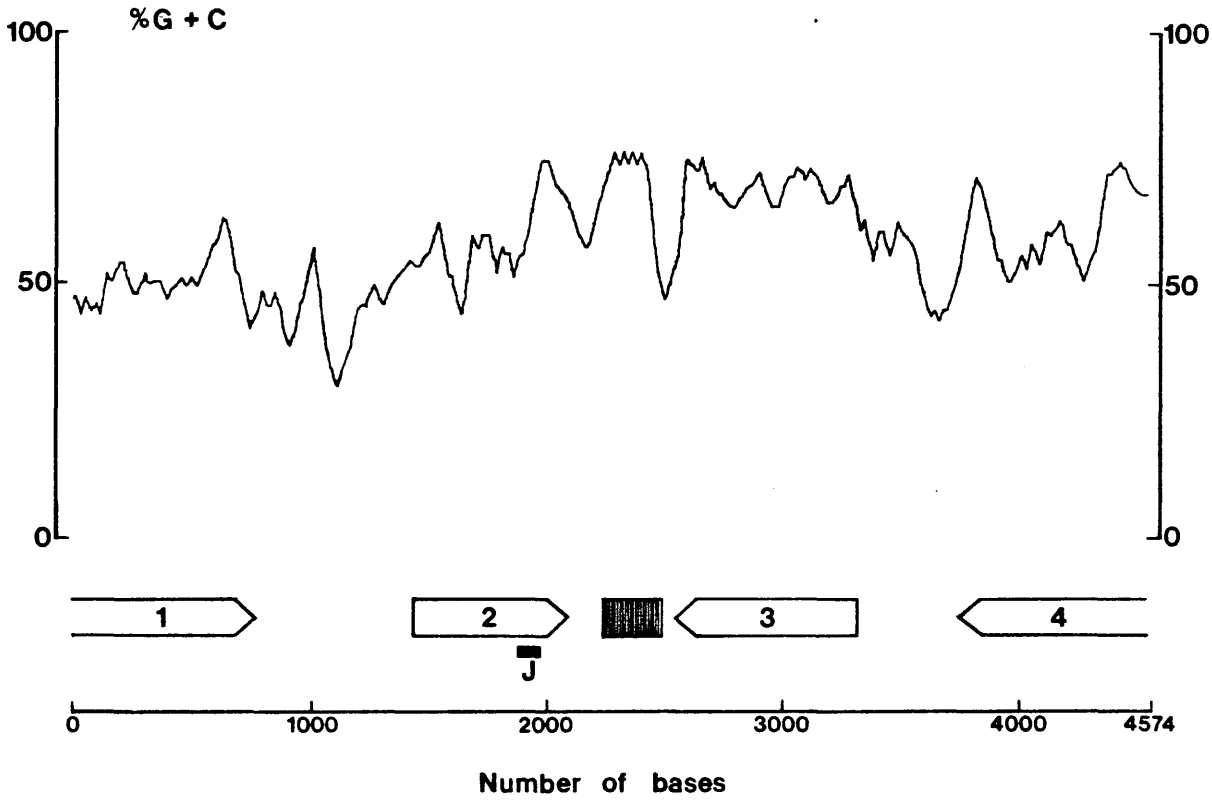
CGGTTTACGTCTGGGCGCCACGGCGGAGCGCAGAACGCTCCCGGCCCCCGAGACAGGTACGGGCGCAGCGACCTCGGCGAGAGCGGCAGG 4410
R N V D P R W P P A C F A G P G R S L Y P R L S R P S L P L    56

CCGTATTCCGGATGGTCAGAGCCGCCGGGAAGCCTGGCGGGCACGCGCACGGAGTCTATGCGCTGGCTGGAGCGTGGGGATGGGTTCCCA 4500
G Y E P H D S G G P L R A P V R V S D I R Q S S R P S P N G    26

CCAGATGAGGGGGACAGGGAGGGTATGATGGGGCTGGTGGACGGCGCCCCCCGCTGTGACATGCGGCAGGATCC                 4574
G S S P S L S P I I P S T S P A G R Q S M R C S G              1
```

FIGURE 5.5

A graph of the G+C content of Bam HI 1 of EHV-1 subtype 2. The ORFs are represented by rectangles numbered 1,2,3 and 4 the pointed ends of which indicate the direction of transcription. The reiterated sequence between ORFs 2 and 3 is shown by a series of vertical lines and the approximate location of the TR_S/U_S junction is indicated by a black box and the letter J.


%G + C
100
50
0
100
50
0
1
2
3
4
J
0
1000
2000
3000
4000
4574
Number of bases

mRNAs, and of two copies of the variant ATTAAA are shown in Figure 5.4.

The ORFs were designated ORFs 1, 2, 3 and 4 from left to right in Bam HI 1 and predicted proteins were designated proteins 1, 2, 3 and 4 (Figure 5.5). Hydrophobicity plots of the predicted proteins were computed using the 'hydropathicity' parameters of Kyte and Doolittle (1982). This program evaluates the average hydropathy within a moving window as it advances through the amino acid sequence. The results are represented graphically. The amino acid sequences of the predicted proteins were compared with those of VZV and HSV-1 using the matrix program of Pustell and Kafatos (1982). This program also uses a moving window approach. It scores the number of residues in a window from one sequence which are identical to those in a window from a second sequence. The windows which are of equal length are shifted by intervals of one residue, and the results obtained as a matrix plot. The alignment program, HOMOL, (Taylor, 1984) was used to compare the homologous amino acid sequences detected using the matrix program. This program aligns amino acid sequences by inserting blank characters in such a way as to maximise the number of matched pairs. The alignment produced depends on the weighting values assigned to inserted mismatched residues and inserted blank characters. Lastly, the hydrophobicity of the predicted EHV-1 proteins was compared with that of their HSV-1 and VZV homologues. Counterparts of the four EHV-1, subtype 2 genes were detected in both HSV and VZV.

FIGURE 5.6

An autoradiograph of the hybridization of EHV-1 subtype 2 DNA, cloned in M13 to a Bam HI digest of EHV-1 subtype 2 DNA blotted onto nitrocellulose. Hybridization was in aqueous solution at 68^{o}C overnight.

Location of the U_S/TR_S Junction

Bam HI 1 contains the TR_S/U_S junction (Figure 3.15). To locate the junction more accurately M13 clones -40, -117, -93, -60, -37 and -14 were radiolabelled and hybridized to a Bam HI digest of EHV-1 subtype 2 DNA. An autoradiograph of the results is shown in Figure 5.6. Clones -6, -37 and -14 hybridized strongly to Bam HI d and 1 which contain the IR_S/U_S and TR_S/U_S junctions respectively (Figure 3.15). Clones -40, -117 and -93 hybridized strongly to Bam HI 1 only, indicating that the U_S/TR_S junction is located between 1898 and 1969bp . (Figure 5.5). This implies that the TR_S and IR_S are each approximately 10.9kbp in size and that U_S is approximately 13.1kbp in size. These values correlate well with previous estimates based on electron microscopy studies and restriction endonuclease analysis (Chapter 3).

The high G+C content of the TR_S portion of Bam HI 1 differs significantly from the U_S portion of the fragment (Figure 5.5). The base composition of the EHV-1 subtype 2 genome is unknown, but the relatively high G+C content of repeat regions compared with that of unique sequences has been documented in other herpesviruses (Wadsworth et al., 1975; Delius and Clements, 1976; Kilpatrick and Huang, 1977; Davison and Wilkie, 1981; Murchie and McGeoch, 1982; Davison, 1983; Ruyechan et al., 1985; McGeoch et al., 1986a). McGeoch et al. (1986a) proposed that mutation rate alone might explain the variation in herpesvirus whole genome G+C content but that recombination must be invoked to explain the high G+C content of the repeat sequences

TABLE 5.1 PROTEIN 1

AMINO ACID COMPOSITION

Ala	6.7%	Val	5.5%	Leu	11.8%	Ile	3.5%
Gly	4.3%	Pro	9.8%	Cys	1.6%	Met	0.4%
His	3.9%	Phe	4.7%	Tyr	3.9%	Trp	2.0%
Asn	2.7%	Gln	2.4%	Ser	9.4%	Thr	7.5%
Lys	2.4%	Arg	5.9%	Asp	5.9%	Glu	5.9%

CODON USAGE

TTT Phe	2.7%	TCT Ser	2.0%	TAT Tyr	2.0%	TGT Cys	0.4%
TTC Phe	2.0%	TCC Ser	1.2%	TAC Tyr	2.0%	TGC Cys	1.2%
TTA Leu	0.8%	TCA Ser	2.3%	TAA ---	0.0%	TGA ---	0.4%
TTG Leu	4.7%	TCG Ser	0.8%	TAG ---	0.0%	TGG Trp	2.0%
CTT Leu	2.0%	CCT Pro	1.2%	CAT His	2.0%	CGT Arg	0.4%
CTC Leu	0.8%	CCC Pro	1.2%	CAC His	2.0%	CGC Arg	1.2%
CTA Leu	2.7%	CCA Pro	4.3%	CAA Gln	1.6%	CGA Arg	0.8%
CTG Leu	0.8%	CCG Pro	3.1%	CAG Gln	0.8%	CGG Arg	1.2%
ATT Ile	0.8%	ACT Thr	1.2%	AAT Asn	0.4%	AGT Ser	1.6%
ATC Ile	2.0%	ACC Thr	2.0%	AAC Asn	2.3%	AGC Ser	1.6%
ATA Ile	0.8%	ACA Thr	2.3%	AAA Lys	0.8%	AGA Arg	1.2%
ATG Met	0.4%	ACG Thr	2.0%	AAG Lys	1.6%	AGG Arg	1.2%
GTT Val	0.8%	GCT Ala	1.2%	GAT Asp	1.6%	GGT Gly	0.4%
GTC Val	1.6%	GCC Ala	1.2%	GAC Asp	4.3%	GGC Gly	1.2%
GTA Val	1.2%	GCA Ala	2.0%	GAA Glu	3.1%	GGA Gly	2.0%
GTG Val	2.0%	GCG Ala	2.3%	GAG Glu	2.7%	GGG Gly	0.8%

FIGURE 5.7

Comparative data for ORF1, HSV-1 US8 and VZV US4

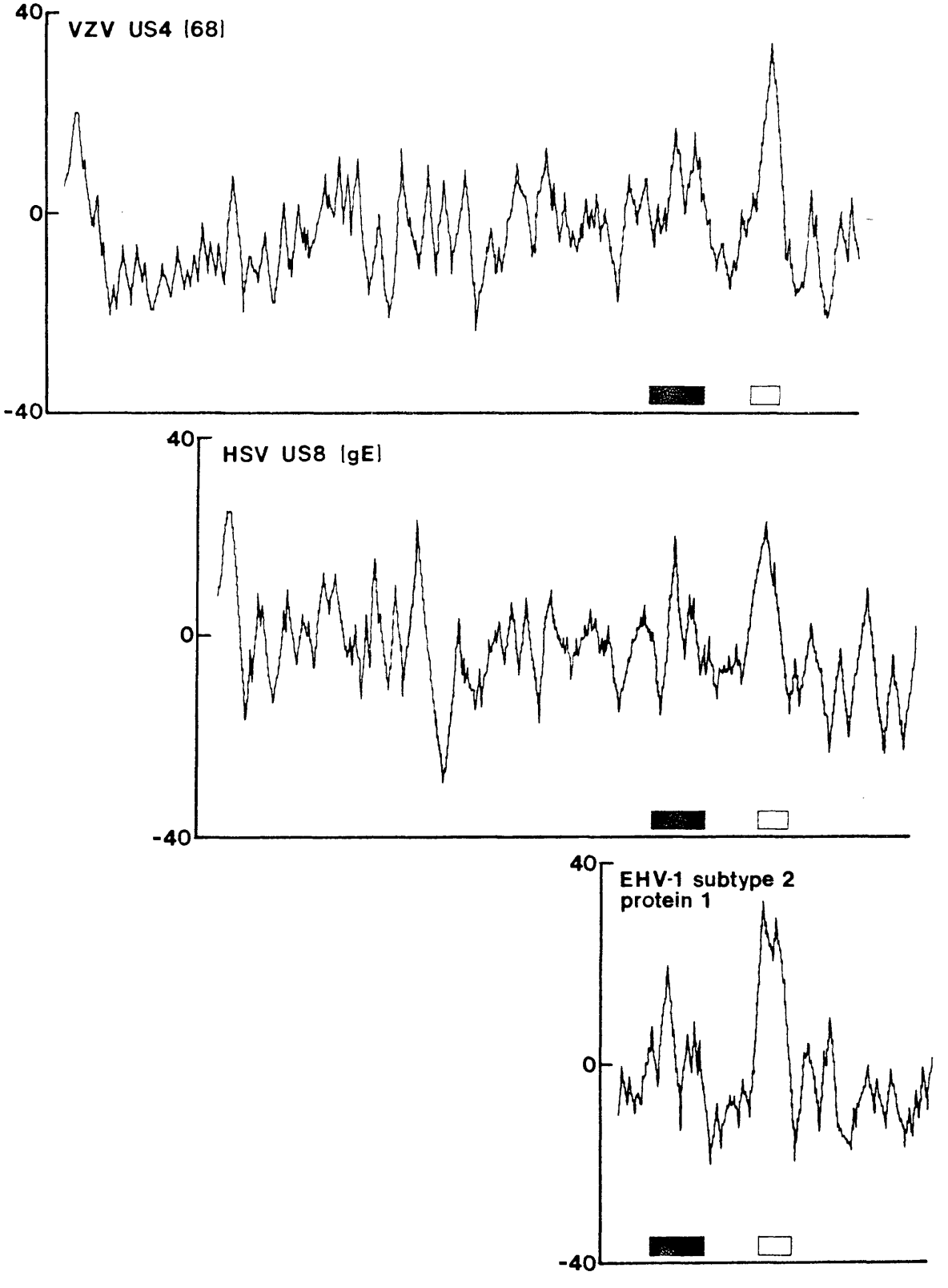
a. Hydrophobicity plots

The peaks represent the hydrophobic regions. The hydrophobicity values for each amino acid were summed over a window of nine residues which was moved along the sequence one residue at a time. The major hydrophic domain of each protein is represented by an empty rectangle and the best conserved region is denoted by a filled rectangle.

b. Direct alignment of residues in the conserved region.

The weighting values used throughout this study were 5 for each mismatched residue and 3k + 7 for k consecutive blanks.

a.



b.

```
342 VNLEFRDASPQHSGLYLCVVYVNDHIHAWGHITISTAAQYRN  HSV-1
461 TTLKFVDTPESLSGLYVFVVYFNGHVEAYAYTVVSTVDHFVN  VZV
 27 VDLLFKDIPESATGLYVFVLLYNGHPEAWTYTLLSTANHFMN  EHV-1
                                                  subtype 2
```

relative to adjacent unique sequences.

ORF1

ORF1 extends into the left end of Bam HI 1 and terminates at a TGA stop codon located at residue 766 (Figure 5.4). The protein-coding sequence is positioned in U_S and has a G+C content of 51.5%. The codon usage of ORF1 and the amino acid composition of the encoded portion of protein 1 are shown in Table 5.1. The predicted protein has a marked hydrophobic region near the carboxy terminal, as indicated in Figure 5.7a). This is a characteristic feature of integral membrane glycoproteins. The hydrophobic domain spans the membrane lipid bilayer, the amino-terminal domain being exposed to the exterior of the cell (Tomita and Marchesi, 1975; Rose et al., 1980). Using the matrix program a region in the predicted amino acid sequence of protein 1 was found to be related to those of HSV-1 glycoprotein E (gE) encoded by gene US8 (McGeoch et al, 1985) and VZV glycoprotein gp I encoded by gene US4 (Davison, 1983). The VZV and EHV-1 proteins are marginally more closely related in this region than HSV-1 gE is to either protein. The region showing the greatest similarity in the three proteins is 42 residues long. Of these 13 are shared by all three proteins, 11 by VZV gp I and protein 1, 3 by HSV-1 gE and VZV gp I only, and 12 by none (Figure 5.7b). The conserved sequence starts at residue 342 of HSV-1 gE, 461 of VZV gp I and 27 of the portion of protein 1. Figure 5.7a shows that the conserved region immediately precedes the major hydrophobic domain in each protein. A

shorter sequence at residues 175 to 192 of protein 1 is also partially conserved in the HSV-1 VZV homologues. The lack of significant conservation of amino acid sequence in the hydrophobic domains indicates the limitations of the methods of analyses used here to determine amino acid homology. Non-identical residues can be homologous in hydrophobicity, charge or structure. The mRNA containing ORF1 probably forms a 3'-coterminal set with that of ORF2, the 3' end mapping close downstream from the AATAAA at residues 2176. This arrangement is similar to that in HSV-1, where the mRNA containing US8 forms a 3'-coterminal family with that containing US9 (Rixon and McGeoch, 1985). Alternatively, the 3' end of ORF1 may be located near the ATTAAA a variant of AATAAA, at residue 1131.

ORF2

ORF2 is located from residue 1441 to 2110 and has an AATAAA sequence located close downstream at 2176 (Figure 5.4). It contains the TR_S/U_S junction and has a G+C content of 58.7%. Codon usage within ORF2 and the amino acid composition of protein 2, which has a predicted molecular weight of 23,000 (23K) are shown in Table 5.2. Protein 2 was shown to be homologous to proteins encoded by HSV-1 US9 (McGeoch et al., 1985) and VZV US1 (Davison, 1983). The EHV-1 protein is approximately twice the size of the VZV and HSV-1 proteins which have the predicted molecular weights of approximately 10,000 (10K) and 11,000 (11K) respectively. The hydrophobicity plot for each of these proteins exhibits a large hydrophobic region at the carboxy terminus (Figure 5.8a). In the HSV-1 (McGeoch et

TABLE 5.2 PROTEIN 2

AMINO ACID COMPOSITION

Ala	13.6%	Val	10.9%	Leu	6.8%	Ile	5.0
Gly	7.3%	Pro	5.5%	Cys	3.6%	Met	0.9%
His	1.8%	Phe	1.4%	Tyr	2.7%	Trp	0.0%
Asn	4.1%	Gln	1.8%	Ser	9.5%	Thr	5.5%
Lys	0.5%	Arg	5.9%	Asp	5.5%	Glu	7.7%

CODON USAGE

TTT Phe	0.5%	TCT Ser	1.4%	TAT Tyr	0.5%	TGT Cys	1.8%
TTC Phe	0.9%	TCC Ser	2.7%	TAC Tyr	2.3%	TGC Cys	1.8%
TTA Leu	0.0%	TCA Ser	1.8%	TAA ---	0.0%	TGA ---	0.5%
TTG Leu	1.4%	TCG Ser	0.5%	TAG ---	0.0%	TGG Trp	0.0%
CTT Leu	0.5%	CCT Pro	0.9%	CAT His	0.9%	CGT Arg	0.0%
CTC Leu	1.8%	CCC Pro	2.3%	CAC His	0.9%	CGC Arg	1.8%
CTA Leu	0.9%	CCA Pro	0.9%	CAA Gln	0.5%	CGA Arg	0.9%
CTG Leu	2.3%	CCG Pro	1.4%	CAG Gln	1.4%	CGG Arg	1.8%
ATT Ile	1.8%	ACT Thr	1.8%	AAT Asn	0.9%	AGT Ser	0.9%
ATC Ile	2.3%	ACC Thr	1.4%	AAC Asn	3.2%	AGC Ser	2.3%
ATA Ile	0.9%	ACA Thr	1.4%	AAA Lys	0.5%	AGA Arg	0.0%
ATG Met	0.9%	ACG Thr	0.9%	AAG Lys	0.0%	AGG Arg	1.4%
GTT Val	0.9%	GCT Ala	5.0%	GAT Asp	3.6%	GGT Gly	1.8%
GTC Val	2.7%	GCC Ala	3.6%	GAC Asp	1.8%	GGC Gly	2.7%
GTA Val	3.2%	GCA Ala	0.9%	GAA Glu	3.6%	GGA Gly	0.0%
GTG Val	4.1%	GCG Ala	4.1%	GAG Glu	4.1%	GGG Gly	2.7%

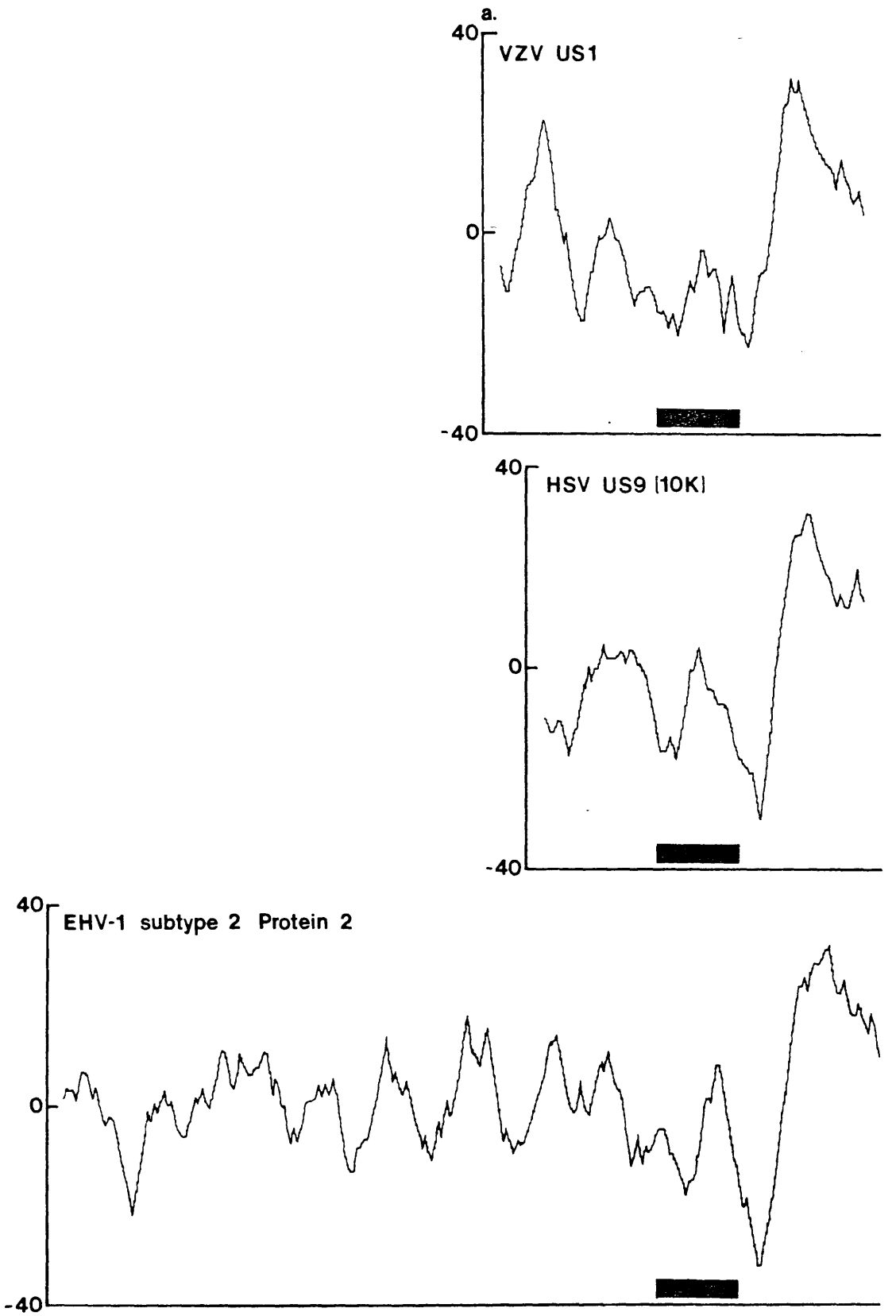
FIGURE 5.8

Comparative data for ORF 2, HSV-1 US9 and VZV US1

a. Hydrophobicity plots

The peaks represent the hydrophobic regions. The hydrophobicity values for each amino acid were summed over a window of nine residues which was moved along the sequence one residue at a time. The best conserved region is denoted by a filled rectangle.

b. Direct alignment of residues in the conserved region.



b.

```
31  AYYSESEDEAANDFLVRMGRQQ  HSV
    |||| | | | | | | | | |
43  CYYSDSENETADEFLRRIGKYQ  VZV
    |||| | | | | | | | | |
161 CYYSESDNETASLFIQRVGRRQ  EHV-1
```

al., 1985) and EHV-1 proteins this region is preceded by 6 arginine residues (RRRRRR). In EHV-1 these are interrupted by a histidine residue after the first two arginine residues (RRHRRRR). While the VZV protein does not possess an equivalent arginine-rich sequence, it does have a basic region preceding the hydrophobic carboxy terminus (Davison, 1983).

HSV-1 10K has been identified as a tegument phosphoprotein by the use of antisera against a synthetic oligopeptide corresponding to the amino terminus (Frame et al., 1986b). The high proportion of serine and threonine in HSV-1 10K is consistent with the extensive phosphorylation characteristic of this protein. The amino terminal portion of the protein is 22% serine, and serine and threonine together account for more than 26% of the amino acid residues. VZV 11K is similar to HSV-1 10K in containing 20% serine and threonine in the amino-terminal portion of the protein. In EHV-1 protein 2, serine is only the third most abundant residue (9.5%) and serine and threonine account for 15% of the amino acid residues. There is no significant clustering of serine and threonine residues in the amino-terminal portion of sequence. However, the portion of sequence which aligns with the amino-terminal portion of VZV 11K and HSV-1 10K (Figure 5.8a) is rich in serine and threonine. Together, they comprise 22.6% of the residues from residue 111 to 173.

The best conserved amino acid sequence is close to the basic and hydrophobic regions in each protein (Figure 5.8a). Ten of the 22 residues in this region are shared by

T A B L E 5.3 P R O T E I N 3

AMINO ACID COMPOSITION

Ala	15.8%	Val	6.2%	Leu	8.5%	Ile	1.9%
Gly	8.9%	Pro	9.3%	Cys	3.1%	Met	3.1%
His	2.7%	Phe	3.9%	Tyr	3.1%	Trp	1.2%
Asn	0.4%	Gln	1.2%	Ser	6.6%	Thr	3.9%
Lys	0.4%	Arg	10.0%	Asp	4.6%	Glu	5.4%

CODON USAGE

TTT Phe	1.5%	TCT Ser	0.0%	TAT Tyr	0.4%	TGT Cys	0.4%
TTC Phe	2.3%	TCC Ser	1.9%	TAC Tyr	2.7%	TGC Cys	2.7%
TTA Leu	0.0%	TCA Ser	1.5%	TAA ---	0.4%	TGA ---	0.0%
TTG Leu	0.0%	TCG Ser	1.5%	TAG ---	0.0%	TGG Trp	1.2%
CTT Leu	1.2%	CCT Pro	0.4%	CAT His	0.4%	CGT Arg	0.4%
CTC Leu	2.3%	CCC Pro	5.8%	CAC His	2.3%	CGC Arg	5.0%
CTA Leu	1.9%	CCA Pro	0.8%	CAA Gln	0.4%	CGA Arg	1.5%
CTG Leu	3.1%	CCG Pro	2.3%	CAG Gln	0.8%	CGG Arg	0.8%
ATT Ile	0.4%	ACT Thr	0.0%	AAT Asn	0.0%	AGT Ser	0.4%
ATC Ile	0.8%	ACC Thr	2.3%	AAC Asn	0.4%	AGC Ser	1.2%
ATA Ile	0.8%	ACA Thr	0.0%	AAA Lys	0.0%	AGA Arg	1.5%
ATG Met	3.1%	ACG Thr	1.5%	AAG Lys	0.4%	AGG Arg	0.8%
GTT Val	0.8%	GCT Ala	1.2%	GAT Asp	0.8%	GGT Gly	1.2%
GTC Val	0.4%	GCC Ala	5.4%	GAC Asp	3.8%	GGC Gly	3.1%
GTA Val	1.5%	GCA Ala	0.8%	GAA Glu	1.9%	GGA Gly	1.2%
GTG Val	3.5%	GCG Ala	8.5%	GAG Glu	3.5%	GGG Gly	3.5%

FIGURE 5.9

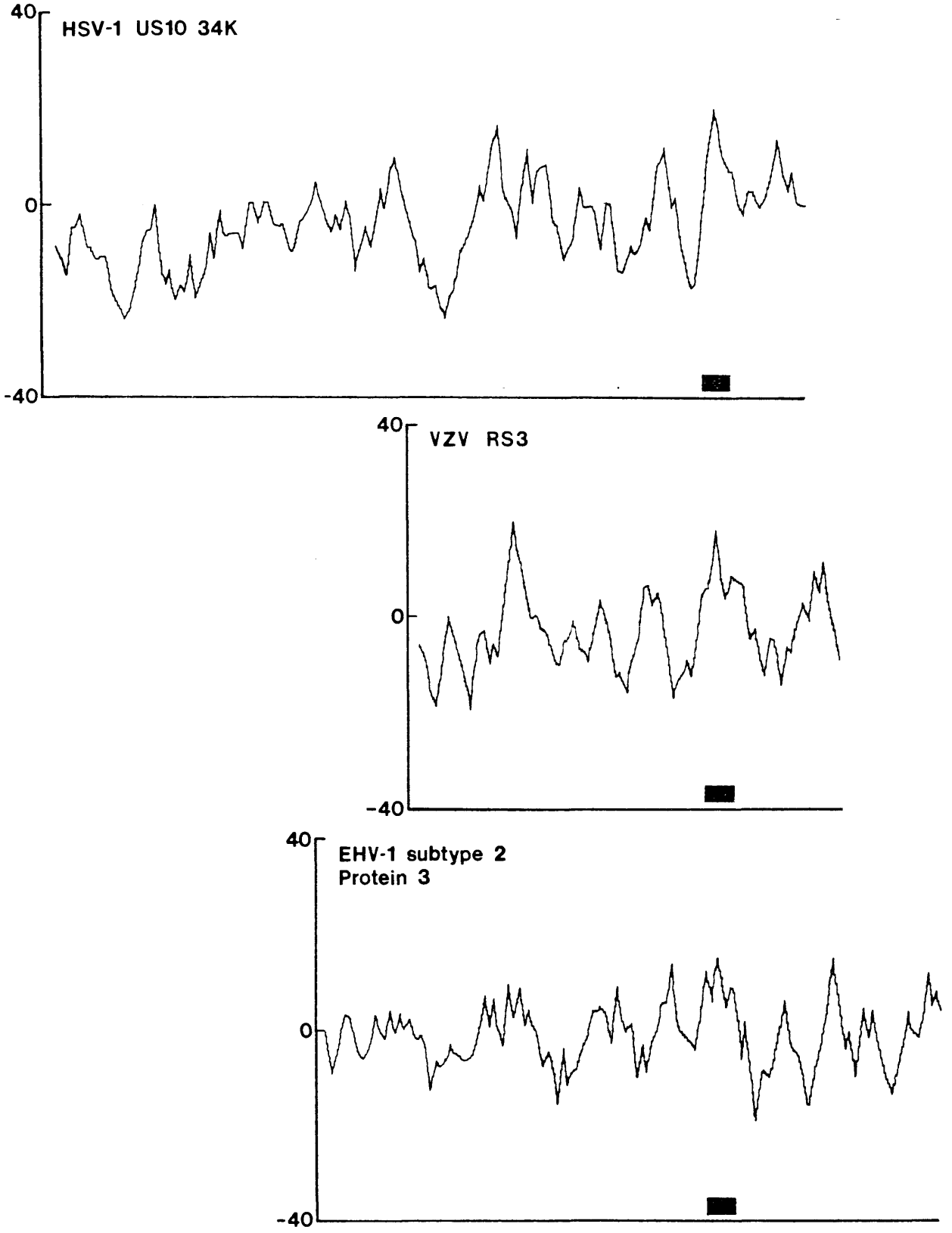
Comparative data for ORF3, HSV-1 US10 and VZV RS3

a. Hydrophobicity plots

The peaks represent the hydrophobic regions. The hydrophobicity values for each amino acid were summed over a window of nine residues which was moved along the sequence one residue at a time. The best conserved region is denoted by a filled rectangle.

b. Direct alignment of residues in the conserved region.

a.



b.

268	LMACAFWCCLTHA	HSV
120	LILMAYWCCLGHA	VZV
159	LLFCAHWCCLGHA	EHV-1 subtype 2

all three proteins (Figure 5.8b). Of the remaining 12, protein 2 and VZV 11K share 3 residues, protein 2 and HSV-1 10K share 2 and VZV 11K and HSV-1 10K share 2. The EHV-1 and HSV-1 proteins also share a hydrophobic sequence (LVAVL) at residues 200 to 204 and residues 74 to 78 respectively. This sequence is not conserved in VZV 11K.

ORF3

ORF3 is transcribed leftwards from residue 3322 to 2545. It is contained entirely within the TR_S and has a G+C content of 68.3%. The high G+C content is reflected in codon usage and in the predicted amino acid composition of protein 3. The protein is rich in amino acids specified by G+C-rich codons such as alanine, arginine, proline and glycine (Table 5.3). The sequence AATAAA is located in the bottom strand within the codon sequence at residue 2555 (Figure 5.4). Protein 3 is partially homologous to the products of HSV-1 US10 (McGeoch et al., 1985) and VZV RS3 (Davison and Scott, 1985). The best conserved region (Figure 5.9b) of 13 residues contains eight residues shared by all three proteins. The sequence GKT at residues 57 to 59 of the proteins 3 is conserved at residues 170 to 172 of the HSV-1 protein and 17 to 19 of the VZV protein.

The product of HSV US10 is a protein with an approximate molecular weight of 34,053 (34K) (McGeoch et al., 1985). The mRNA containing US10 shares a common 3' terminus with those of two upstream genes, US11 and US12, and US10 has a 110 codon out-of-frame overlap with the coding sequence of US11 (Rixon and McGeoch, 1984; McGeoch

FIGURE 5.10

Comparative data for ORF4 HSV-1 US1 and VZV RS2.

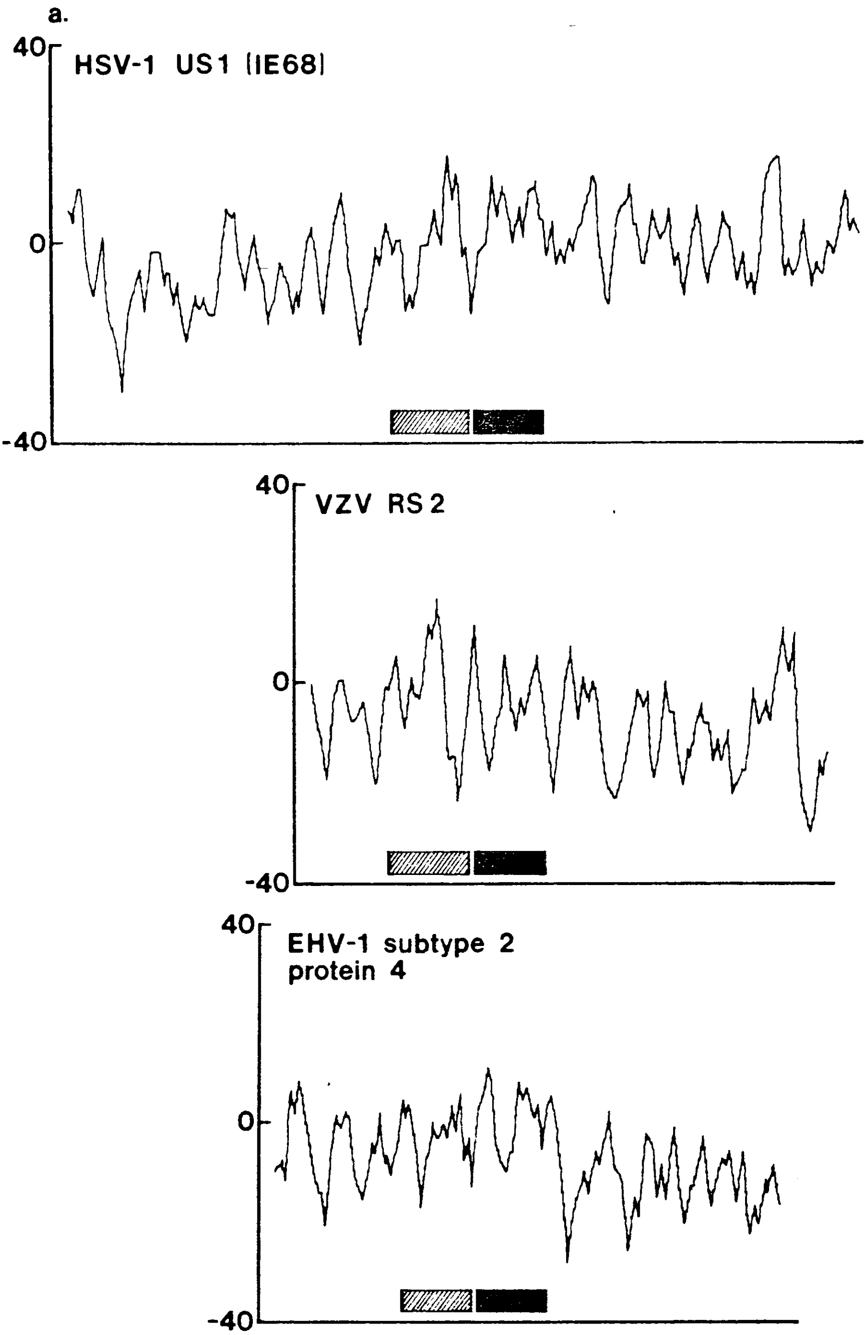
a. Hydrophobicity plots

The peaks represent the hydrophobic regions. The hydrophobicity values for each amino acid were summed over a window of nine residues which was moved along the sequence one residue at a time. The best conserved region is denoted by a filled rectangle. A region conserved in the VZV and EHV-1 proteins but not in the HSV-1 protein is indicated by a cross-hatched rectangle.

b. Direct alignment of residues in the best conserved region.

c. Direct alignment of residues in the region conserved in the VZV and EHV-1 subtype 2 proteins but not in the HSV-1 protein.

a.



b.

```
218  L R H L I R D C Y L M G Y C R A R L A P R T W C R L L Q V S G G T W G M H L R N T   HSV-1
     | |         |   | | | |     |   |           |     | | |                   | |   |
 85  L R R I C L D F Y L M G R T R Q R P T L A C W E E L L Q L   Q P T Q T Q C L R A T   VZV
     | |         |   |   | |         |           |     | | |                   | |   |
111  L R R V L L D F Y A M G Y T H A R P T L E C W G A L L Q L   M P E Q S L P L R A T   EHV-1
                                                                                       subtype 2
```

c.

```
176  V R Q A Q R R S S A R W T P D L G Y M R Q C I N Q L F R V L R V A R D P H G S A N   HSV-1
                           |                               | |     |
 42  S R G P G A F C T P G W E I H P A R L V E D I N R V F L C I A Q S S G R V T R D S   VZV
     | | | | | | | |   |   |           | |     |   |     |     |     | |     | |   | |
 68  S R G P G A F C A P P W R P D V N R L A G D V N R L F R G I S T S S I H V T E D S   EHV-1
                                                                                       subtype 2
```

et al., 1985). With the overlapping sequence there are three copies of an 18bp tandem repeat sequence plus a partial copy of 6bp (McGeoch et al., 1985). The reiterated sequence encodes a partially repeated amino acid sequence in 34K, composed of seven copies of the sequence PGX, where X is L, S or P (Rixon and McGeoch, 1984). This feature is in the region specific to HSV-1 and is therefore not present in the VZV or EHV-1 homologues.

ORF4

ORF4 originates from beyond the right end of Bam HI 1 and terminates at 3756. It is contained entirely within TR_S, and has a G+C content of 62.5%. The codon usage in ORF4 and amino acid composition of the predicted protein are shown in Table 5.4. The sequence AATAAA is positioned in the lower strand at residue 3617, and the variant ATTAAA is found at position 3753. The amino acid sequence of the predicted primary translation product of ORF4 shows homology to that of VZV RS2 (Davison and Scott, 1985), and somewhat less homology to HSV-1 US1 (McGeoch et al, 1985). The best conserved region is 41 residues long, within which 14 are shared by all three proteins (Figure 5.10a,b). Of the remaining 27 residues the EHV-1 protein shares two with the HSV-1 protein and 13 with the VZV protein. A second sequence from residue 68-108 in the EHV-1 protein is partially conserved in the VZV protein (residues 42 to 82) but not in the HSV-1 protein (Figure 5.10c).

HSV-1 US1 encodes the immediate early protein V_{mw}IE 68. The temporal class of the EHV-1 and VZV proteins is unknown. The VZV gene is not preceded by the consensus sequence TAATGARAT, which is present upstream from HSV-1

and HSV-2 immediate early genes (Whitton and Clements, 1984). The upstream sequence of the EHV-1 gene lies outside Bam HI, and so it is not known whether it is preceded by a TAATGARAT sequence.

Reiterated Sequences

In EHV-1 subtype 2 Bam HI 1 the sequence GGAGGTGG is tandemly repeated 31 times from residue 2255 to 2508. There is a partial copy of 6bp at the right end of the reiteration in Figure 5.4. Tandemly repeated DNA sequences are a feature of many eukaryotic genomes, and demonstrate a high frequency of sequence variation due to sequence duplication or deletion of single copies of the repeating unit (Farabaugh et al., 1978; Spritz, 1981; Bell et al., 1982). A number of different tandem reiterations, in which the repeat unit ranges from 1bp-37bp, have been identified in the HSV genome. They occur both in polypeptide-coding regions (McGeoch, 1984; Rixon and McGeoch, 1984; Rixon et al., 1984; McGeoch et al., 1985) and non-coding regions (Davison and Wilkie, 1981; Mocarski and Roizman, 1981; Watson et al., 1981; Murchie and McGeoch, 1982; McGeoch, 1984; Rixon et al., 1984; Whitton and Clements, 1984). Four unique G+C-rich tandemly reiterated sequences have been identified in the VZV genome (Davison and Scott, 1986a). Three of these are positioned in U_L and appear to encode repeated amino acid sequences. The fourth, in TR_S/IR_S, is non-coding. The VZV reiterations are not related to those of HSV-1 either in location or in sequence. As is typical of HSV-1 and VZV reiterations, the

FIGURE 5.11

Summary of the reiterated DNA sequences in the S segment of HSV-1 and VZV genomes and the reiterated EHV-1 subtype 2 DNA sequence identified in this study.

In HSV-1 reiteration 1 lies within the a sequence, reiteration 2 and 3 lie between the a sequence and gene RS1 which codes for $V_{mw}175$; reiteration 4 lies within the common intron of the IEmRNA-5 which contains US1; reiteration 5 lies within the coding sequence of gene US7; reiteration 6 lies between genes US9 and US10; reiteration 7 lies within the overlapping coding regions of genes US10 and US11 (Figure 5.12).

The VZV reiteration lies between the genes RS1 and RS2 (Figure 5.12).

The EHV-1 subtype 2 reiteration lies between ORFs 2 and 3 (Figures 5.4 and 5.5).

Reiterated DNA Sequences in the S Segments of HSV-1 and VZV and in Bam HI 1 of EHV-1 Subtype 2

HSV-1

		bp	coding
1	GGAGCGGGGGGA	12	NO
2	GCCGGGGAGGGCTGGG	16	NO
3	CGAGGGGCGGGAGGGGG	17	NO
4	CCCCCTCCTCCGCCCCCGCGTC	22	NO
5	CCTCCACCCCCTCGACCACCA	21	YES
6	TGGGTGGGTGGGGAG	15	NO
7	CCGGGGCTCCCGGGGAGA	18	YES

VZV

1	CCCCGCCGATGGGGAGGGGGCGCGGTA	27	NO

EHV-1

1	GGAGGTGG	8	NO

EHV-1 sequence is G+C rich. This non-coding reiteration occupies most of the region between ORFs 2 and3. Figure 5.11 shows the DNA sequences of the reiterations in the HSV-1 and VZV S segment. Of the seven HSV-1 reiterated sequences four (reiterations 1-4 in Figure 5.11) lie in the TR_S/IR_S and are non-protein coding (McGeoch et al., 1986). VZV has far fewer reiterations than HSV-1 and only one of these is located in the S segment (Davison and Scott 1986a). Five entire copies and a partial copy of a tandemly repeated 27bp sequence are located between the RS1 and RS2 of VZV (Figures 5.11 and 5.12). Of the three reiterated sequences in HSV-1 U_S (Figure 5.11) two lie within coding regions (McGeoch et al., 1985). The third, a 15bp sequence, TGGGTGGGTGGGGAG, is located in a similar intergenic position to the EHV-1 subtype 2 reiteration, between the coding sequence for the 10K and 34K proteins.

The marked copy number variability in at least some HSV tandem reiterations is reflected in the heterogeneity of electrophoretic mobility of certain restriction endonuclease fragments (Lonsdale et al., 1980; Davison and Wilkie, 1981; Murchie and McGeoch, 1982). This type of mobility variation in restriction enzyme fragments has also been observed for isolates of VZV (Straus et al., 1983) and EHV-1 subtype 1 (Allen et al., 1983). Variability in the number of repeated units in the coding sequence of $V_{mw}21$ is thought to be responsible for variation in the mobility of protein in different strains of HSV-1 (Rixon and McGeoch, 1984).

The significance of reiterations in herpesvirus

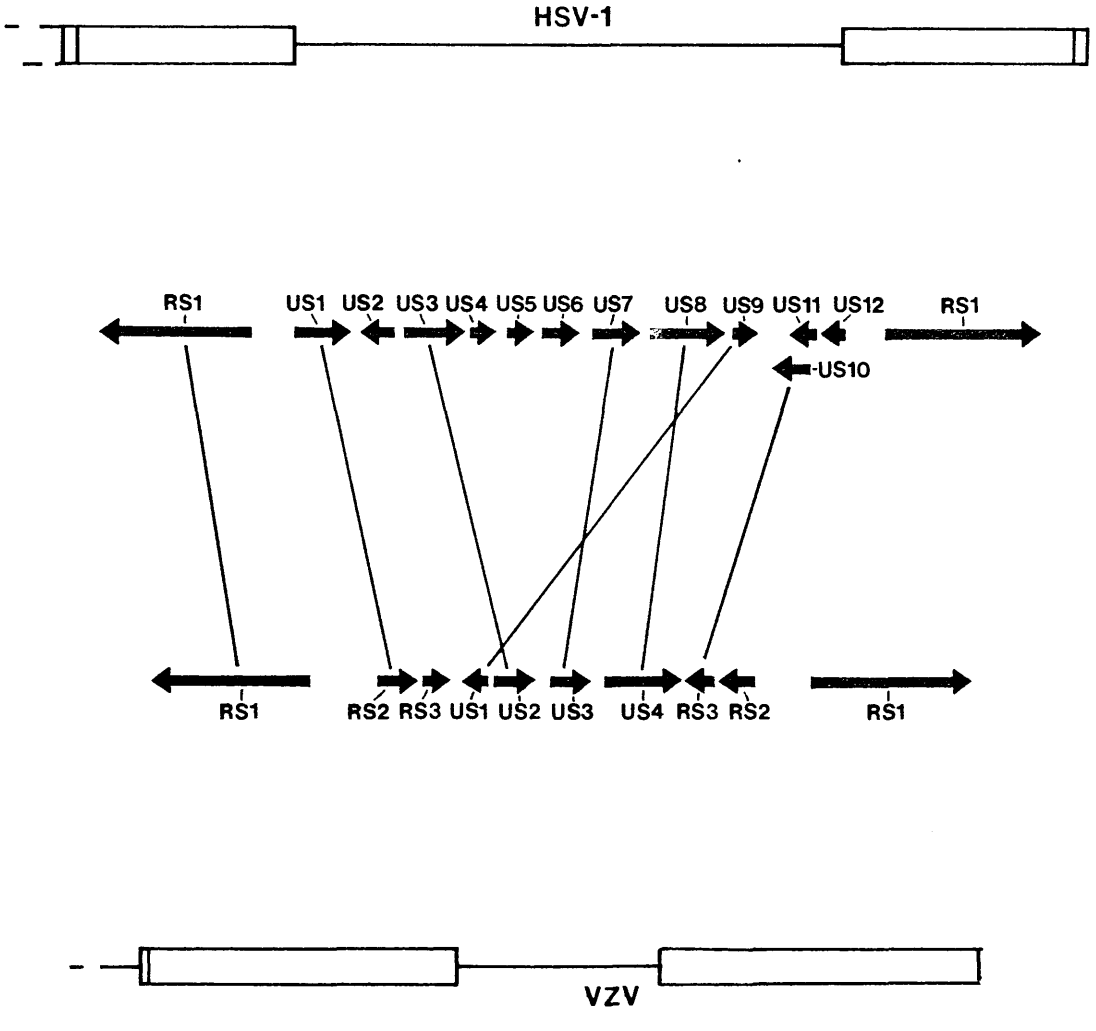
genomes is unknown. Several HSV-1 reiterations have no counterparts in HSV-2 or VZV (Davison and Wilkie, 1981; Rixon et al., 1984; Davison and Scott, 1986a). This would suggest that they encode no essential functions, but are simply regions of the genome which are readily amplified in random unequal crossover (Smith, 1976). It is likely that non-coding reiterations accumulate at locations in herpesvirus genomes which are not subject to rigorous selective pressure. Protein-coding reiterations are obviously constrained by requirements for protein function but it is not known if they have a functional role. The carboxy terminal portion of the HSV-1 protein $V_{mw}21$ contains 24 repeats of the tripeptide XPR which extend well beyond the reiterated DNA sequence. This suggests that, in this case, the repeated amino acid sequence is of functional importance and that it, rather than the nucleotide sequence, is under selective pressure (Rixon and McGeoch, 1984).

Comparison of Gene Arrangement in EHV-1 Subtype 2, HSV-1 and VZV

The DNA sequences of the S segments of HSV-1 (Davison and Wilkie, 1981; Murchie and McGeoch, 1982; McGeoch et al., 1985, 1986a) and VZV (Davison, 1983; Davison and Scott, 1985) have been determined. HSV-1 U_S contains ten entire genes plus major parts of two others. Each inverted repeat contains one entire gene and the 5' portion of one other. VZV U_S contains two entire genes plus the major parts of two more, and each inverted repeat contains three

FIGURE 5.12

Comparison of gene layout in the S segments of HSV-1 and VZV. Relationships are indicated for only one copy of genes in the inverted repeats (Davison and McGeoch, 1986).


HSV-1
RS1
US1
US2
US3
US4
US5
US6
US7
US8
US9
US11
US12
RS1
US10
RS1
RS2
RS3
US1
US2
US3
US4
RS3
RS2
RS1
VZV

entire genes. The relationships between genes in the S segments of HSV-1 and VZV are illustrated in Figure 5.12 (Davison and McGeoch, 1986). The seven genes in the VZV S segment all have homologues in the HSV-1 S segment (Davison and McGeoch, 1986). Homology was detected at the level of predicted amino acid sequence, and was not apparent in hybridization studies, except for the large gene in TR_S/IR_S (Davison and Wilkie, 1983). The HSV-1 S segment contains six genes which have no VZV homologues (Davison and McGeoch, 1986). The S segment of EHV-1 subtype 2 is considerably larger than that of VZV and HSV-1 (Figure 5.1) suggesting that the EHV-1 S segment may contain more genes than the equivalent regions of HSV-1 and VZV. If this is so, these genes are not contained in Bam HI 1. All four ORFs identified in this fragment have HSV-1 and VZV counterparts.

The relationships between genes in the S segments of HSV-1 and VZV and Bam HI 1 of EHV-1 subtype 2 is shown in Figure 5.13. EHV-1 subtype 2 has a gene arrangement in Bam HI 1 intermediate between that of the corresponding regions of the HSV and VZV genomes. EHV-1 ORFs 3 and 4 are larger than their VZV homologues, RS3 and RS2, but smaller than their HSV-1 counterparts, US10 and US1. These EHV-1 genes are more closely related in location to the VZV genes than to the HSV genes. EHV-1 ORF3 and 4 are positioned in the same tandem arrangement as VZV RS3 and RS2 in TR_S/IR_S (Figure 5.13). The HSV-1 homologue of ORF4, the gene encoding V_{mw}IE 68 is positioned across the IR_S/U_S junction (Marsden et al., 1978; McGeoch et al., 1985), and the

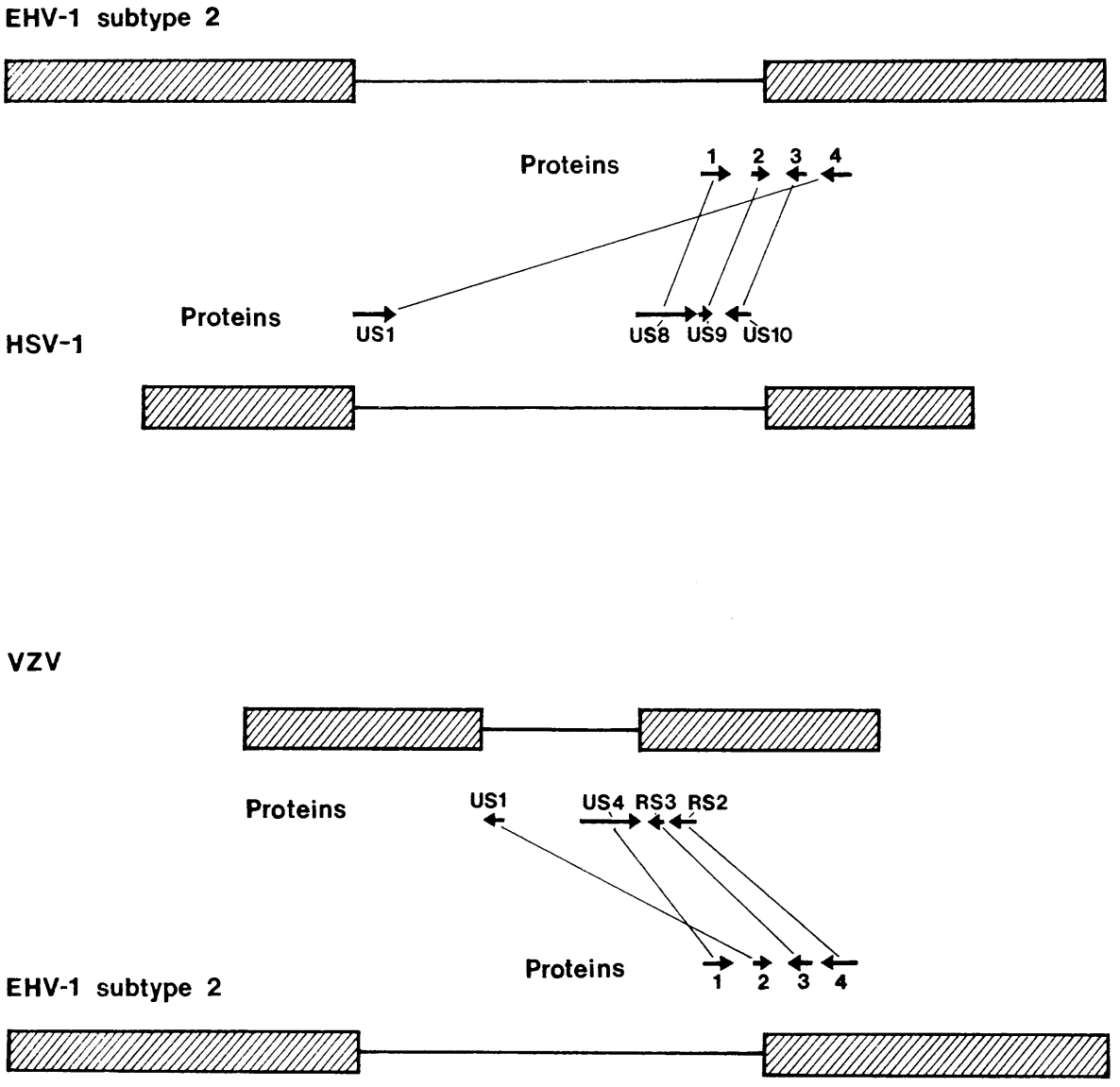
coding sequence is contained entirely within U_S (Rixon and Clements, 1982; Watson and Vande Woude, 1982; Murchie and McGeoch, 1982). The HSV-1 homologue of ORF3 is also located in U_S. While the EHV-1 genes and the two VZV homologues are adjacent and transcribed in the same direction the HSV-1 genes are at opposite ends of U_S and transcribed in the opposite directions (Figure 5.13). A reiterated sequence is located between HSV-1 US9 and US10. Unlike VZV, EHV-1 subtype 2 also possesses a tandem reiteration between the counterparts of these HSV-1 genes. However, apart from their equivalent location and typically high G+C content, the two reiterated sequences show little homology in length, copy number or nucleotide sequence.

EHV-1 ORF1 and ORF2 bear closer resemblance to their HSV-1 than their VZV counterparts in position and orientation (Figure 5.13). Like HSV-1 US8 and US9, they are positioned tandemly in the right half of U_S and are transcribed rightwards. The sequence between ORF1 and 2 lacks a significantly large ORF and is considerably longer than that between HSV-1 US8 and US9. EHV-1 ORF2 differs from the HSV-1 homologue in that the 3' coding end of the sequence apparently extends into the TR_S. In VZV, the homologue of ORF1 (US4) contains the U_S/TR_S junction (Figure 5.13); the stop codon is located within TR_S. The VZV homologue of ORF2 (US1) is located at the other end of U_S (Figure 5.13) and the stop codon spans the junction between U_S and IR_S. In HSV-1, the U_S/R_S junctions are located 8 and 40 nucleotides, respectively, from the initiator ATG codons of US12 and US1 (Murchie and McGeoch,

FIGURE 5.13

Relationships between genes in the S segments of HSV-1 and VZV and in Bam HI 1 of EHV-1 subtype 2. For clarity only one copy of genes in the inverted repeats is illustrated.

Gene orientation in the S segments of HSV-1, VZV and EHV-1 subtype 2



1982). The junctions in HSV-2 are located 1 and 33bp from the corresponding ATG codons (Whitton and Clements, 1984). Whitton and Clements (1984) suggested from the HSV-1 and HSV-2 results that the TR_S/IR_S are capable of expansion during evolution to include sequences from the U_S, but that the extent of expansion is limited by adjacent coding sequences. Thus, the location of IR_S in EHV-1 subtype 2 is likely to be defined by a gene at the other end of the U_S. Comparison of gene organisation in the S segments of HSV-1 and VZV indicate that not only can TR_S/IR_S expand to include short sequences of U_S (Whitton and Clements, 1984) but they can include or expel entire genes (Davison and McGeoch, 1986). Davison and McGeoch (1986) have proposed an evolutionary model for the descent of the S segments of HSV-1 and VZV from a common ancestor by a series of recombinational events involving expansion or contraction of TR_S/IR_S. The homology of the predicted EHV-1 subtype 2 proteins to HSV-1 and VZV proteins specified by genes in U_S and TR_S/IR_S suggests that EHV-1 subtype 2 is also a product of this evolutionary pathway. Elucidation of the full extent of divergence of EHV-1 subtype 2 from HSV-1 and VZV awaits the determination of the complete DNA sequence of the S segment.

CHAPTER 6

GENERAL DISCUSSION

APPLICATION OF MOLECULAR ANALYSIS TO THE UNDERSTANDING AND CONTROL OF EHV-1 DISEASE IN THE HORSE

Although the genome structures of the two subtypes of EHV-1 are similar, and the cross-hybridization data indicate that they share a common gene arrangement, the restriction endonuclease maps are quite different. This is in accord with the existing body of evidence that EHV-1 is not a single entity but two genetically distinct viruses, one of which could be renamed EHV-4 as suggested by Studdert et al. (1981). The recognition of two subtypes, only one of which, despite their overlapping disease potential, appears to be associated with epizootic abortion and neurological disease, is altering the thinking on EHV-1 disease throughout the horse industry. Dogmas unchallenged since the 1960s are currently under renewed investigation. The long-accepted hypothesis that annual outbreaks of EHV-1 respiratory disease in yearlings in Kentucky are the usual source of virus which induces abortion in mares (Doll and Bryans, 1963b) is questionable in the light of molecular epizootiological studies conducted in the same area which have demonstrated the relative paucity of subtype 1 virus isolated from cases of upper respiratory tract disease (Allen et al., 1983a). Also, virus challenge studies in seronegative foals showed intersubtypic cross-protection. Animals experimentally infected at 6-monthly intervals with EHV-1 subtype 2 were resistant to challenge with subtype 1 (Allen and Bryans, 1986). This confirms the hypothesis originally put forward by Doll and Bryans (1963b) that

repeated EHV-1 respiratory infections stimulate protective immunity against abortigenic EHV-1. This hypothesis arose from the observation that the most devastating abortion storms tended to occur on studs which had been free of EHV-1 respiratory disease in their young stock for an extended period or where mares were separated from younger animals.

At present there is no safe effective vaccine against EHV-1. The existence of two distinct virus subtypes, both capable of causing abortion and epizootics of respiratory disease, and their ability to cross-protect would suggest that the EHV-1 disease complex would be best controlled by a bivalent vaccine containing both virus subtypes (Fitzpatrick and Studdert, 1984). All the currently available vaccines, whether live attenuated or killed, consist only of EHV-1 subtype 1 (Mumford and Bates, 1984). According to the manufacturers, one live attenuated vaccine on sale in the U.K. cannot be guaranteed to protect against abortion, but is claimed to protect against respiratory disease. However, this vaccine contains no subtype 2 virus. Several vaccines have been developed against EHV-1, ranging from the "planned infection" program with a live virus adapted to hamsters (Doll and Bryans, 1963a), to the use of an adjuvanted chemically-inactivated virus (Moore and Koonse, 1977). All these vaccines were ineffective to a greater or lesser extent, and many were dangerous. For example, use of an inactivated virus vaccine derived from the tissues of equine foetuses increased the incidence of haemolytic icterus of new-born foals (Doll et al., 1952). Also, hamster-adapted live virus caused abortion in a

percentage of its recipients (Doll and Bryans, 1963a), and a chemically inactivated vaccine produced from virus propagated in hamsters lacked immunogenic potency and gave rise to anaphylactoid reactions (Doll et al., 1956; Doll and Bryans, 1963a). In the molecular epizootiological surveys in Kentucky (Allen et al., 1983a), several viruses isolated from aborted foetuses had the distinctive electropherotype of vaccine strains. Pneumabort-K is an inactivated adjuvanted EHV-1 vaccine initially developed by Bryans in Kentucky and now available in Great Britain. The vaccine was shown to protect against abortion in challenge studies in America (Bryans, 1978; Moore and Koonse, 1977) and to elicit a satisfactory immune response in field trials in France, Ireland and Kentucky (Bryans, 1980). The vaccine received a manufacturing license in the United States in 1980, and the results of its use have been closely monitored in Kentucky. Allen and Bryans (1986) reported a decrease of more than 50% in the incidence of EHV-1 abortion during the first four years after the vaccine was licensed. However, trials in Great Britain have not shown this vaccine to be efficacious in the control of either subtype of EHV-1 (Burrows et al., 1984; Mumford and Bates, 1984).

Much of the current thinking in vaccine development centres on the virus glycoproteins, which stimulate protective immunity (Mertz et al., 1984). Very little is known about EHV-1 glycoproteins, but the existence of HSV glycoproteins gB, gC, gD, (Spear, 1976), gE (Baucke and Spear, 1979), gH (Buckmaster et al., 1984) and gG (Marsden

et al., 1978, 1984; Roizman et al., 1984; Richman et al., 1986; McGeoch et al., submitted) has been established. The existence of additional glycoproteins has been predicted from the DNA sequence analysis of HSV-1 U_S (McGeoch et al., 1985). HSV gB plays an important role in virus penetration and cell fusion (Manservigi et al., 1977; Sarmiento et al., 1979); gC, a non-essential component of the virion, has an inhibitory effect upon cell fusion and acts as a receptor for a complement component, C3b, following infection (Friedman et al., 1984; Manservigi et al., 1977; Ruyechan et al., 1979); gD is thought to play a role in the fusion of infected cells (Nobel et al., 1983); gE acts as an Fc receptor for γ-globulin (Baucke and Spear, 1979; Para et al., 1980; Machtiger et al., 1980); gH may have a role in virus maturation or egress, as anti-gH antibodies can block plaque formation (Buckmaster et al., 1984). HSV glycoproteins stimulate the production of neutralizing antibodies (Powell et al., 1974; Cohen et al., 1978; Norrild, 1980; Para et al., 1982), are implicated in both antibody-dependent complement-mediated cytotoxicity and antibody-dependent cellular cytotoxicity (Glorioso et al., 1978; Norrild et al., 1979), and may function as targets for T cell-mediated lysis of HSV-infected cell (Carter et al., 1981; Eberle et al., 1981). Antisera (Honess and Watson, 1974; Powell et al., 1974; Norrild, 1980) or monoclonal antibodies (Showalter et al., 1981; Pereira et al., 1982; Rector et al., 1982; Cranage et al., 1983) to individual glycoproteins neutralize virus in vitro. Specific

monoclonal antibodies have been used to purify individual HSV glycoproteins by immunoadsorption chromatography, and purified gD (Eisenberg et al., 1982a; Chan, 1983; Long et al., 1984), gC (Schrier et al., 1983; Roberts et al., 1985) and gB (Chan, 1983; Roberts et al., 1985) were able to immunize mice against challenge with virulent HSV-1.

Similarly, EHV-1 envelope glycoproteins serve as target antigens against which the host's immune response is mounted (Papp-Vid and Derbyshire, 1978, 1979). Six major and six minor glycoproteins have been identified within the envelope of both subtypes of EHV-1 (Turtinen and Allen, 1982; Turtinen, 1983), but it is possible that some of these proteins may differ only in their carbohydrate moieties or be related by modification of a primary translation product, as has been shown for some HSV glycoproteins (Spear and Roizman, 1980; Cohen et al., 1980; Pereira et al., 1982). Convalescent horse sera were found to be immunoreactive with five of the six major glycoproteins using the Western blot technique, and studies using hyperimmune rabbit serum demonstrated that four of these glycoproteins contained subtype cross-reactive antigenic determinants (Turtinen, 1983).

No information has been published on the location or DNA sequence of genes coding for EHV-1 glycoproteins. The colinearity of the genomes of HSV-1 and the two subtypes of EHV-1 discussed in Chapter 4 makes it possible to predict the location of glycoprotein genes in the EHV-1 genome. This is particularly true for glycoprotein genes present in the L segment. Genes for gB and gH, or their counterparts,

have been located in the genomes of two members of the Alphaherpesvirinae (HSV and VZV), one member of the Betaherpesvirinae (CMV) and one member of the Gammaherpesvirinae (EBV) (Pellet et al., 1985; Davison and Scott, 1986a; McGeoch and Davison 1986; Davison and Taylor, 1986; Tony Kouzarides, personal communication) by DNA sequence analysis. Similarly, a counterpart of the HSV gC gene has been identified in an equivalent location in the VZV genome (Davison and Scott, 1986a). However, the predictive value of genome colinearity is far less in the S segment, the least homologous region of the Alphaherpesvirinae. U_S has been shown to contain glycoprotein genes in several members of the Alphaherpesvirinae (Mettenleiter et al., 1985; Rea et al., 1985; Ellis et al., 1985; Davison et al., 1985; McGeoch et al., 1985). These include HSV gD, for which no counterpart is present in the VZV genome (Davison and McGeoch, 1986). Thus the location or even the presence of an EHV-1 homologue to HSV gD cannot be predicted with any degree of certainty. In Chapter 5, the observation that the EHV-1 S segment is larger than that of HSV and VZV led to the suggestion that it may contain more genes than the latter. The HSV-1 S segment contains 6 genes with no counterparts in the VZV S segment (Davison and McGeoch, 1986). One encodes the HSV-1 equivalent of HSV-2 gG (Frame et al., 1986a; McGeoch et al., submitted), and the amino acid sequences of the predicted products of two others suggest that they also encode glycoproteins (McGeoch et al., 1985). It is thus, possible that the EHV-1 S

sequence may contain genes encoding glycoproteins which have no homologues in either HSV-1 or VZV. However, the glycoprotein gene identified by DNA sequence analysis of Bam HI 1 (Chapter 5) encodes the EHV-1 homologue of HSV gE and VZV gpI. This homology was detected by comparisons of predicted amino acid sequence, and was not indicated by comparative hybridization experiments.

Genetically engineered vaccines may offer a safer and more effective approach to the control of EHV-1 disease than the more conventional methods of vaccine development. The present orientation of research on subunit vaccines is based on the idea that immunogenic glycoproteins can be prepared in limitless quantities at a relatively low cost by cloning and expression in a number of vector systems including mammalian cells, bacteria and yeast. It is possible that a very concentrated preparation of the appropriate antigens might stimulate an immune response which would offer as much, if not more, protection than that elicited by a natural infection. Subunit vaccines are free of risks associated with the presence of residual live virus, potentially harmful cellular products or infectious contaminants. In HSV much of the earlier work on subunit vaccine development concentrated on gD, which elicits formation of potent cross-reactive neutralizing antibodies (Sim and Watson 1973; Honess and Watson, 1974; Cohen et al., 1978; Eisenberg et al., 1982a). HSV-1 gD has been successfully expressed in E. coli (Watson et al., 1983; Weis et al., 1983) and as a non-glycosylated fusion protein stimulates development of neutralizing antibodies in

rabbits (Weis et al., 1983). It has also been expressed in mammalian cells (Berman et al., 1983) where the protein is correctly processed and incorporated into the cell membrane (Gething and Sambrook, 1981). Deletion of the hydrophobic membrane-anchor region at the carboxy terminus of gD allowed the expressed glycoprotein to be secreted into the medium. This product protected mice against lethal challenge with the homologous virus (Lasky et al., 1984). The synthesis and assembly in yeast of a model hybrid particle containing both hepatitis B surface antigen and HSV-1 gD is an interesting approach to polyvalent vaccine development described by Valenzuela et al. (1985). Another approach to subunit vaccine development utilises chemically synthesized peptides representing amino acid sequences for the immunogenic domains of the glycoprotein (Bittle et al., 1982; Shinnick et al., 1983). The immunologically important domains of gD were mapped using monoclonal antibodies (Eisenberg et al., 1982b, 1985). Cohen et al. (1984) described the localisation and synthesis of an antigenic determinant of gD that induces type-common neutralizing antibodies. Immunisation with this synthetic peptide protected mice against virus challenge (Eisenberg et al., 1985).

Live vaccines are often superior to inactivated or subunit vaccines because they are able to replicate and stimulate a stronger and longer-lasting humoral and cell-mediated immune response. However, the pathogenesis of EHV-1 is still poorly understood, and the inherent risk of live vaccines has resulted in a reluctance to use such

prophylactic measures in the U.K., where the problem of EHV-1 abortion has never been as severe as in the United States and some European countries. An approach to live vaccination which circumvents the risk of inducing EHV-1 abortion is to insert EHV-1 glycoprotein genes into the DNA of a non-pathogenic vector virus such as vaccinia (Mackett et al., 1982, 1984; Panicali and Paoletti, 1982; Smith et al.,1984; Smith and Moss, 1984). Expression of the HSV-1 gD gene by a vaccinia vector has been achieved (Paoletti et al., 1984), and the recombinant virus protected mice against lethal challenge with HSV-1. Vaccinia has a large capacity for foreign genes. More than 20kbp of exogenous DNA has been inserted into vaccinia without affecting virus viability (Smith et al., 1984). It should be possible not only to express a number of different glycoproteins in one vector but also to engineer a vaccinia recombinant which would act as a vehicle for the immunogenic components of several viruses. Paoletti et al. (1985) have described a vaccinia recombinant which expresses the influenza virus haemagglutinin, the hepatitis-B virus surface antigen and HSV-1 gD. This virus elicited the appropriate immunological responses on injection into laboratory animals. Thus, the potential exists to develop a vaccinia vector vaccine offering protection against both subtypes of EHV-1 and equine influenza virus.

The characterization of the genome of EHV-1 subtype 2 discussed in Chapters 3 and 4 allows one to make predictions of the possible locations of suitable glycoprotein genes for expression in the vector systems

mentioned above. EHV-1 subtype 2 Bam HI c, c and f, and g are predicted to contain sequences coding for the homologues of HSV-1 gB, gH and gC respectively. HSV-1 gH is an essential glycoprotein (Weller et al., 1983; McGeoch and Davison, 1986) and a monoclonal antibody against it, neutralizes virus infectivity (Buckmaster et al., 1984). HSV-1 gB is an essential glycoprotein which appears to play an important role in the stimulation of cell mediated immunity. Human HSV-specific T cells can be activated by gB (Yasukawa and Zarling, 1985), and lysis of HSV-1-infected target cells by human natural killer cells appears to correlate with the expression of gB and gC on the cell membranes (Bishop et al., 1983, 1984). HSV-1 gB expressed in mammalian cells has been shown to protect guinea pigs against initial HSV-2 infection (Stanberry et al., 1985a). The conservation of gB and gH in the Alphaherpesvirinae, Betaherpesvirinae and Gammaherpesvirinae indicates the conservation of some essential functions in the herpesviruses. The EHV-1 homologue of HSV gC might also be a candidate for the development of an EHV-1 vaccine but, although the HSV glycoprotein elicits a neutralizing antibody response, it does not appear to be an essential component of the virion (Heine et al., 1974; Zezulak and Spear, 1984; Centifanto-Fitzgerald et al., 1982). There is some evidence that gD is the most important protective HSV immunogen (Holland et al., 1983; Showalter et al., 1981; Para et al., 1985). gpI, the homologue of HSV gE, is the most abundant and most immunogenic of the VZV glycoproteins (Weigle and Grose 1983; Keller et al.,

1984. The genes encoding these proteins are positioned in the U_S, and thus the location of their EHV-1 counterpart is difficult to predict confidently. However, protein 1 specified by EHV-1 subtype 2 Bam HI 1 (Chapter 5) shows similarities to the predicted amino acid sequences of VZV gpI and HSV gE. This EHV-1 subtype 2 homologue of HSV gE is another potentially useful candidate for vaccine development.

Over the last two decades, significant advances have been made in the development of antiviral agents. The short-lived protective immunity induced by natural infection with EHV-1 suggests that antiviral chemotherapy may be a useful alternative or adjunct to a vaccination programme. Three of the genes located on the EHV-1 subtype 2 genome by comparative hybridization encode the DNA polymerase and the two subunits of the ribonucleotide reductase. The HSV-1 DNA polymerase is a target for nucleoside analogues such as acyclovir (ACV: 9 -(2-hydroxyethoxymethyl) guanine), the triphosphate derivative of which acts as a competitive inhibitor with dGTP for viral DNA polymerase (Elion et al., 1977). ACV has proven activity against a number of human herpesviruses (Schaeffer et al., 1978; Collins and Bauer, 1979; Brigden et al., 1981; Prober et al., 1982; Balfour et al., 1983), and has been licensed for use in man since 1981. ACV is active against EHV-1 in Syrian hamsters (Rollinson and White, 1983), but the pharmacokinetics of this drug in the horse indicate that it is unlikely to be suitable for use in the treatment of EHV-1 disease (Elizabeth Rollinson, personal

communication). However, a close analogue of ACV, DHPG, (9 - [2-hydroxy - 1 (hydroxymethyl) ethoxymethyl] guanine), appears to be far more potent than ACV against EHV-1 in plaque reduction experiments and in the hamster disease model (Rollinson and White, 1983). DHPG also appears to be less cytotoxic than ACV (Smith et al., 1983). Neither the bioactivation of these two drugs nor the reasons for the greater antiviral effect of DHPG compared with ACV are fully understood. The localization of the DNA polymerase gene is a first step in its further characterization and the elucidation of drug action and resistance (Coen et al., 1985).

Concern exists about the likelihood of nucleoside analogues causing damage to host cell DNA. DHPG has been tentatively associated with testicular atrophy in rats, a side-effect which would be totally unacceptable in a thoroughbred stallion. In view of this, the recent identification by Dutia et al. (1986) of a synthetic peptide which specifically inhibits the activity of herpesvirus-induced ribonucleotide reductase may offer an exciting alternative in the field of antiherpetic agents. The mechanism of inhibition probably involves interference with the normal interaction between the two HSV-1 ribonucleotide reductase subunits. Nothing is yet known about the components of EHV-1 ribonucleotide reductase or their interactions. Antisera raised against the carboxy-terminal 7 amino acids of the small ribonucleotide reductase subunit of HSV-1 and HSV-2, a proposed site of interaction between the two subunits and a target for

inhibitory oligopeptides, failed to precipitate the EHV-1 ribonucleotide reductase. However, the approximate localisation of the genes encoding this EHV-1 enzyme is a first step towards fine mapping and DNA sequencing studies. It is likely that a modified synthetic peptide will have an inhibitory effect on the EHV-1 ribonucleotide reductase similar to that reported for HSV-1.

Many questions relating to the pathogenicity and interrelationships of the two subtypes of EHV-1 remain to be answered. The recently proven ability of the virus to establish a latent infection (Edington et al., 1985) increases the difficulties anticipated in the control of the disease by vaccination or anti-viral therapy. An understanding of latency and reactivation is a prerequisite for a logical approach to the control of the disease. It is not known whether latent infection with one subtype precludes the establishment of latency with the second (Gerdes and Smith 1983; Nettleton et al., 1984), or whether superinfection with one subtype may induce reactivation of the other latent subtype, as is possibly the case for HSV-1 and HSV-2 (Thomas et al., 1985). The site of latency of EHV-1 has not yet been identified. Much speculation surrounds the role of leukocytes in EHV-1 infection. EHV-1 is isolated only from intact leukocytes (Gleeson and Coggins, 1980; Scott et al., 1983), suggesting that the virus may persist in these cells in a latent state (Gleeson and Coggins, 1980). Leukocytes have recently been implicated as a site of latency for another member of the Alphaherpesvirinae, PRV (Rziha et al., 1984). It has been

suggested that in an abortigenic EHV-1 infection the lymphocytes harbour the virus until the infected cells or the released virus cross the placenta (Gleeson and Coggins, 1980). However, virus has not been isolated from leukocytes beyond the 24th day after inoculation (Bryans and Prickett, 1969), and other sites for the virus during the period between the nasopharyngeal infection and abortion have been suggested (Kendrick, 1969). Jackson et al. 1977 suggested that in the neurological form of the disease the virus spreads directly from leukocytes to vascular endothelial cells, where it induces a characteristic vasculitis.

DNA hybridization techniques have a major role to play in an investigation of the pathogenesis of EHV-1. The cloned DNA fragments of EHV-1 subtype 2 may be used as specific probes to identify virus-specific DNA of both EHV-1 subtypes in equine tissues. Probes of superior specificity and sensitivity may be obtained by radiolabelling M13 clones used in the DNA sequence analysis of Bam HI 1 of EHV-1 subtype 2. Efstathiou et al. (1986) used an 800bp fragment of the HSV-1 thymidine-kinase coding region to detect HSV-specific DNA sequences at concentrations as low as 0.01 copy per cell in human trigeminal ganglia. DNA hybridization also permits the identification of integrated, non-replicating or subvirion DNA which is not detectable by other means. Little is known about the molecular state of latent herpesviruses. It has been suggested that the latent HSV genome persists in a static non-infectious form rather than as a low-level

chronic infection. Virus cannot be isolated from homogenized latently infected tissue, but may be detected only after a long lag phase by explant cocultivation. Also, treatment with inhibitors of viral DNA replication did not affect the rate of virus reactivation from latently infected ganglia (Field et al., 1979; Blyth et al., 1980). However, although Puga et al. (1978) observed no viral RNA in latently infected cells, indicating that latent virus may not be transcribed, limited expression of viral mRNA has been demonstrated in the nervous system of latently infected humans (Galloway et al., 1982) and rabbits (Green et al., 1981). Moreover, HSV-1 thymidine kinase activity was detected in latently infected mouse ganglia (Tenser and Dunstan, 1979), even though this enzyme may not be essential for establishment of latency (Stanberry et al., 1985b; Sears et al., 1985).

The elucidation of the genome structure of latent virus is of fundamental importance to our understanding of the mechanisms involved in latency and reactivation. Rock and Fraser (1983) showed that free genome termini were not detectable in mouse brain and trigeminal ganglia harbouring latent HSV-1. These workers later concluded that the genome termini join to form long linear concatemers or circles which may or may not integrate (Rock and Fraser, 1985). This work was substantiated by that of Efstathiou et al. (1986) who, in the acute phase of infection in mice, could detect DNA fragments from the L-S joint and free termini but one month later could detect only the L-S joint fragments. Similarly, on hybridization analysis of DNA

from latently infected human trigeminal ganglia, no free termini were detected, but fragments corresponding to the L-S joint were present (Efstathiou et al., 1986). Thus, the use of cloned EHV-1 DNA fragments to screen equine tissues may not only throw some light on the pathogenesis of the virus and the role that leukocytes play in the disease, but also may help to elucidate the molecular state of latent EHV-1.

The study and control of EHV-1 disease is severely curtailed by the lack of quick sensitive diagnostic techniques. While the indirect immunofluorescence technique (IFT) is a satisfactory means of identifying cases of EHV-1 abortion (Thomson et al., 1976), in the face of an abortion storm it is impossible to decide conclusively from serological or hormonal data whether or not a mare is going to abort. Diagnosis of an EHV-1 subtype 2 respiratory infection can take up to two weeks (Mumford, 1984). Detection of the neurological form of the disease is often difficult, as by the time clinical signs are manifest the antibody titre is rising and the peak of virus excretion from the upper respiratory tract is usually past. Virus is sometimes isolated from the blood leukocytes by co-cultivation (Mumford, 1984). Seroepidemiological studies have demonstrated that EHV-1 is endemic in horse populations worldwide (Matumoto et al., 1965), and that the majority of horses, in Great Britain at least, experience an infection within the first year of life (Thomson 1978). One might presume from this that a large proportion of the equine population is latently

infected with EHV-1. As yet there is no means of diagnosing latent infections nor of identifying those animals likely to suffer reactivation and thus be a source of infection to their companions.

Nucleic acid hybridization procedures are not used only in basic research, but also as an alternative tool in diagnostic microbiology. Spot hybridization tests can be used to detect viruses, for example EBV (Brandsma and Miller, 1980), CMV (Chou and Merrigan, 1983; Spector et al., 1984) and hepatitis B virus (Brechot et al., 1985), bacteria such as enterotoxigenic E. coli (Mosley et al., 1982; Echeverria et al., 1984, 1986) and parasites like Plasmodium falciparum (Franzen et al., 1984). Stalhandske and Pettersson (1982) were able to differentiate between HSV-1 and HSV-2 infected cells using a cloned restriction enzyme fragment of HSV-1 DNA as a probe.

Cloned DNA fragments from both subtypes of EHV-1 are now available for development as diagnostic probes. Molecular hybridization may offer a relatively fast and sensitive method of detecting virus in nasal scrapings, tracheal washes and leukocytes of infected horses. Also, in the case of a latently infected carrier, DNA probes may identify virus which is otherwise undetectable. The development of a rapid and accurate subtyping system for EHV-1 isolates is of primary importance. The isolation of a subtype 1 virus from a foal on a stud calls for far more stringent isolation procedures than the isolation in similar circumstances of a subtype 2 virus. Use of a

cloned fragment from a heterologous region of the genomes should enable differentiation between the two subtypes in a hybridization assay. Alternatively, as shown in Chapter 2, the hybridization pattern of the cloned fragments of subtype 2 to a Bam HI or an Eco RI digest of subtype 1 is now known and could be utilized to type an isolate, although less rapidly.

Nucleic acid probes labelled with radioisotopes are not ideal for routine use in a diagnostic laboratory. A number of non-radiographic methods of hybridization detection have been described, many of which use biotinylated DNA (Langer et al., 1981; Renz, 1983; Tchen et al., 1984). Polynucleotides substituted with biotin-labelled analogues of TTP or UTP have been reported to exhibit similar reassociation kinetics to biotin-free polymers (Langer et al., 1981). Hybridization can be detected by immunofluorescent or immunohistochemical techniques in the presence of antibiotin antibody or by an avidin-enzyme conjugate combined with a colour-producing substrate (Leary et al., 1983). A biotin-labelled DNA probe kit for the rapid detection of HSV is now commercially available (Fung et al., 1985).

The problems of EHV-1 disease have been baffling horse-owners, scientists and veterinarians for decades. The application of recombinant DNA technology to Veterinary Virology is still in its infancy but it is of vital importance to the horse industry that equine herpesvirologists exploit the ongoing revolution in applied molecular genetics. The data presented in this thesis

constitute the groundwork for such an approach to the study of EHV-1 and the prophylaxis of the diseases with which it is associated.

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