RESTRICTION ENZYME ANALYSIS OF BOVINE HERPESVIRUS-1 DNA

THESIS

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TO

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To My

Beloved Parents

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CERTIFICATE

Certified that the research work embodied in this thesis entitled "RESTRICTION ENZYME ANALYSIS OF BOVINE HERPESVIRUS 1 DNA" submitted by Dr.Praveen Kumar Gupta for the award of Master's Degree of Indian Veterinary Research Institute, is the original work carried out by the candidate himself under my supervision and guidance.

It is further certified that Dr.Praveen Kumar Gupta has worked for more than 24 months in the Institute and has put in more than 150 days' attendance under me from the date of registration for Master's Degree of the University as required under the relevant ordinance.

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We have carefully gone through the contents of the thesis and are fully satisfied with the work carried out by the candidate, which is being presented by him for the award of M.V.Sc. Degree of the institute.

It is further certified that the candidate has completed all the prescribed requirements governing the award of M.V.Sc. Degree of Deemed University, Indian Veterinary Research Institute.

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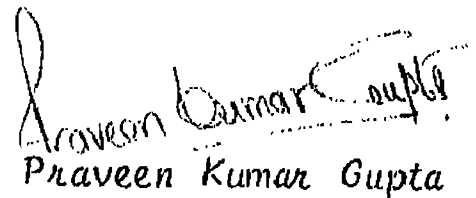

Praveen Kumar Gupta

ABBREVIATIONS

ATP	:	Adenosine-5'-triphosphate
BHV-1	:	Bovine herpesvirus 1
Ci	:	Curie
CPE	:	Cytopathic effect
CsCl	:	Cesium chloride
dATP	:	2 -deoxyadenosine-5'-triphosphate
dCTP	:	2 -deoxycytidine-5'-triphosphate
dGTP	:	2'-deoxyguanosine-5'-triphosphate
dNTP	:	2 -deoxynucleoside-5'-triphosphate
dTTP	:	2-deoxythymidine-5'-triphosphate
EDTA	:	Ethylenediamine tetraacetic acid
GMEM	:	Glasgow modified minimum essential medium
h	:	hour
HSV-1	:	Herpes simplex virus 1
IBR	:	Infectious bovine rhinotracheitis
IBRV	:	Infectious bovine rhinotracheitis virus
IPTG	:	Isopropyl thio galactoside
IPV	:	Infectious pustular vulvovaginitis
IPVV	:	Infectious pustular vulvovaginitis virus
Kb	:	Kilobase
LMT	:	Low-melting temperature
LR	:	Latency related
M	:	Molar
MCS	:	Multiple cloning site
MDBK	:	Madin-Darby bovine kidney
Min	:	Minute(s)
mM	:	Millimolar
moi	:	Multiplicity of infection
MW	:	Molecular weight
nm	:	nanometer
OD	:	Optical density
PAGE	:	Polyacrylamide gel electrophoresis

PI	:	Postinfection
pr	:	Polyrepetitive
RE	:	Restriction endonuclease
RF	:	Replicative form
SDS	:	Sodium dodecyl sulfate
sec	:	Seconds
$TCID_{50}$	:	50% tissue culture infective dose
TE	:	Tris-EDTA
Tk	:	Thymidine kinase
TNE	:	Tris-sodium chloride-EDTA
UV	:	Ultra violet
w/v	:	Weight/volume
X-gal	:	5-bromo-4-chloro-3-indoyl β-D-galacto-pyranoside
μci	:	Microcurie
μg	:	Microgram
μl	:	Microliter

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INTRODUCTION

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INTRODUCTION

Bovine herpesvirus 1 (BHV-1) is associated with a variety of clinical syndrome in cattle worldwide. These are primarily respiratory, but may also include genital infection, abortion, ocular carcinoma, encephalitis and fatal systemic infection in new born calves. The BHV-1 has been isolated or antibody detected from cattle in all parts of the world (Gibbs and Rweyemamu, 1977). In addition to cattle, virus has been reported in sheep, goat (Whetstone and Evermann, 1988), swine, water buffalo and in a variety of wild ruminants throughout the world (Gibbs and Rweyemamu, 1977; Kahrs, 1977).

Besides cross-breeding programme launched in our country, import of cattle and semen have made our livestock population susceptible to many new diseases. Among them BHV-1 infection, associated with clinical syndrome like pyrexia, increased respiratory rate, persistent harsh cough, slight anorexia, depression, and in milking cows a severe drop in milk production, has emerged as a major economic disease in bovines. In India, the major economic losses due to BHV-1 are in terms of milk production and draught power. BHV-1 was first recorded in India serologically by Mehrotra et al. (1976). Subsequently, isolation of the virus from the case of abortion in cows was also reported (Mehrotra, 1977). The serum samples were found positive from Andhra Pradesh, Karnataka, Orissa, Tamil Nadu and West Bengal (Suribabu et al., 1984). Suribabu and Mallick (1983) reported viral abortion in exotic cattle (Jersey) in India due to BHV-1 and Singh et al. (1983) reported its presence in buffaloes.

Restriction enzyme analysis of BHV-1 DNA has been used to classify it into subtypes and to resolve diagnostic, epidemiologic and vaccination problems. As like other herpesviruses, BHV-1 can also cause latent infections and that can be reactivated by natural or corticosteroid-induced stress. Reactivated viruses are shed from the mucous membranes and in semen from bulls. This raises questions on the use of live virus vaccination since such vaccines can establish

latent state carriers. The study of the structural polypeptides of BHV-1, involved in immune response, can be very well used to distinguish the genomic sequences for immunogenic polypeptides. This immunogenic gene of BHV-1 can be inserted into a vector to elicit protective neutralizing antibodies in the host. An alternate strategy may be used to insert these immunogenic gene sequences into an avirulent virus, creating a Chimeric live vaccine strain (Palca, 1986).

While significant achievements in this field of genetic engineering of animal viruses have been made abroad, limited work has been taken up in this field in India. Moreover, no work has been done with indigenous isolate of BHV-1 in this country in respect of restriction enzyme analysis. Keeping these facts in view, the present investigation was taken up to isolate the BHV-1 DNA from cell culture grown virus and to analyse the BHV-1 DNA using restriction enzymes.

REVIEW OF LITERATURE

REVIEW OF LITERATURE

PROPERTIES OF BHV-1

Bovine herpesvirus 1 (BHV-1), also known as infectious bovine rhinotracheitis and infectious pustular vulvovaginitis viruses, is associated with respiratory and genital tract infections. It has got easy transmission and latent infection which can be reactivated under stress conditions. The distribution of the virus is world wide and it is of major economic importance in many countries (Gibbs and Rweyemamu, 1977).

BHV-1, belongs to the group alphaherpesvirus, possesses a linear double stranded DNA genome with molecular weight 89.6×10^6 (IBR 'LA'V) (Engels et al., 1981), molecular length 45.9±1.5 µm, genome size 142.7±4.65 kb, G+C content 71.5% and Tm value (0.1xSSC)85.5°C. The genome of BHV-1 is composed of a unique long segment U_L (100 kb), an internal repeat I_R (11 kb), a unique short segment U_S (13kb), and a terminal repeat T_R (11kb), which is inverted with respect to I_R (Farley et al., 1981). Engels et al. (1986/87) reported the lengths of the inverted repeats 11.45 kb for strain LA, 12.15 kb for k22, 11.5 kb for N569 and 11.3 kb for A663 and the lengths of the short unique sequences ranged between 10.8 and 11.4 kb. Due to this type of genome structure, it has the ability to invert U_S relative to U_L, leading to two isomeric structures of the DNA molecules. This genome structure exemplifies a class-D herpesvirus and is also found in pseudorabies virus, equine herpesvirus 1 and 3, and varicella-zoster virus. The linear double-stranded DNA genome needs to be circularized after infection of host cell and before DNA replication to form replicative concatemer (Hammerschmidt et al., 1988).

The mature virion, measuring 150 nm in diameter, is an enveloped nucleocapsid with icosahedral symmetry made up of 162 capsomeres. The buoyant density of the virus has been investigated to be 1.249 to 1.254 g/cm^3 in cesium chloride (Bagust, 1972) and 1.22 g/cm^3 in potassium tartrate (Talens and Zee, 1976).

BHV-1 multiplies in a wide variety of cell cultures of the bovine and other species. For virus isolation, the cell cultures derived from bovine tissues are considered to be the most susceptible, and most of the workers have employed either fetal or neonatal kidney or testis cultures (Gibbs and Rweyemamu, 1977). The culture systems used for virus preparation by different workers are Madin-Darby bovine kidney (MDBK) cells (Owen and Field, 1988; Mittal and Field, 1989; Bandyopadhyay et al., 1990), Georgia bovine kidney (GBK) cells (Mayfield et al., 1983; Hammerschmidt et al., 1986), Bovine turbinate cells (Mittal and Field, 1989), bovine fetal testis cells (Andino et al., 1987), primary calf kidney cells (Edwards et al., 1990) and embryonic bovine lung cells (Engels et al., 1981).

The gross cytopathology induced by BHV-1 includes the cell rounding, frequently ballooned with small refractile syncytia being formed, and as the cytopathic effect (CPE) progresses, strands of cytoplasm are seen linking affected cells. This type of CPE is typified by herpes simplex virus type 1 and most strains of BHV-1. All herpesviruses seem to be able to induce the formation of large intranuclear, often eosinophilic (Cowdry type A) inclusion bodies in infected cells. The CPE produced in MDBK is characterized by granulation and rounding followed by formation of grapelike clusters (Gibbs and Rweyemamu, 1977).

RESTRICTION ENZYME ANALYSIS OF BHV-1 DNA

In restriction enzyme analysis, the loss or acquisition of endonuclease cleavage sites, consequent to DNA base sequence alterations, results in change in size and hence in migration of restriction fragments during electrophoresis in agarose gels. Thus, comparison of electrophoretic patterns (fingerprints) of the restriction fragments from different virus isolates allows one to conclude that two isolates with different patterns are genetically distinct.

The restriction endonuclease digestion fragment pattern or fingerprint of BHV-1 DNA was first described by Skare et al. (1975) who used the K22 strain as a heterologous control for their studies of herpes simplex virus. Engels et al. (1981) examined four IPV, three respiratory (IBR) and one neurological isolates of bovine herpesvirus and reported specific differences in the restriction endonuclease (HpaI and HindIII) cleavage pattern of IBR and IPV virus strains. Similar finding was again reported by Ludwig (1983) and Gregersen et al. (1985). This preliminary study by Engels et al. (1981) indicated that there were atleast two patterns of fingerprint which they equated with IBR and IPV isolates. A close inspection of the sources of their isolates did not support such a distinction in that some of their so called IPV strains were isolated from respiratory disease cases.

The restriction enzyme analysis of a respiratory isolate of BHV-1 (Cooper strain) for four restriction endonucleases HindIII, BamHI, EcoRI and HpaI was reported by Mayfield et al. (1983). The virus grown in Georgia bovine kidney cells, at a multiplicity of infection (m.o.i.) 0.01 to 0.1, was pelleted and then purified by centrifugation in 10 to 40% potassium tartrate density gradient. The BHV-1 DNA was isolated by phenol extraction from 1% sodium dodecyl sulfate-disrupted virus and followed by banding on cesium chloride density gradients. Purified DNA was cleaved with restriction enzymes and HindIII fragments were cloned into pBR322 plasmid with E. coli strain HB101 as host cell. They prepared cleavage maps of different cloned BHV-1 HindIII fragments using single and double digestion with EcoRI, HpaI, BamHI and HindIII restriction enzymes (Fig.1, Table 1).

Misra et al. (1983) attempted to determine whether distinct types of BHV-1 are responsible for causing specific syndromes. For this they analysed polypeptides and DNA of 93 BHV-1 - isolates by polyacrylamide gel electrophoresis (PAGE) and restriction enzyme analysis, respectively. The polypeptide patterns showed that only six isolates contained polypeptides that varied from the norm in

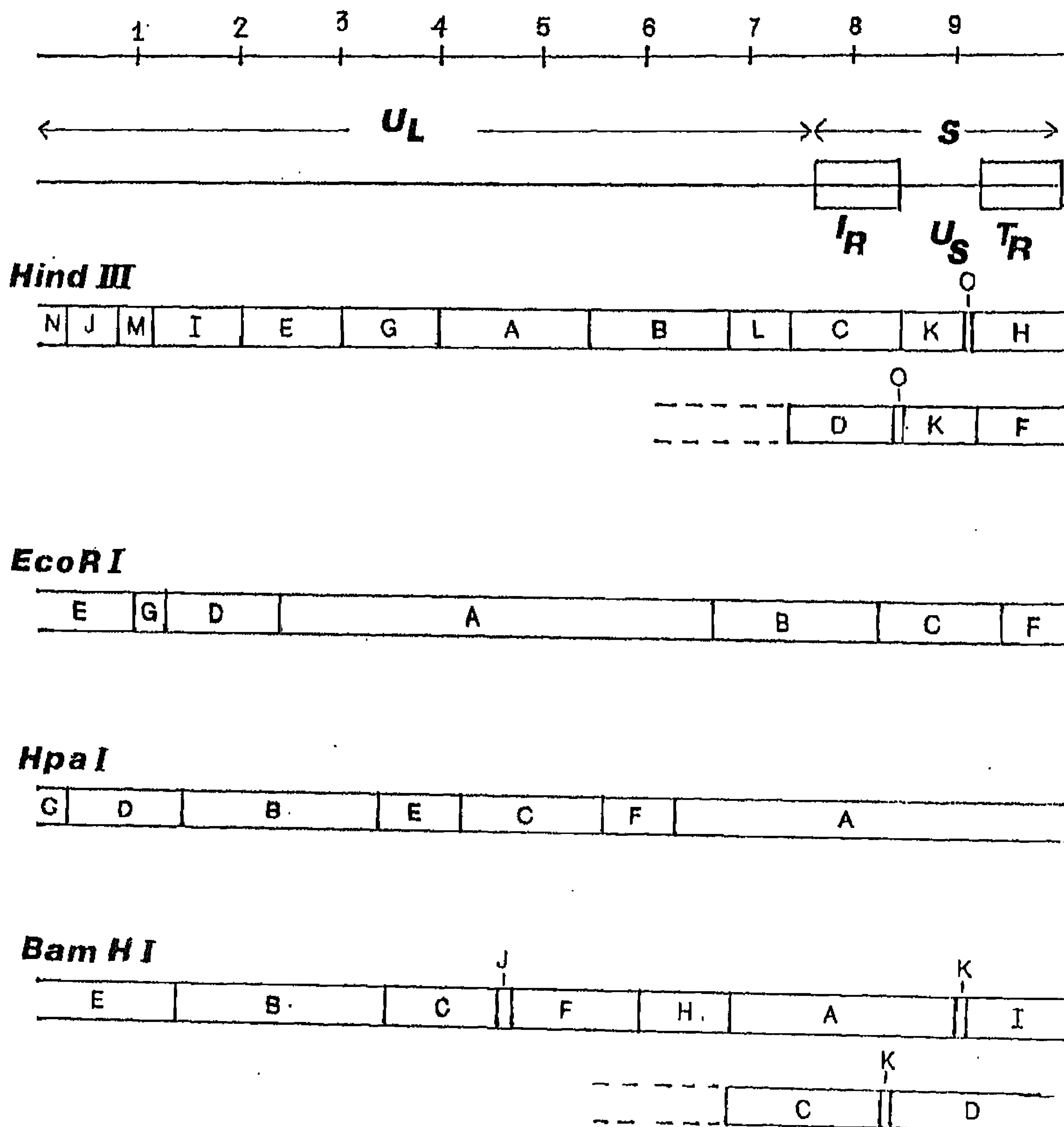

Fig.1 Restriction endonuclease map of BHV-1 DNA corresponding to the Cooper map (adapted from Mayfield et al., 1983).

Table 1. Restriction endonuclease digestion fragment sizes of BHV-1 (Cooper strain) DNA (Mayfield *et al.*, 1983).

HindIII		BamHI		EcoRI		HpaI	
Fragment	Measured size (kb)	Fragment	Measured size (kb)	Fragment	Measured size (kb)	Fragment	Measured size (kb)
A	21.4	(½)A	29.2	A	55	A	55
B	19.3	B	29.2	B	22.5	B	26.5
(½)C	15.9	(½)C	21.2	C	15.8	C	18.7
(½)D	14.8	(½)D	21.2	D	15.3	D	15.4
E	13.5	E	18.2	E	12.9	E	10.2
(½)F	12.8	F	17.3	F	8.4	F	8.7
G	11.9	G	15.7	G	2.9	G	4.0
(½)H	11.7	H	12.3				
I	11.7	(½)I	12.3				
J	9.0	J	0.9				
K	8.4	K	0.25				
L	7.7						
M	3.6						
N	2.4						
O	0.36						

their molecular weight, or were absent. For restriction enzyme analysis, they used EcoRI, HindIII, and HpaI restriction endonucleases. With EcoRI, BHV-1 DNA yielded six/seven fragments into three patterns. The restriction endonuclease pattern with Hind III yielded 11 high molecular weight (MW) fragments and 3 to 4 low MW fragments into three patterns and with HpaI two patterns were reported. On the basis of these results, they categorized 109 BHV-1 isolates, including commercial vaccines, into three strains and 9 substrains. According to them HpaI did not distinguish between genotypes of BHV-1. But Mayfield et al. (1983), Kennedy et al. (1986) and Edwards et al. (1990) could clearly distinguish the viral types using the same enzyme. It was because Mayfield et al. (1983) found that all HpaI digested fragments were equimolar due to absence of cleavage sites in the short region of viral genomes.

Thiry et al. (1983) found that a number of Belgian field isolates of BHV-1 were indistinguishable by DNA fingerprinting from the IBR Los Angeles strain, but did differ from one vaccine strain which was originally from an IPV case. Mayfield et al. (1983) compared BHV-1 Cooper strain with K22 strain and indicated a difference in base sequence of approximately 5% between two strains. This was confirmed by Seal et al. (1985). They analysed 5 respiratory isolates, 4 vaginal isolates, one isolate from an aborted fetus, one from bovine ocular carcinoma, and one from encephalitis using restriction endonuclease HindIII. On the basis of DNA liquid hybridization results between BHV-1 DNA fragment transferred to nitrocellulose paper and nick-translated IBR virus LA isolate DNA probe, they reported high percentage (95%) of homology between isolates of BHV-1 regardless of the clinical manifestation. Although there were differences in restriction enzyme pattern of viral DNA, there was no correlation between the types of pattern obtained and the corresponding clinical manifestation. They supported the findings of Misra et al. (1983) that it might be the routes of infection and/or other environmental factors that determine whether a particular isolate causes a typical respiratory tract infection or some other type of disease such as an encephalitis or genital infection.

Brake and Studdert (1985) analysed 14 genital and 16 respiratory BHV-1 isolates and 3 bovine encephalitogenic, 2 caprine and 2 buffalo herpesviruses using restriction enzyme HindIII, EcoRI, HpaI and BamHI. On comparison, they found that restriction enzyme pattern of 3 encephalitogenic viruses were similar to each other but were totally different from BHV-1 respiratory and genital isolates. On the basis of these results they proposed that bovine encephalitis herpesvirus is a prototype of a new herpesvirus type.

Osorio <u>et al</u>. (1985) compared reference strains and field isolates of BHV by restriction endonuclease analysis using EcoRI, HindIII and BglII restriction endonucleases and the indirect fluorescent antibody test (IFAT). IFAT results showed no cross-reactivity of BHV-1 and BHV-2 with any other types of BHV tested. Restriction enzyme analysis showed 5 major biotypes of bovine herpesviruses namely (i) IBR (BHV-1), (ii) bovine herpes mammillitis virus (BHV-2), (iii) malignant catarrhal fever (MCF) virus (herpesvirus alcelaphinae), (iv) the group of slow-growth isolates represented by the prototype strain Movar 33/63 (bovine cytomegalovirus candidate), and (v) the syncytia forming Pennsylvania 47 strain.

Metzler <u>et al</u>. (1985) compared eleven European isolates of BHV-1 together with two reference virus strains (LA and K22 as type 1 and type 2 respectively) using restriction endonuclease digestion with HindIII, SDS-PAGE and their reactivity with monoclonal antibodies. Of 15 fragments with HindIII digestion (A to O), fragments K and L displayed molecular weight (MW) of 5.7×10^6 and 5.3×10^6 with type 1 (BHV-1 type 1) respectively and 5.2×10^6 and 4.8×10^6 with type 2 (BHV-2 type 2) respectively. For subtype of type 2, HindIII fragment I displayed MW 7.2×10^6 with type 2a and 7.9×10^6 with type 2b. On the basis of results they showed that among 13 isolates of BHV-1, 5 isolates were of type 1 (Cooper-type, IBR like), 5 isolates were of type 2a and 3 isolates type 2b.

Metzler et al. (1986) recognized a new variant virus of BHV-1 on analysing 5 virus isolates recovered in Argentina following the above three parameters - restriction endonuclease analysis, SDS-PAGE and monoclonal antibody. They showed that two isolates were new variant of BHV-1 on the basis of HindIII restriction enzyme pattern. Using the enzyme BstEII or PstI, one strain among these two isolates could be distinguished from other. On the basis of these restriction enzyme analysis results, they identified new variant isolates which were type 3 (BHV-1 type 3) and with BstEII or PstI, type 3 isolates could be further subdivided into subtype 3a and subtype 3b.

Engels et al. (1986/87) compared BHV-1.3 (exhibiting neuropathogenic potential) with the other BHV-1 strains and constructed restriction site maps. For detailed studies they constructed recombinant plasmids with HindIII or EcoRI. The HindIII double digested fragments of strain K22 were inserted into plasmids pBR 322, pDS71, pUC9 and these plasmids were used to transform competent E. coli HB101 host cells. Restriction enzyme analysis results with HindIII and BstEII supported the findings of Metzler et al. (1985, 1986). Of 7 frag ments with EcoRI digestion (A to G), fragments C and D of BHV 1.2 were slightly larger than those of BHV 1.1. They also constructed restriction endonuclease maps using combination of double digestion, Bal 31 digestion and hybridization experiments.

BHV-1, like other herpesviruses can cause latent infections, preferably in the trigeminal ganglion and that can be reactivated by natural or corticosteroid induced stress (Homan and Easterday, 1980; Misra et al., 1981). This raises question on the use of live-virus vaccinations, since such vaccines can establish latent state carriers. Whetstone et al. (1986) analysed restriction endonuclease patterns for IBRV vaccines and compared it with restriction pattern of field samples of IBRV, previously vaccinated with IBRV vaccines, using PstI, BglII, HpaI, BamHI, HindIII and SalI.

Using PstI and BglII they found difference in restriction enzyme patterns among vaccinal strains due to changes in the virus occuring through animal passage. Although BHV-1 is stable in vitro (Whetstone _et al._, 1989). For 2 field samples, showing same restriction endonuclease profile, they concluded that either vaccine virus had been reisolated or field viral isolate could have the same restriction enzyme pattern as a vaccinal strain. For 4 field samples, showing different restriction endonuclease profile, they concluded that it might be due to deletion or acquisition of sequences or due to changes that could occur within the internal repeat region and the left terminus of unique long region of BHV-1 genome (Hammerschmidt _et al._, 1988).

Andino _et al._ (1987) prepared diagnostic probes using A-663 strain DNA for the detection of Argentine isolates of BHV-1 using dot-blot hybridization. They hybridized extracted DNA from cultured cells, fixed on nitrocellulose membrane with denatured radiolabelled cloned BamHI restriction fragments of A-663 strain in pAT 153 plasmid as probe. They reported that 10 picograms of viral DNA was the detection limit of probe having cloned Bam HI fragments (8.9, 15.5 and 17.8 kb A-663 strain BamHI fragments).

Whetstone _et al._ (1989) observed changes in restriction pattern for IBRV vaccines, IPVV vaccine strain FI and k22 both during acute infection and after virus reactivation or virus reactivation followed by superinfection. For studies they collected samples during the acute phase of infection, postinfection (PI) days 0-10, vaginal swabs, nasal swabs and buffy coat cells from each animal daily and inoculated into MDBK cells for viral DNA isolation. They observed restriction enzyme pattern change with PstI for IBRV vaccines, IPVV strains FI and K22 and concluded that changes could occur in the BHV-1 genome during host animal passage, and that the changes could vary from tissue to tissue with the same animal. They also reported that restriction endonuclease analysis using PstI could be used in both the

of subtype (BHV-1.1 and BHV 1.2a/b). On the basis of restriction enzyme pattern they concluded that (i) the BHV-1 genome is variable in vivo, (ii) cattle latently infected with one subtype of BHV-1, can coexpress these viruses both in nasal and vaginal tissue, (iii) in diagnostic and epidemiologic studies of BHV-1, restriction enzyme analysis has application that is limited to analysis between type and subtype, but is not applicable in the examination of isolate from within a BHV-1 subtype.

Tenser et al. (1979) showed that the lack of viral TK activity resulted in loss of ability of HSV-1 (Herpes Simplex Virus-1) - to maintain the latent state. So, for studying latency of BHV-1, map location of the non-essential Thymidine kinase (TK) gene was found in a 1.1 kilobase Bgl II-Sal I fragment (map unit 0.47-0.48) in Hind III A fragment (Bello et al., 1987). Tk gene of BHV-1 has been compared to HSV-1, HSV-2, Varicella-zoster virus, Epstein-Barr virus, marmoset herpesvirus (MarHV) (Mittal & Field, 1989) and its activity has been shown in E. coli tk^- host (TK deficient mutant) (Liu and Manning, 1986) and in bovine cells (Bello et al., 1987). Rock et al. (1987) mapped latency-related (LR) gene of BHV-1 in rabbit trigeminal ganglia. They used HindIII cloned fragments of BHV-1 as probe to identify the fragment of LR gene using in situ hybridization. Ten probes of various lengths of HindIII fragment D, (hybridizing to LR gene, subcloned in pUC18 or pGEM1 vectors using RE XhoI, EcoRI, KpnI and SalI) was used to map LR gene on BHV-1 genome (map unit 0.734 to 0.748).

Whetstone and Evermann (1988) analysed DNA of 10 herpesvirus field isolates from 6 sheep and 4 goats using restriction endonuclease PstI and also examined their relatedness to one other; to bovine herpesvirus 6 (BHV-6); and to 2 strains of BHV-1 (IBR/IPV). They found that 5 of 6 sheep isolates and 3 of 4 goat isolates yielded patterns that differed from each other by one or more bands. Sheep isolate DNA patterns were different from goat isolate patterns and all restriction endonuclease analysis patterns were similar to the pattern of BHV-I IBRV but different from that for BHV-1

IPVV or for BHV-6. Restriction patterns from 3 sheep and 2 goat isolates were indistinguishable from patterns reported for 3 modified live IBRV vaccines. On the basis of these results, they concluded that none of the herpesvirus isolates belonged to either the BHV-6 (caprine herpesvirus), or the BHV-1/IPVV type but belonged to BHV-1/IBRV. They recommended sheep and goats as potential hosts for BHV-1.

Restriction endonuclease analysis of DNA of a BHV-1 isolate (FI) from an aborted fetus was compared to other BHV-1 isolates (Colorado-1, Iowa and K22) by Miller et al. (1988). Using restriction endonuclease PstI and HindIII, they found difference in restriction enzyme pattern with Colorado-1, Iowa and K22 BHV-1 isolates. On the basis of results, they concluded that the FI isolate appeared to be a type-2 (IPV) virus specifically subtype a.

Edwards et al. (1990) did the genotypic classification of 84 U.K. isolates of BHV-1 by restriction enzyme analysis using EcoRI, HindIII, HpaI, BamHI, PstI and BstEII. They found that HindIII and HpaI gave the clearest pattern and they grouped all isolates as IBR like and IPV like.

RESTRICTION ENZYME ANALYSIS OF HERPESVIRUS DNA OTHER THAN BHV-1 DNA

Ehlers et al. (1985) analysed polyrepetitive DNA (prDNA) of all six strains of bovine cytomegalovirus (BHV-4) and found similar restriction profiles. They cloned complete unit sequence of strain 66-P-347 (ClassII) in bacteriophage vector M13mp7 and mapped using restriction enzymes EcoRI, BamHI, BglI, NaeI, SstII and PstI. Dubuisson et al. (1988) compared BHV-4 isolates V.Test and LVR140 (from genital disease) and the reference strain Movar 33/63 and DN599 using restriction endonucleases EcoRI, BamHI and HindIII and classified genital strains V.Test and LVR 140 into class III and II respectively. Bublot et al. (1990) constructed restriction map of BHV-4 genome. For studies, they constructed a plasmid library in pSP18

vector into EcoRI site and a phage library into lambda Gem-11 vector using EcoRI digested and Sau3 AI partially digested products of BHV-4 - genome. Using these libraries they constructed restriction maps using single and multiple digestion with EcoRI, BamHI and HindIII. They also constructed restriction maps of prDNA for 14 restriction enzymes. On the basis of differences in restriction pattern, and maps of the four BHV-4 strains, they classified BHV-4 strains into two groups.

Paul et al. (1982) analysed genome of six virulent and three attenuated vaccine strains of pseudorabies virus (Suid herpesvirus 1) using 15 restriction endonucleases. Restriction enzyme BamHI showed distinct electrophoretic patterns for each of the nine SHV-1 strains. Several other workers also reported BamHI as the enzyme of choice for restriction analysis (Gielkens et al., 1985; Tisdall et al., 1988; Christensen, 1988). Tisdall et al. (1988) analysed 14 isolates of Aujeszky's disease virus (SHV-1) by restriction endonuclease analysis using BamHI, XhoI, and kpnI restriction enzymes. On the basis of BamHI patterns they grouped all isolates as Group 1. Christensen (1988) grouped 11 European isolates of SHV-1 into 4 types by restriction fragment pattern analysis using restriction endonuclease BamHI and Southern blot hybridization.

Viruses causing respiratory disease and abortion in horses were classified as equine herpesvirus 1 immunologically. But Kovler et al. (1981) and Studdert (1983) analysed viral DNAs from animals with respiratory disease (Respiratory strain) and from aborted fetus (fetal strain) using restriction endonucleases and reported similar restriction patterns among the strain and different patterns between the strains. On the basis of these results, they designated respiratory strain and fetal strain as equine herpesvirus-1 (EHV-1) and EHV-4, respectively. The genome of EHV-1 has been analysed using restriction endonuclease analysis (O'Callaghan et al., 1981; Whalley et al., 1981). Whalley et al. (1981) also constructed physical map of an Australian isolate of EHV-1 for EcoRI, BglII and BamHI restriction enzymes. Allen et al. (1983) analysed 297 field

isolates of EHV-1 by digestion with BamHI, EcoRI, BglII, SalI and Sst I restriction enzymes and reported two markedly different DNA cleavage patterns among isolates designated as subtype 1 and subtype 2. Similar findings have been reported by Cullinane et al. (1988) using restriction enzymes EcoRI and BamHI. Browning and Studdert (1989a) partially cloned low equine cell passage equine cytomegalovirus (EHV-2) and determined physical maps for the restriction endonucleases BamHI, EcoRI, HindIII and SalI. Using cloned fragments as a probe, Browning and Studdert (1989b) further constructed physical maps of the genomes of 14 isolates of EHV-2 for restriction endonucleases BamHI, EcoRI and HindIII. Colacino et al. (1989) determined the molecular structure of the genome of EHV-1 by restriction enzyme mapping and molecular hybridization using cloned BamHI, EcoRI and HindIII fragments of the viral genome as a probe. Sullivan et al. (1984) analysed genome of equine coital exanthema (EHV-3) using BamHI, BclI, BglII, EcoRI and HindIII restriction endonucleases. Like BHV-1 genome, they found four half molar fragments that confirmed two isomeric forms of EHV-3 genome. Similar work was reported by Jacob et al. (1985) using XbaI, BglI, EcoRI and Hind III restriction endonucleases. They got one XbaI cleavage site in the unique sequence of the S component. Meyer et al. (1987) investigated two equine herpesvirus isolates from horses with clinical signs of neurological disorders using restriction endonuclease analysis. They characterized one isolate as a typical EHV-1 subtype 1 while another isolate as EHV-1 subtype 2 (EHV-4) strains.

Hirai and Ikuta (1981) analysed DNA from virulent strain BC-1 and avirulent strain C_2(A) of Marek's disease virus (MDV) by restriction enzyme analysis using BamHI, SalI, SmaI enzymes and reported very similar restriction patterns with some significant differences. Ross et al. (1983) showed that a strain of MDV HPRS24 (serotype 2) shared less than 10% homology with either HPRS16 (serotype 1) or herpesvirus of turkey (HVT) (serotype 3) by using

restriction enzyme analysis and nucleic acid hybridization procedures. Later, Fukuchi et al. (1985) found homology between MDV and HVT, throughout the MDV genome except in the long inverted repeat regions (TR_L). Further, Fukuchi et al. (1984) constructed restriction maps for BamHI, BglI, and SmaI on Marek's disease virus strain GA genome. For studies they could clone 27 fragments out of the 29 fragements into bacterial plasmid vectors. They also reported its genome structure similarity with herpes simplex type 1 and 2. Kotiw et al. (1982) differentiated infectious laryngotracheitis virus (ILTV) strains using restriction endonuclease HindIII.

Restriction endonuclease analysis of human herpes simplex virus has been done using restriction endonuclease EcoRI (Skare et al., 1975; Hayword et al., 1975; Lonsdale, 1979). Lonsdale (1979) reported that HpaI could be used to show the distinction between HSV-1 and HSV-2 most clearly. Nosocomial outbreaks of herpes simplex have been investigated using restriction endonuclease analysis (Halperin et al., 1980; Filatov et al., 1982; Buchman et al., 1978). Restriction endonuclease analysis has been reported as a simple method for unambiguously typing herpes simplex virus (Senkevich et al., 1982; Ueno et al., 1982; Arens and Swierkasz, 1983; Du et al., 1988). On comparison with enzyme linked immunosorbent assay (ELISA), restriction enzyme analysis showed superiority for rapid identification and differentiation between HSV-1 and HSV-2 (Mith et al., 1984). Variation within the strain of HSV-1 from epidemiological isolates has been reported using 4 base pair recognizing restriction enzymes HaeIII, HhaI, Sau3AI and 6-base pair recognizing restriction enzymes BamHI, kpnI, SalI (Senkevich et al., 1981; Umene et al., 1984; Sakaoka et al., 1987; Umene, 1987). Umene (1987) found that 4 base pair recognizing restriction enzymes were suitable for analysing closely related strains. Smith et al. (1981) and Maitland et al. (1982) analysed genital isolates of HSV-2 using restriction endonuclease EcoRI, BglII, HindIII, KpnI, BamHI and SstI. On the basis of sequence variation occurring in the terminal and subterminal BamHI fragments, Maitland et al. (1982) suggested that each individual patient might induce specific variation in the herpes

simplex virus-2 genome. Jarrett et al. (1989) prepared restriction maps for human herpes virus-6 (HHV-6) genome and demonstrated that one earlier reported virus HTLHV (human T-lymphotropic herpesvirus) was nothing but isolate of HHV-6.

Cytomegalovirus genome has been analysed with Hind III and EcoRI enzymes (Kilpatrik et al., 1976; Spector, 1983; Grillner and Blomberg, 1984). Mishra et al. (1984) constructed physical maps of Varicella-zoster virus DNA with 11 restriction endonucleases using double digestion and hybridization to cloned BamHI fragments. Arrand et al. (1989) analysed DNA sequence of the 11 kb SacI-EcoRI region on the EcoRI-J fragment of four strains of Epstein-Barr virus and compared with the prototype sequence from strain B95-8. On the basis of restriction endonuclease analysis, they grouped these 4 strains and 26 EBV positive cell lines into two families (1 and 2).

RESTRICTION ENZYME ANALYSIS OF PARVOVIRUS DNA

Burd et al. (1983) analysed bovine parvovirus replicative form (RF) DNA using 12 restriction enzymes and reported no cleavage site for these enzymes within the 3' terminal hairpin and no cleavage sites for HindIII, SalI, SmaI, SstI and XorII restriction enzymes on entire genome. They also compared the restriction site locations for bovine parvovirus with those of other autonomous parvovirus. Ridpath et al. (1987) determined genomic relationship between porcine parvovirus and several other autonomous parvoviruses using restriction mapping and hybridization analysis. They prepared restriction site maps of porcine parvovirus genome and compared with the genome of canine parvovirus, feline panleukopenia virus, minute virus of mice, H-1 virus and bovine parvovirus. Krell et al. (1988) analysed DNA of porcine parvovirus strain NADL-2 using restriction endonuclease analysis and reciprocal southern blot hybridization. A PstI/HindIII double digested 3.0kb DNA was cloned into a plasmid vector pUC19. They developed a diagnostic probe using this 3.0 kb cloned fragment recovered from transformed E. coli strain TB1.

RESTRICTION ENZYME ANALYSIS OF PAPOVAVIRUS DNA

Morgan _et al_. (1981) constructed restriction maps of bovine papilloma virus type-1 (BPV-1) DNA using 15 restriction endonucleases. Using restriction enzymes, Spradbrow and Ford (1983) analysed 6 DNA samples isolated from cutaneous bovine papilloma virus type 2 (BPV-2). Campo and Coggins (1982) cloned genomic DNA of BPV-1, BPV-2 into HindIII sites and BPV-4 into BamHI sites of pAT13, using restriction enzyme analysis and hybridizaton techniques they reported broad homology between BPV-1 and BPV-2 and no homology of BPV-4 with either BPV-1 or BPV-2 DNA.

RESTRICTION ENZYME ANALYSIS OF ADENOVIRUS DNA

Shinozaki _et al_. (1988) grouped 19 strains of fecal human adenovirus as adenovirus type 40 and 13 strains as adenovirus 41 using restriction endonuclease SmaI. They further analysed DNAs of adenovirus 40 and 41 using HindIII, EcoRI, BglI and BamHI and found two different restriction profiles of adenoviruses 40 DNA for BamHI. Adrian _et al_. (1989a,b) analysed 168 strains of adenovirus 7 and 138 strains of adenovirus 3 using 6 restriction endonucleases and grouped adenovirus 7 into 9 genome types and adenovirus 3 into 18 genome types. They divided adenovirus 3 genome types into two groups D1 or D3 using BglIII restriction endonuclease. Benko _et al_. (1988) compared eight reference strains of bovine adenovirus using BamHI, EcoRI, HindIII and PstI restriction enzymes and classified all strains into restriction enzymes and classified all strains into two subgroups.

Benko _et al_. (1990) cloned DNA of bovine adenovirus 4 (BAV-4) into plasmid vector pKH47 into ClaI and SalI sites and subcloned with enzyme BamHI. Using these cloned fragments they mapped genome with 11 restriction enzymes and determined DNA homology among bovine, human and porcine adenoviruses. They showed strong sequence homology among the members of subgroup 2, but no homology to subgroup 1, or any of these human (HAV) and porcine adenoviruses.

Spibey _et al_. (1989) cloned canine adenovirus type 1 (CAV-1) DNA and prepared restriction maps. They compared restriction enzyme maps of CAV-1 with those of CAV-2 and again characterized left end of both genomes using restriction enzymes. Spibey and Caranagh (1989) cloned a field isolate (CAV-2, Glasgow) and a vaccine strain of canine adenovirus (CAV-2) into pBR322 plasmid vector and constructed physical maps of these two viral genomes through molecular hybridization of PstI, EcoRI, SmaI, BamHI and KpnI digests of the viral DNA fragments with the cloned PstI fragments as probe. They showed that CAV-2 Glasgow field isolate was virtually identical to those of the two prototype CAV-2 strains, Toronto A26/61 and Manhattan.

RESTRICTION ENZYME ANALYSIS OF IRIDOVIRUS DNA

Wesley and Pan (1982) analysed wild type, Vero-cell adapted and plaque purified African swine fever virus (ASFV) DNA using restriction endonucleases SmaI and EcoRI and showed similarity in restriction profiles. Tabares _et al_. (1987) analysed ASFV DNA grown in pig leukocytes (strain E70 L6) and after adaptation in MS monkey kidney cells (E70 MS14) using restriction enzymes ClaI, SalI and SmaI and reported decrease in size of the ASFV genome after adaptation, due to deletion and additions mainly in the terminal regions of the genome. Santurde _et al_. (1988) reported this cell culture adaptation change in viral genome from 156 kb to 170 kb using Hind III restriction endonuclease. Dixon (1988) analysed ASFV DNA from RBCs using enzyme BamHI, ClaI and SalI and prepared restriction enzyme maps. They reported close relationship with isolates from Europe, Caribbean and Cameroon by Southern blot hybridization using radiolabelled fragments of ASFV Malawi in bacteriophage and pUC18 plasmid vectors as probes.

RESTRICTION ENZYME ANALYSIS OF POXVIRUS DNA

Among orthopoxviruses, vaccinia virus is the most studied virus. Gangemi and Sharp (1976) analysed WR and CV-1 vaccinia strain DNA using Hind III enzyme. They reported that 14 fragments of Hind III cleaved DNA could reveal small molecular weight differences among strains. De Fillipes (1982) constructed restriction site maps of vaccinia virus (WR strain) for the restriction enzymes BglI, HindIII, KpnI, SalI, SmaI and XhoI using single and double digestion. They showed that BglI and SmaI cleaved fragments provided a convenient means to group fragments produced by other enzymes as these two enzymes cleaved DNA into only 5 segments.

Muller et al. (1977) compared DNA of different pox viruses DNA using HindIII, BamHI and EcoRI restriction endonucleases and reported that cowpox and ectromelia viruses showed some degree of relatedness to each other as rabbitpox and vaccinia virus. However, fowlpox virus DNA showed no similarities with those of orthopoxvirus DNAs indicating a very low degree of homology.

Esposito & Knight (1985) analysed 38 orthopoxviruses and prepared restriction maps using HindIII, SmaI, BglII, SalI and KpnI restriction enzymes. Variation of HindIII maps in middle and terminal regions provided a basis for differentiating species, strains and variants of 38 pox viruses.

Black et al. (1986) reported a high degree of sequence homology for 10 isolates of capripoxvirus DNA on analysing DNA of 12 field and vaccine isolates of capripoxvirus from cattle, sheep and goats. They reported 27-33 fragments of DNA on digestion with HindIII. Gershon and Black (1987) constructed restriction site maps for a cattle isolate of capripox virus (KC-1) using PstI, AvaI and SalI restriction enzymes. Gershon and Black (1988) compared the genomes of capripox virus isolates using restriction enzymes HindIII, PstI, AvaI and SalI. They constructed restriction maps for PstI, AvaI and SalI on DNA of InS-I and IrG-I isolates, comparing with restriction profile of KC-1 DNA.

Schnitzlein et al. (1988) compared the genomes of different avipox isolates including juncopox, pigeonpox and,field and vaccine strain of fowlpox viruses using BamHI and HindIII endonucleases. On the basis of restriction endonuclease analysis, they reported that avipoxviruses appeared to be closely related. Coupar et al. (1990) analysed fowlpox virus genome using PstI, SalI, SmaI and NotI restriction enzymes and identified partial maps for EcoRI and BamHI. On the basis of restriction enzyme analysis , they reported 100 kb large size of genome on comparison with orthopoxvirus vaccinia. They reported the presence of a non-essential region and potential insertion site for foreign DNA as it was previously used in the construction of recombinant fowlpox virus as vectors for poultry vaccines (Boyle and Coupar, 1988).

RESTRICTION ENZYME ANALYSIS OF HEPADNAVIRUS DNA

The complete genome of hepatitis B virus (HBV) has been cloned into the BamHI site of the pBR322 plasmid by Bichko et al. (1982). They also constructed physical maps for 13 restriction endonucleases and reported that cloned DNA is similar but not identical to the HBV DNA of ayw subtype. Lo et al. (1985) isolated HBV DNA from Dane particles of 9 patients' plasma and cloned into the EcoRI or BamHI site of the pUC 8 plasmid. They obtained 2 plasmids with full length HBV DNA and four plasmids with HBV surface antigen gene.

RESTRICTION ENZYME ANALYSIS OF RNA VIRUSES

For restriction enzyme analysis and mapping of RNA viruses, first cDNA of RNA genome is synthesized using reverse-transcriptase enzyme, then cDNA is analysed using restriction endonucleases. Tracy et al. (1985) cloned cDNA of Coxsackie virus B3 genome into PstI site of pBR322 and prepared restriction maps of clones using double digestion with restriction endonucleases. On the basis of restriction maps and Southern hybridization results, they showed that CB3 genome was different from poliovirus genome.

Huismans et al. (1987) cloned genome segment of blue-tongue virus serotypes 2,4,6,7,8,9 and 10 into pBR322 and investigated genetic homology by hybridization, restriction endonuclease mapping and sequencing of the terminal ends.

Marx et al. (1979) analysed unintegrated DNA of Schmid-Ruppin Rous Sarcoma virus, subgroup D using SmaI restriction endonuclease digestion and found 5 cleavage sites on DNA, 2 of which were localized at the ends of the provirus. They also reported no cleavage site of SmaI in src and env genes of Rous Sarcoma virus. Devaux et al. (1985) cloned 78AI isolate of Moloney murine sarcoma virus 1 (78AI Mo-MuSV) and prepared restriction site maps using restriction endonucleases. They also compared restriction maps with HT-1 provirus and showed that difference lied in the gag-pol junction region of Mo-MuSV. Using restriction enzymes, Gray et al. (1986) mapped mouse mammary tumor virus (MMTV) proviral loci (M+V-1 and M+V-2), associated with virus expression and tumorogenicity. Fukasawa et al. (1987) analysed proviral genome of two isolates of human T-cell leukemia virus Type 1 (HTLV-1) from lymphocyte cultures and found that these two isolates had homologous restriction enzyme analysis pattern to prototype HTLV-1. Yoshiyama et al. (1987) reported human immunodeficiency virus (HIV_{yu-6}) as a mixture of Yu-6a and Yu-6b viruses on the basis of restriction analysis. They found that Yu-6a had one more kpnI site than Yu-6b. Krause et al. (1989) cloned unintegrated circular proviral DNA of a type D retrovirus (PMFV) from human cells into the bacteriophage vector L47.1 and again subcloned in the plasmid vector pGEM-2. Using these cloned fragments they prepared restriction endonuclease map of PMFV DNA for 10 different restriction enzymes using single and multiple digestions. They also compared cloned long terminal repeat (LT_R) of SAIDS retrovirus type-1 (SRV-1) and type 2 (SRV-2), retrovirus type D and Masson-Pfizer monkey virus (MPMV).

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MATERIALS AND METHODS

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MATERIALS AND METHODS

BOVINE HERPESVIRUS 1 (BHV-1)

The bovine herpesvirus-1 (BHV-1) isolate No.216, was obtained, in freeze-dried form from the Division of Standardization, I.V.R.I., Izatnagar-243122 (U.P.). The virus was isolated from nasolachrymal duct of calves suffering from spontaneous cases of conjunctivitis in India and adapted in bovine kidney cell culture at passage no.17. This Indian BHV-1 respiratory isolate was characterized using bovine antiserum against Colorado strain of BHV-1, procured from Dr.R.G.Marshall, National Animal Disease Centre, Ames, Iowa-50010 (Mehrotra, 1977).

Cell culture

Madin-Darby Bovine Kindey (MDBK) cells were used for cultivation of BHV-1 in bulk in this study. MDBK cells were grown in GMEM (Glasgow modified minimal essential medium) obtained from Microlab, Bombay. For growth medium, GMEM was supplemented with 10% heat inactivated new born calf serum while the maintenance medium was devoid of serum.

Host bacteria

Escherichia coli TG-1 (Amp^-, $LacZ^-$) bacterial cells were used as host cell bacteria in transformation with recombinant pUC9 plasmids.

Vector

The pUC9 plasmid (Fig. 2) was used as a cloning vector. *E. coli* bacterial cells containing pUC9 plasmid were grown and plasmid isolated from these cells was used for construction of recombinant plasmids.

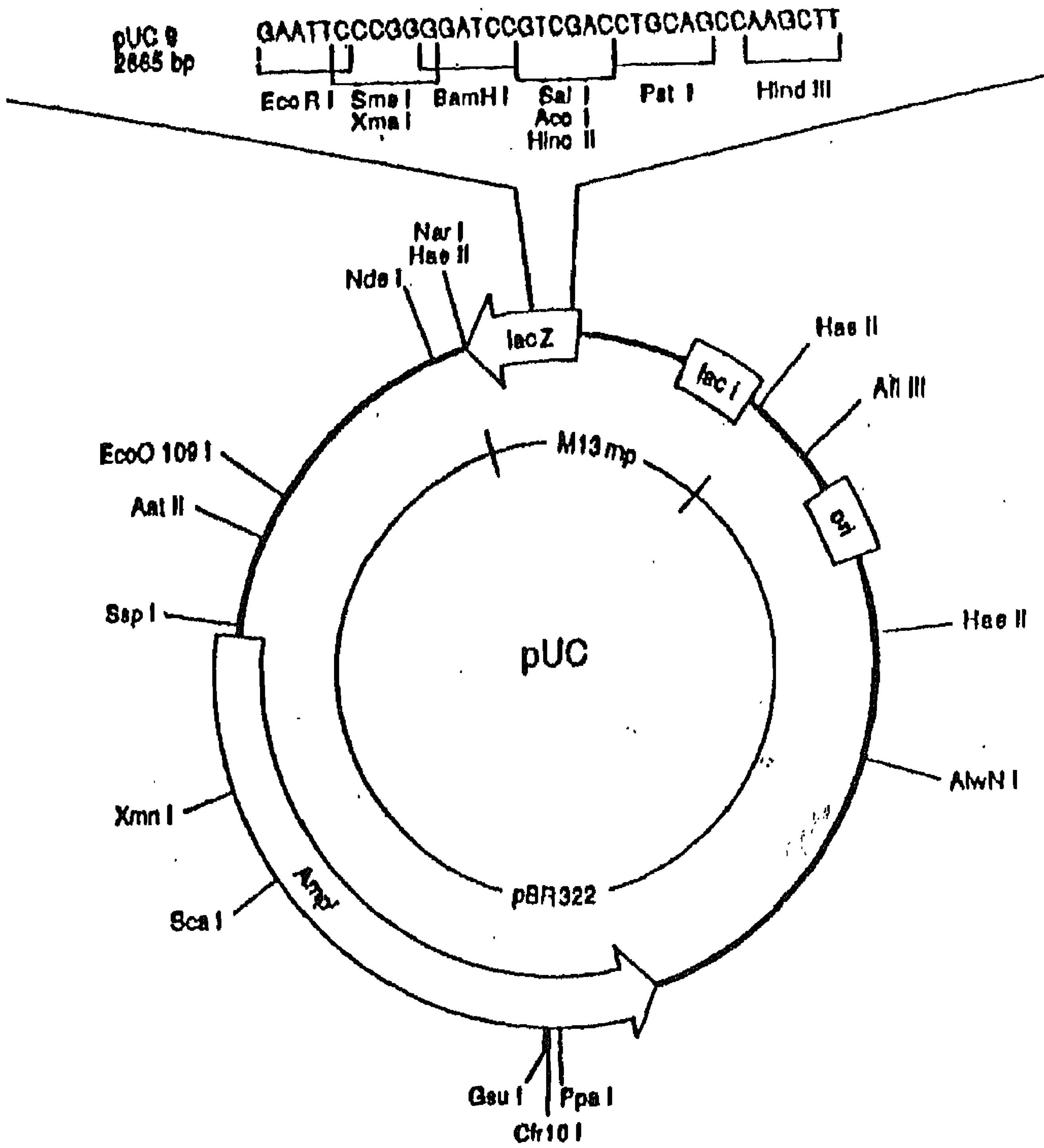

Fig.2 Restriction endonuclease map of pUC9 plasmid DNA, showing multiple cloning site (MCS) in lacZ region. Amp^r is ampicillin resistance gene.

Chemicals

The following chemicals were used:
Trypsin (1:250), Tryptose-phosphate broth, Bacto-Yeast extract, Bacto-tryptone Agar Noble (all DIFCO), L-glutamine, Tris, Ethylene diamine tetraacetic acid (EDTA), Potassium tartrate, 8-hydroxy quinoline, Acetic acid (glacial), Sodium dodecyl sulphate (SDS), Dithiothreitol (all BDH), Agarose-Low melting temperature (LMT), $MgCl_2.6H_2O$, Albumin bovine (Fraction V), Ampicillin, 5-bromo-4-chloro-3-indoyl β-D-galactopyranoside (X-gal) (all Sigma), Isopropyl thio galactoside (IPTG) (Boehringer Mannheim), Agarose NA (Pharmacia), Sodium acetate, Sodium chloride (all Merck).

Isotope

Tri-ethyl ammonium salt of alpha $[^{32}P]$ dATP (3000 Ci/mmol) in aquous solution containing 5mM 2-mercaptoethanol was obtained from BRIT (Board of Radiation and Isotope Technology, BARC, Trombay, Bombay).

Enzymes

All restriction enzymes (HindIII, PstI, EcoRI, BamHI, HpaI, HaeIII, BglI, BglII, XhoI, SmaI, SalI, RsaI, StuI, DraI) used in this study were obtained from Boehringer Mannheim. Other enzymes Klenow fragment of DNA polymerase I, Proteinase K, T4 DNA ligase and Calf intestinal alkaline phosphatase (CIP) (Boehringer Mannheim), DNase I and RNase A (Sigma) were also used.

Glassware and Plasticware

All glassware were Borosil make and plasticware were from Flow Lab. and Nunc. These were used for cell culture, virus cultivation, bacterial culture and other laboratory works.

BUFFERS AND REAGENTS

Cell culture medium

Glasgow modified minimum essential medium (Eagle's)

GMEM	1 vial
Penicillin (100 IU/ml)	1,00,000 IU
Streptomycin (100 μg/ml)	100 mg
Autoclaved triple glass distilled water (TGDW)	ad. 1000 ml

pH was adjusted to 7.2-7.4 using $NaHCO_3$ and sterilized by filtration.

Tryptose phosphate broth (TPB) 10X

Tryptose Phosphate Broth (Difco)	20 g
Autoclaved tripple glass distilled water	ad. 500 ml

Sterilized by autoclaving at 15 lb/sq.in. for 15 min.

L-glutamine (100X)

L-glutamine (GIBCO)	2.92 g
TGDW (autoclaved)	ad. 100 ml

Sterilized by filtration.

Finally the cell culture growth medium was prepared as follows:

GMEM	790 ml
TPB (10X)	100 ml
L-glutamine (100X)	10 ml
New born calf serum	100 ml

Trypsin-versene solution (TVS)

NaCl	5 g
KCl	0.125 g

Na_2HPO_4	0.95 g
KH_2PO_4	0.125 g
Trypsin (Difco 1:250)	0.85 g
EDTA	0.70 g
0.4% Phenol red	0.5 ml
TGDW	ad. 500 ml

The solution was sterilized by filtration.

Agarose Gel Electrophoresis Buffer

Tris-acetate buffer (TAE) 50X

Tris-base	242 g
Acetic acid (Glacial)	57.1 ml
0.5M EDTA (pH 8.0)	100 ml
TGDW	ad. 1000 ml

Tris-borate buffer (TBE) 5X

Tris-base	54 g
Boric acid	27.5 g
0.5M EDTA (pH 8.0)	20 ml
TGDW	ad. 1000 ml

Ethidium bromide (2000X)

Ethidium bromide	10 mg
Distilled water	1 ml

Loading buffer (10X)

Orange G	0.25%
Ficoll	25%
EDTA	0.25 M

Plasmid Isolation and Transformation

Luria-Bertani (LB) broth (per liter)

Bacto-tryptone	10 g
Bacto-yeast extract	5 g
Sodium chloride	10 g

The pH was adjusted to 7.0 with 5N NaOH and sterilized by autoclaving.

Luria-Bertani (LB) agar (per liter)

LB broth	1000 ml
Bacto-agar	15 g

Sterilized by autoclaving.

SOB medium (per liter)

Bacto-tryptone	20 g
Bacto-yeast extract	5 g
Sodium chloride	0.5 g

Dissolved in 950 ml of deionized TGDW and 10 ml of a 250 mM solution of KCl was added. The pH was adjusted to 7.0 with 5N NaOH. The volume of the medium was made 1 liter with deionized TGDW and sterilized by autoclaving for 20 min at 15 lb/sq. in. on liquid cycle. Just before use, sterile solution of 1M $MgCl_2$ and 1M $MgSO_4$ (5 ml each) were added.

SOC medium

To make SOC medium, 20 ml of a sterile 1M solution of glucose was added in 1 liter SOB medium.

X-gal (1000X)

X-gal	25 mg
Dimethyl formamide (DMF)	1 ml

Stored at -20°C in dark bottle.

IPTG (1000X)

IPTG	25 mg
TGDW (autoclaved)	1 ml
Stored at -20°C	

T4 DNA Ligase buffer (10X)

Tris HCl (pH 7.6)	0.5 M
$MgCl_2$	100 mM
Dithiothreitol	100 mM
Bovine serum albumin (Fraction V:Sigma)	500 µg/ml

T4 DNA ligase mixture (2X)

1M Tris HCl (pH 7.6)	1.0 µl
100 mM $MgCl_2$	1.0 µl
200 mM dithiothreitol	1.0 µl
10 mM ATP	1.0 µl
TGDW (autoclaved)	4.0 µl
T4 DNA ligase	2 µl

CIP dephosphorylation buffer (10X)

$ZnCl_2$	10 mM
$MgCl_2$	10 mM
Tris HCl (pH 8.3)	100 mM

PREPARATION OF MDBK CELL CULTURE

Preformed monolayer was subcultured using trypsin-versene solution and then cells were suspended in the growth medium containing 10% heat inactivated new born calf serum, 2 mM L-glutamine, 0.4% tryptase phosphate broth in GMEM with antibiotic. Suspended cells were incubated at 37°C for 2-3 days to get a complete monolayer.

ADAPTATION OF BHV-1 IN MDBK CELL CULTURE

An uniform complete monolayer of MDBK cells was prepared in milk dilution bottle. The growth medium was decanted and monolayer was washed twice with maintenance medium. For inoculum preparation, the contents of two freeze-dried ampules were reconstituted in 0.5 ml maintenance medium with antibiotics (Penicillin 200 IU/ml and Streptomycin 200 μg/ml). Then it was incubated at 37°C for 30 minutes. Using this prepared inoculum, the monolayer was infected and kept at 37°C for 1 h with intermittent rotation for adsorption of the virus after which the inoculum was removed and replaced with 10 ml maintenance medium with the pipette using pipetus (Flow lab, Germany) and bottle was incubated at 37°C. The monolayer was examined daily for cytopathic effect (CPE) and the virus was harvested after 4 days of infection. This harvested virus was used as inoculum for the next passage after freezing and thawing twice. After 5 passages in MDBK cell culture, the virus was titrated in 24 well plates alongwith control. 0.1 ml of each of the serial ten fold diluted virus, from 10^{-1} to 10^{-6}, was added in each well and after adsorption, 1 ml maintenance medium was added in each well. The plates were incubated at 37°C for 5 days and CPE was recorded. The titre of the virus was calculated according to the method of Reed and Muench (1938). The fifth passaged virus in MDBK cells was used to infect MDBK monolayer cells for bulk preparation of virus.

PURIFICATION OF BHV-1

The BHV-1 was concentrated and purified following the method described by Talens and Zee (1976) and Owen and Field (1988) with slight modification.

The two times freeze thawed infected MDBK culture fluid was pooled and clarified at 3000 rpm (1500xg) for 20 min at 4°C in Sorvall GSA rotor. The virus in the clarified supernatent was pelletted at 33,000 rpm (1,21,000xg) for 90 min at 4°C in A-641 fixed angle rotor (OTD75B Sorvall ultracentrifuge). The resultant virus pellet was

suspended in 0.5 ml TNE buffer, pH 7.5 [0.01 M Tris HCl, pH 7.5, 0.15M NaCl, 0.001 M EDTA, pH 8.0] per tube and kept overnight at 4°C.

The resultant virus suspension was layered onto a 3.0 ml cushion of 40% sucrose solution in TNE buffer, pH 7.5 and centrifuged at 31,000 rpm (1,60,000xg) for 75 min at 4°C in RPS-40T swinging bucket rotor (SCP70H Hitachi ultracentifuge). The crude virus pellet was resuspended in 0.5ml TNE buffer, pH 8.0 [0.1M Tris HCl (pH 8.0), 0.15M NaCl, 0.001M EDTA (pH 8.0)] and kept overnight at 4°C. The resuspended crude-virus suspension was incubated at 37°C for 60 min with DNase at a final concentration of 50 µg/ml and layered onto a 10-50% linear potassium tartrate gradient in TNE buffer (pH 8.0). This density gradient centrifugation was performed at 28,000 rpm (1,00,000xg) in RPS-40T swinging bucket rotor (SCP70H Hitachi ultracentrifuge) for 10 h at 4°C. The virus band was aspirated with a Pasteur pipette, diluted approximately 4-fold with TNE buffer, pH 8.0, and centrifuged in RPS-40T rotor at 28,000 rpm (1,00,000xg) for 2 hours. The concentrated and purified virus pellet was finally resuspended in 0.5 ml of TNE buffer, pH 8.0 [0.05M Tris HCl (pH 8.0), 0.1M NaCl, 0.001M EDTA (pH 8.0)] and kept overnight at 4°C.

ISOLATION OF BHV-1 DNA

BHV-1 DNA was isolated from purified virus suspension according to method described by Owen and Field (1988). The purified virus suspension was incubated at 37°C for 30 min with RNase at a final concentration of 40 µg/ml and then incubated with Proteinase K at a final concentration of 250 µg/ml at 37°C for 15 min. The resultant virus suspension was treated with sodium dodecyl sulfate (SDS) to a final concentration of 0.5% at 37°C for 1 h. The virus DNA was extracted with phenol:chloroform:isoamylalcohol (25:24:1) twice, then once with chloroform and precipitated with 2.5 volumes of chilled ethanol in the presence of 0.3M sodium acetate (pH 5.2). Precipitated virus DNA was washed with 70% ethanol. The DNA pellet was dried and dissolved in TE buffer, pH 7.5 [0.01M Tris HCl (pH 7.5), 0.001M EDTA (pH 8.0)] and stored at 4°C. The absorbance of DNA sample was recorded in

Gilford UV-VIS Spectrophotometer at 260 nm and 280 nm and DNA concentration was estimated.

RESTRICTION ENZYME DIGESTION OF BHV-1 DNA

The digestion of viral DNA with restriction endonucleases was performed in the manufacturer's recommended buffer and assay conditions. With all restriction enzymes, used in the work, the digestion was performed overnight at 37°C in Julabo F20/LKB2219 multitempII thermastatic circulator water bath. In all cases the restriction enzyme digestion was stopped either by addition of loading buffer or stored at -20°C till it was used.

AGAROSE GEL ELECTROPHORESIS

All digested DNA, including digested alpha [^{32}P] dATP end labelled samples were analysed using standard procedures of agarose gel electrophoresis (Sambrook et al., 1989).

The agarose was boiled in microwave oven (Deltawave, Toshiba, U.K.) for 1-2 min and cast on gel trays. The submarine horizontal slab gels of various concentrations (0.5%, 0.7% or 0.8% agarose, w/v) and various dimensions (7x10cm, 15x20 cm, 13.5x25 cm) were run with Atto Digi-Power or LKB 2301 Macrodrive 1 power supplies. Tris-borate EDTA or tris-acetate EDTA running buffers were used for running the slab gels for 2 to 24 h at 60 to 100 V depending upon the agarose concentration, gel size and size and number of DNA fragments generated by restriction enzymes. The gel was monitored with Chromato VUE Lamp (Model UVM-57, San Gabriel, USA) while running. After agarose gel electrophoresis, DNA bands were visualized and photographed in a UV-transilluminator (Fotodyne Inc., U.S.A.) using Polaroid MP-4 Camera and Polaroid Black and White films, type 57, 667 and 665 (Polaroid Corp., U.S.A.).

ALPHA [^{32}P] dATP END LABELLING OF RESTRICTION DIGESTS

End labelling of digested fragments using alpha [^{32}P] dATP was carried out immediately following restriction enzyme digestion. Each digested sample , having approximately 1 μg DNA in 10 μl reaction volume was diluted to 25 μl with 1X restriction enzyme buffer. To each 25 μl diluted sample , 2 μCi alpha [^{32}P] dATP was added and pulse spun down to collect radioactive material at bottom. One microliter of 2mM unlabelled deoxyribonucleoside triphosphates (d NTPs) dCTP, dGTP, dTTP in TE buffer [3mM Tris Cl pH 7.5, 1mM EDTA], was added to only BamHI, BglII and EcoRI restriction digested samples. The reaction mixture was kept for 20 min at room temperature after adding 1 unit of Klenow fragment of DNA polymerase I.

End labelled restriction enzyme digested fragments were separated on a 0.7% agarose gel run at 60V for 20-24 h using tris-borate EDTA running buffer system. After running, the agarose gels were dried on a slab gel drier (Biotec-Fischer) and exposed to Kodak X-OMAT S, X-ray film in Cronex (DU Pont) casettes (20x25 cm) with intensifying screen lightning plus on both sides. The casettes were kept at -20°C for 4 days or -70°C for 2 days. Autoradiograms were scanned in LKB Ultrascan XL Laser densitometer attached with LKB Laboratory Computer.

PREPARATION OF pUC9 PLASMID DNA AS CLONING VECTOR

Preparation of pUC9 plasmid DNA as cloning vector was done following the method described by Sambrook et al. (1989). *E. coli* strain containing pUC9 plasmid was taken from glycerol culture kept at -20°C. After thawing at room temperature, the culture was streaked on LB/Amp plates (60 μg/ml ampicillin) and incubated at 37°C overnight to get individual colonies. A single colony from the plate was picked up and incubated in LB/Amp broth overnight at 37°C in Gallen Kamp cooled orbital incubator. The plasmid DNA was isolated by alkali lysis method.

The bacterial pellet, obtained after centrifugation at 12,000 rpm for 1 min at 4°C in Tomy high speed micro refrigerated centrifuge MTX-150, was resuspended in 100 μl of ice-cold lysis buffer [50mM glucose, 20 mM Tris.Cl, pH 8.0, 10 mM EDTA] containing lysozyme (4 mg/ml). Freshly prepared 200 μl of alkaline SDS (0.2N NaOH, 1% SDS) was added and kept in ice for 5 min. Then 150 μl of ice cold 3.0 M sodium acetate, pH 4.8 was added and again kept in ice for 5 min. It was centrifuged at 12,000 rpm for 5 min at 4°C and the supernatent was extracted twice with phenol: Chloroform (1:1), once with Chloroform: isoamyl alcohol (24:1) and precipitated with 2 volumes of ethanol at room temperature. The DNA pellet, obtained by centrifugation at 12,000 rpm for 5 min at 4°C was washed once with 70% ethanol and resuspended in 50 μl TE buffer [10 mM Tris-Cl, pH 8.0, 1mM EDTA] containing RNase (20 μg/ml).

Closed circular plasmid DNA (10 μg) was incubated overnight with PstI restriction endonuclease in water bath at 37°C. The completely digested DNA sample was extracted with phenol:chloroform and precipitated with 2 volumes of ethanol for 15 min at 0°C. Recovered DNA pellet after centrifugation was resuspended in 10 mM Tris.Cl, pH 8.3. The linearized pUC9 PstI digested DNA was dephosphorylated with 10 μl calf intestinal alkaline phosphatase (CIP) and incubated at 37°C for 15 min. Then another aliquot of CIP was added and continued incubation for a further 45 min at 55°C. At the end of the incubation period, 0.5% SDS, 5 mM EDTA, pH 8.0 and proteinase K at a final concentration of 100 μg/ml were added and the tube was incubated for 30 min at 56°C. After cooling the tube to room temperature, the dephosphorylated linearized DNA was extracted once with phenol:chloroform and precipitated with 2 volumes of ethanol at 0°C in presence of 0.1 volume of 3M sodium acetate pH 7.0. The DNA, recovered by centrifugation at 12,000 rpm for 10 min at 4°C was washed with 70% ethanol and redisolved in 100 μl TE, pH 7.6. The concentration of DNA was estimated on the basis of absorbance at 260 nm using Gilford UV-Vis-Spectrophotometer.

CONSTRUCTION OF RECOMBINANT pUC9 PLASMID

Recombinant pUC9 plasmids were constructed through ligation of cohesive termini of PstI digested pUC9 plasmid DNA and viral DNA following the procedure described by Sambrook et al. (1989). The PstI digested fragments of viral DNA were extracted twice with phenol:chloroform, once with chloroform, and precipitated with 2 volumes of ethanol. After washing with 70% ethanol, DNA pellet was redissolved in TE, pH 7.6. To a fresh microfuge tube, 50 μg (2 μl) of dephosphorylated pUC9 DNA was added alongwith 8 μl PstI digested BHV-1 DNA (to get vector:insert ratio 1:5) 10 mM ATP, 1 unit bacteriophage T4 DNA ligase and ligase buffer. This ligation mixture was incubated at 16°C for 10 h alongwith ligation control for the plasmid vector. After incubation, the ligated mixture was kept at -20°C till transformation was done in E. coli TG-1 competent cells

TRANSFORMATION OF E. COLI TG-1 WITH RECOMBINANT PLASMIDS

Fresh competent cells preparation and transformation was performed using protocol described by Sambrook et al. (1989). E. coli TG-1 strain was taken from glycerol culture kept at -20°C and streaked on LB agar plates without antibiotics just after thawing to room temperature. Single colony was obtained after keeping the plates at 37°C overnight. A single colony, 2-3 mm in diameter was transferred from plate to 100 ml LB broth and incubated for 2 to 3 h at 37°C with vigorous shaking (300 cycles/min) in Gallenkamp Cooled orbital incubator. When OD reached approximately 0.6, TG-1 cells were pelleted at 5000 rpm (3000xg) for 10 min at 4°C in SS-34 fixed angle rotor in a Sorvall RC5B high speed centrifuge. The supernatant was discarded and the cells were kept in ice for 10 min after suspending in 10 ml freshly prepared ice cold 0.1M $CaCl_2$ solution. The cells were again centrifuged and resuspended in 2ml ice cold 0.1M $CaCl_2$. The aliquots of 200 μl of these freshly prepared competent TG-1 cells were dispensed in sterile microfuge tubes and kept for 12-24 h at 4°C.

Recombinant pUC9 plasmid was added in one microfuge tube containing competent TG-1 cells alongwith controls which consisted of circular pUC9 plasmid, dephosphorylated pUC9 DNA in separate microfuge tubes containing competent TG-1 cells and competent cells that received no plasmid DNA. All these tubes were kept on ice for 30 min and then heat shocked at 42°C for 90 sec without shaking. Again, tubes were kept on ice bath for 1-2 min and then 800 μl SOC medium was added. The tubes were incubated at 37°C for 45 min and 100 μl or 200 μl volume of samples from each tube were plated on SOB agar plates containing ampicillin (60 μg/ml), X-gal (25 μg/ml) and IPTG (25 μg/ml). All the plates were incubated at 37°C for 12-16 h.

ISOLATION AND SCREENING OF RECOMBINANT PLASMIDS

The recombinant plasmids in the bacterial colonies were identified by alpha-complementation and restriction analysis of miniprepared plasmid DNA (Sambrook et al., 1989). All white (recombinant) colonies were transferred from SOB plates to LB/Amp broth and incubated at 37°C overnight. Minipreparation of plasmid DNA from this culture was made. The plasmid DNAs were then digested with PstI restriction endonuclease at 37°C overnight and run on 0.7% agarose gel alongwith lambda molecular weight marker and PstI digested viral DNA to identify the insert fragment.

SELECTIVE CLONING OF BHV-1 DNA FRAGMENT

The viral PstI digested DNA fragments were ligated with PstI digested dephosphorylated pUC9 plasmid DNA (Pst I digested) in the melted slabs of agarose following the method described by Sambrook et al. (1989).

The PstI digested BHV-1 DNA were separated on 0.7% low-melting-temperature agarose gel containing 0.5 μg/ml ethidium bromide, run in 1x TAE buffer system for about 20 h. A viral DNA band was cut, using surgical blade from the gel visualized in the long wave length UV

light, and collected in a sterile microfuge tube. This microfuge tube was heated to 70°C for 10-15 min and an aliquot of 9 μl from this melted agarose was added in a 37°C prewarmed microfuge tube containing 1 μl of dephosphorylated pUC9 DNA. After incubation for 5-10 min at 37°C, 10 μl of ice-cold 2X bacteriophage T4 DNA ligase mixture was added to this 10 μl reaction mixture (plasmid DNA and insert DNA) and incubated again for 12-16 h at 16°C for ligation in LKB multitemp thermostatic circulator water-bath. After ligation, transformation of fresh competent _E. coli_ TG-1 cells with recombinant plasmids was done as per the method of Sambrook _et al_. (1989).

RESTRICTION ENZYME ANALYSIS OF RECOMBINANT PLASMID DNA

The recombinant plasmid DNAs were analysed with restriction endonucleases using single or multiple digestion methods. The restriction endonucleases PstI, EcoRI, BamHI, SmaI, SalI, HindIII, BglI, BglII, HaeIII, XhoI, RsaI and StuI were used for this study. The digested plasmid DNAs were separated on 0.8% agarose gels using tris-borate EDTA running buffer system (Sambrook _et al_., 1989).

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RESULTS

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RESULTS

GROWTH OF BHV-1 IN MDBK CELL CULTURE

The BHV-1 induced cytopathic effect (CPE) was manifested by granulation and rounding of cells within 24 h postinfection. After 24 h and upto 48 h postinfection ballooning of the cells with small refractile syncytia followed by formation of grapelike clusters or aggregation of infected cells was observed. In later stage, after 48 h postinfection, strands of cytoplasm linking affected cells and detached cells floating in the medium were observed. On further incubation, the infected cells showed marked degeneration with large number of cells detached from the monolayer (Fig. 3 to 5). The fifth passaged BHV-1 - in MDBK was titrated on the preformed monolayer in 24 well plates and the titre was $10^{6.7}$ $TCID_{50}$/ml.

The MDBK cell culture grown virus, when pelleted through sucrose solution and purified on a linear 10% to 50% potassium tartrate gradient, yielded a clear purified virus band.

RESTRICTION ENZYME ANALYSIS OF BHV-1 DNA

The DNA obtained from density gradient purified BHV-1 was found to have the concentration of 0.31 µg/ml on the basis of absorbance at 260 nm. The absorbance ratio of 260 nm/280 nm (OD_{260}/OD_{280}) was 1.80, which showed the purity of isolated BHV-1 DNA.

The BHV-1 DNA was analysed using restriction endonucleases HindIII, EcoRI, BamHI, SalI, BglI, PstI, HpaI, BglII, KpnI, RsaI, XhoI, DraI and SmaI. The DNA was cleaved with restriction endonucleases following the manufacturer's recommended buffers and assay conditions and the resultant DNA fragments were separated on 0.5% or 0.7% agarose gel. Molecular sizes of restriction digested fragments were estimated by comparison with electrophoretic mobilities of HindIII digested lambda DNA fragments of known

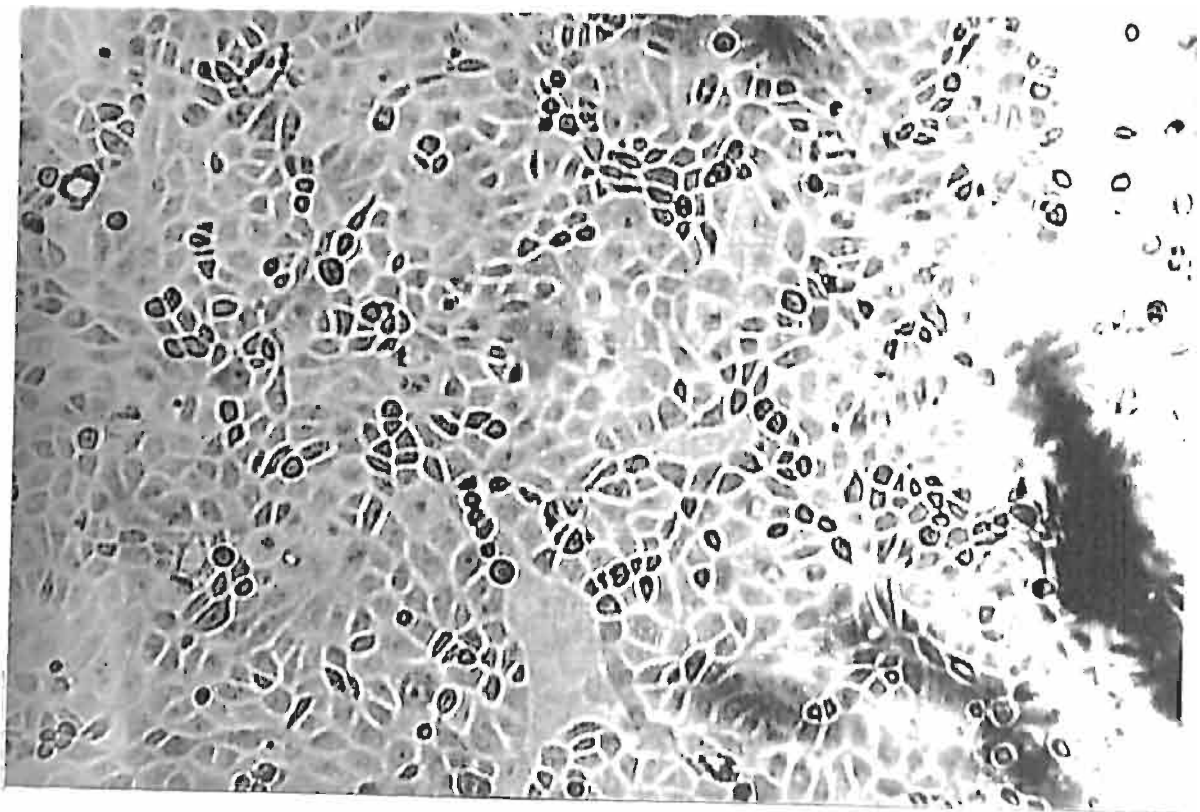


Fig.3 Healthy MDBK cell culture monolayer, 48 h of growth. Unstained living culture, x75.

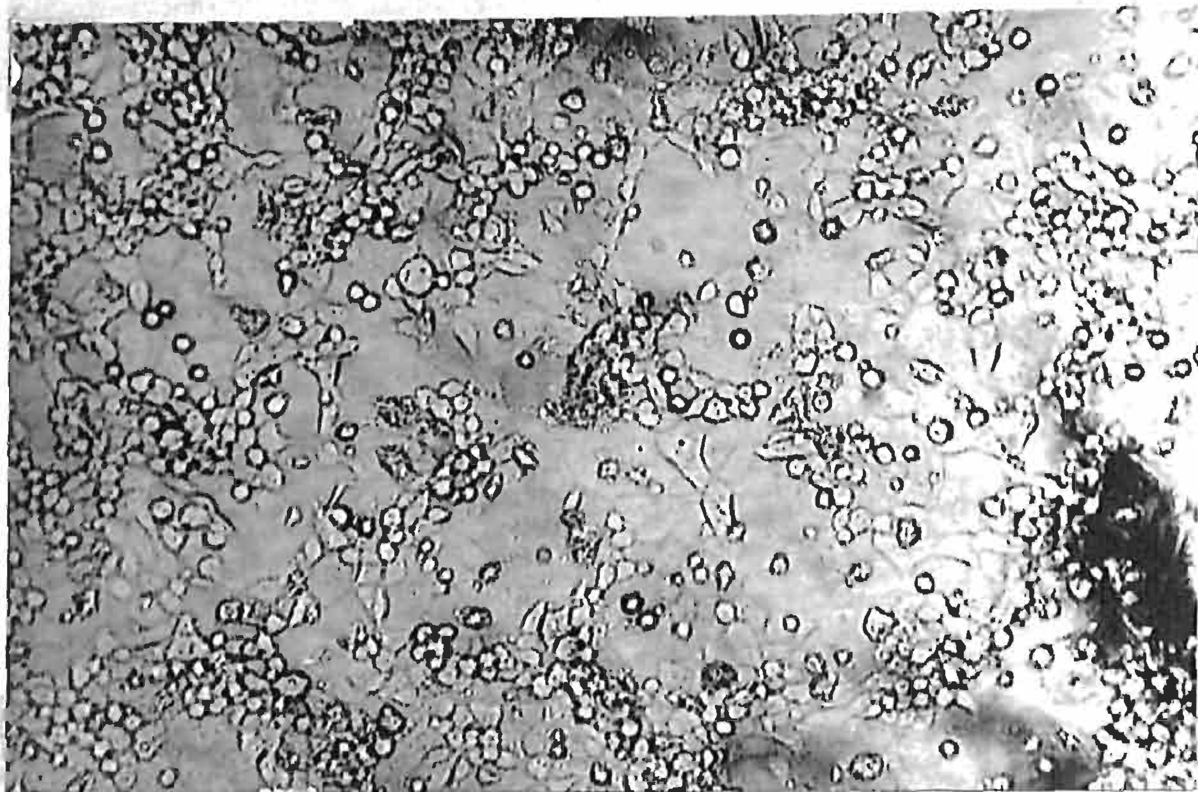


Fig. 4

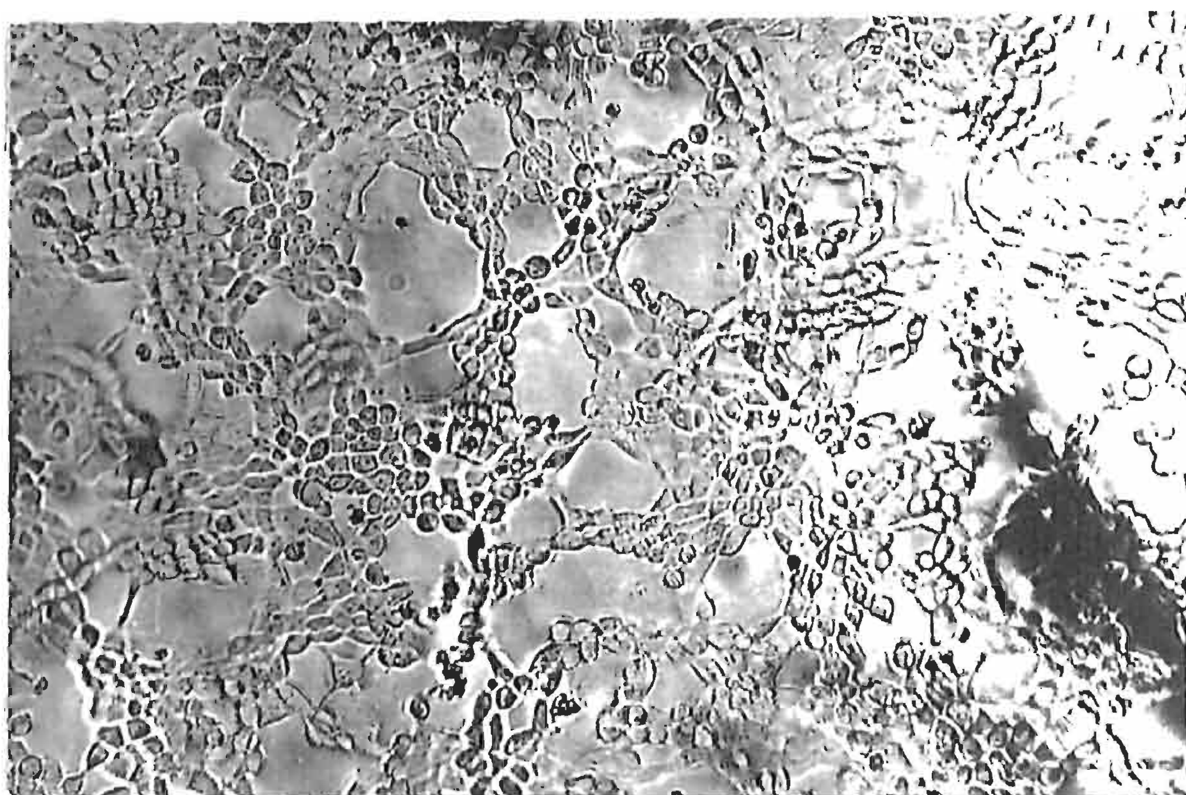


Fig. 5

Fig.4 BHV-1 infected MDBK cell culture, 24 h postinfection. Unstained living culture, x75.

Fig.5 BHV-1 infected MDBK cell culture, 48 h postinfection. Unstained living culture, x75.

molecular sizes. Restriction fragments with increased fluorescence on gels with ethidium bromide or increased intensity with alpha [^{32}P] dATP end labelling on autoradiograms were considered to be present in more than equimolar ratio while those with decreased fluorescence on gel or decreased intensity on autoradiogram were considered to be of half molar ratio. All the restriction fragments were designated alphabetically with decreasing molecular sizes in terms of kilobase pairs.

HindIII

The HindIII digested BHV-1 DNA fragments were separated on 0.7% agarose gel using ethidium bromide staining. On 0.5% agarose gel, the fragments were well separated and half molar fragments were identified on the basis of decreased intensity of the fluorescence. The maximum number of fragments, that could be identified on 0.5% agarose gel were 15. The genomic size was estimated to be 138.8 kb or 138.6 kb (Table 2, Fig.6, 7).

EcoRI

The EcoRI restriction enzyme digested BHV-1 DNA fragments and end labelled digested fragments with [^{32}P] dATP were separated on 0.7% agarose gel. On 0.5% agarose gel, the higher molecular size fragments could be better separated. On the basis of restriction profile on agarose gel stained with ethidium bromide and end labelled fragments with [^{32}P] dATP exposed to Kodak X-ray film, the maximum number of fragments was 7 and molecular size was found to be 137.0 kb. Four submolar fragments, F_1 (8.0kb), F_2 (4.7kb), F_3 (4.1kb) and G1(1.4kb), were observed on autoradiogram, but these fragments could not be visualized on agarose gel stained with ethidium bromide. However, their fragment sizes were not included in total genome size of BHV-1 DNA (Table 2, Fig. 6,7,14).

BamHI

The BHV-1 DNA fragments digested with BamHI was analysed on 0.7% and 0.5% agarose gel using ethidium bromide staining

and on 0.7% agarose gel using end labelling with alpha [^{32}P] dATP. The fragments A to I were not separated clearly on 0.7% agarose gel due to their large genome sizes and large numbers but the same could be clearly separated on 0.5% agarose gel run for about 36 h in TBE running buffer system. The total number of restriction fragments was 11. Three sub-molar fragments I_1 (3.4kb), I_2 (2.7kb), I_3 (1.4kb) were identified on autoradiogram only (Table 3, Fig.6,8,14).

SalI

The restriction endonuclease analysis of BHV-1 DNA using SalI produced a large number of fragments below 18.5 kb. There were few fragments below 0.4 kb that could not be visualized using ethidium bromide staining and end labelling with alpha [^{32}P] dATP. The total number of 26 fragments could be identified using alpha [^{32}P] dATP end labelling and scanning results (Table 3, Fig.6,8,13,15,16).

BglII

The restriction fragments, on digestion of BHV-1 DNA with BglII, were separated on 0.7% agarose gel. The analysis revealed 11 fragments of different sizes ranging from 32.0 kb to 1.5kb. The fragments B and C did not separate on 0.7% agarose gel but could be identified on the basis of increased fluorescence on ethidium bromide stained gel (Table 4, Fig.6,9).

KpnI

The digestion of BHV-1 DNA with KpnI generated 14 fragments. The restriction fragments C and D did not separate on 0.7% agarose gel. The presence of four half molar fragments could be identified on the basis of reduced fluorescence with ethidium bromide in the gel. The genome size of BHV-1 DNA was calculated to be 137.8 kb excluding 12.3 kb G and K half molar fragments or 138.2 kb excluding 11.9 kb H and K half molar fragments (Table 4, Fig.6,9).

XhoI

The restriction enzyme digestion of BHV-1 DNA with XhoI generated numerous fragments. The small fragments below approximately 1.0 kb size made a smear. Only 26 fragments could be identified on 0.7% agarose gel stained with ethidium bromide (Fig.6,10).

RsaI

The BHV-1 DNA yielded large number of numerous small fragments on digestion with RsaI restriction endonuclease. The numerous small fragments sized below 1.0 kb made a smear on 0.7% agarose gel stained with ethidium bromide (Fig.6,10).

DraI

The BHV-1 DNA digested with DraI yielded 11 fragments as analysed on 0.7% agarose gel. The sum of individual fragment size accounted for a total genome size of 138.2 kb. The fragments B, C and E, F could not be separated on 0.7% agarose gel (Table 5, Fig.6,10).

SmaI

The SmaI digested BHV-1 DNA fragments, separated on 0.7% agarose gel, revealed 26 fragments. The numerous small fragments below 0.5 kb sizes made a smear (Fig.6,10,13,17).

PstI

The cleavage products of PstI digested BHV-1 DNA were analysed on 0.7% agarose gel in TBE running buffer system using ethidium bromide staining. The individual fragments, that could not be distinguished on ethidium bromide stained gel, were delineated by scanning the negative of Polaroid 665 film. The alpha [^{32}P] dATP end

labelling yielded distinguishable fragments of PstI digested BHV-1 DNA. The restriction enzyme PstI had yeilded more than 48 fragments of varying sizes. The fragments below 0.40 kb size could not be distinguished easily. The BHV-1 DNA size was estimated to be of 135.05 kb (Table 6, Fig.6,11,12,15).

BglI

The BHV-1 DNA digested with BglI showed only one large 25.3 kb fragment and rest of the fragments were below 3.0 kb as revealed on 0.7% agarose gel (Fig. 6).

HpaI

The restriction endonuclease digestion of BHV-1 DNA with HpaI was partial. So no restriction profile could be obtained (Fig.6, 13).

CLONING OF PstI FRAGMENTS OF BHV-1 DNA

For shotgun cloning, the plasmid pUC9 was digested with PstI and then treated with calf intestinal alkaline phosphatase. The concentration of dephosphorylated pUC9 linear plasmid was estimated to be 25 ng/μl after extraction with phenol: chloroform. Two microliter of dephosphorylated plasmid was ligated with 8 μl PstI digested BHV-1 DNA in 1:5 ratio and the ligated mixture was used to transform freshly prepared competent E. coli TG-1 cells. The recombinant plasmid containing bacterial colonies were amplified and minipreparation was done to isolate recombinant plasmids. The inserts of BHV-1 DNA were identified on restriction enzyme digestion of recombinant plasmids with PstI and running on 0.7% agarose in tris-borate EDTA running buffer system alongwith lambda DNA cut with HindIII marker and BHV-1 DNA digested with PstI. There were many recombinant plasmids containing the same insert. Few colonies (P1, P6, P11, P26) did not contain any insert while two colonies P42 and P31D contained recombinant plasmid with two inserts. One recombinant plasmid in lane 33 (Fig.19) showed partial digestion (Table 7, Fig.18,19,20,21).

For selective cloning, the BHV-1 DNA digested with PstI was separated on 0.7% low melting temperature (LMT) agarose gel run in TAE running buffer system. The fragment K was cut from the gel and collected in a microfuge tube. An aliquot of 9 μl of melted agarose with fragment K was ligated with 25 ng PstI digested dephosphorylated pUC9 plasmid. This ligated plasmid (recombinant plasmid) was used to transform freshly prepared E. coli TG-1 competent cells. The recombinant plasmid containing bacterial colonies were amplified and recombinant plasmid DNA was isolated. The isolated recombinant plasmids were digested with PstI and run on 0.7% agarose gel along with lambda DNA HindIII digested marker and pUC9 DNA linearized with PstI digestion(Fig.22). The insert was identified on the basis of migration of DNA fragments in agarose gel.

RESTRICTION ENZYME ANALYSIS OF RECOMBINANT PLASMID DNA

The recombinant plasmids pUC9P18(G), pUC9P20(S) and pUC9P49(K) containing BHV-1 DNA inserts were analysed using single and multiple digestion with restriction endonucleases (Table 8).

The pUC9P18(G) recombinant plasmid, having insert G-5.9kb fragment of PstI digested BHV-1 DNA, yielded complete insert when double digested with any of the restriction endonuclease, EcoRI, BamHI, BglII and XhoI after initial digestion with PstI. This showed no restriction site on the insert itself for these restriction endonucleases. The digestion with StuI and SalI separately after initial digestion with PstI, yielded two fragments of the insert showing one restriction site on the insert fragment for these endonucleases. More than 5 sites could be identified for BglI restriction enzyme. For mapping StuI and SalI restriction site on the insert, pUC9P18(G) recombinant plasmid was first digested with StuI to get linear 8.5 kb recombinant plasmid, then this linearized plasmid was again digested with SalI. This yielded three fragments - 6.7kb fragment (having total pUC9 plasmid and 4.1 kb insert), 0.8kb fragment and 1.0 kb fragment. Finally restriction sites for StuI and SalI were mapped on the recombinant plasmid (Fig.23,24).

The recombinant plasmid pUC9P20(S) yielded complete insert (S-2.35kb) on double digestion with HindIII, EcoRI, BamHI, BglII and StuI restriction enzymes after initial digestion with PstI. This showed no restriction site for these restriction enzymes on the insert. The restriction endonuclease RsaI and BglI showed 3 sites and 2 sites, respectively whereas restrcition endonucleases SmaI, XhoI and SalI showed one restriction site each on the insert fragment. The restriction sites for XhoI and SmaI were mapped on the insert fragment. For this, the recombinant plasmid was digested first with XhoI and SmaI separately. On XhoI digestion, the recombinant plasmid yielded linear 5.0 kb fragment while with SmaI it yielded 4.3 kb and 0.7 kb fragments showing only one site on the insert. Through double digestion with SmaI and XhoI these sites could be mapped on the recombinant plasmid (Fig.25,26).

The restriction endonuclease analysis of recombinant plasmid pUC9P49(K) revealed no restriction site for EcoRI, BamHI and BglII but 3 sites for SmaI and 5 sites for RsaI. There was only one restriction site on the insert fragment for each of the restriction enzymes XhoI, StuI and SalI. For BglI, more than 3 restriction sites could be identified on the insert. The restriction enzyme digestion of the recombinant plasmid with SalI, yielded two fragments because one site for SalI existed on the insert and the other on the MCS of pUC9 plasmid. To map restriction site for StuI and XhoI on the insert, the recombinant plasmid was first linearized with HindIII and BamHI separately and then again digested with StuI and XhoI separately. These yielded two fragments in each case. The double digestion of the recombinant plasmid with StuI and XhoI yielded 1.9 kb fragment and 4.5 kb fragment which contained 2.665 kb plasmid pUC9 DNA also. On the basis of these results, the restriction sites for StuI and XhoI could be mapped on the recombinant plasmid. Out of 3 sites of SmaI, only one site could be mapped on the recombinant plasmid since on digestion with SmaI the recombinant plasmid yielded 4 fragments of which the 3.5 kb fragment contained pUC9 plasmid as well as 0.9 kb fragment of the insert. Remaining 3 fragments were 0.8kb, 1.4 kb and 1.4 kb size (Fig.27,28).

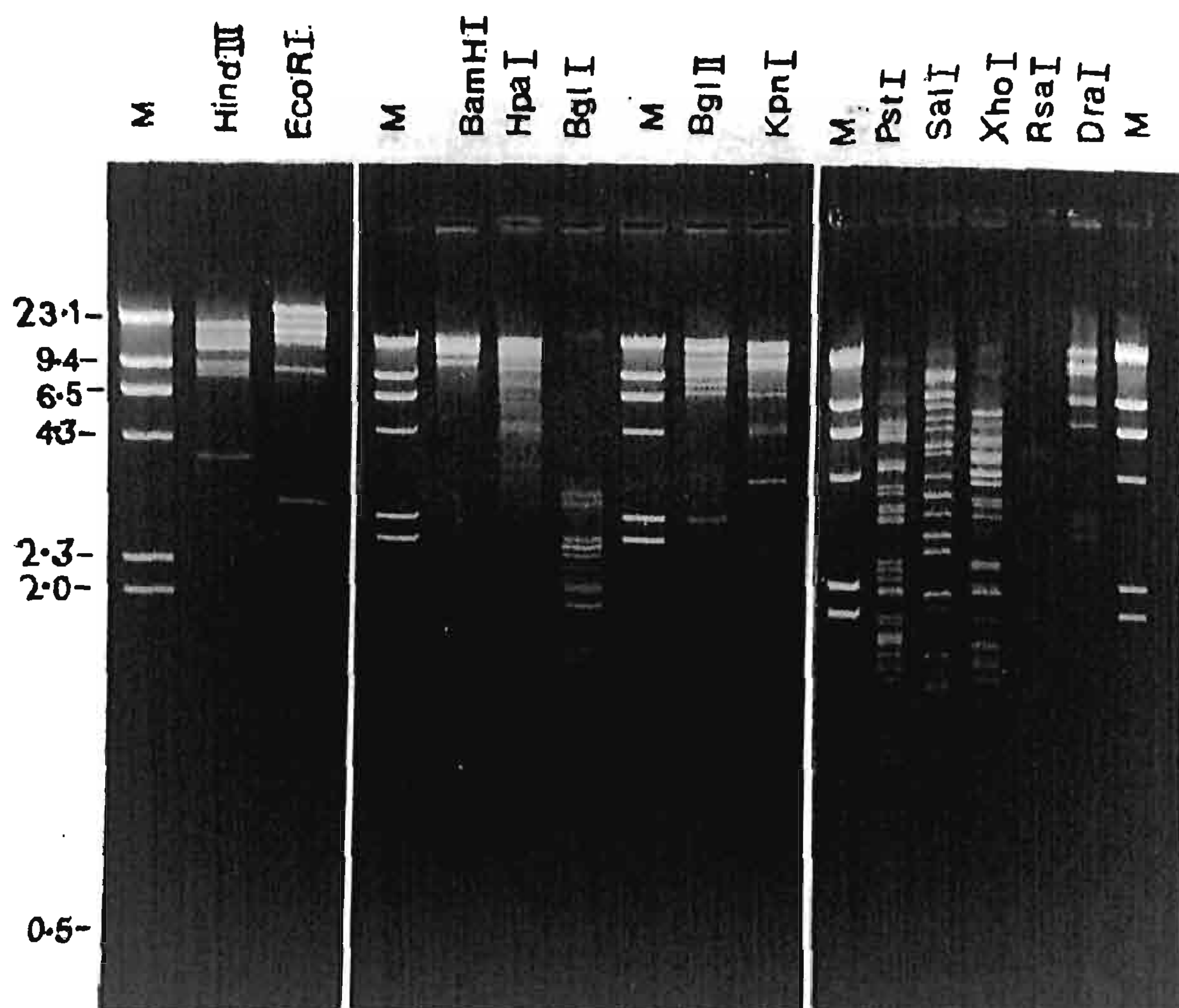


Fig.6 Restriction endonuclease profile of BHV-1 DNA on ethidium bromide stained 0.7% agarose gel.

M: HindIII cut lambda DNA marker (size in kb).

Table 2. Molecular size of HindIII and EcoRI digested BHV-1 DNA fragments*

Hind III		EcoRI	
Fragment	Size (kb)	Fragment	Size (kb)
A	21.2	A	52.5
B	20.5	B	23.5
C½	15.6	C	17.2
D½	14.7	D	17.2
E	13.2	E	14.7
F½	13.2	F	8.8
G	12.1	G	3.1
H½	12.1		
I	12.1		
J	9.0		
K	8.4		
L	7.6		
M	3.8		
N	2.7		
O	0.36		
Sum of fragment sizes	166.56		137
Total fragment number	15		7
Genome size excluding half molar fragments			
C, H	138.86		
D, F	138.66		

½ indicates half molar fragments

*The digested fragments were sized by comparison with standard HindIII cut lambda DNA marker on agarose gel.

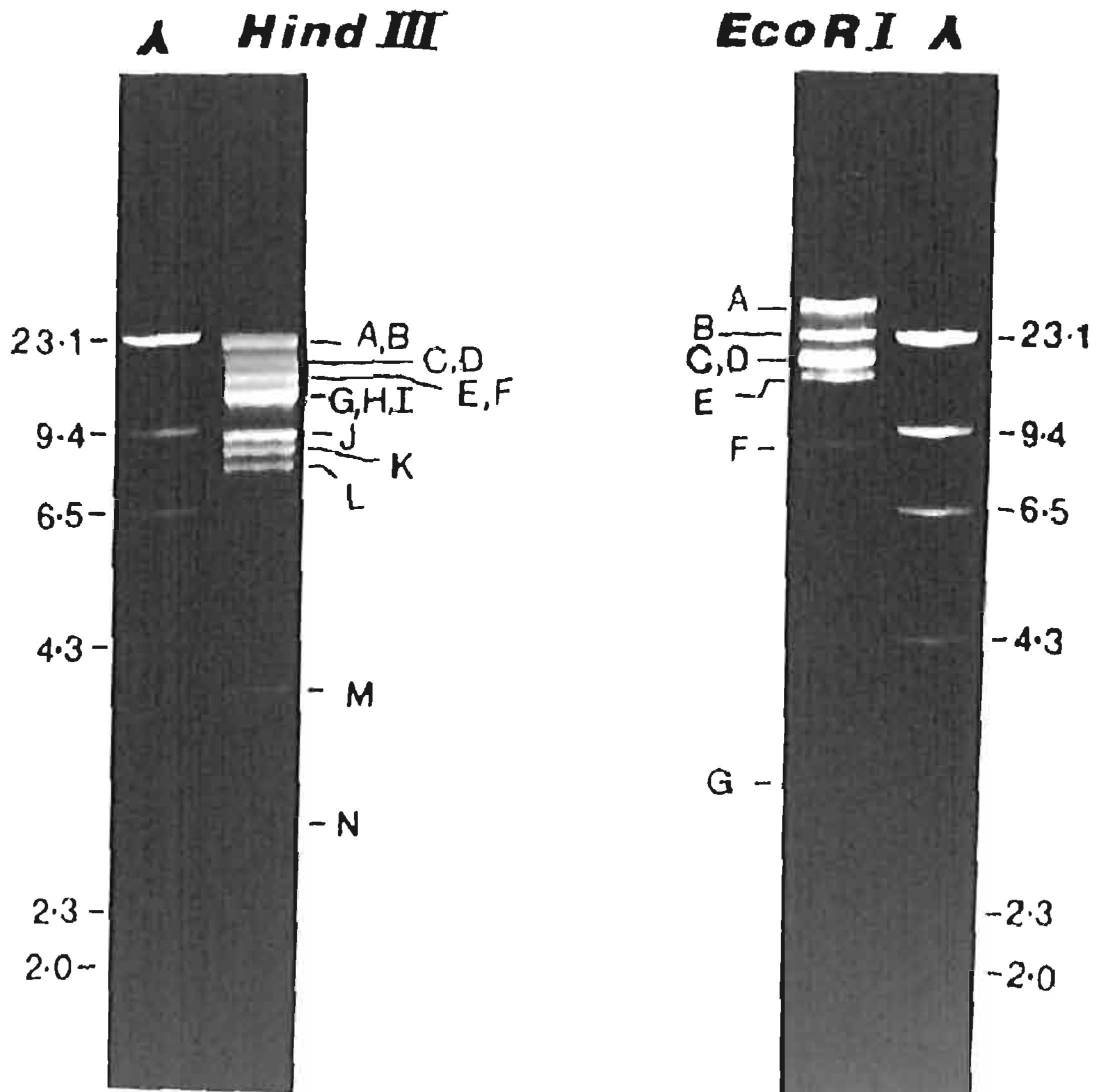


Fig.7 Restriction endonuclease profile of HindIII and EcoRI digested BHV-1 DNA on ethidium bromide stained 0.5% agarose gel.

λ : HindIII cut lambda DNA marker (size in kb).

Table 3. Molecular size of BamHI and SalI digested BHV-1 DNA fragments*

BamHI

Fragment	Size (kb)
$A^{\frac{1}{2}}$	30.20
B	30.20
$C^{\frac{1}{2}}$	21.70
$D^{\frac{1}{2}}$	21.70
E	17.50
F	16.80
G	15.70
H	12.50
$I^{\frac{1}{2}}$	12.50
J	0.88
K	0.25
Sum of fragment sizes	179.93
Total number of fragments	11
Genome size excluding half molar fragments	
A,I	137.23
C,D	136.53

SalI

Fragment	Size (kb)
A	18.50
B	18.50
C	17.00
D	11.00
E	9.60
F	8.00
G	7.00
H	6.00
I	5.40
J	4.30
K	3.90
L	3.50
M	3.10
N	2.90
O	2.20
P	2.10
Q	1.70
R	1.50
S	1.45
T	1.40
U	1.30
V	0.97
W	0.90
X	0.80
Y	0.70
Z	0.40
Small fragments closely located	approx. 1.0
Sum of fragment sizes	135.12

* The digested fragments were sized by comparison with standard HindIII cut lambda DNA marker on agarose gel.

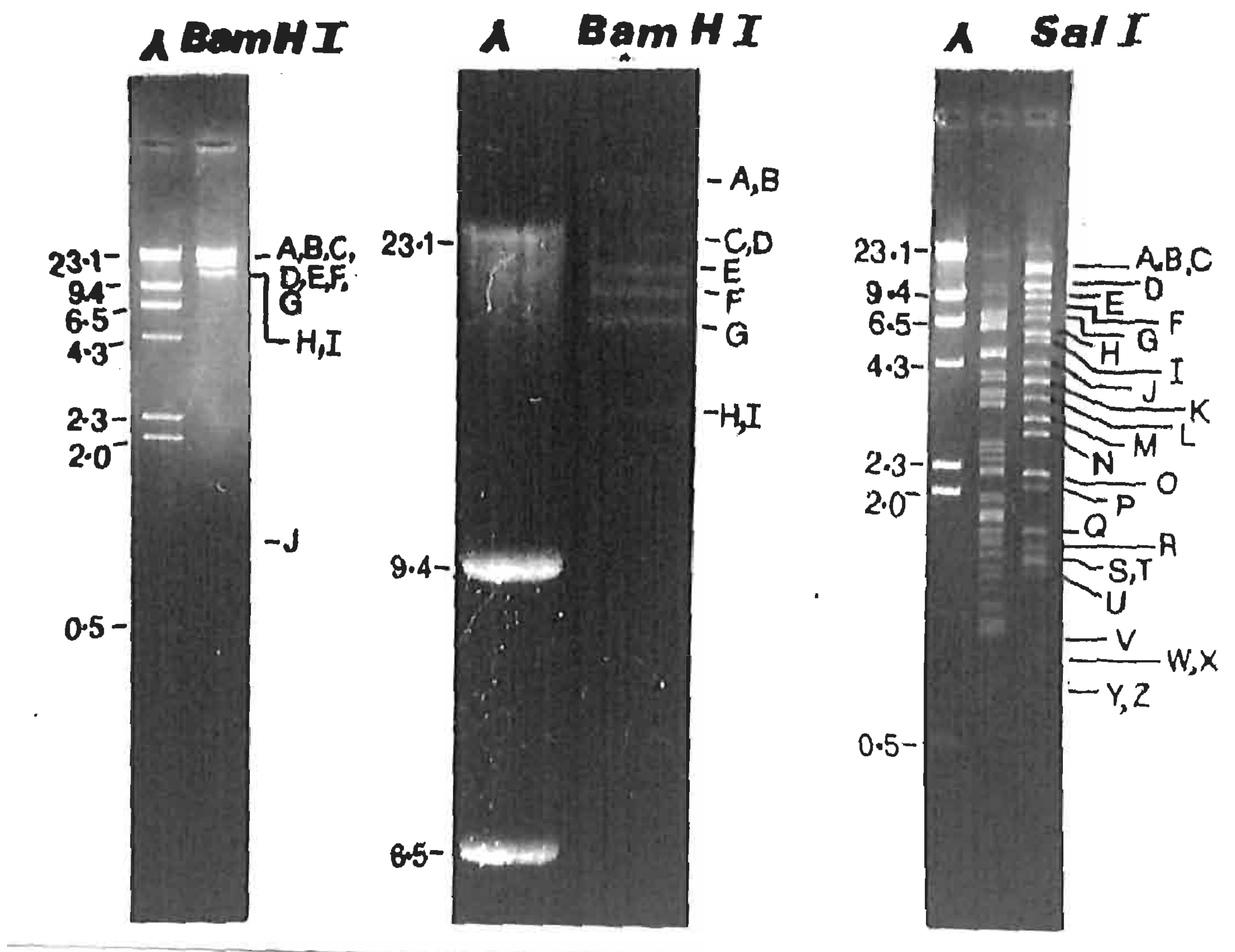


Fig.8 Restriction endonuclease profile of BamHI and SalI digested BHV-1 DNA on ethidium bromide stained agarose gel.

λ : HindIII cut lambda DNA marker (size in kb).

Table 4. Molecular size of BglII and KpnI digested BHV-1 DNA fragments*

BglII		KpnI	
Fragment	Size (kb)	Fragment	Size (kb)
A	32.0	A	28.0
B	23.0	B	23.1
C	23.0	C	17.2
D	19.0	D	17.2
E	10.4	E	15.0
F	8.4	F	12.5
G	8.0	G $^{\frac{1}{2}}$	8.0
H	6.8	H $^{\frac{1}{2}}$	7.0
I	2.3	I	6.8
J	1.8	J $^{\frac{1}{2}}$	4.9
K	1.5	K $^{\frac{1}{2}}$	4.3
		L	3.0
		M	1.9
		N	1.2
Sum of fragment sizes	136.2	Sum of fragment sizes	150.1
Total number of fragments	11	Total number of fragments	14
		Genome size excluding half molar fragments	
		G,K	137.8
		H,J	138.2

$\frac{1}{2}$ indicates half molar fragments

* The digested fragments were sized by comparison with standard HindIII cut lambda DNA marker on agarose gel.

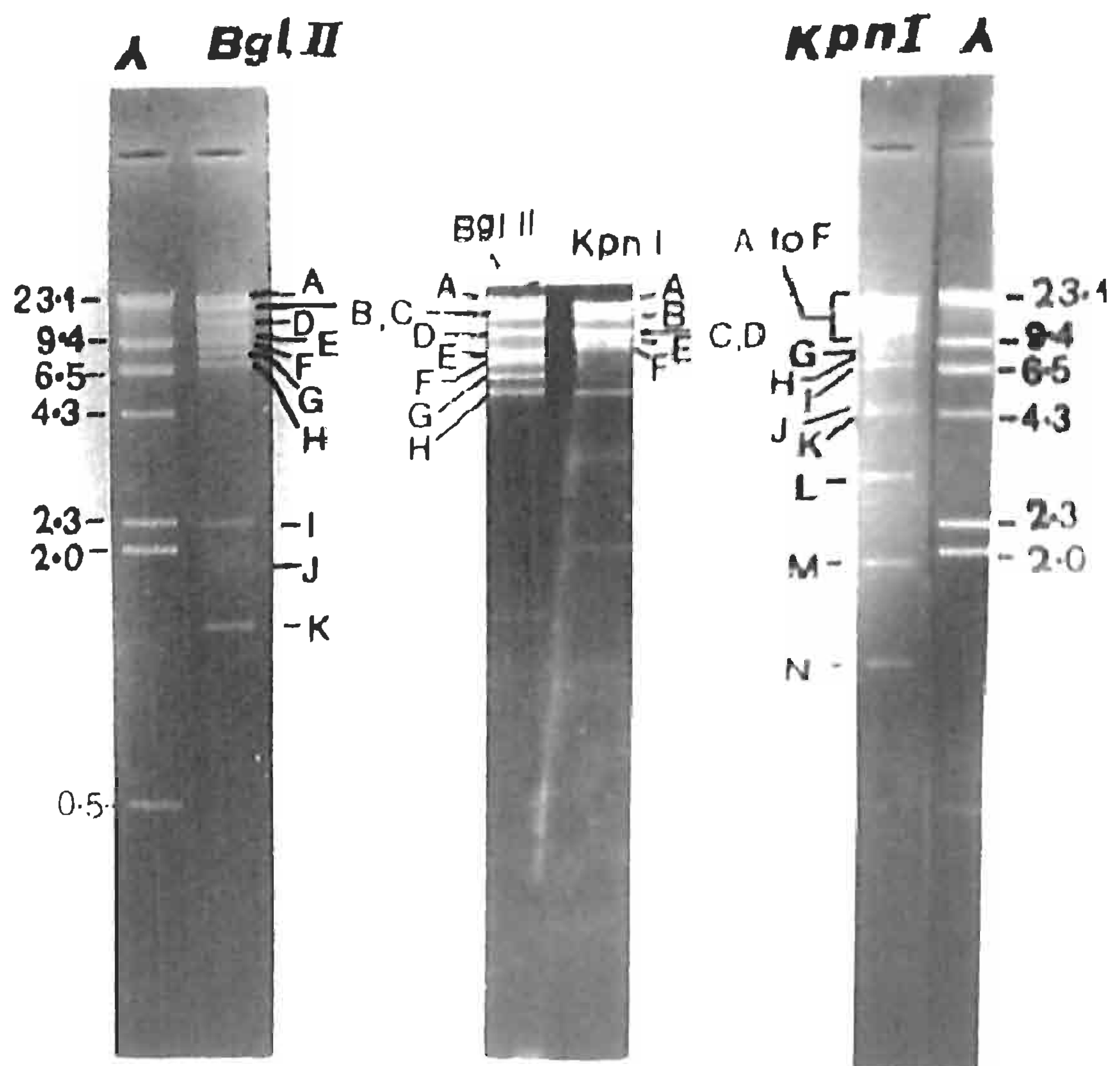


Fig.9 Restriction endonuclease profile of BglII and KpnI digested BHV-1 DNA on ethidium bromide stained 0.7% agarose gel.

λ : HindIII cut lambda DNA marker (size in kb).

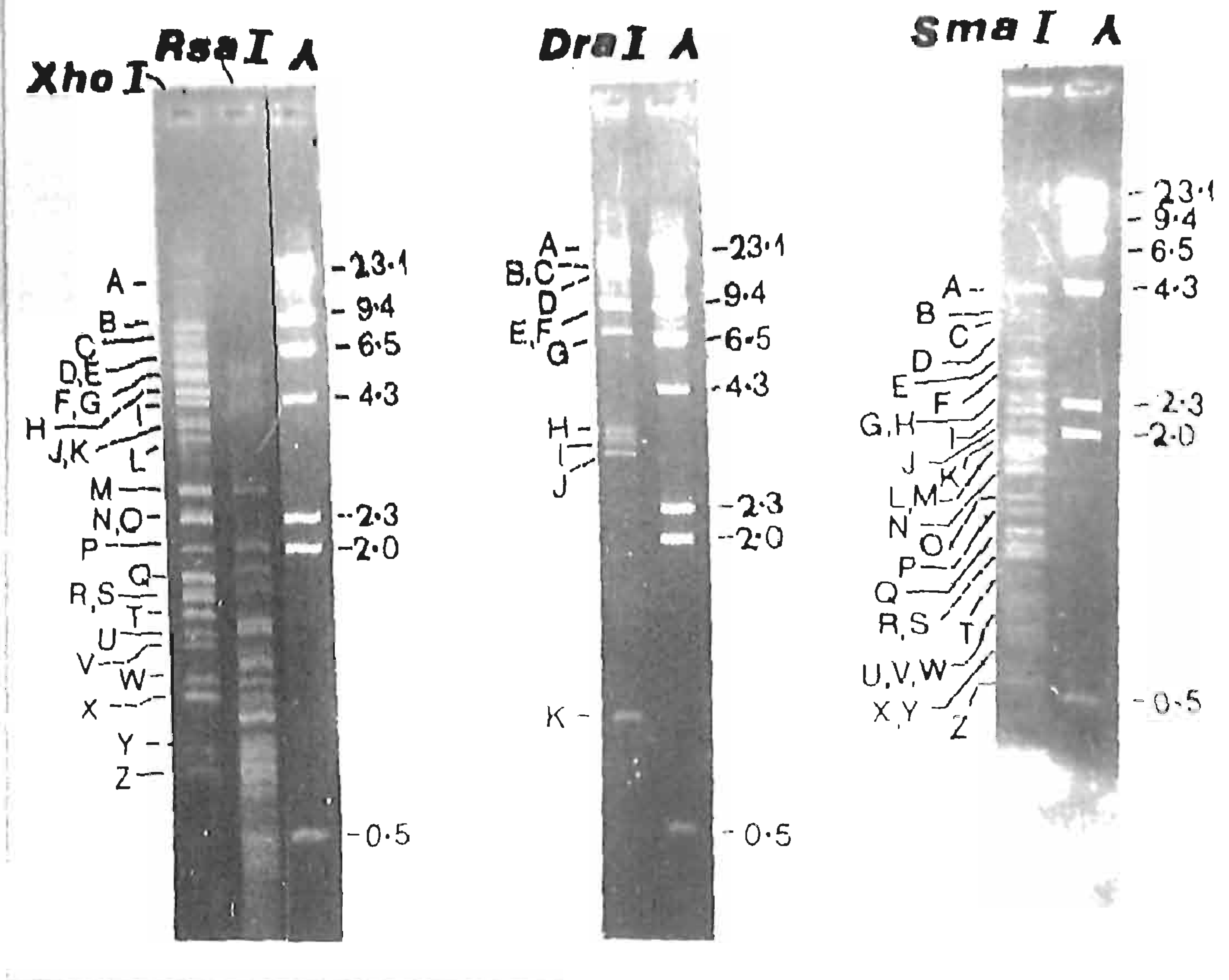


Fig.10 Restriction endonuclease profile of XhoI, RsaI, DraI and SmaI digested BHV-1 DNA on ethidium bromide stained 0.7% agarose gel.

λ: HindIII cut lambda DNA marker (size in kb).

Table 5. Molecular size of DraI digested BHV-1 DNA fragments*

Fragments	Size (kb)
A	39.1
B	20.5
C	20.5
D	18.5
E	10.2
F	10.2
G	7.4
H	3.8
I	3.6
J	3.4
K	1.0
Sum of fragment sizes	138.2
Total number of fragments	11

*The digested fragments were sized by comparison with standard HindIII cut lambda DNA marker on agarose gel.

Table 6. Molecular size of PstI fragments of BHV-1 DNA*

Fragment	Size (kb)	Fragment	Size (kb)
A	14.0	AF	1.25
B	12.0	AG	1.20
C	7.8	AH	1.10
D	7.0	AI	1.00
E	6.2	AJ	0.95
F	6.2	AK	0.90
G	5.9	AL	0.87
H	4.7	AM	0.70
I	4.5	AN	0.65
J	4.5	AO	0.60
K	3.8	AP	0.58
L	3.7	AQ	0.53
M	3.5	AR	0.50
N	3.4	AS	0.45
O	3.3	AT	0.40
P	3.2	AU	0.32
Q	2.5	AV	0.30
R	2.4	Very small fragments closely located	approx. 1.0
S	2.35		
T	2.20		
U	2.20		
V	1.90	Sum of fragment sizes	135.05
W	1.85		
X	1.80		
Y	1.80		
Z	1.70		
AA	1.60		
AB	1.55		
AC	1.50		
AD	1.40		
AE	1.30		

The digested fragments were sized by comparison with standard HindIII cut lambda DNA marker on agarose gel.

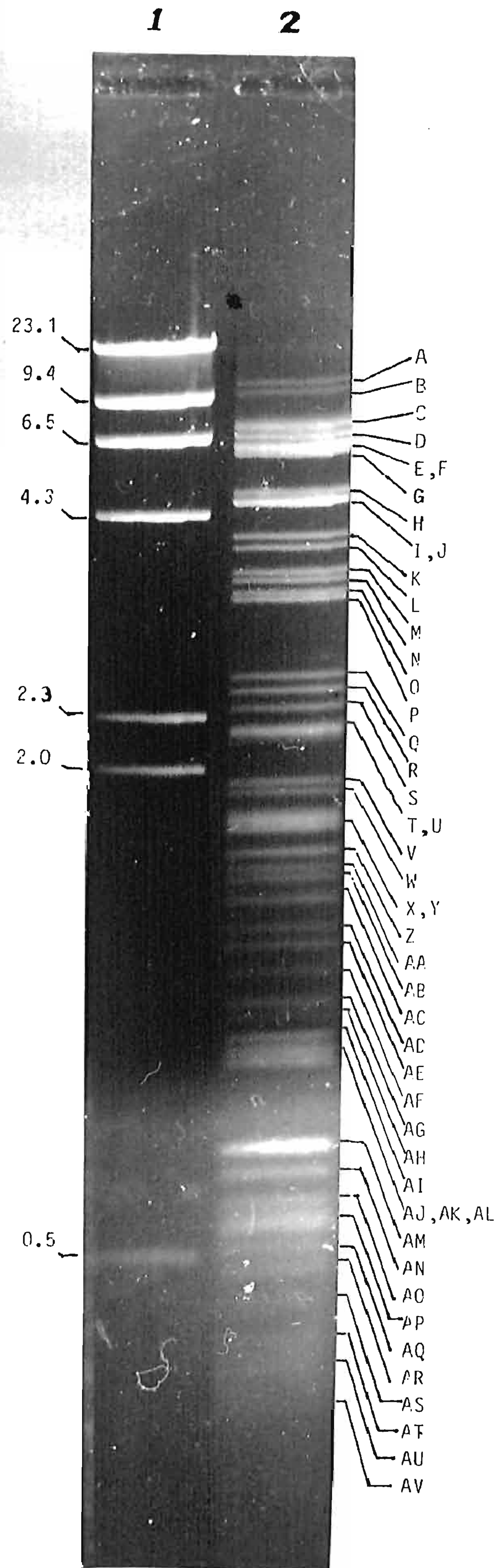


Fig.11 Restriction endonuclease profile of PstI digested BHV-1 DNA on ethidium bromide stained 0.7% agarose gel. Lane 1: HindIII cut lambda DNA marker (size in kb), Lane 2: PstI cut BHV-1 DNA.

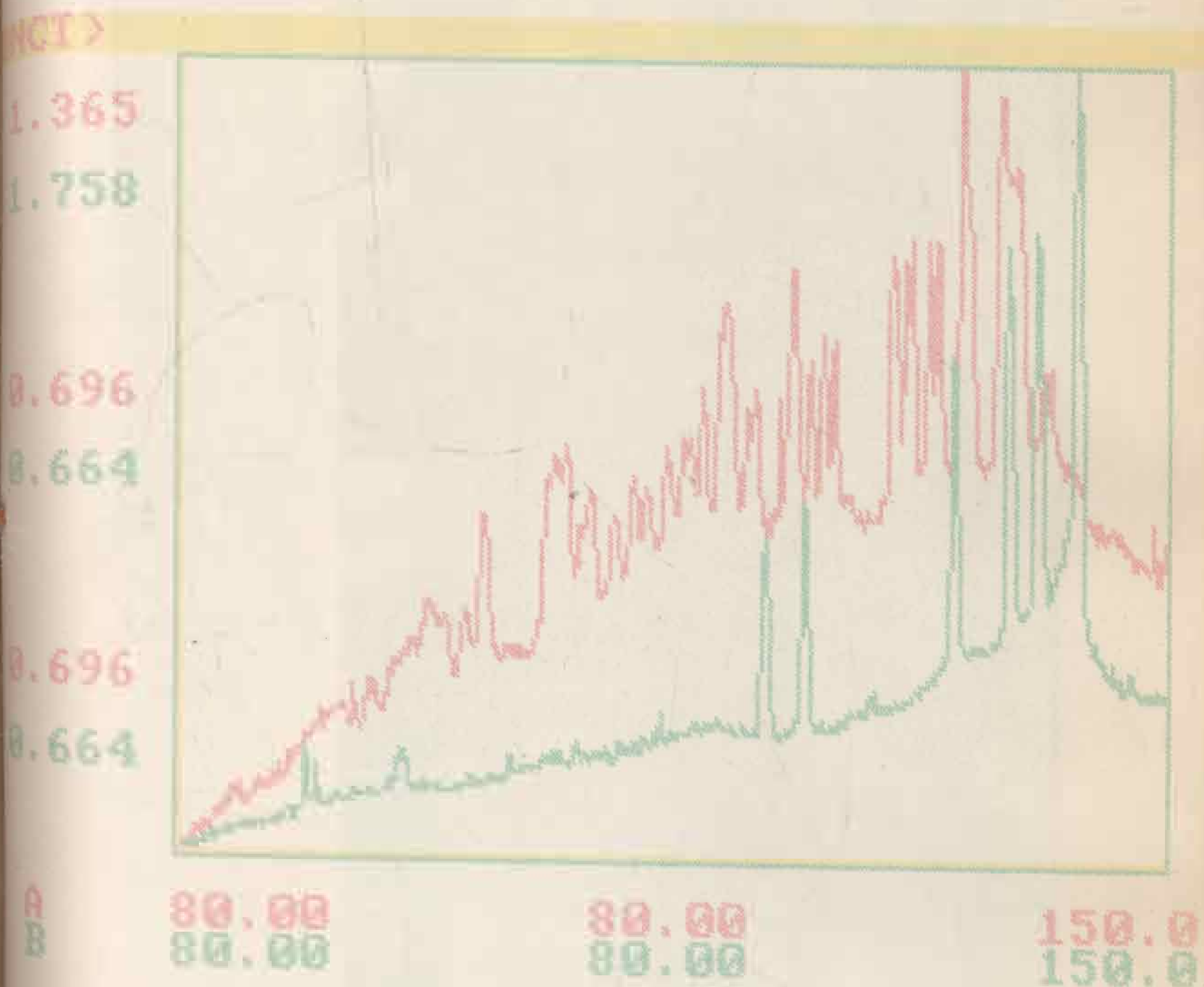


Fig.12 Scan of Polaroid 665 film negative of PstI digested BHV-1 DNA fragments separated on ethidium bromide stained 0.7% agarose gel. Peaks show PstI cut BHV-1 DNA fragments (red colour) and HindIII cut lambda DNA marker (green colour).

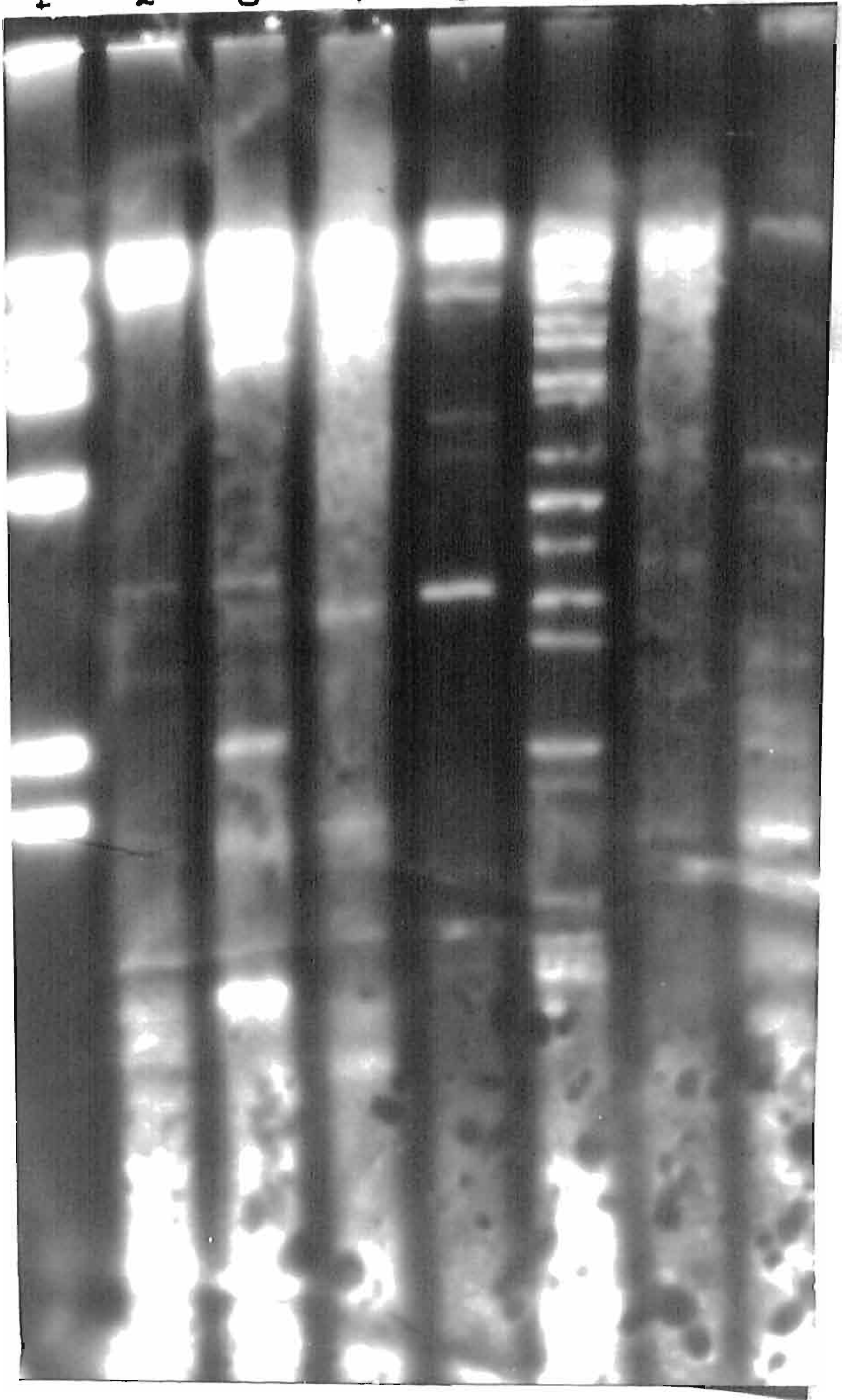


Fig.13 Autoradiogram of electrophoretically separated [^{32}P] end labelled restriction fragments. Direct contact print: HindIII cut lambda DNA marker (lane-1), BHV-1 DNA fragments digested with BamHI (lane-2), BglII (Lane-3), KpnI (Lane-4), EcoRI (lane-5), SalI (lane-6), HpaI (lane-7), SmaI (lane-8).

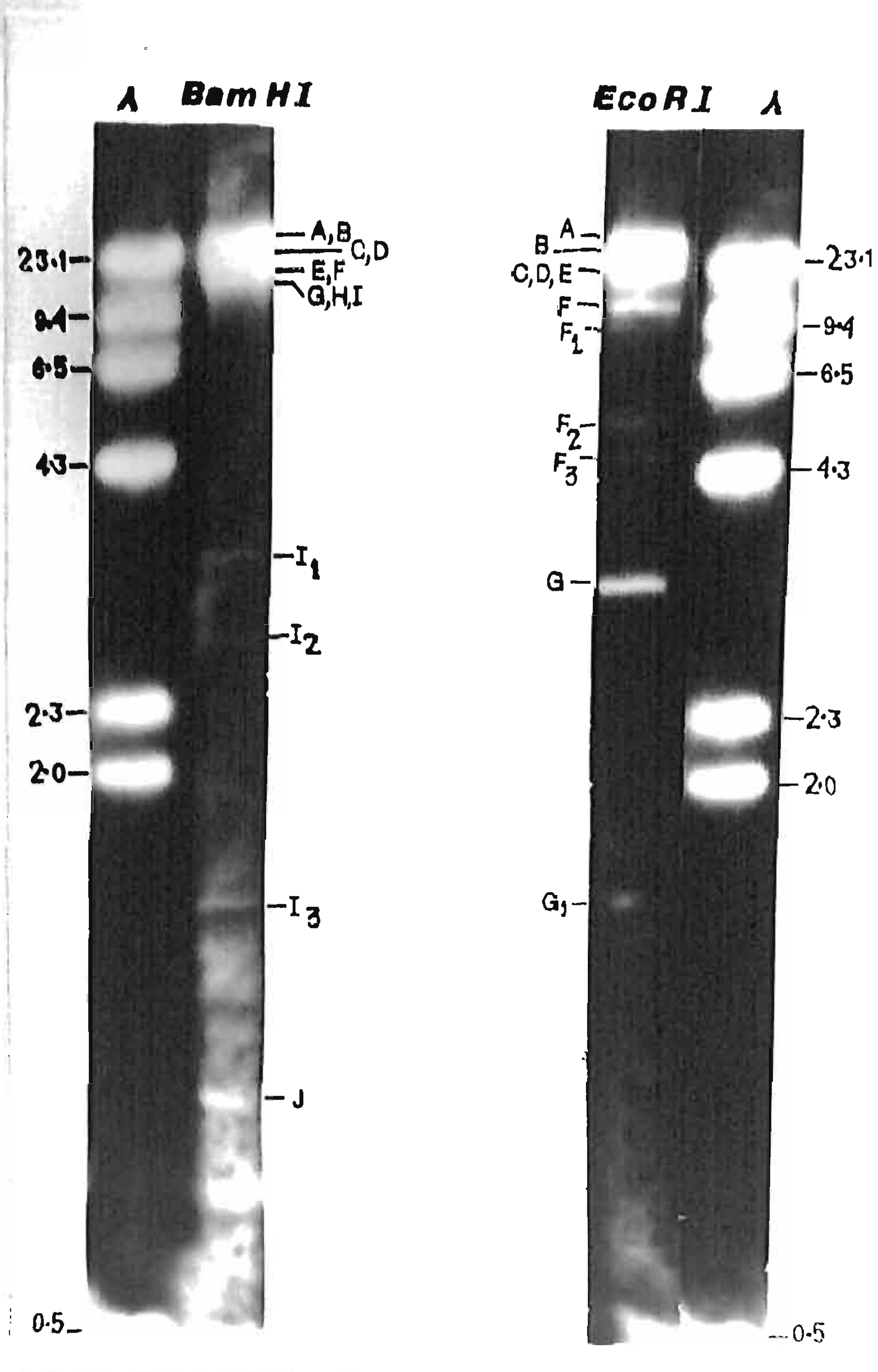


Fig.14 Autoradiogram of [^{32}P] end labelled BamHI and EcoRI restriction fragments of BHV-1 DNA. Direct contact print.

λ: [^{32}P] end labelled HindIII cut lambda DNA marker (size in kb).

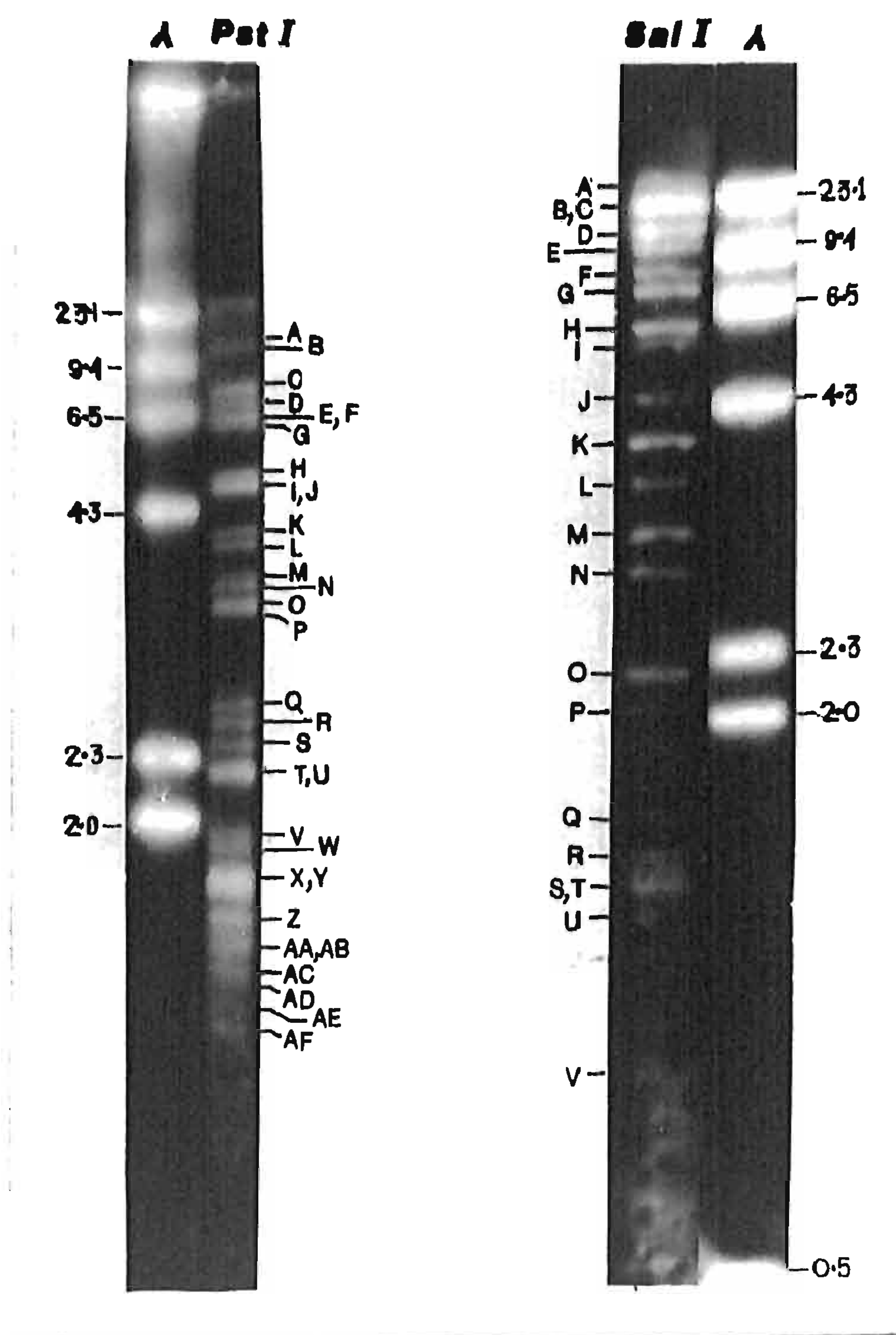


Fig.15 Autoradiogram of [^{32}P] end labelled PstI and SalI restriction fragments of BHV-1 DNA. Direct contact print.

λ : [^{32}P] end labelled HindIII cut lambda DNA marker (size in kb).

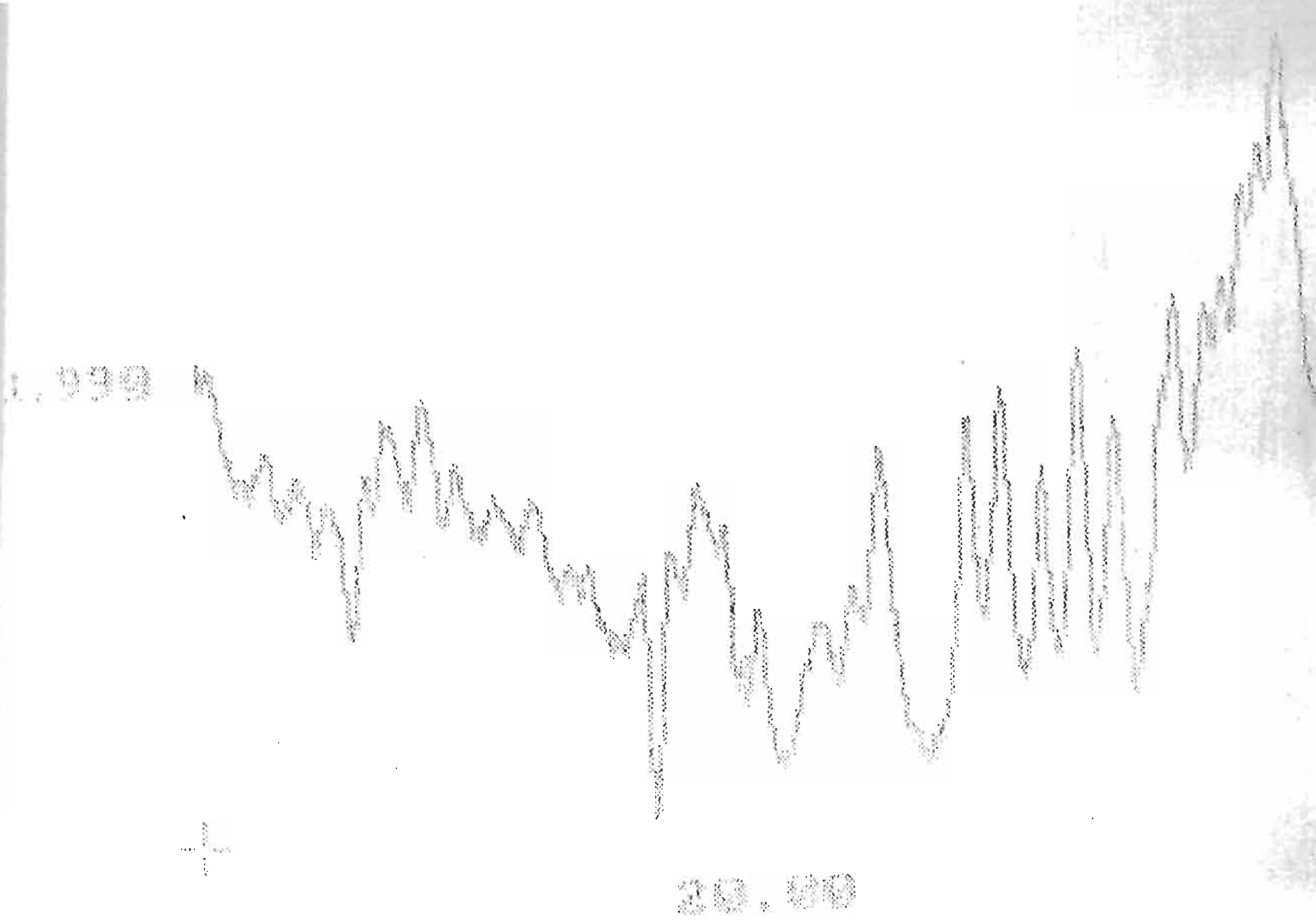


Fig.16 Scan of autoradiogram of [^{32}P] end labelled SalI restriction fragments of BHV-1 DNA.

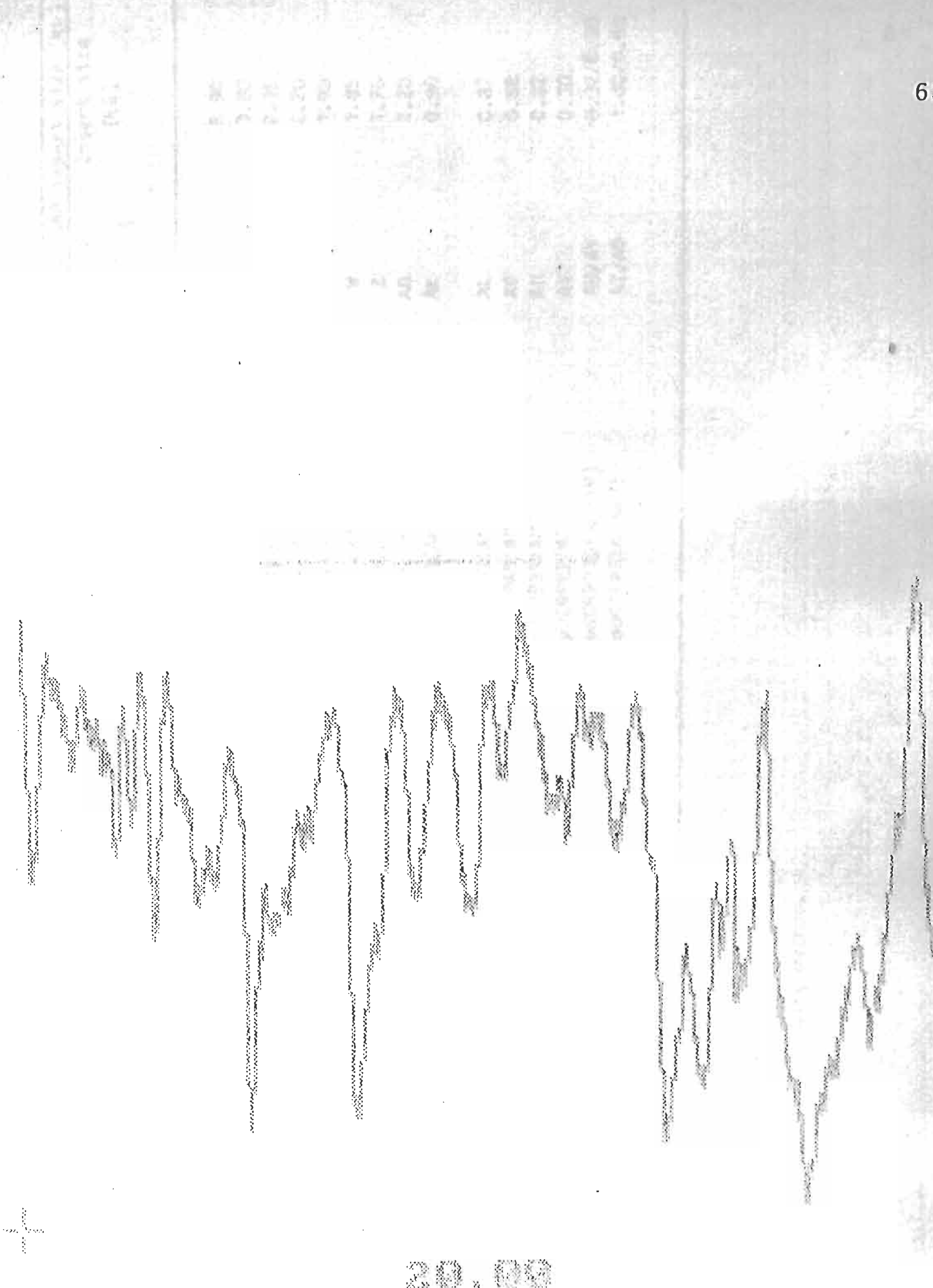


Fig.17 Scan of autoradiogram of [^{32}P] end labelled SmaI restriction fragments of BHV-1 DNA.

Table 7. Cloning of PstI fragments of BHV-1 DNA in pUC9 plasmid vector

No.of white colonies transferred from plates to broth	Colonies having no viral DNA insert	No.of colonies having viral DNA insert	Groups of colonies based on recombinant plasmid	No.of colonies in each group	Representative colony	Recombinant plasmids based on viral DNA insert size (kb)		
						Recombinant plasmid	Viral DNA insert	Insert size (kb)
P1 to P50 (50)	P1,P6,P11, P26 (4)	46	(1) P18, P19, P47	3	P18	pUC9P18(G)	G	5.90
			(2) P49*, P50*	2	P49	pUC9P49(K)	K	3.80
			(3) P16, P20	2	P20	pUC9P20(S)	S	2.35
			(4) P9	1	P9	pUC9P9(U)	U	2.20
			(5) P4, P5, P23, P24, P25, P35	6	P4	pUC9P4(V)	V	1.90
			(6) P41, P43	2	P43	pUC9P43(W)	W	1.85
			(7) P32, P38, P46	3	P38	pUC9P38(Z)	Z	1.70
			(8) P10, P14, P15, P17, P27, P28, P44	7	P15	pUC9P15(AG)	AG	1.20
			(9) P12, P13, P30, P36, P45	5	P13	pUC9P13(AK)	AK	0.90
			(10) P2, P3, P7, P8, P21, P33, P34, P37, P49	9	P33	pUC9P33(AL)	AL	0.87
			(11) P29, P40	2	P40	pUC9P40(AP)	AP	0.58
			(12) P39	1	P39	pUC9P39(AU)	AU	0.32
			(13) P22	1	P22	pUC9P22(AV)	AV	0.30
			(14) P31D	1	P31D	pUC9P31D(AU/AV)	AU/AV	0.32/0.30
			(15) P42	1	P42	pUC9P42(AI/AO)	AI/AO	1.00/0.60

No. of viral DNA fragments cloned = 15

No.of recombinant plasmids obtained= 15

* Colonies obtained using selective cloning method.

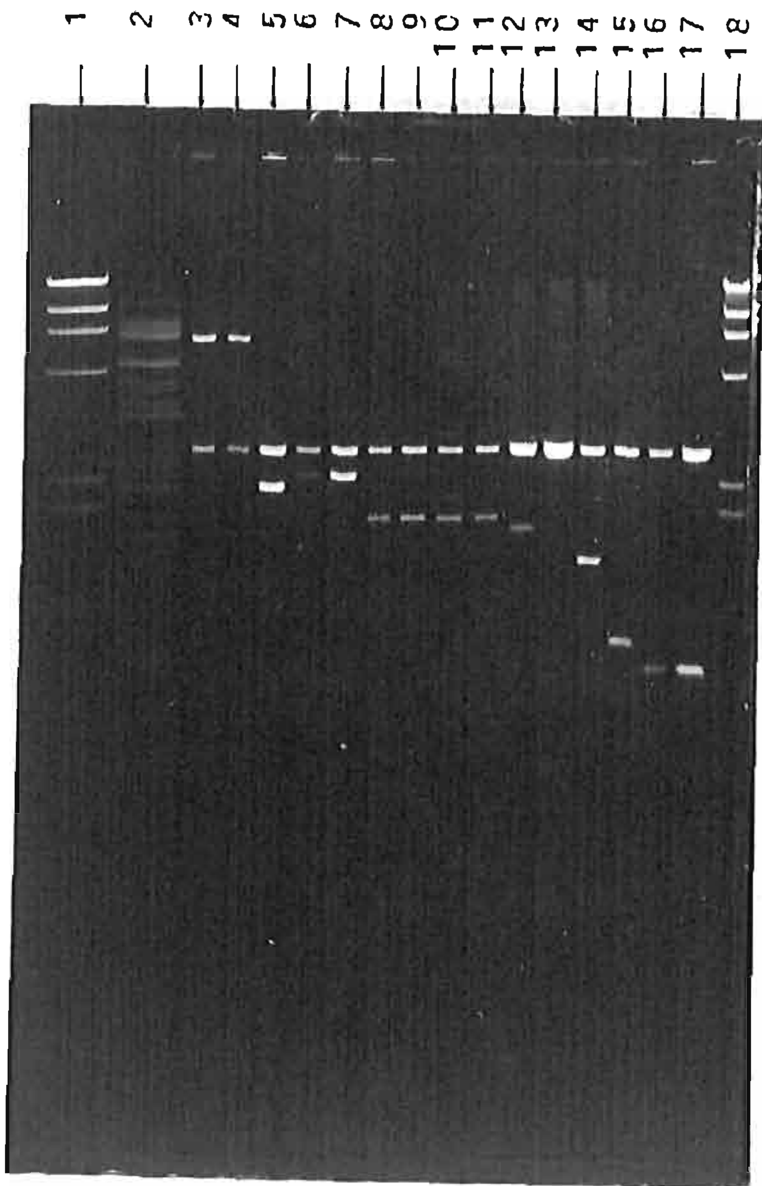


Fig.18

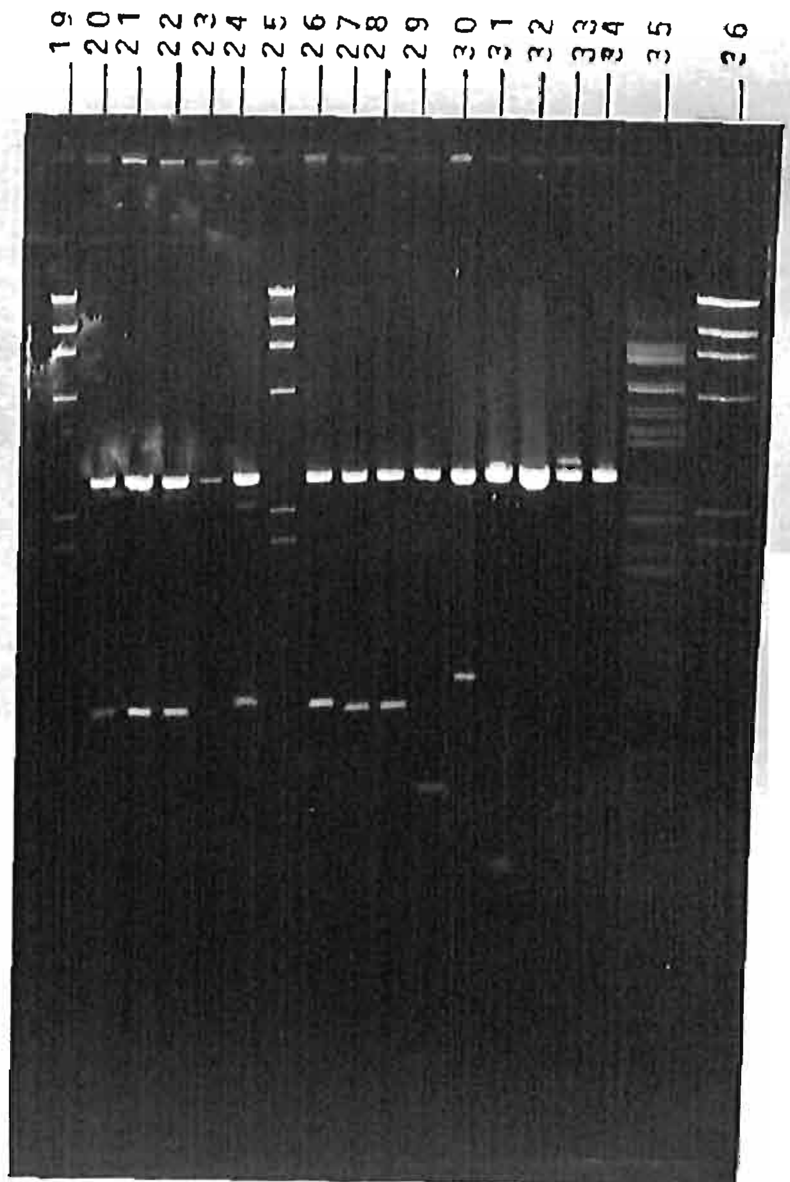
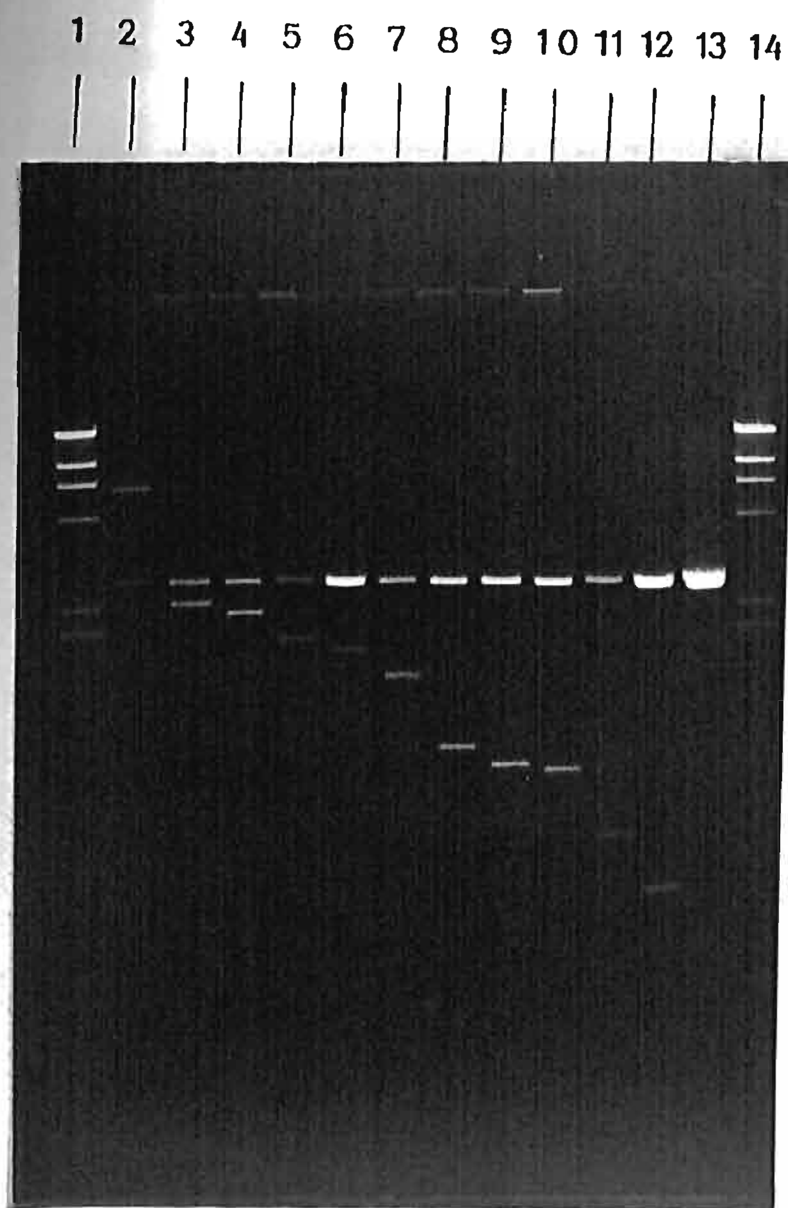


Fig.19

Fig.18 & 19 Screening of recombinant plasmids for BHV-1 DNA inserts after digestion with PstI.

HindIII cut lambda DNA marker (lane 1,18,19,25,36), PstI digested BHV-1 DNA (lane 2,35), PstI digested recombinant plasmids, isolated from bacterial colonies P19 (lane3), P18 (lane 4), P9 (lane 5), P16 (lane 6) P20 (lane 7), P4 (lane 8), P5 (lane 9), P24 (lane 10), P25 (lane 11), P43 (lane 12), P29 (lane 13), P38 (lane 14), P15 (lane 15), P44 (lane 16), P10 (lane 17), P7 (lane 20), P8 (lane 21), P2 (lane 22), P3 (lane 23), P12 (lane 24), P13 (lane 26),. P33 (lane 27), P37 (lane 28), P40 (lane 29), P14 (lane 30), P39 (lane 31), P22 (lane 32), P11 (lane 33) and P6 (lane 34).



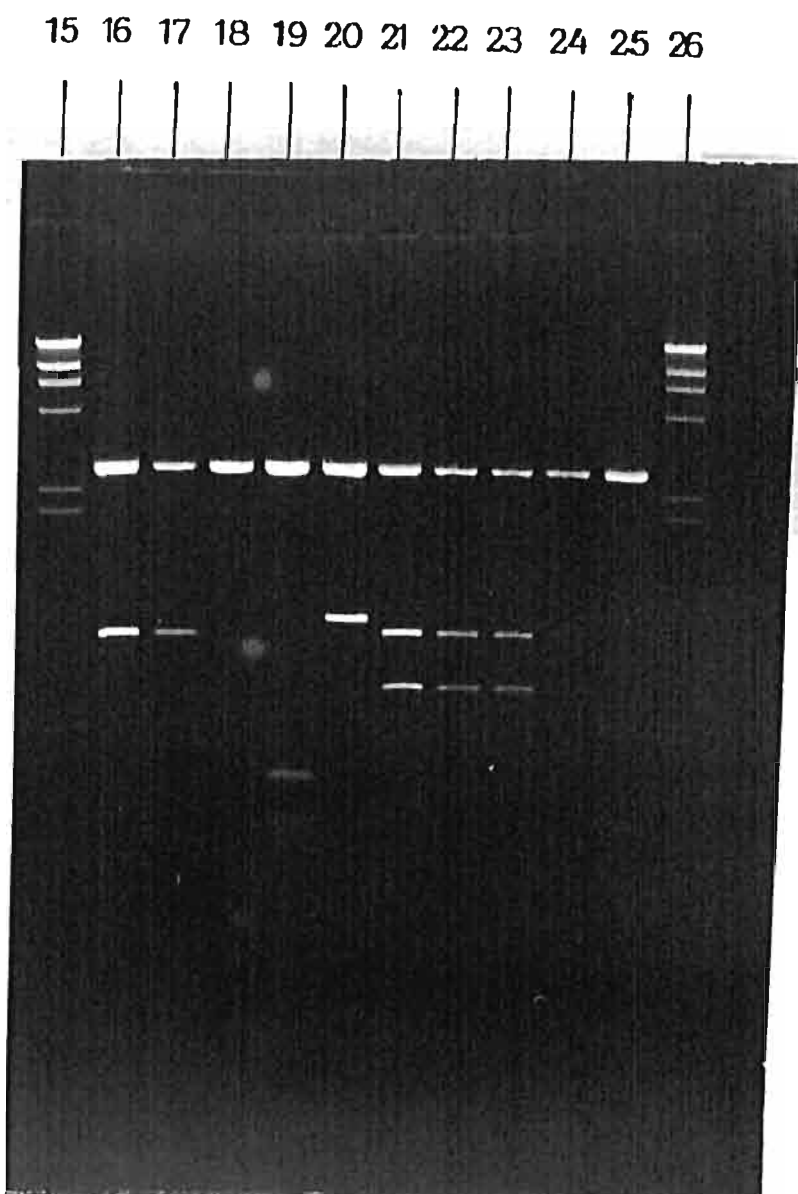


Fig.20

Fig.21

Fig.20 & 21 Recombinant plasmids showing different BHV-1 DNA inserts. HindIII cut lambda DNA marker (lane 1,14,15,26). PstI digested recombinant plasmids pUC9P18(G) (lane 2), pUC9P20(S) (lane 3), pUC9P9(U) (lane 4), pUC9P4 (V) (lane 5), pUC9P43 (W) (lane 6); pUC9P38 (Z) (lane 7), pUC9P15 (AG) (lane 8), pUC9P13 (AK) (lane 9), pUC9P33 (AL) (lane 10), pUC9P40 (AP) (lane 11), pUC9P39 (AU) (lane12), pUC9P22 (AV) (lane 13), pUC9P31D (AU/AV) (lane 19), pUC9P42 ([illegible]) (lane [illegible]).

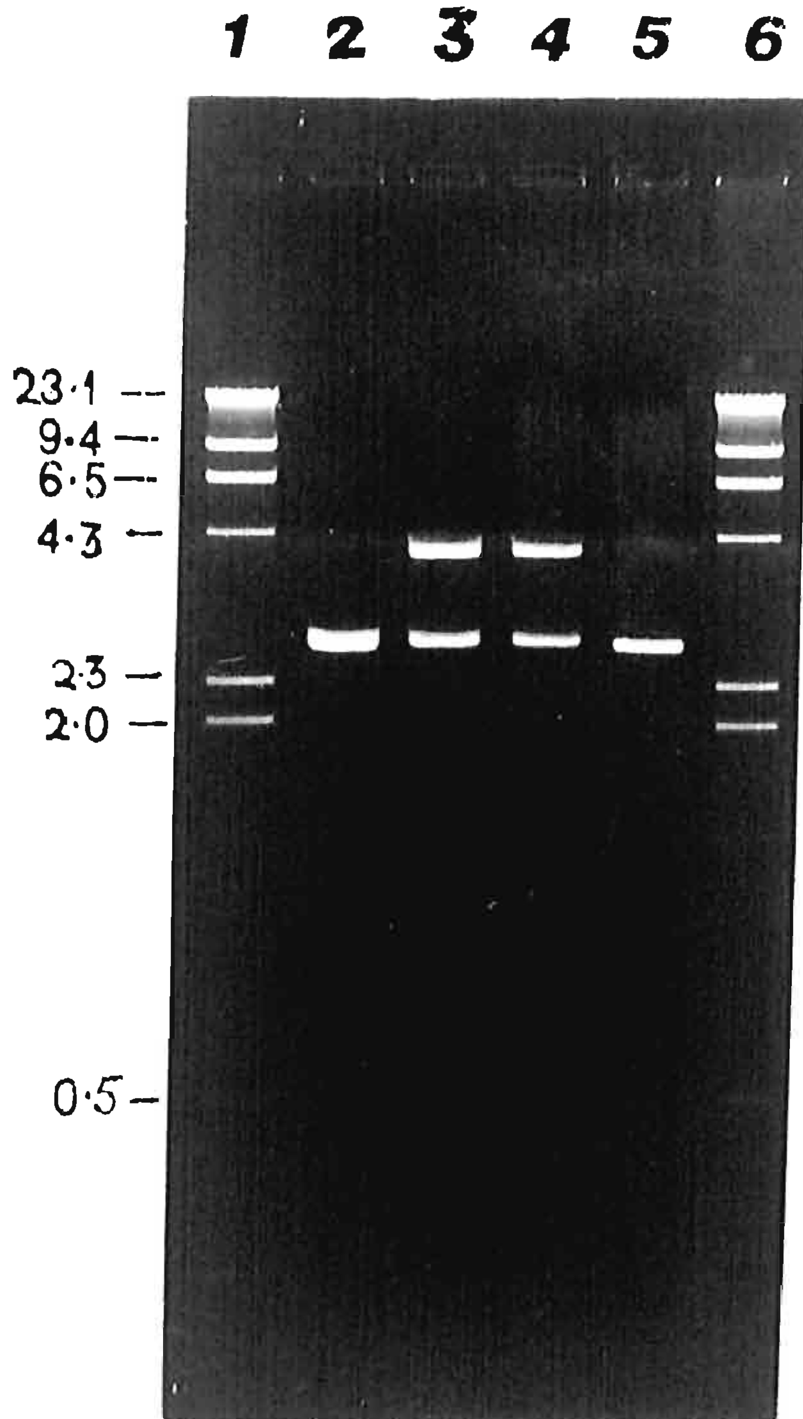


Fig.22 Selective cloning of PstI-'K' fragment of BHV-1 DNA. HindIII cut lambda DNA marker (lane 1,6), PstI cut pUC9 plasmid DNA (lane 2,5), PstI cut pUC9P49 (K) recombinant plasmid DNA (lane 3), PstI cut pUC9P50 (K) recombinant plasmid DNA.

Table 8. Restriction endonuclease analysis of recombinant plasmids

Restriction endonuclease	No.of restriction site(s) on BHV-1 DNA insert in recombinant plasmids		
	pUC9P18(G)	pUC9P20(S)	pUC9P49(K)
HindIII	NA	0	0
EcoRI	0	0	NA
BamHI	0	0	NA
SmaI	6	1	3
BglI	>5	2	>3
BglII	0	0	NA
XhoI	0	1	1
RsaI	4	3	5
StuI	1	0	1
SalI	1	1	1

'0' = No restriction site on insert fragments; NA = Not analysed

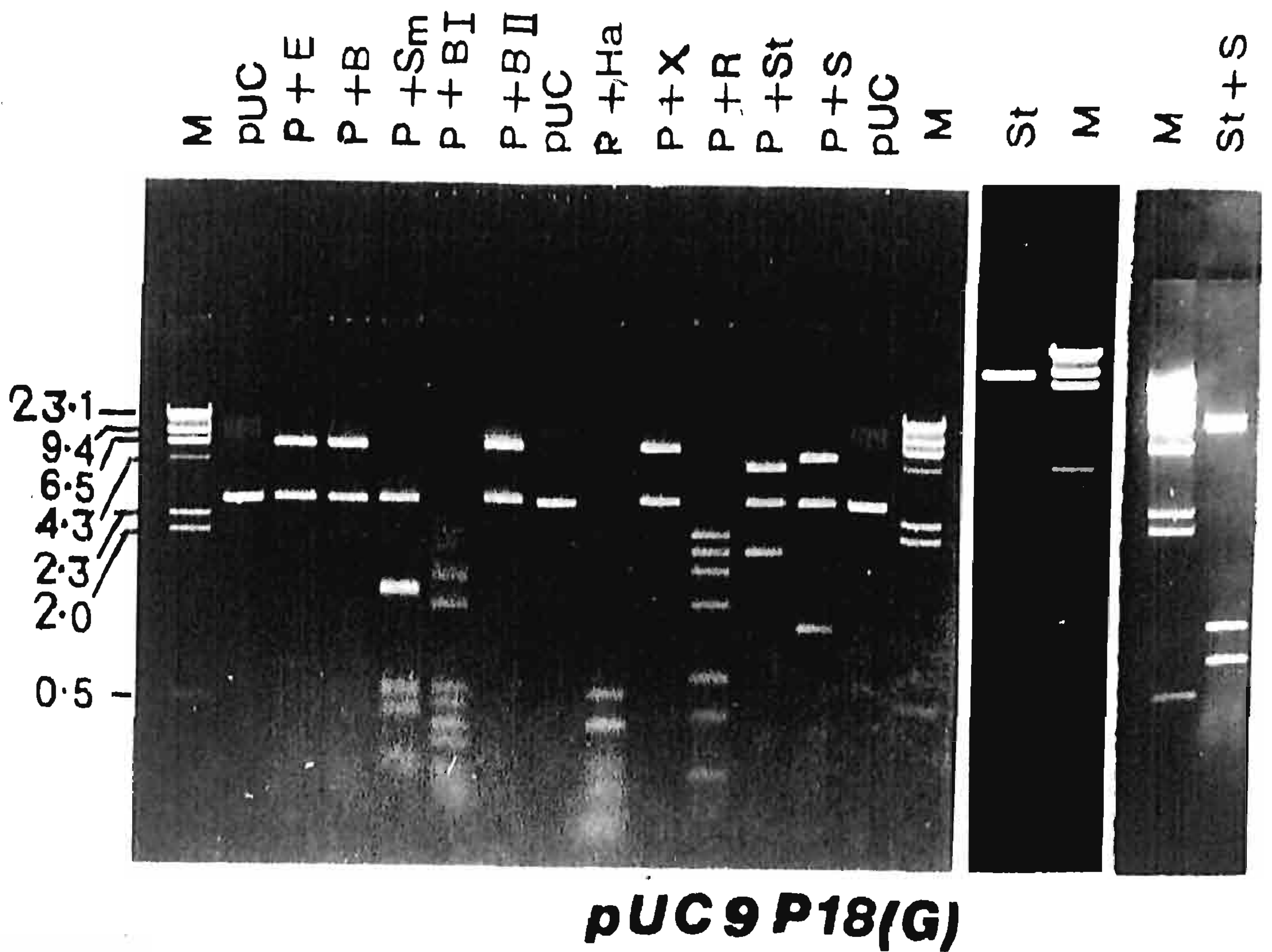


Fig.23 Restriction endonuclease analysis of pUC9P18 (G) recombinant plasmid using restriction endonucleases PstI (P), EcoRI (E), BamHI (B), SmaI (Sm), BglI (BI), BglII (BII), HaeIII (Ha), XhoI (X), RsaI (R) and StuI (St). 'M'- HindIII cut lambda DNA marker (size in kb). 'pUC' - pUC9 DNA cut with PstI.

Fig.24 Restriction site map of pUC9P18 (G) recombinant plasmid for restriction endonucleases *StuI* (St), *PstI* (P), *HindIII* (H) and *SalI* (S).

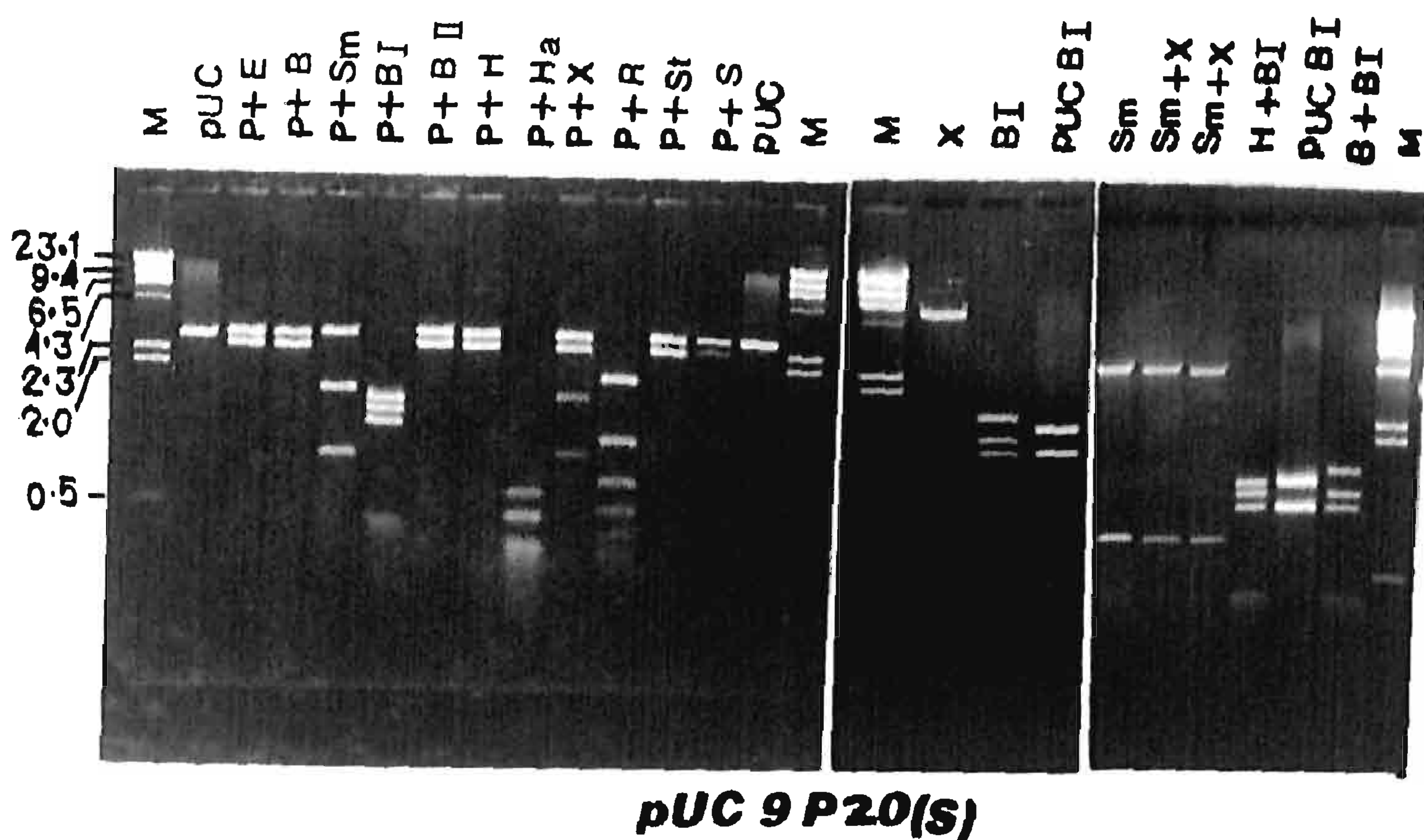


Fig.25 Restriction endonuclease analysis of pUC9P20 (S) recombinant plasmid using restriction endonucleases, PstI (P), EcoRI (E), BamHI (B), SmaI (Sm), BglI (BI), BglII (BII), HaeIII (Ha), XhoI (X), RsaI (R), HindIII (H) and StuI (St). 'M'-HindIII cut lambda DNA marker (size in kb). 'pUC'- pUC9 DNA cut with PstI. 'pUCBI'-pUC9 DNA cut with BglI.

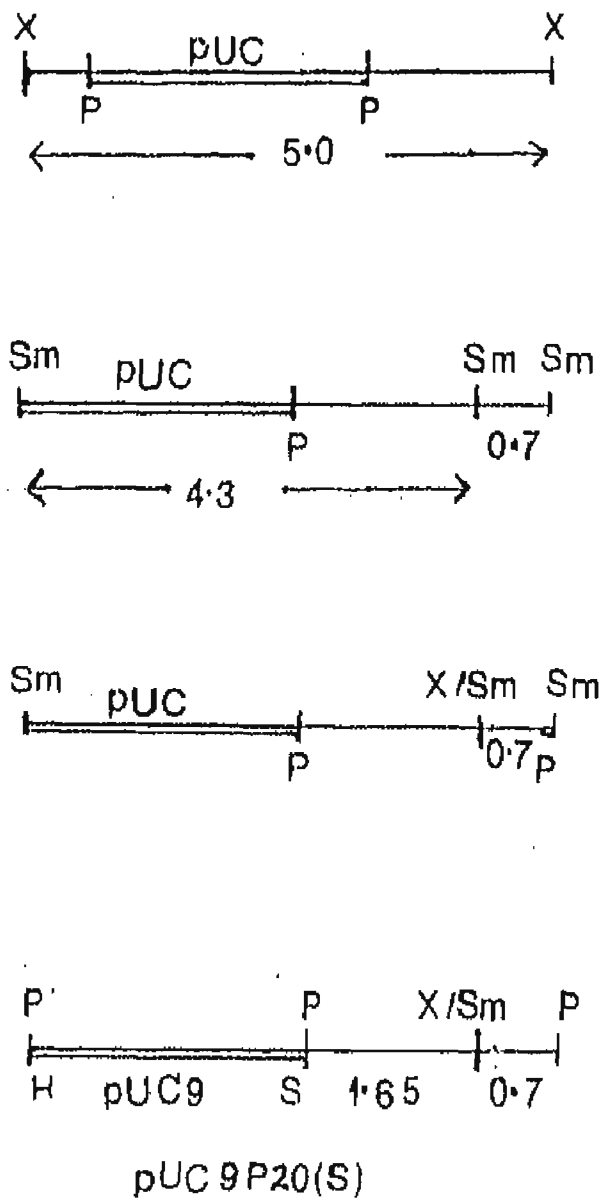


Fig.26 Restriction site map of pUC9P20 (S) recombinant plasmid for restriction endonucleases XhoI (X), PstI (P), SmaI (Sm), SalI (S) and HindIII (H).

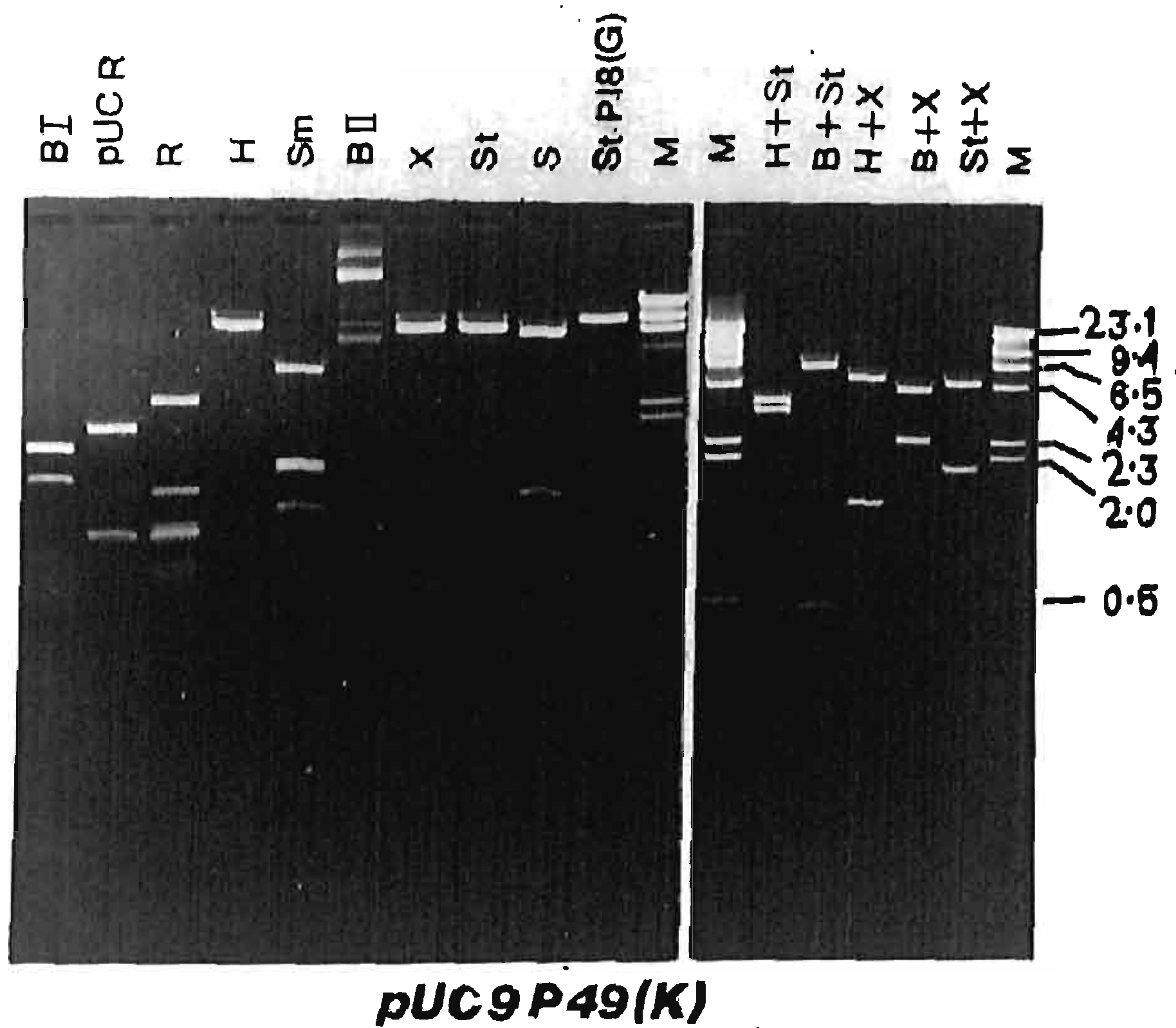


Fig.27 Restriction endonuclease analysis of pUC9 P49 (K) recombinant plasmid using restriction endonucleases BglI (BI), RsaI (R), *HindIII* (*H*), *SmaI* (*Sm*), *BglII* (BII), XhoI (X), StuI (St), and SalI (S). 'M'-HindIII cut lambda DNA marker (size in kb). 'pUC R'-pUC9 DNA cut with RsaI. 'St P18 (G)'-pUC9P18 (G) recombinant plasmid cut with StuI.

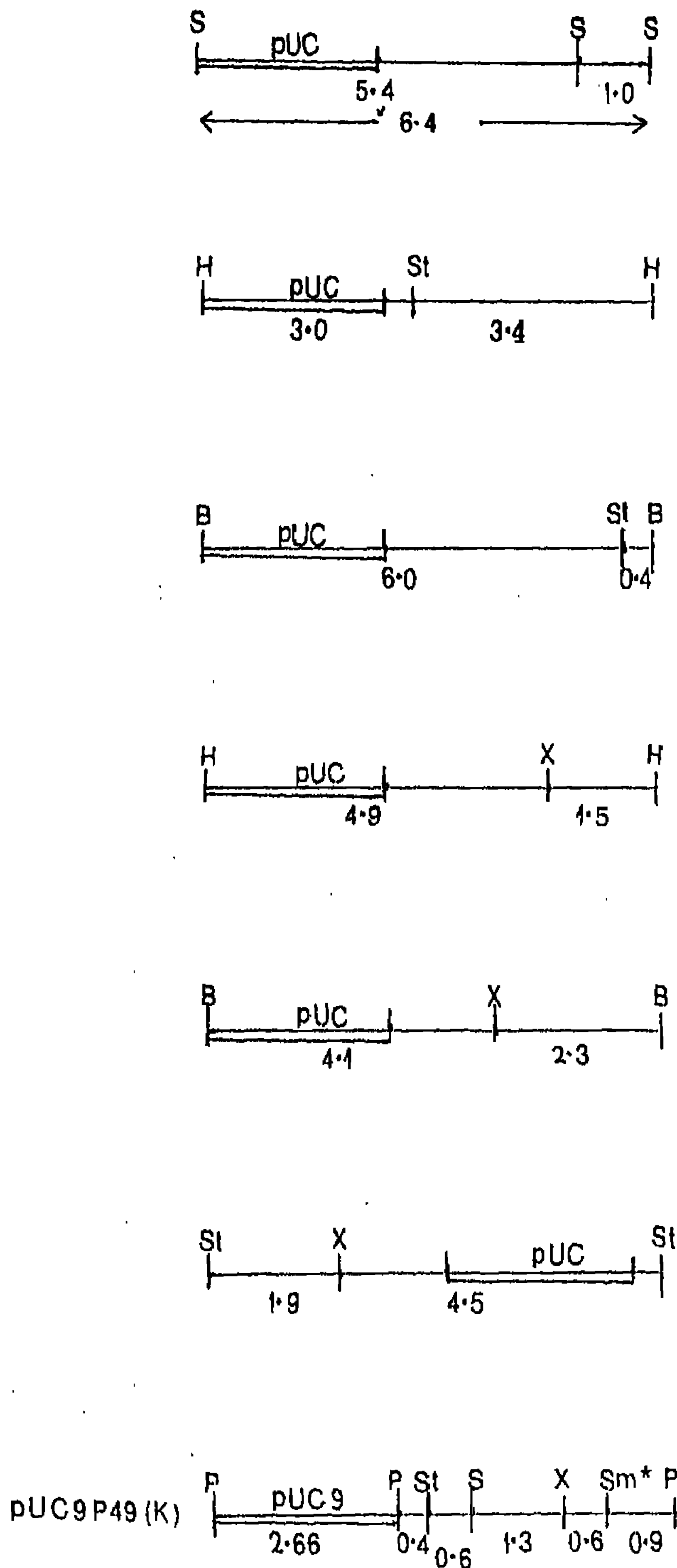


Fig.28 Restriction site map of pUC9P49(K) recombinant plasmid for restriction endonucleases PstI (P), StuI (St), SalI (S), XhoI (X) and SmaI (Sm).

* Out of 3 restriction sites for SmaI, only one could be mapped.

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DISCUSSION

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DISCUSSION

Keeping the economic importance of the BHV-I disease in our livestock in view, the present investigation was taken up to characterize the DNA from an indigenous respiratory isolate of BHV-1 - by restriction enzyme analysis.

The respiratory isolate of BHV-1, initially adapted in bovine kidney cell culture, could be readily adapted in MDBK cell culture in which it produced characteristic cytopathic effect reaching to its maximum within 48-72 h post infection. This observation is in agreement with the observations of earlier workers (Gibbs and Rweyemamu, 1977; Shukla, 1987; Owen and Field, 1988; Mittal and Field, 1989; Bandyopadhyay et al., 1990).

The BHV-1, concentrated and purified following the method described by Talens and Zee (1976) and Owen and Field (1988) with slight modification, yielded highly purified virus. An additional step of DNase treatment was given before potassium tartrate density gradient centrifugation to remove any cellular DNA contaminant from the viral suspension, obtained after 40% sucrose pelleting. The density gradient centrifugation was done for 10 h at 28,000 rpm (1,00,000xg) since the method described by Owen and Field (1988) did not yield viral band when the density gradient centrifugation was done for 3 h at 35,000 xg. The 10 h time given for density gradient centrifugation in the present study is in agreement with the findings of Talens and Zee (1976) who also used 10-40% potassium tartrate gradient for 12 h to purify the BHV-1. The BHV-1 DNA was isolated from density gradient purified virus following the method described by Owen and Field (1988) and the isolated DNA was found to be pure since it gave OD_{260}/OD_{280} ratio of 1.80 and an intact band on the agarose gel electrophoresis. Therefore, the present method of virus purification and DNA isolation, which is based on the methods described by Talens and Zee (1976) and Owen and Field (1988) could be advantageously used for such work and may be recommended for further use.

The BHV-1 DNA was analysed by restriction enzymes HindIII, EcoRI, BamHI, SalI, SmaI, KpnI, BglII, DraI, PstI and XhoI. Each restriction enzyme produced a unique restriction pattern of BHV-1 DNA on agarose gel. In restriction digestion with HindIII, EcoRI, BamHi, BglII, KpnI, DraI and PstI, co-migrating fragments of same or almost same molecular sizes were identified on the basis of either their increased fluorescence with ethidium bromide on agarose gel or their increased intensity obtained on the exposed X-ray film which corroborates the findings of earlier workers also identified co-migrating fragments using the above techniques (Mayfield et al., 1983; Misra et al., 1983). Digestion of the BHV-1 DNA with HindIII, EcoRI, BamHI restriction endonculeases and measurement of the fragment sizes by gel electrophoresis gave an estimated genome size between 137.0 kb to 138.8 kb, which is in agreement with the molecular size reported by earlier workers (Mayfield et al., 1983; Seal et al., 1985; Engels et al., 1986/87). However, the genome size of BHV-1 could also be estimated using other restriction endonucleases like BglII, KpnI and DraI and found to be 136.2 kb, 137.8/138.2 kb, and 138.2 kb, respectively. The approximate genome size of BHV-1 DNA with SalI and PstI could be estimated and found to be 135.2 kb and 135.05 kb, respectively since the small fragments below 0.40 kb could not be exactly located on the agarose gel.

Restriction enzyme analysis of BHV-1 DNA yielded results in which the DNA restriction patterns were the same for HindIII, EcoRI and BamHI restriction endonucleases as reported by earlier workers (Mayfield et al., 1983; Misra et al., 1983; Seal et al., 1985). Restriction endonuclease HindIII produced 15 fragments ranging in size from 21.2 kb to 0.36 kb. The 0.36 kb fragment was very faint and could not be reproduced in the photography. The restriction endonuclease pattern of HindIII cleaved BHV-1 DNA was found to be correlated with pattern A, among the four patterns (A, B, Ca, Cb) described by Mishra et al. (1983). Misra et al. (1983) further correlated this pattern A of Hind III cleaved BHV-1 DNA, with strain I, with respiratory isolates of BHV-1. The restriction

pattern of HindIII cleaved BHV-1 DNA was found to be correlated with the pattern described by Seal et al. (1985) and Engels et al. (1986/87) for respiratory isolates including IBR LA isolate and Metzler et al. (1985) for IBR Cooper type. With EcoRI and BamHI restriction endonucleases, the BHV-1 DNA was fragmented into 7 fragments ranging from 52.5 kb to 3.1 kb and 11 fragments ranging from 30.2 kb to 0.25 kb sizes, respectively. The fragment K of BHV-1 DNA with BamHI could not be clearly made out on 0.7% agarose gel stained with ethidium bromide. The restriction pattern of BHV-1 DNA with EcoRI was found to be related with patterns Aa, Ab, Ac and B reported by Misra et al. (1983) for respiratory isolates including IBR LA isolate. Thus the results obtained with the BHV-1 respiratory isolate is in close agreement with the above reports. The Restriction profile of BamHI digested BHV-1 DNA fragments yielded pattern similar to the pattern reported by other workers (Mayfield et al., 1983; Misra et al., 1983; Seal et al., 1985).

Only two restriction endonucleases PstI and BglII, among the restriction endonucleases used in this study, revealed the restriction pattern distinct from reported patterns. Whetstone et al. (1986) also reported that PstI and BglII restriction endonucleases could be used to distinguish vaccinal virus from the field isolates. The DNA from the BHV-1 respiratory Indian isolate produced 11 fragments on digestion with BglII ranging from 32.0 Kb to 1.5 kb in which fragment H (6.8 kb) was found to be additional from the pattern reported by Whetstone et al. (1986). However, the size of the BHV-1 DNA was found to be 136.2 kb which was in good agreement with the observed genome size of BHV-1 DNA thus showing that only the restriction pattern was different and the fragment observed was the viral fragment. Similarly with PstI digestion, the BHV-1 DNA produced two new fragments A and B sized 14.0 kb and 12.0 kb respectively. These two fragments showed fluorescence less than equimolar fragment in ethidium bromide stained agarose gel and intensity was also found to be submolar on autoradiogram exposed with $[^{32}P]$ dATP end labelled PstI digested fragments of BHV-1 DNA. The PstI digested BHV-1 DNA fragments below fragment G showed

almost similar pattern as reported by other workers (Whetstone et al., 1986, 1989; Miller et al. 1988). However the fragments C,D,E,F and G showed different migration pattern on 0.7% agarose gel that might be due to differences in size of these fragments. The genome size of the BHV-1 DNA estimated from this fragment pattern was 135.05 kb suggesting that these new fragments were from the viral DNA. The reason of variation in the restriction pattern with these two restriction endonuclease could be due to the fact that the Indian isolate might have slight variation from the strain reported abroad. The BHV-1 strain used by Whetstone et al. (1986) was the modified live infectious bovine rhinotracheitis vaccine strain virus. Various workers have already reported variation in the restriction pattern of BHV-1 DNA from different isolates/strains (Engels et al., 1981; Misra et al., 1983; Thiry et al., 1983; Seal et al., 1985; Brake and Studdert, 1985; Osorio et al., 1985; Metzler et al., 1985; Engels et al., 1986/87) and hence the observations in the present study are in agreement with the above reports. Few submolar fragments were observed on autoradiogram for BamHI and EcoRI digested BHV-1 DNA but could not be detected in ethidium bromide stained agarose gel. There is strong need to confirm the nature and origin of these submolar DNA fragments using Southern blot hybridization. The sum of size of EcoRI submolar fragments was approximately 18 kb so the possibility of the extra fragments observed in the present investigation being of mitochondrial origin may not be ruled out. Since in a valuable study, Obom et al. (1988) were able to demonstrate presence of mitochondrial DNA in vaccinia and Shope fibroma virus DNAs, extracted from cesium chloride purified virion tested with DNase I and the same could be detected in viral DNA after restriction enzyme digestion.

The restriction endonuclease pattern of BHV-1 DNA with KpnI, DraI, BglI, SmaI and XhoI were found to be unique restriction pattern for BHV-1 DNA. Restriction endonuclease KpnI produced 14 fragments of BHV-1 DNA, ranging from 28.0 kb to 1.2 kb. Four half molar fragments could be identified on the basis of fluorescence produced on 0.7% agarose gel stained with ethidium bromide. Restriction endonuclease DraI yielded 13 fragments of BHV-1 DNA ranging from 39.1 kb to 1.0 kb.

For cloning of PstI fragments of BHV-1 DNA, pUC9 plasmid was used as cloning vector because of the presence of PstI restriction site in the multiple cloning site of pUC9 plasmid vector, easy identification of the recombinant plasmid based on LacZ insertional inactivation marker and presence of ampicillin resistance gene. The recombinant plasmid containing TG-1 cells could be easily identified on the agar plates producing white colonies in the presence of IPTG/X-gal since their Lac Z gene was inactivated by insertion of PstI digested fragments of BHV-1 DNA. Out of 50 white colonies transferred from plates to broth, 46 colonies yielded recombinant plasmids with viral insert fragment. Few colonies,P42 and P31D, yielded recombinant plasmids with two viral insert fragments. Mayfield et al. (1983) also reported a recombinant plasmid containing two HindIII fragments L and D and on digestion with HindIII, this recombinant plasmid yielded both the inserts and plasmid pBR322 vector. They explained that it might be due to insertion of an L-D partial digest fragment into the plasmid. The present observation supports the findings of Mayfield et al. (1983). The colonies P42 and P31D, containing insert fragments AI/AO (1.0 kb/0.60 kb) and AU/AV (0.32 kb/0.30 kb) respectively, might have originated due to insertion of partial digest fragments of BHV-1 DNA with PstI restriction endonuclease since the possibility of two colonies being transferred from plate to broth was ruled out by further streaking of these colonies on LB/Amp plates and then again ~~single~~ transferring the single colony to broth. On the basis of these findings the position of fragments AI and AO as well as AU and AV could be located close to each other on the BHV-1 genome.

The results of shotgun cloning with PstI digested fragments showed that a large number of smaller fragments could be cloned into pUC9 vector. To clone a large fragment, fragment K (3.80 kb) was selectively taken out from the LMT agarose gel and ligated into PstI cloning site of pUC9 plasmid in MCS region and a recombinant plasmid containing this fragment could be obtained successfully.

Out of 15 recombinant plasmids obtained, three plasmids could be mapped for restriction enzymes SalI, StuI, SmaI and XhoI using single and double digestion. For the recombinant plasmid pUC9P18(G) the restriction site for SalI and StuI was found to lie near MCS of pUC9 plasmid. The restriction site for XhoI and SmaI was found to be close to each other on the pUC9 P20(S) recombinant plasmid. This was confirmed by single and double digestion with these two restriction endonucleases. On digestion with SmaI, the recombinant plasmid yielded two fragments showing one restriction site on the insert fragment itself and another on MCS region. The 0.7 kb small fragment obtained after digestion of pUC9P20(S) with SmaI, showed that the restriction site for SmaI on the insert fragment existed near SmaI site of MCS region. The double digestion of pUC9P20(S) with SmaI and XhoI also yielded two fragments similar to single digest fragments with SmaI, showing that the restriction site for SmaI and XhoI lies very near to each other on the insert fragment in pUC9P20(S). On restriction endonculease digestion with SalI, this recombinant plasmid yielded two fragments showing one restriction site on the insert fragment. Restriction endonucleases StuI and XhoI also showed one restriction site on the insert fragment as recombinant plasmid became linear on digestion with these restriction endonucleases. The restriction site for SalI, StuI and XhoI restriction endonucleases could be mapped on the insert fragment. The restriction sites for StuI and XhoI were located on the insert fragment in pUC9P49(K) recombinant plasmid linearized with HindIII and BamHI. These restriction sites were further confirmed by digesting recombinant plasmid pUC9P49(K) first with StuI and then followed by XhoI. Similarly, the restriction sites for SalI and SmaI could be located on the insert fragment on the recombinant plasmid pUC9p49(K).

The recombinant plasmids, having PstI digested BHV-1 DNA inserts may be used as a diagnostic probe to detect various isolates of BHV-1 from denatured DNA samples from cell cultures. As Andino <u>et al</u>. (1987) reported that three recombinant plasmids having

insert fragment of BamHI digested BHV-1 DNA, could detect 10 ng to 1pg BHV-1 DNA in dot-blot hybridization using nick translated ^{32}P-labelled recombinant plasmids as a probe. Similarly recombinant plasmid containing PstI digested BHV-1 DNA insert could be used to detect BHV-1 DNA from cell culture samples using dot-blot hybridization procedure. Furthermore pUC9P18(G), pUC9P2(S), and pUC9P49(K) mapped recombinant plasmids could be used to prepare restriction map for BHV-1 DNA using these ^{32}P labelled recombinant plasmids as probe. Thus restriction endonuclease analysis, cloning and mapping results obtained in the present study would further help in understanding the molecular nature of the BHV-1 DNA.

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SUMMARY

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SUMMARY

An Indian respiratory isolate of BHV-1, initially adapted in bovine kidney cell culture; was propagated in MDBK cell culture and purified by ultracentrifugation. Restriction enzyme analysis was performed using BHV-1 DNA isolated from potassium tartrate density gradient purified virus.

To characterize the DNA of BHV-1, it was cleaved with HindIII, EcoRI, BamHI, SalI, BglI, BglII, KpnI, XhoI, RsaI, DraI, SmaI and PstI restriction endonucleases. The restriction cleavage products were analysed by agarose gel electrophoresis. All the restriction enzymes used in the present study, generated a unique restriction profile of BHV-1 genome. The total molecular size of BHV-1 DNA was estimated to be within 135 kb to 138 kb which was in good agreement with the reports for other BHV-1 strains.

The restriction endonculease pattern of BHV-1 DNA with HindIII, BamHI and EcoRI was found to be almost similar with the reports for other BHV-1 strains of respiratory isolate. Restriction endonucleases HindIII, BamHI and EcoRI cleaved BHV-1 DNA into 15, 11 and 7 fragments, respectively. Restriction profile of BHV-1 DNA for restriction endonculeases KpnI, DraI, BglI, SmaI and XhoI could also be produced. Few restriction endonuclease, PstI and BglII, yielded distinct restriction profile of BHV-1 DNA from the restriction profile reported for other respiratory isolates of BHV-1 from abroad.

For further analysing the BHV-1 DNA, the PstI fragments of the BHV-1 DNA was cloned into the PstI site of pUC9 plasmid vector. Using shotgun cloning approach of cloning, 14 PstI fragments of viral DNA could be cloned into the PstI site of the plasmid vector pUC9. One PstI-K (3.8 kb) fragment of BHV-1 DNA could also be selectively cloned into the PstI site of the plasmid vector pUC9. Through restriction enzyme analysis of the recombinant plasmids, it

was observed that the PstI fragments AI an AO were located close to each other on the BHV-1 genome. Similarly the PstI fragments AU and AV were also observed to be located close to each other on the BHV-1 genome. The viral fragments G, K and S, cloned in the recombinant plasmids pUC9P18(G), pUC9P49(K) and pUC9P20(S), respectively, were mapped for SalI, StuI, XhoI and SmaI restriction endonuclease sites.

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