BIOCHEMICAL CHARACTERIZATION OF THE NUCLEIC ACIDS OF SOME HUMAN AND ANIMAL VIRUSES

Ronald Terence Mew

Thesis submitted in fulfilment of the requirements for the degree of Doctor of Philosophy (Medicine) in Chemical Pathology, Faculty of Medicine, University of Cape Town.

Cape Town, January 1982

Promoted by Professor E.H. Harley, M.D., Ph.D.

The University of Cape Town has been given the right to reproduce this thesis in whole or in part. Copyright is held by the author.

The copyright of this thesis vests in the author. No quotation from it or information derived from it is to be published without full acknowledgement of the source. The thesis is to be used for private study or non-commercial research purposes only.

Published by the University of Cape Town (UCT) in terms of the non-exclusive license granted to UCT by the author.

University of Cape Town

Table of contents

1. ABSTRACT -3

2. PART I -6
CHARACTERIZATION OF NEW POLYOMAVIRUS ISOLATES -6

3. HUMAN POLYOMAVIRUS LITERATURE REVIEW -7
3.1. ISOLATION OF HUMAN POLYOMAVIRUSES -9
3.1.1. Prototype BK Virus -9
3.1.2. Isolation of Other Human Polyomaviruses -11
3.1.3. JC Virus -11
3.1.4. GS Virus -12
3.1.5. DW Virus -12
3.1.6. MM Virus -13
3.1.7. RF Virus -13
3.1.8. MG Virus -14
3.1.9. JM Virus -14
3.1.10. BK (Dun) Strain -14
3.1.11. JL Virus -15
3.2. PROPERTIES OF HUMAN POLYOMAVIRUSES -15
3.2.1. Origin and Distribution -15
3.2.2. Growth Characteristics in Cell Culture -16
3.2.3. Cell Culture of Human Polyomaviruses -17
3.2.4. Transformation and Tumour Induction -20
3.2.4.1. On the Nature of Tumour (T) Antigens -23
3.2.5. Is BK DNA Present in Human Tissue? -25
3.3. THE BK VIRUS GENOME -29
3.4. Transcription -30

4. ANALYSIS OF BK VIRUS DNA -32
4.1. Restriction Endonuclease Mapping -32
4.2. PREPARATION OF DNA -32
4.2.1. Cell culture propagation -32
4.2.2. Recombinant DNA Amplification -33
4.2.2.1. pBR322, a Plasmid Cloning Vector -34
4.2.2.2. Containment of Recombinant DNA Molecules -35
4.2.2.3. Theory of Recombinant Plasmid Formation -36

5. RESULTS -42
5.1. ISOLATION OF BK-LIKE VIRUSES -42
5.2. DETAILS OF ELECTROPHORETIC ANALYSIS -47
5.3. RESTRICTION ANALYSIS -48
5.3.1. Handling of Restriction Enzymes -48
5.3.2. Isolate SN -48
5.3.3. Isolate WW -51
5.4. Molecular Weight Markers -52
5.4.1. BK Virus Prototype -53
5.4.2. BK(MM) DNA Extraction -55
5.5. MOLECULAR WEIGHTS OF SN AND WW HINDIII FRAGMENTS -63
5.6. Cloning of BK DNA into pBR322 -66
5.6.1. MM DNA -68
5.6.2. WW DNA -70
5.6.3. Colony Hybridization with an MM DNA Probe -71
5.6.4. Characterization of Recombinant Plasmids -71
5.6.4.1. Probing for Putative MM Inserts -73
5.6.5. Probing for BK DNA Sequences in Human Isolates -75

Table of contents

6. ADDENDUM - LATEST RESULTS -82
6.1. Restriction Mapping of New Isolates -82
6.2. Cloning of a Clinical Isolate -84

7. DISCUSSION -95

8. METHODS -106
8.1. Cell Culture Propagation of MM Virus -106
8.2. Purification of DNA from Urine Pellets -106
8.3. Purification of BK DNA from Cell Culture -107
8.4. DNA Restriction -108
8.5. GEL ELECTROPHORESIS -109
8.5.1. Agarose Cylindrical Gels -109
8.5.1.1. Gel Photography -109
8.5.2. Vertical Agarose Slab Gels -110
8.5.3. Horizontal Agarose Slab Gels -111
8.5.4. Polyacrylamide Cylindrical Gels -111
8.5.5. Vértical Polyacrylamide Slab Gels -112
8.6. RECOMBINANT DNA TECHNIQUES -113
8.6.1. Transformation -113
8.6.1.1. Transformation procedure -114
8.6.2. Characterization of Transformed Colonies -115
8.6.2.1. Contaminants -115
8.6.2.2. Spontaneous Mutants -115
8.6.2.3. Vector Plasmid Transformants -115
8.6.3. Replica Plating -116
8.6.4. Toothpick Transfer -116
8.6.5. Colony Hybridization -117
8.6.6. Nick Translation -119
8.6.7. Southern Gel Transfer -120
8.6.8. Autoradiography -121
8.7. PLASMID EXTRACTION -122
8.7.1. Microscale Screening of Recombinant Colonies -122
8.7.2. Gentle Lysis Procedure -124
8.7.2.1. Preparation of Dialysis Tubing -125
8.7.2.2. Dialysis -126

9. REFERENCES FOR PART I -127

10. PART II -138
CHARACTERIZATION OF ROTAVIRUS RNA -138

11. ROTAVIRUS LITERATURE REVIEW -139
11.1. Discovery of Rotaviruses -139
11.1.1. EDIM -139
11.1.2. Neonatal Calf Diarrhoea Virus -140
11.1.3. SA11 -140
11.1.4. "O" Agent (OA) -141
11.1.5. Infantile Gastroenteritis Virus (IGV,HGV) -141
11.1.6. Other Host Species -142
11.2. Epidemiology (Human Rotavirus) -142
11.2.1. Clinical Features -143
11.2.2. Diarrhoeal Mechanism -143
11.3. Gastroenteritis Epidemics in Livestock -144
11.4. Morphology of Rotaviruses -144
11.4.1. Morphogenesis -146
11.5. Antigenic Properties: Detection of Rotaviruses -148

Table of contents

11.5.1. Cell Culture as a Diagnostic Tool -149
11.6. Cell Culture Characteristics of Rotaviruses -150
11.6.1. Plaque Assay and the Effect of Trypsin -151
11.7. THE ROTAVIRUS GENOME -153
11.7.1. Molecular Weight Determination -154
11.8. Analysis of Rotavirus Polypeptides -158
11.9. Coding Assignments for Rotavirus Genome Segment -159

12. RESULTS AND DISCUSSION -162
12.1. Cell Culture Propagation of SA11 and OA -162
12.1.1. Newman Tytell Medium -165
12.1.2. Effect of Trypsin on Rotavirus Propagation -167
12.1.2.1. Addition of VT to Culture Medium -167
12.1.2.2. Use of Pure Trypsin -169
12.1.3. Infections -170
12.2. Buoyant Density of SA11 Virus -178
12.3. Buoyant Density of SA11 RNA -179
12.4. Extraction of EDIM and IGV -183
12.5. Molecular Weights of Rotavirus Genome Segments -183
12.5.1. Molecular Weight Estimations by 32P-Labelling -186
12.6. Voltage Effects on RNA Electrophoresis -190
12.7. Digestion of SA11 RNA with RNase and DNase -191

13. METHODS -209
13.1. Propagation of Rotavirus -209
13.1.1. Routine Subculture -209
13.1.2. Infection with Rotavirus -209
13.2. 32P-Labelling of Rotavirus Nucleic Acids -210
13.3. Purification of Rotavirus from Cell Culture -210
13.3.1. Analysis of CsCl Gradients -211
13.4. Extraction of dsRNA from Infected Cell Cultures -211
13.4.1. Extraction of Cells Attached to Flask -211
13.4.2. Extraction of Cell Debris -212
13.4.3. Extraction of Free Virus -212
13.4.4. Extraction of Faecal Samples -212
13.5. Caesium Sulphate DGC of Nucleic Acids -213
13.6. Gel Electrophoresis of dsRNA -213
13.6.1. Analysis of Gels Containing 32P-Labelled RNA -213
13.6.2. Fluorometric Scanning of EtdBr Stained Gels -214
13.7. Sensitivity of Rotavirus Nucleic Acid to RNase -214

14. REFERENCES FOR PART II -216

15. BUFFERS AND SOLUTIONS -225

16. ABBREVIATIONS -231

Acknowledgements

I would like to thank Eric Harley for giving me the opportunity to come to Cape Town. It has been very exciting to see the local involvement in molecular genetics evolve so rapidly, from a complete ignorance of restriction enzymes to full-blown recombinant DNA experiments. Professor Harley is a man of ideas, and has a rare talent for serendipidy. He has also an unmatched optimism - an essential weapon against the disappointments which bedevil researchers, especially inexperienced ones. My thanks are due also to Prof. O. Prozesky, Mike Lecatsas and Barry Schoub who supplied me with virus samples, to Prof. W. du T. Naude for assistance with viral cultures, to Louise Olliver for collaboration in the polyomavirus DNA cloning experiments, to P. Howley for the gift of MM virus, to Prof. M.C. Berman for use of the facilities of the UCT Membrane Research Unit, and to all the staff in the Dept. of Chemical Pathology, UCT, for providing an unusually stimulating environment. Bacteriological P2 containment facilities for cloning experiments were kindly provided by Richard Ambrosio at the Dept. of Medical Microbiology, University of Stellenbosch, and later by Dr. R. Moodie at the Dept. of Medical Microbiology, UCT. I would like to thank Langley Purves for instructing me in the use of the Dept. of Chemical Pathology's computer terminal editing and documentation program, which was used to prepare this manuscript.

I am grateful to the Medical Research Council and the University of Cape Town Postgraduate and Jagger Scholarship Committees for financial assistance.

1.. ABSTRACT

In Part I, the isolation and partial characterization of human polyomaviruses from a number of renal transplant patients is described. These isolates proved refractory to cell culture propagation by the methods used, and were thus extracted directly from large volumes of patient's urine. This approach has the advantage that the virus cannot undergo any possible genomic modification, as tends to occur during adaptation to cell culture. Human polyomavirus DNA is very susceptible to mutation during cell passage. Four isolates from different patients yielded sufficient DNA for limited restriction endonuclease characterization. Surprizingly, all four gave the same patterns with EcoRI, BamHI and HindIII. Two isolates that were also digested with PstI gave an identical pattern. These patterns are similar to, but distinct from, other strains of the human polyomavirus BK that have been described. Our isolates had a similar-sized genome to BK, but only 3 HindIII sites compared with 4 in the prototype, and 2 PstI sites compared with only 1 in the prototype.

The quantity of DNA obtained directly from urine was usually very limited. In order to produce adequate DNA for complete analysis, viral DNA was recombined with a bacterial vector (pBR322) and cloned into Escherichia coli strains HB101 and C600. Initially, the well-studied strain BK(MM) was successfully cloned. This clone was used to prepare radioactively-labelled DNA probes for the detection of BK-specific sequences in urine isolates and in subsequent recombinants with patient material. Such cloned material is easier to prepare in bulk than DNA from virus passaged in cell culture. Early attempts to clone DNA

from clinical isolates failed, but BK-specific DNA from a patient (P.R.) has recently been cloned successfully. These clones are presently being used to investigate the differences in sequence between our isolates and the known strains of BK. It is hoped that this will shed light on the mechanisms of gene expression of these potentially oncogenic viruses.

In Part II, the genomes of four rotaviruses were studied. "Simian agent 11" (SA11) and "offal agent" (OA) were cell culture-adapted strains, whereas "epizootic diarrhoea of infant mice virus" (EDIM) and "infantile gastroenteritis virus" (IGV) were isolated from stool specimens. Experiments were performed to confirm the double-stranded RNA (dsRNA) nature of the SA11 genome. It ran at a characteristic density of 1.595g/ml in caesium sulphate density gradients, and was resistant to DNase and RNase at high ionic strengths, but susceptible to RNase at low ionic strength. At the start of the project few or no polyacrylamide gel pictures of the nucleic acids of these viruses had been published, although it was known they resembled reovirus in consisting of segmented double-stranded RNA. Such pictures were obtained, and molecular weight estimations made by comparison with dsRNA markers of known MW from a cytoplasmic polyhedrosis virus (CPV) from Heliothis armigera (Harley, Rubenstein, Losman and Lutton, 1977. Virology 76: 210-216). The difficulties in obtaining precise MW values for rotavirus genome segments are discussed. All four genomes consist of 11 dsRNA segments. The pattern of bands produced by PAGE is very similar, and high resolution gels are required to detect the small mobility differences between some segments. Gel systems were developed to improve on the resolution obtained in

co-electrophoresis experiments.

During attempts to culture SA11 and OA viruses in cell culture, it was observed that treatment of the cells and/or virus with versene-trypsin solution during infection gave a marked increase in virus yield. While this effect was being investigated, reports appeared on the potentiating effect of trypsin on the cell culture of previously refractory rotaviruses. We confirmed that trypsin, when present in the culture medium, greatly increased the yield of progeny SA11 virus.

2. PART I

CHARACTERIZATION OF NEW HUMAN POLYOMAVIRUS ISOLATES

3. HUMAN POLYOMAVIRUS LITERATURE REVIEW

Table 3-1. POLYOMAVIRUSES

SPECIES	NATURAL HOST
Polyoma virus (Py)	Mouse
K-papovavirus (K)	Mouse
Rabbit kidney vacuolating virus (RKV)	Rabbit
Simian virus 40 (SV40)	Rhesus macaque
Stumptailed macaque virus (STMV)	Stumptailed macaque
Simian agent 12 (SA12)	Chacma baboon
Human polyomavirus BK	Man
Human polyomavirus JC	Man

After Howley, (1980).

The polyomavirus group consists of small spherical mammalian viruses with a circular double-stranded DNA genome. Well studied examples are listed in table 3-1. Most polyomaviruses were initially isolated from the urinary tract of their respective hosts. Exceptions include JC virus, which was isolated from human brain tissue, and the murine K virus which replicates in pulmonary endothelial cells. The polyomavirus genus is part of the papovavirus family, which also includes the papillomaviruses responsible for benign warts. The two groups may be distinguished by the larger size of the papillomaviruses. Their virions are 52-55nm in diameter compared with 40-45nm for polyomaviruses, and the genome is about 5Md compared with around 3.5Md for polyomavirus. It has proved impossible so far to propagate papillomaviruses in cell culture, whereas most polyomaviruses can be adapted to cell culture, albeit with low efficiency in some cases. All polyomaviruses are similar in structure, consisting of 72 capsomeres arranged in icosahedral symmetry. 88% of the virus particle is protein, the remainder being DNA, (Howley, 1980).

There is some confusion in the literature over nomenclature. Many authors refer to this group as "papovaviruses", which should perhaps be reserved for the family as a whole. The more specific name "polyomavirus" for the group can lead to confusion with the murine virus, "polyoma virus", after which the group was named. Nevertheless, the name "polyomavirus" will be used throughout this work.

Both the molecular biology and the mechanisms of replication of several polyomaviruses have been studied intensively in recent years. The entire nucleotide sequences of SV40 and several BK strains have now been published, enabling a comparison in minute detail to be made between the genomes of these related viruses, in the hope, ultimately, of understanding the detailed relationship between nucleotide sequence and phenotypic expression in a eukaryotic system, and in particular the mechanism of cell transformation by tumour viruses.

SV40 has undergone the most intensive study of all the papovaviruses, and has become the model organism for studying all aspects of DNA virus morphology, genetics, biochemistry, oncology, and molecular biology. Perhaps most importantly of all, SV40 has been used to study the mechanisms of eukaryotic gene expression and regulation in general.

The initial stimulus for all this effort was possibly the discovery of its role in eukaryotic cell transformation and its ability to induce tumours in laboratory animals, at a time when investigations into the causes of cancer were gaining world attention (and liberal funding!). It was also easy to work with, growing well in cell culture.

A review of the vast amount of literature on SV40 would leave little room for anything else, so attention will be focussed

upon the BK-like human polyomaviruses. However, SV40 is the reference organism for practically all investigations into the other polyomaviruses, and will feature in comparisons of their properties (Lebowitz and Weissman, 1980).

3.1. ISOLATION OF HUMAN POLYOMAVIRUSES

3.1.1. Prototype BK Virus

The isolation of the first human polyomavirus, from a patient B.K., was made by Gardner et al. (Gardner et al., 1971) in 1970 at St. Mary's Hospital, London. Virus particles were detected by electron microscopy in the urine of the patient 3 months after he underwent a kidney and ureter transplant operation and was placed on immunosuppressive drug therapy. The transplanted ureter became blocked and showed signs of rejection 15 weeks after implantation, and was removed. At this time many epithelial cells bearing basophilic inclusions were shed into the urine, but their numbers gradually diminished to zero by 16 days post operation. Virus-like particles were also detected in the nuclei of many epithelial cells bordering the lumen of the exised donor ureter. There was a marked and progressive increase in BK virus antibody titre in the patient's serum at the time of this rejection episode, continuing for 3 months. Antibody titre was estimated as a function of complement fixation (CF), haemagglutination inhibition (HI), and immune electron microscopy. Virus excretion continued for only about one month after removal of the donor ureter. Virus from urine specimens taken later than two weeks after this second operation, when examined by electron microscopy, was found to be

associated with antibody. Early isolates of virus were successfully propagated in Vero cells, cultured in vitro, and all the BK "prototype" virus used in subsequent detailed characterizations originated from these lines.

For the purpose of screening patients for BK virus infection, electron microscopy (EM) examination of urine samples was found to be more sensitive than a cytological investigation of epithelial cells shed in the urine (Coleman et al., 1973). EM was too laborious for a large number of samples. Maintaining cell cultures inoculated with urine extracts for up to 8 months was also prohibitive on time and space, and not all extracts containing virus particles could be cultured. Cytology was thus the recommended primary screening procedure.

Since the initial isolation of BK virus, Dr. Gardner has identified 89 patients actively infected with polyomavirus, but only managed to culture BK-like or JC-like virus from 59 of these, or 66% (1981, Personal communication). The initial incubation period required was sometimes prolonged (7 months in the case of one JC virus strain!), which indicated that virus reproduction was being repressed in some way. A number of factors can affect productive viral infection, including the presence of antibody, defective particles, and the number of particles present in the urine originally. However, the development of "cell culture-adapted" strains seems to indicate genetic modifications which increase replication efficiency.

3.1.2. Isolation of Other Human Polyomaviruses

Table 3-2. ISOLATION OF CULTIVABLE HUMAN POLYOMAVIRUS STRAINS

VIRUS	SOURCE	CELLS USED	REFERENCE
BK(WT)	Urine, renal tr.	VERO	Gardner et al., 1971
JC	Brain, PML	PHFG	Padgett et al., 1971
GS	Urine, renal tr.	HEL	Wright et al., 1976
DW	Urine, renal tr.		Wright et al., 1976
MM	Brain, WAS	HEF/VERO	Takemoto et al., 1974
RF	Urine, renal tr.	HEK	Gardner, 1973
MG	Urine, renal tr.	HEF	Lecatsas and Prozesky, 1975
JM	Urine, WAS		Howley et al., 1975a
BK(dun)	Urine, WAS		Howley et al., 1975a; Manaker et al., 1979
JL	Urine,	HEF	Pauw and Choufoer, 1978

PHFG = Primary human foetal glial cells.
HEL = Human embryonic lung cells.
HEF = Human embryonic kidney cells.
PML = Progressive multifocal leucoencephalopathy.
tr. = Transplant recipient.
WAS = Wiscott-Aldrich syndrome.

3.1.3. JC Virus

Padgett et al., (1971) described the first successful cultivation of virus from brain tissues of a patient suffering from a rare demyelinating disease known as progressive multifocal leucoencephalopathy (PML). Papovavirus-like particles had previously been observed by electron-microscopic examination of brain cells from PML patients, but no biological activity was demonstrated. Brain extracts were inoculated onto cultures of PHFG cells containing astrocytes, or astrocytes and spongioblasts. Both cell types showed CPE in 10-12 days, although spongioblasts were more severely affected. JC virus failed to grow in primary mouse embryo, BSC-1, VERO or primary

African green monkey cells, thus distinguishing it from SV40 or BK, for which these cells are normally permissive. During the following 5 years, JC virus was isolated exclusively from patients with PML (Cathala et al., 1973; Field et al., 1974; Gardner, 1977a; Padgett et al., 1976; Weiner et al., 1973). Recently, Gardner has twice isolated JC from the urine of renal allograft patients, neither of whom showed any of the neurological symptoms of PML (Gardner, 1977a). JC has also been isolated from the urine of a woman in the third trimester of an apparently normal pregnancy (Coleman et al., 1977).

3.1.4. GS Virus

GS virus was isolated by Gardner in London. Wright et al., (1976) showed that GS and BK were serologically identical, and that the tryptic digests of the two major virion proteins of GS and BK were indistinguishable. A small region of non-homology between the genomes of the two viruses has since been identified (Pater et al., 1979).

3.1.5. DW Virus

DW was isolated during a screening of 25 kidney transplant patients at the University of Illinois Medical Centre (Wright et al., 1976). Like GS, it was indistinguishable from BK antigenically and by tryptic digest analysis. No analysis of the genome has been reported, to my knowledge.

3.1.6. MM Virus

The isolation of MM was described by Takemoto et al. (1974) from a brain tumour identified histologically as a reticulum cell sarcoma. The patient was an 11-year old boy with WAS, who was being studied at the NIH Clinical Centre. Co-cultivation of minced tumour fragments with WI-38 (human) and VERO (African green monkey kidney) cells failed to show cytopathic changes during 2 month's observation. Human foetal brain cultures containing tumour fragments developed characteristic CPE after 6 weeks. Also, clarified supernatants of all three culture types caused vacuolization and cell destruction 3 weeks after inoculation into fresh foetal brain cell cultures. This was found to be caused by the same virus, which was serologically and morphologically identical to BK.

The MM genome has since been extensively characterized, (Yang and Wu, 1978c) and finally sequenced (Yang and Wu, 1979c), pinpointing the differences between this strain and BK(WT).

3.1.7. RF Virus

RF was isolated from the urine of a renal transplant recipient (Dougherty and DiStephano, 1974). RF was also immunologically indistinguishable from BK prototype, but was more oncogenic when injected into newborn hamsters (Dougherty, 1976). The peptide patterns of two major capsid proteins were slightly different to BK (Wright et al., 1976). Although DNA reassociation saturation tests showed an 88% homology to BK, and 29% homology to SV40 (Miao and Dougherty, 1977) the arrangement of the genome has recently been found to be unique (Pater et

al., 1980). (See section 3.2.5).

3.1.8. MG Virus

A polyomavirus which differed serologically from BK and other previously described strains was isolated in South Africa by Lecatsas and Prozesky (1974), from the urine of a renal allograft patient. Seeding onto primary human foetal fibroblasts produced a CPE after 30 days. Crystalline aggregates of virus particles in the nucleus were extremely common, and differed from those described for BK (Lecatsas et al., 1974) and JC (Zu Rhein, 1969). MG also differed from BK or JC by HAI testing (Lecatsas et al., 1976a) and in minor detail by tryptic digest mapping of the two major virion proteins, VP1 and VP3 (Wright et al., 1976).

The MG genome has been cloned recently into a bacterial plasmid, allowing full characterization (Pater et al., 1981).

3.1.9. JM Virus

Isolated by Takemoto et al. (unpublished observation) from the urine of a patient with Wiskott-Aldridge syndrome, JM was found to be identical to BK(WT) by all tests, including restriction endonuclease analysis (Howley et al., 1975a).

3.1.10. BK (Dun) Strain

The Dunlop strain of BK was isolated by Takemoto in similar circumstances to JM (see above, and Manaker et al., 1979). Its only significant difference from BK lies in a 43 base sequence

in the BK(WT) genome at 0.70 map units in a non-coding region which is absent in BK(Dun). However, this strain is far less susceptible to the development of defective genomes during cell passage, and so was used as a basis for sequencing the entire BK genome (Seif et al., 1979). It is remarkable that an apparently inessential difference can cause such a change in biological properties. There is as yet no explanation for this.

3.1.11. JL Virus

This strain was distinguished from BK by its different restriction endonuclease pattern (Pauw and Choufoer, 1978). It was isolated from the urine of a 24 year old female patient in 1975 who had undergone bone marrow transplantation for acute myeloid leukemia. The virus had the same antigenic properties as BK and was less oncogenic in newborn hamsters than RF.

3.2. PROPERTIES OF HUMAN POLYOMAVIRUSES

3.2.1. Origin and Distribution

Despite the fact that no disease has ever been conclusively associated with BK, it is almost certainly a human virus. The BK-type viruses have only been isolated from human beings, and about 70% of adult populations studied possess specific anti-BKV antibodies. BK has never been isolated from immunocompetent humans, but only from patients undergoing immunosuppressive therapy, or receiving drugs with immunosuppressive side-effects, or with genetically determined insufficiency (WAS) (Blaese et al., 1968).

Lecatsas detected polyomavirus particles in the urine of 37.5% of 96 patients who had undergone renal transplantation (Lecatsas and Van Wyk, 1978). Excretion did not appear to be continuous, and variable quantities were seen, even in specimens from the same patient taken at different times after the start of therapy. The mechanism of activation is still not understood. All except one of the polyoma viruses detected by Lecatsas were of the typical BK serotype. The exception was named MG virus, and was also the only isolate that was successfully adapted to cell culture by inoculating human foetal fibroblast cells with urine extracts. It would appear that BK-type strains require more persistence to grow in culture than the six-week period routinely applied with these isolates, as Dr. Gardner successfully grew two strains supplied by Dr. Lecatsas (Gardner, 1981, personal communication). Also, fibroblasts may not be as permissive to viral growth as HEK cells used in other laboratories (Martin and Chou, 1975).

Further evidence for the human origins of BK is its preferential growth in human cells in vitro (Dhar et al., 1978). Most papers published on human polyomaviruses have dealt with strains which had been successfully adapted to culture. However, these represent only a proportion of isolates, and many may not be so amenable to propagation in this fashion.

3.2.2. Growth Characteristics in Cell Culture

BK has been grown in a number of cell lines. It was initially propagated in VERO cells, a continuous line derived from African green monkey kidney, but the virus had a strong tendency to develop defective genomes with deletion mutations in

these cells, possibly because growth was inefficient, so that high MOI's were required (Howley et al., 1975b). The most suitable cell type tried to date has been human embryonic kidney (HEK), either primary or secondary (Seehafer et al., 1978; Purchio and Fareed, 1979; Lecatsas et al., 1974; Gardner et al., 1971). A successful plaque assay has been developed using third to fifth passage level primary HEK cells (Seehafer et al., 1978). The virus has also been propagated in other human embryonic cell types, including fibroblasts, muscle and lung (Lecatsas et al., 1974).

Growth, even under the most favourable conditions, was slow compared with other polyomaviruses such as SV40, with CPE developing only after 7 days at best. Great care needed to be taken, at least with the wild type strain, to avoid the development of heterogeneous viral DNA species, by keeping the MOI low (Howley et al., 1975b).

The virus has a tendency in some cell lines to remain strongly associated with the cell membrane, even after the development of extensive cytopathology. This explains why these cell types were found unsuitable for plaque assays of BK (Seehafer et al., 1978), which rely on the release of free virus particles from infected cells to infect neighbouring cells, in order to form a plaque which can be picked out by staining.

3.2.3. Cell Culture of Human Polyomaviruses

One of the most striking features of the human polyomaviruses is that many of them grow poorly or not at all in cell culture (Van der Noordaa, 1976; Taguchi et al., 1980). This may be a reflection of the fact that free virus is very rarely detected

in nature, and has never been detected in normal people. This could mean that continuous virus production is not critical to the survival of the polyomavirus entity, or to be more specific, its genome, and that cryptic existence of the DNA within the cell is the normal state. One could envisage the virus waging eternal war against the body's defence mechanisms, but considering that most people produce anti-BK antibodies and never show other signs of infection it is surprizing that this war has not already been lost if survival depended upon productive virus infection. Since the virus has difficulties growing in the "de-repressed" environment of in vitro cell culture, the possibility that human polyomavirus normally exists in some state other than as infective virus particles needs to be considered.

At the experimental level, more easily definable reasons may be proposed for the failure of some isolates to replicate in culture.

(1) The virus may have been incompatible with the cell type chosen. Coding for so few genes, polyomaviruses are dependent on host enzymes for replication, transcription and translation. Conceivably, viral DNA is not readily recognized by the replicative machinery of some cells. However, the basis of host specificity in polyomaviruses is not understood.

(2) The virus may have been inactivated by antibody present in the urine. Some electronmicrographs of urine pellets appear to show the presence of antibody coating the polyomavirus particles (Reese et al., 1975; Gardner et al., 1971; Lecatsas et al., 1973). Gardner et al. (1973) demonstrated a dramatic increase in anti-BK antibody in the patient after renal transplantation,

which supports the idea that the shedding of BK virus soon elicits an antibody response, so that virus particles in the urine become increasingly associated with antibody molecules. Normal human urine contains small amounts of immunoglobulins of various classes (Reese et al., 1975). Specifically anti-BK antibodies were found in low titre in a proportion of normal urines, but especially in urine from heavily immunosuppressed adult patients. Antibodies in urine could have arisen from glomerular leakage of serum antibodies or from local synthesis. None of the sIgA class synthesized in the urinary tract were found to be BK specific, however (Reese et al., 1975). Most specific antibodies seem to be of the IgG class, which is the predominant class present in urine. This does not rule out the possibility that specific antibodies result from the activation and multiplication of BK in the urinary tract of immunosuppressed patients. It may be interesting to note that immunosuppressive treatment does not prevent the production of these antibodies.

(3) Other non-specific virus inhibitors may have been present in the urine.

(4) The virus particles may for some reason be genuinely non-viable, either due to faulty assembly, or to degradation during and/or subsequent to excretion (Lecatsas and Prozesky, 1975).

To test whether the viral genome is competent to replicate itself and fully express viral functions, the infectivity of the naked viral nucleic acid must be tested. Several methods have been described for infecting cells with purified viral DNA, but I have been unable to find any reports of these methods being

used with BK virus isolates which had not first been successfully cell passaged. Howley et al. (1975b) used the DEAE-dextran method of McCutchan and Pagano (1968) to investigate the infectivity of a heterogeneous population of viral DNA molecules resulting from serial cell passage of the prototype BK(WT) strain. It was found that only the largest DNA species was infective, the other smaller molecules presumably being deletion mutants, sustained by high MOI's.

A number of viable deletion mutants have also been isolated (Yang and Wu, 1978c, Pater et al., 1980). For example, RF virus has been found recently to consist of two complementary deletion mutants, one with a 50% deletion of the late region, the other with about a 40% deletion of the early region. Both species are required for active infection (Pater et al., 1980).

Some isolates such as the BK(Dun) strain, which has only very minor differences in DNA sequence to the BK prototype, are not subject to the development of deletion mutants by cell passage. Nevertheless, because so many BK strains are so liable to develop mutants with altered biological properties when cultured in vitro, it is important to study the virus in its original state. Our investigations thus centred on DNA obtained from virus extracted directly from the urine of renal transplant recipients, in order to determine whether there were any important differences between such material and culture-adapted strains.

3.2.4. Transformation and Tumour Induction

A number of cell types, both animal and human, have been successfully transformed with BK virus (Van der Noordaa, 1976).

A few cells may survive infection with virus, and start to exhibit properties typical of transformed cells (see below). However, the state of the viral DNA within the transformed cell appears to be quite variable. Depending on the cell line used, the viral strain used, the culture history of the transformed line, and the laboratory performing the work, the viral DNA either becomes integrated into the cell genome, or remains in an episomal state, and portions of the genome are sometimes lost. Virus inocula containing defective virions with genetic deletions tend to produce more fully transformed cells than plaque purified stocks (Seehafer et al., 1979). Transformed cell cultures containing non-integrated BK DNA tend to shed virus continuously, and are cured in some cases by treatment with anti-BK antiserum (Takemoto et al., 1979b).

Human cells may also be transformed with BK. Human foetal brain cells, consisting mainly of spongioblasts and astrocytes, and transformed with BK, contained only non-integrated BK DNA and began to shed BK virus in the absence of BKV antiserum. They were T- and V-antigen negative when antiserum was present (Takemoto et al., 1979b). No defective genomes were detected. Transformed secondary human embryonic kidney cells also contained episomal DNA, and shed virus, but were T-antigen positive (Purchio and Fareed, 1979). This may have been due to productive virus infection, as the culture medium did not contain BK antiserum. Clones were obtained from these cultures that contained no detectable free DNA, were positive for large T- and small t-antigens, and presumably had BK early sequences integrated into the cell DNA. These cells were not as fully transformed as other papovavirus-transformed animal cells, in that they grew well in medium containing 2% serum, but were

unable to form colonies in soft agar or cause tumours in athymic nude mice. This contrasts with studies of SV40-transformed rat cells which associated small t-antigen production with the ability of transformed cells to grow in semi-solid medium (Purchio and Fareed, 1979).

Transformation of hamster cells (Howley and Martin, 1977), or tumour induction in hamsters by subcutaneous inoculation of virus (Ter Schegget et al., 1980; Dougherty, 1976) produced cells which contained either fully integrated BK sequences (Howley and Martin, 1977) or episomal DNA (Ter Schegget et al., 1980; Dougherty, 1976). Newly transformed cells with integrated DNA had the whole BK genome equally represented (Howley and Martin, 1977), but older lines seemed to lose late region sequences (Botchan et al., 1974). The episomal DNA in transformed human foetal brain cells remained indistinguishable from the prototype stock (Takemoto et al., 1979b), whereas that in virus-induced hamster tumour cells developed a number of free BK DNA molecules all lacking part of the late region of the genome (Ter Schegget et al., 1980), despite the fact that the original stock contained no detectable defective DNA.

Similar results have been obtained with SV40, JC, and the BK variants, such as RF. The degree and state of transformation achieved by polyomaviruses depend on the presence of defective genomes. From work with SV40, which has been more extensively studied, the T antigens seem to play a critical role in transformation, and deletion mutations affecting these proteins have reduced transforming ability (Sleigh et al., 1978; Lewis and Martin, 1979; Bouck et al., 1978; but see Israel et al., 1979). Mutations elsewhere in the genome seem to enhance transformation, if anything. The exact role of the T antigens

is still under intensive study (Lai et al., 1979; Lane and Hoeffler, 1980; Lowy et al., 1980). It would appear, for instance, that little-t antigen is not essential for transformation, since strains of BK which apparently lack this antigen, such as the BK(MM) strain, are still able to transform cells with comparable efficiency to other strains (Yang and Wu, 1980; Seif et al., 1979).

A recent report (Watanabe et al., 1979) described a mutant of BK with a deletion at about 0.72mu which specifically induced insulinomas in hamsters, whereas a wild-type BK strain produced brain tumours and osteosarcomas in these animals. This deletion lay near the origin of late region transcription.

This type of result raises the question of how mutations in sequences coding for late mRNA (or its control region) can affect the specific types of tumour which develop in infected animals (Uchida et al., 1979). This is of key interest in the quest to understand transformation mechanisms.

3.2.4.1. On the Nature of Tumour (T) Antigens

Early in the lytic cycle of productive BK infection, before the onset of DNA replication, at least two proteins can be detected by immunoprecipitation in the cell nuclei (Takemoto and Mullarkey, 1973). The largest is known as large T antigen, and corresponds to the SV40 large T antigen, with a size of between 86,000 and 97,000d. Both proteins are phosphorylated (Farrell et al., 1978).

BK infected cells also produce a small t antigen of molecular weight 17,000, which is also precipitable with BK or SV40 T antisera (Simmons and Martin, 1978). Small t and large T

proteins have amino acid sequences in common, resulting from the common RNA sequence coding for both, which is spliced differently to form one or the other mRNA (see below).

The functions of these early gene products have not been fully elucidated, despite their apparently important role in cell transformation. They are presumably involved in the initiation of viral DNA synthesis and the switch to late gene transcription for virion synthesis and cell lysis (Howley, 1980). SV40 DNA replication requires large T antigen, and takes 15min to complete (Lebowitz and Weissman, 1980). Large T antigen is located in the nucleus of transformed cells, but outside the nucleolus. It is known to bind to the origin of DNA replication. Little t antigen does not bind DNA, does not affect the lytic cycle of viral reproduction, and apparently promotes, but is not essential for, transformation (Lebowitz and Weissman, 1980). The little t antigens of both SV40 and BK contain sequences of the type Cys X Cys X X Cys which also occur in FSH, LH, TSH and chorionic gonadotrophin. It has been speculated that t antigen may act as a "false hormone" (Lebowitz and Weissman, 1980; Seif et al., 1979).

BK T antigen can complement an early mutant of SV40 (tsA58) with a temperature sensitive T antigen, allowing it to replicate at a non-permissive temperature (Mason and Takemoto, 1976; Lai et al., 1979) This supports the relatedness between the functions of the two proteins. BK T antigen is probably responsible for the "helper" function of BK virus which, like SV40, allows adenovirus 2 to overcome its normal host range restriction and replicate in AGMK cells (Bradley and Dougherty, 1978; Crawford et al., 1978; Miyamura and Takemoto, 1979).

3.2.5. Is BK DNA Present in Human Tissue?

There are conflicting reports about the presence of BK DNA in normal and neoplastic human tissue. Fiori and DiMajorca (1976) detected DNA sequences homologous to BK DNA in 5 out of 12 human tumour tissues. Israel et al. (1978), in contrast, could detect no hybridizable sequences to BK in any of 5 tumours and 16 tumour cell lines examined. Wold et al. (1978) re-examined three of the human malignant cell lines reported positive by Fiori and Di Majorca, as well as 5 other such lines, 166 human tumours, and a variety of normal human tissues. No sequences which hybridized to a BK DNA probe were detected. This negative result is supported by the general failure to detect BK virus or T antigen in any normal human tissues or tumours (Israel et al., 1978). Similar results have been reported with other polyomaviruses (Botchan et al., 1976; Israel et al., 1979; Wold et al., 1980).

These examples illustrate the complexity of the transformed state and of the role of BK viruses in causing it. The possible role of BK viruses in human cancer is thus still obscure, as is the mode of existence of the virus and/or its DNA in nature, which have thus far remained undetected unless induced by immunosuppressed conditions. Its presence is inferred from the detection of BK specific antibodies in a high proportion of the human population.

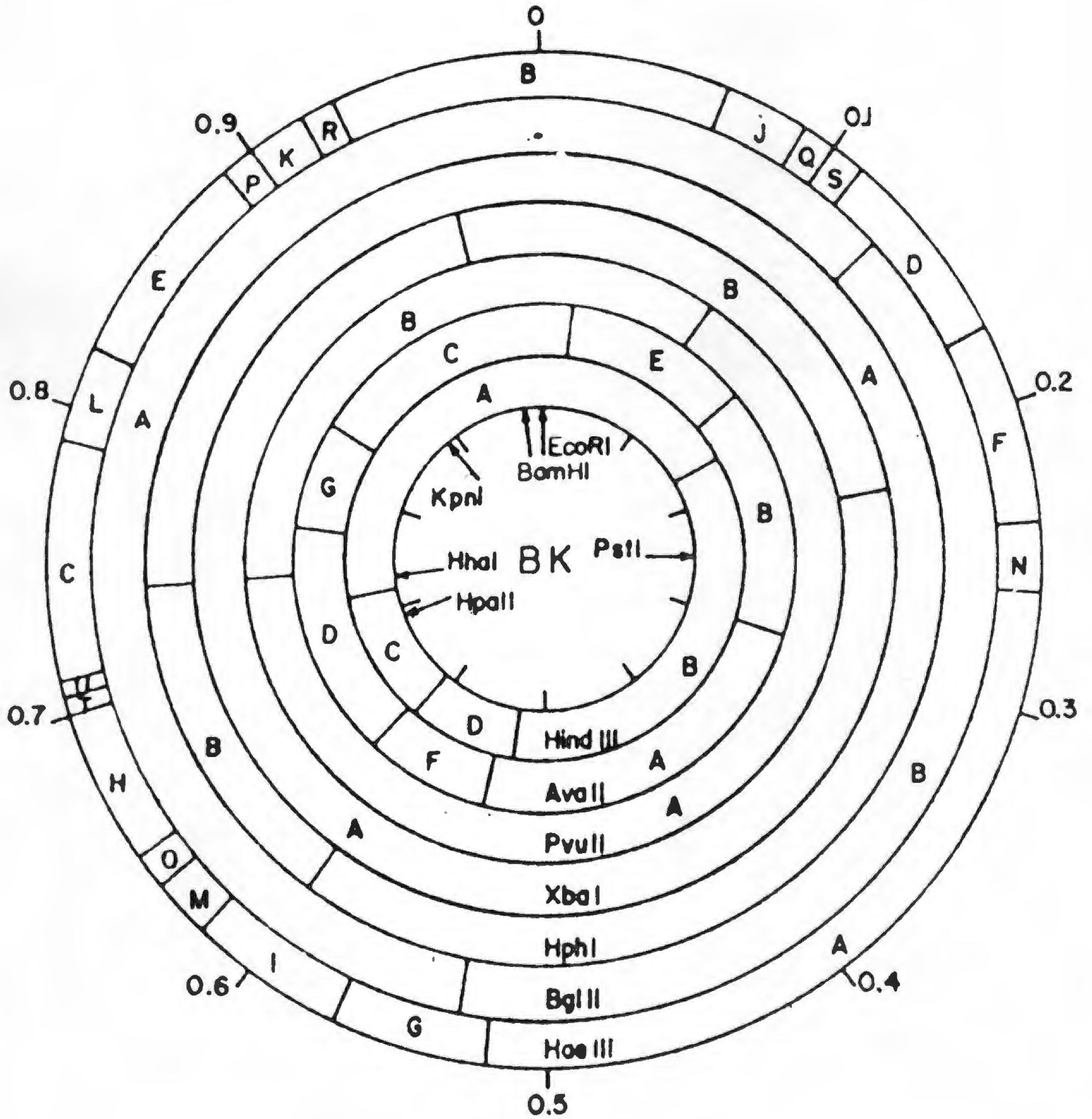


Figure 3-1. Restriction Endonuclease Map of BK(WT) DNA

The unique EcoRI site has been arbitrarily designated as the 0 map position (Howley, 1980).

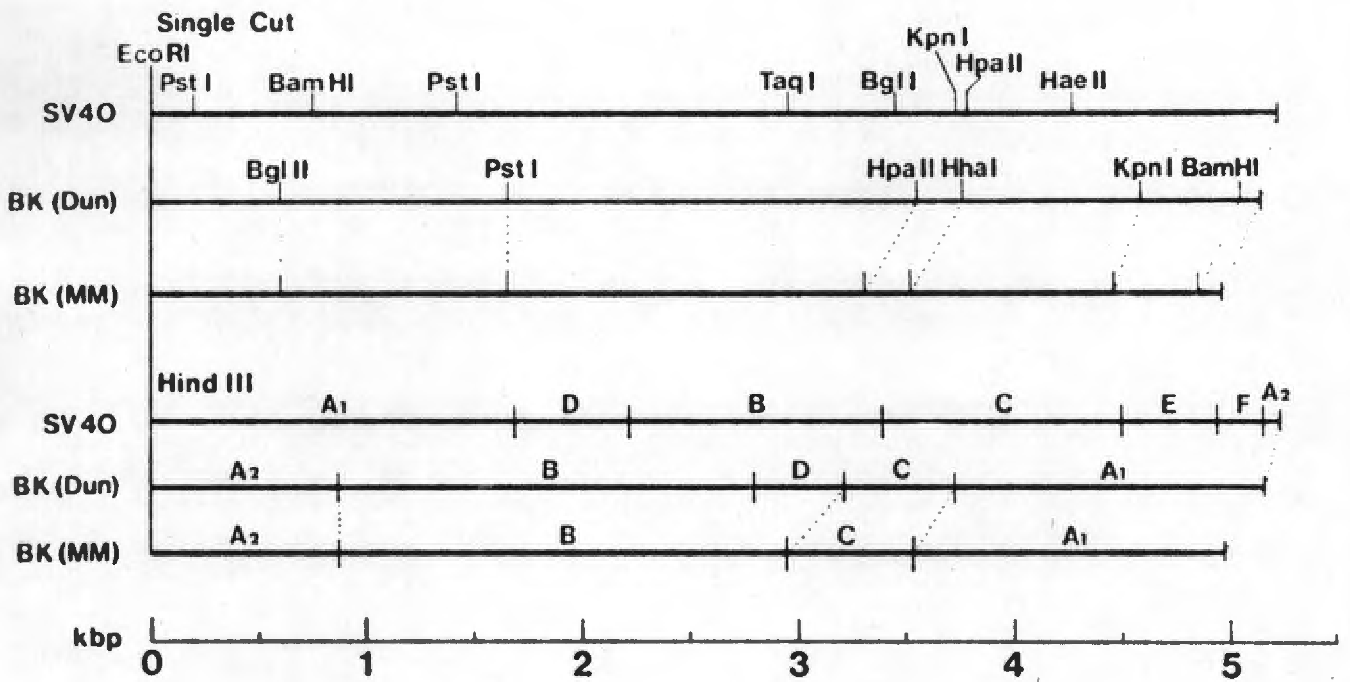


Figure 3-2. Comparison of Restriction Maps of SV40, BK(Dun) and BK(MM).

The maps have been opened and aligned at the unique EcoRI site in each molecule.

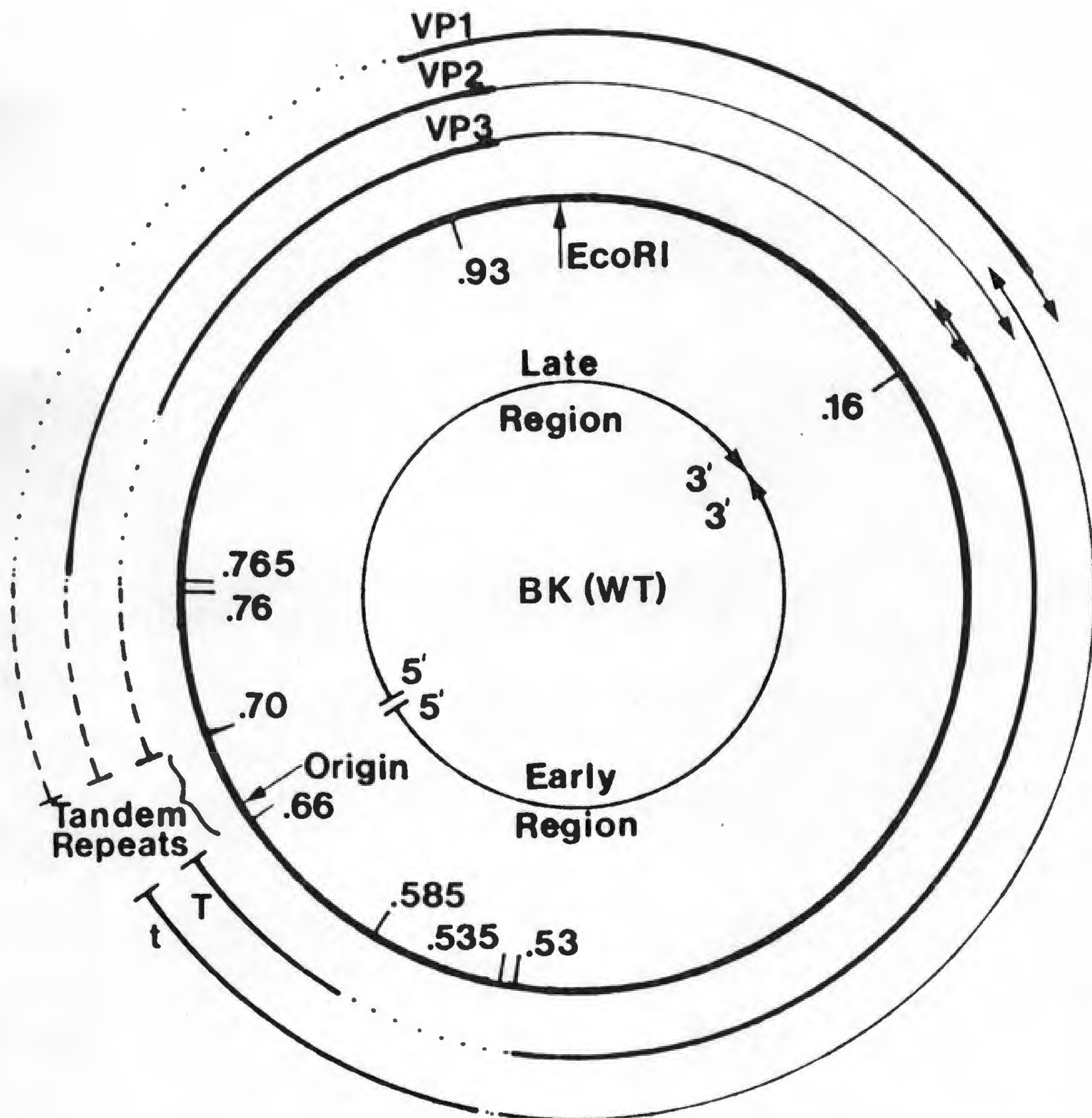


Figure 3-3. Genetic Organization of BKV DNA

Map positions are oriented clockwise from the EcoRI cleavage site at 1.0/0.0. The origin of DNA replication is shown at 0.67 map units (mu) (Howley et al., 1979a). The stable mRNAs are indicated in the outer arcs. The early mRNAs have regions from 0.585 to 0.53 (large T antigen) and from 0.535 to 0.53 (small t antigen) spliced out (dotted lines). The stable late mRNA species map clockwise from near the origin of replication. Dashed lines represent late leader sequences initiating between 0.70 and 0.76, dotted lines are spliced out to form the late 16S RNAs coding for VP1 and 19S RNAs coding for VP2 and VP3. Coding regions are shown as heavy lines. (Howley, 1980; Lebowitz and Weissman, 1980).

3.3. THE BK VIRUS GENOME

The BK virus genome consists of circular double-stranded DNA. Extracted DNA consists mainly of covalently closed supercoiled DNA (CCC, form I) with negative superhelical turns, and a proportion of nicked open circular (NOC, form II) DNA resulting from the nicking of one of the strands, which allows relaxation of the supercoiling to occur. SV40 DNA similarly, has been shown to be an underwound supercoiled helix with ±26 negative turns. DNA from infected cells and virions extracted under gentle non-deproteinizing conditions is relaxed, due to association of histone molecules forming nucleosomes as in cellular eukaryotic DNA (Lebowitz and Weissman, 1980; Meneguzzi et al., 1978b). The BK prototype is slightly smaller than SV40, being 5,196 base pairs (bp) compared with 5,227bp (3.6Md) (Fiers et al., 1978; Howley et al., 1975b; Osborn et al., 1974; Yang and Wu, 1979c; Reddy et al., 1978).

SV40 and most human polyomavirus DNAs have a single EcoRI restriction site, which was arbitrarily given the zero position in restriction endonuclease maps. Comprehensive restriction maps of SV40 (Danna et al., 1973; Yang et al., 1975), BK(WT) (Howley et al., 1975a; Freund et al., 1979), BK(MM) (Yang and Wu, 1978c) and JC virus (Osborn et al., 1974; Martin et al., 1979) have been prepared as a prelude to sequencing the genomes (Figs. 3-1 and 3-2). Complete sequences have been published for SV40 (Fiers et al., 1978; Reddy et al., 1978), BK(Dun) (Seif et al., 1979) and BK(MM) (Yang and Wu, 1979c). The prototype BK(WT) sequence has also been determined, and compared with the BK(Dun) sequence from which it differs mainly in a 43bp deletion in the BK(Dun) genome at 0.70mu in a non-coding region (Yang and

Wu, 1979d). BK(MM) differs from the prototype in two regions, and is shorter by 233bp. A 262bp sequence between 2,745 and 3,006 of BK(WT) is deleted in BK(MM) (Yang and Wu, 1979c). This maps between 0.527 and 0.576mu on the BK(WT) genome and means that MM is lacking part of the carboxy-terminal portion of its small t antigen. This deletion is spliced out of the large T mRNA so does not affect the translation product. The region between 0.665 and 0.714mu is characterized by a series of tandem repeats, but the sizes, arrangements and sequences of these repeats differs between MM, BK(WT) and SV40 (Dhar et al., 1978; Howley et al., 1979a; Newell et al., 1978). The region thus appears to be under little evolutionary constraint.

These differences will be described further in the Discussion.

3.4. Transcription

BK transcription in productively infected cells has been much less intensively studied than in SV40, although the two seem to be very similar. Prior to the onset of DNA synthesis the two T antigen mRNAs are synthesized. From studies with SV40 the negative DNA strand codes for early mRNAs, and the positive strand for late mRNAs (Lebowitz and Weissman, 1980). The 5' ends of both map at around 0.66mu near the origin of replication, and the 3' ends of both species map at 0.16mu (Fig. 3-3). The large T antigen mRNA has an intervening sequence spliced out from 0.535 to 0.53mu, and little t mRNA from 0.585 to 0.53mu (Manaker et al., 1979). Thus the two proteins have a subset of amino acid sequences in common, which explains their partial immunological cross-reactivity. The SV40 arrangement is

similar (Lebowitz and Weissman, 1980). These antigens are also produced in certain polyomavirus-transformed cell lines.

After viral DNA replication has commenced, 16S and 19S RNA transcripts from the opposite strand mapping clockwise from 0.70 to 0.16mu are produced coding for "late" structural proteins (Manaker et al., 1979). Naturally in fully transformed cells there is no productive viral infection, and virion proteins are not synthesized (Takemoto et al., 1979b). The body sequences of the late 16S RNA map between 0.93 and 0.16mu, and the late 19S RNA between 0.765 and 0.16mu. The portion between 0.70 and 0.76mu codes for a 300 nucleotide-long leader RNA sequence. These positions are also analogous to SV40. A leader is spliced to the 16S RNA body sequences at 0.93mu, and a similar leader splices to the 19S RNA body sequences at 0.76mu (Manaker et al., 1979).

The smaller 16S late RNA codes solely for the major capsid protein VP1. The 19S RNA species contains sequences homologous to all three capsid proteins, VP1, and the two minor capsid proteins VP2 and VP3. By analogy with SV40, this RNA probably serves as the transcript for VP2 and VP3 (Howley, 1980).

Both SV40 and BK genomes possess a potential coding frame for a small protein (the "agnogene" or "protein X") which has only recently been detected (Jay et al., 1981). This protein is 66 amino acids long in BK(MM), 62 in SV40, and has 40 amino acids in common between the two species, according to DNA sequence data (Yang and Wu, 1979b). It is a type of DNA binding protein (Jay et al., 1981).

4. ANALYSIS OF BK VIRUS DNA

4.1. Restriction Endonuclease Mapping

The first major step required to sequence the DNA of a BK or other polyomavirus strain is the production of a detailed map of the genome locating the sites of cleavage of selected restriction endonucleases. The cleavage pattern characteristic of each strain also gives a preliminary indication of the relatedness between the strains. Most of the viruses antigenically indistinguishable from BK(WT) have restriction patterns similar to each other, whereas less closely related viruses have more divergent patterns. The small size of the polyomavirus genome makes restriction mapping relatively simple (Fig. 3-1).

4.2. PREPARATION OF DNA

4.2.1. Cell culture propagation

Detailed restriction mapping requires several micrograms of homogeneous, pure viral DNA. Obtaining enough DNA can present a problem. As mentioned already, all detailed characterization reported to date has been carried out on strains that have been amplified by cell culture passage. Many strains, such as JC virus, propagate very inefficiently or not at all in any cell culture system, and tend to develop significant proportions of defective genomes which are liable to confuse analysis (Miyamura _et al._, 1980; Yang and Wu, 1978c; Howley _et al._, 1975b; Pater _et_

al., 1980). Howley et al. (1975b) were forced to isolate full-length BK prototype DNA from agarose gels to use to infect cell cultures, and to keep the subsequent MOI low to prevent the reappearance of defective genomes. Yang and Wu (1978c) found that their stock of the BK(MM) strain developed two viable deletion variants, which could not be eliminated by lowering the MOI. Luckily, the short deletions only occurred in the region of one restriction site, namely, the unique HpaII site, which was lost in the mutants. The resulting patterns were thus reasonably simple to interpret. The fact that three different mutants had a deletion in practically the same site on the genome is significant. This region, around 0.695mu, is within a "highly variable" region (0.52-0.71) noted in comparisons between a number of polyomaviruses. It corresponds to a non-coding sequence spliced out of the mRNA coding for T antigen, and thus does not affect the virus-coded protein sequence directly.

4.2.2. Recombinant DNA Amplification

An alternative approach to the production of adequate quantities of viral DNA is to clone the DNA into a plasmid vector and propagate the recombinant DNA in a bacterial system. This approach could be used to clone either the entire genome intact, or else specific fragments generated by restriction enzyme digestion. Several enzymes, such as BamHI, PstI, and EcoRI, that produce a single cut in both BK and a vector such as the artificially constructed plasmid pBR322, could be used to build recombinant plasmids containing the entire BK genome.

4.2.2.1. pBR322, a Plasmid Cloning Vector

A multipurpose E. coli cloning vehicle needs to manifest the following properties:

(1) Small molecular weight - a smaller plasmid is easier to purify and characterize.

(2) Resistance genes to several antibiotics to allow for easy selection after transformation of sensitive recipients.

(3) Unique sites for a number of restriction enzymes, preferably within the resistance genes, to allow for selection of recombinants acquiring sensitivity to the relevant antibiotic.

(4) Relaxed replication and high copy number to permit amplification with chloramphenicol for the production of high yields.

(5) A lack of transposable elements to increase the stability of a selected plasmid genome, and to decrease the possibility of undesirable recA independent recombinations of drug resistance genes and/or foreign DNA sequences in vivo.

(6) Non-conjugability and a low mobilization frequency, again to improve containment.

pBR322 was developed with these principles in mind (Bolivar et al., 1977). It was derived from pBR313, which in turn derived from a ColEl-like plasmid into which had been grafted ampicillin and tetracycline resistance markers - the ampicillin transposon (TnA) from pRSF2124 (So et al., 1976) and tetracycline from pSC101 (Cohen et al., 1973). The TnA was

altered so that it was no longer transposable. In the construction of pBR322 from pBR313 the colicin immunity gene was destroyed, and the number of PstI restriction sites reduced to a single site within the *amp* gene. This permitted cloning into the PstI site with inactivation of the *amp* gene. In addition, cloning into the unique HindIII, SalI and BamHI sites inactivated the *tet* gene, permitting selection of Ap(R)Tc(S) recombinants. Recombination by blunt-end ligation into a HincII site within the *amp* gene was also possible (Bolivar *et al.*, 1977).

4.2.2.2. Containment of Recombinant DNA Molecules

A question of the possible biohazard of this operation arose, considering that BK is potentially an oncogenic virus. The current philosophy seems to be, however, that viral DNA cloned into a bacterial system is safer than that DNA on its own, either naked or packaged into viral particles. Work on the cloning of murine polyoma virus failed to show that such recombinant manipulations produced any added hazard compared with working on the intact virus or its DNA (Boll and Weissman, 1979).

Nevertheless, up to May 1981, the NIH guidelines, and the South African equivalent issued by SAGENE, still recommended the use of P2 containment facilities, an EK1 *E. coli* strain such as C600, and a plasmid vector such as pBR322 certified for use in EK2 classified strains, for recombinant work with intact tumour virus genomes (Federal Register 43: 60109-60123).

Subgenomic fragments may be handled under less stringent conditions, however, providing that the transforming region of

the genome is cut, and thus inactivated. PstI cuts BK in this region, and our isolates were shown to be sufficiently related to assume that what was initially considered to be a unique PstI site also lay within the region responsible for transformation. Both pBR322 and BK(WT) have unique sites for PstI and EcoRI. The smaller of the two fragments thus produced from pBR322 is not essential for replication, so that PstI/EcoRI digested BK may be recombined with the larger PstI/EcoRI digested pBR322 fragment.

This approach has another advantage. Because double digestion produces molecules with one EcoRI and one PstI sticky end, the pBR322 fragment cannot recircularize by itself, but first must link up with at least one other fragment. This increases the chances of obtaining hybrid plasmids, although it is still possible for two or more pBR322 molecules to link up "back-to-back". The proportion of recombinant plasmids which contain viral DNA is now dependent on the relative concentration of pBR322 and viral DNA fragments.

4.2.2.3. Theory of Recombinant Plasmid Formation

A major problem in optimizing the number of chimeric plasmids produced in a ligation reaction between fragments cut by only one restriction enzyme is the prevention of extreme concatemerisation on the one hand, and recircularization of the original plasmid vector alone on the other. The relative proportion of each type of reaction is governed by a number of factors, including the DNA concentration, and length of the various fragments. A theoretical treatment of this reaction was given by Dugaiczyk _et al._ (1975). They considered two

variables:

i, the total concentration of self-complementary single-stranded ends of a duplex linear DNA, and

j, the effective concentration of one end in the neighbourhood or volume of the other end of the same molecule.

$$j = \left(\frac{3}{2\pi l b}\right)^{3/2}$$

ends/ml....................1

where l = the contour length, b = the random coil length of a DNA molecule, which is a measure of the molecule's stiffness. A value for b of 7.17×10^{-2} μm is obtained from interpretation of sedimentation coefficients of DNAs.

j depends on the length of the DNA molecule in question, and also the ionic strength, which affects factor b. Assuming constant ionic conditions, however, b remains effectively constant.

Now,

$$i = 2N_{\bullet}M \times 10^{-3} \qquad \text{ends/ml} \ldots\ldots\ldots\ldots 2$$

where $N_{\bullet}$ = Avogadro's number, M = molar concentration of the DNA molecules.

Combining (1) and (2):

$$\frac{j}{i} = \frac{\left(\frac{3}{2\pi l b}\right)^{3/2}}{2N_{\bullet}M \times 10^{-3}} \qquad \ldots\ldots\ldots\ldots 3$$

l is proportional to MW. For example for lambda, l_{λ}=13.2μm,

and $MW=30.8x10^6$.

$$l = \frac{MW \times 13.2}{30.8 \times 10^6} \qquad \ldots\ldots\ldots\ldots\ldots\ldots\ldots 4$$

$$M = \frac{[DNA]}{MW} \qquad \ldots\ldots\ldots\ldots\ldots\ldots\ldots 5$$

Substituting,

$$\frac{j}{i} = \frac{\left(\frac{3 \times 30.8 \times 10^6}{2\pi \times 13.2 \times 7.17 \times 10^{-2} \times MW \times 10^{-8}}\right)^{3/2}}{2 \times 6.022 \times 10^{23} \times \frac{[DNA]}{MW} \times 10^{-3}}$$

$$= \frac{51.1}{[DNA]\,(MW)^{\frac{1}{2}}} \qquad \ldots\ldots\ldots\ldots\ldots\ldots\ldots 6$$

(Note: factor of 10^{-8} comes from the conversion of μm to cm for l and b).

Theoretically, when j=i there should be an equal probability of ring closure or linear concatemerization. Thus for a j/i ratio of 1,

$$[DNA] = \frac{51.1}{(MW)^{\frac{1}{2}}} \qquad \ldots\ldots\ldots\ldots\ldots\ldots\ldots 7$$

At greater DNA concentrations i>j, and linear n-mers should be preferentially formed, whereas below this concentration circularization should predominate.

In practice the relationship is not as simple as this. The stability of the reannealed hydrogen bonded "sticky ends" prior to ligation will depend on their length and base composition,

and the ionic conditions, although the circularization and linearization reactions should be equally affected in the absence of steric effects. The b value in equation 1 can be decreased by increasing the ionic strength of the medium. Thus increasing [Na^+] has a twofold effect:

(1) It will increase the stability of the hydrogen-bonded sticky ends due to the electrostatic effect, affecting linear and circular modes of condensation equally, and

(2) It will decrease the random coil segment b and thus increase j, promoting circularization.

However, the DNA ligase may be adversely affected by high concentration.

Dugaiczyk et al. (1975) found experimentally that when j was made equal to i, predominantly linear concatemers of DNA fragments were produced, whatever size DNA fragment was used as the starting monomer. To produce covalently closed circular structures it was necessary to keep the j/i ratio greater than 2. The larger the DNA fragment, the lower the DNA concentration required to maintain the same j/i ratio. Too low a DNA concentration can become experimentally inconvenient. Obviously, as initially short monomers become polymerized, there will be a greater tendency for remaining monomers to circularize, as the concentration of free ends, i, is reduced. This was observed experimentally.

One explanation for the high j/i ratio requirement found by Dugaiczyk et al. is that even the largest fragments examined were relatively short, having contour lengths only 35 times the

random segment length, which may constrain circularization reactions. Also, binding of the ligase may increase b.

These considerations become relevant when the DNA to be cloned is in severely limited quantity. The pBR322 vector (MW = 2.6Md) and the polyomavirus DNA donor (MW = 3.3Md) are both within the size range found by Dugaiczyk et al. to require a j/i ratio greater or equal to 2 for circularization.

The situation is changed when considering ligation of doubly digested fragments. Recircularization of monomers is now prohibited, because the single stranded tails at each end are not complementary, so that only oligomers made up of even numbers of fragments can circularize. In the formation of dimers, each vector fragment has a choice of forming a back-to-back dimer with another vector molecule, or hybridizing with a donor fragment. An equimolar mixture of donor and vector fragments should ligate to produce a population of dimers (initially) in the proportion of

1 vector/vector : 2 vector/donor : 1 donor/donor dimers.

Increasing the proportion of vector would decrease the proportion of donor dimers, but increase vector dimers that, if circularized, would confer the same antibiotic resistance on transformed cells as hybrid recombinants. An excess of donor would reduce vector-only recombinants, but waste donor DNA as "donor-only" plasmids which would be unable to transform the recipient cells. However, these conditions would favour recombinants containing several donor fragments per vector fragment. This may be acceptable, except that donor fragments linked in random orientations could produce somewhat confusing gel band patterns when subsequently analysed with restriction enzymes. Such a plasmid could, however, be amplified and used

as a source of DNA for a subsequent cloning using the same enzymes, which would regenerate the original fragments. Hence a much greater quantity of DNA would be available for making more useful recombinant plasmids. Unfortunately, such conditions might generate clones with intact polyomavirus genomes if the fragments had not been physically separated prior to ligation, which would contradict containment stipulations.

In conclusion, the best compromise would seem to be to use a few-fold excess of donor over vector fragments, at a j/i ratio expected to favour circularization of the donor/vector dimer. This implies as high a j/i ratio as feasable without diluting the DNA excessively, to maximize circularization of the dimer, bearing in mind that, the monomers cannot form circles. New techniques are being developed all the time to increase the chances of recombinant molecule formation. For instance, removal of phosphoryl groups from the 5' termini of vector fragments with bacterial alkaline phosphatase prevents religation of vector-only molecules (see Results, Section 5.6.1).

The best way to achieve the desired result is through sufficient practice with the technology to obtain high recombinant yields from a given quantity of DNA. Given an adequate number of transformed colonies, the required recombinant can then always be screened for post hoc.

5. RESULTS

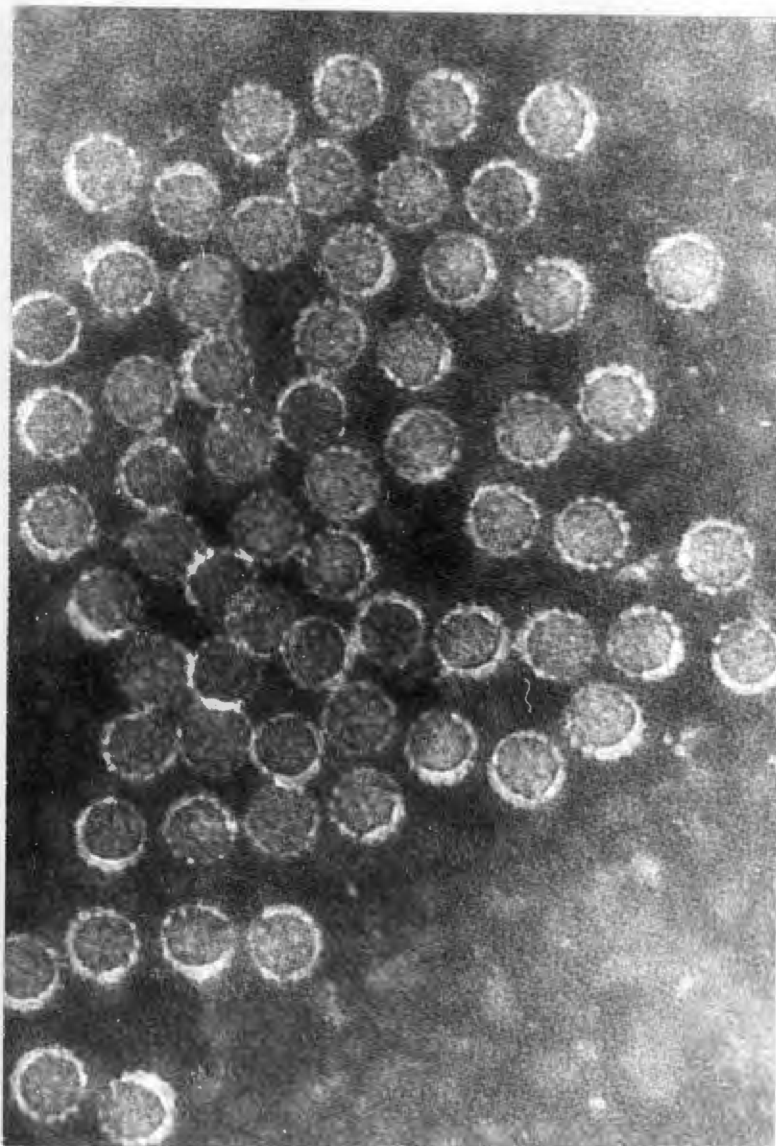


Figure 5-1. Human Polyomavirus Particles in Urine

Virus particles were negatively stained with 3% phosphotungstic acid, pH6, and examined in a Phillips EM 300 electronmicroscope (Lecatsas et al, 1976a). Photographs courtesy of Dr. Lecatsas.

5.1. ISOLATION OF BK-LIKE VIRUSES

Dr. Lecatsas has been using electron microscopy and other techniques for a number of years to look for viruses in urine specimens from kidney transplant patients (Fig. 5-1). A surprizingly high proportion of these patients shed

papovavirus-type particles at some stage after this operation (but never before), that generally exhibit the BK virus serotype. Routine attempts were made to propagate virus from urine extracts in cell culture, usually without success. The main exception to date was a papovavirus of a different serotype to BK, which adapted successfully to growth in primary foetal fibroblasts, and was named MG virus (Lecatsas and Schoub, 1976a). Routinely, cell cultures were inoculated with approximately 2 haemagglutinating units of papovavirus extracted from urine. When these failed to develop characteristic cytopathic effects (CPE) after 6 weeks, the cells were cultured once, and haemagglutination assays performed on frozen and thawed samples before discontinuing attempts at culture (Dr. Lecatsas, personal communication).

Unfortunately, this procedure did not establish categorically that these isolates were incapable of propagating in these cells (Gardner, personal communication). Nevertheless, in order to determine whether any of these isolates differed significantly in their DNA from BK virus prototype (BK(WT)), we characterized DNA purified from virus extracted directly from patient's urine. Virus particles prepared by repeated differential ultracentrifugation from urine collected from patients known to be shedding papovaviruses (Lecatsas and Van Wyk, 1978) were subjected to nucleic acid extraction and purification using a modification of Hirt's method. Aliquots of these extracts were electrophoresed through cylindrical agarose and/or polyacrylamide gels, and visualized by staining with ethidium bromide.

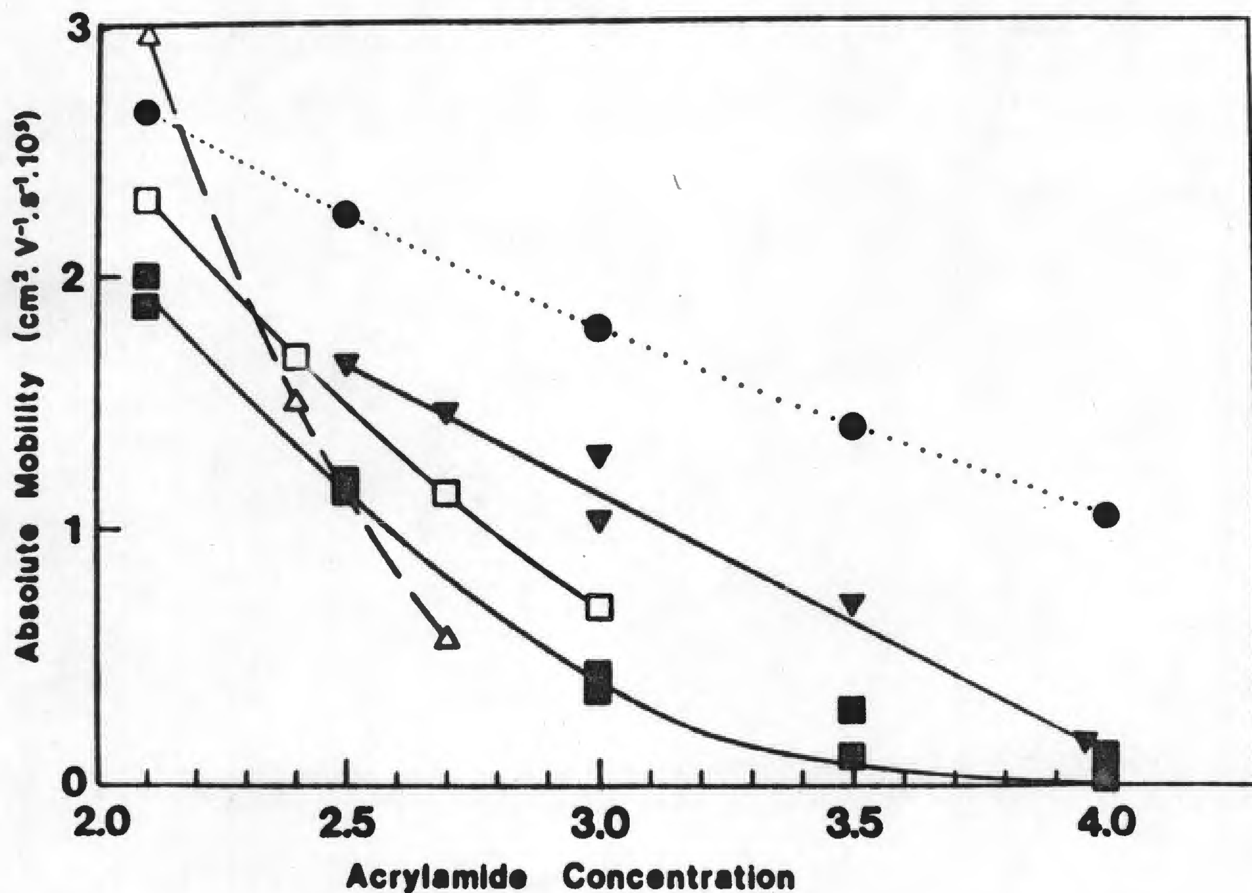
A number of the virus samples proved to have low or undetectable amounts of viral DNA present. The virus pellets

may have degraded in transit from Pretoria to our laboratory, possibly through the action of microbial contaminants present despite the addition of antibiotics, or the DNA may have been lost during extraction. It is also possible that there was insufficient virus present in the original specimen. Out of 10 samples obtained from various patients between 1976 and 1978, 6 produced bands characteristic of a small circular DNA species, 2 gave only one fuzzy band in a position characteristic of chromosomal DNA and 2 gave no, or barely detectable, bands at all (See Table 5-1).

Figure 5-2 shows ethidium bromide stained cylindrical polyacrylamide gels of DNA extracted from the urine of renal transplant recipients. Gels 1, 2 and 4 show material from patient S.N.. Purified material from the supernatant fraction obtained after NaCl precipitation of SDS-treated urine pellets was run on gels 1 and 4, and material extracted from the SDS/NaCl pellet was run on gel 2. Gel 1 shows a strong band running slower than a linear DNA marker (gel 3), and thus consisting of a circular DNA species, presumably polyomavirus CCC DNA. Material at the top of the gel could be NOC DNA having a very low mobility in this 3.5% gel. Gel 2 demonstrated the lack of detectable viral DNA in the Hirt NaCl/SDS pellet of this prep. Gel 4 had a 2.5% polyacrylamide concentration, and shows two bands running slower than linear DNA (Gel 7). The lower band was assumed to be CCC polyomavirus DNA, and the upper, NOC DNA running with greatly increased mobility through the lower concentration gel. Gels 5 and 6 show preparations from another patient (14/11/77) containing barely detectable quantities of DNA.

Figure 5-2. Detection of Human Polyomavirus DNA

Polyacrylamide gel electrophoresis of DNA extracted from urine of renal transplant recipients. Cylindrical gels were run for 5h at 125V.
A:3.5%, B:2.5% acrylamide.
(1) Patient S.N.; 10% of prep from Hirt supernatant.
(2) Patient S.N.; 50% of prep from Hirt SDS/NaCl pellet.
(3) Marker linear DNA.
(4) Patient S.N.; 7% of Hirt supernatant prep.
(5) Patient 7 (14/11/77); 7% of Hirt supernatant prep.
(6) Patient 7 (14/11/77); 100% of Hirt SDS/NaCl pellet.
(7) 0.5μg lambda DNA marker.



<u>Figure 5-3. Relationship Between Nucleic Acid Structure and Mobility in Acrylamide Gels</u>.

Absolute electrophoretic mobility of different nucleic acid species was plotted against gel concentration.

●·····● High MW linear DNA (Harley <u>et al</u>., 1980)
□——□ SV40 NOC DNA " " " "
△——△ 45S ssRNA " " " "
■——■ HPV NOC DNA (my data)
▼——▼ HPV CCC DNA " "

5.2. DETAILS OF ELECTROPHORETIC ANALYSIS

Table 5-1. DNA ANALYSIS OF POLYOMA VIRUS ISOLATES

SPECIMEN	PATIENT	HIGH MW LINEAR DNA	VIRAL DNA	EXTRACTION DATE
1		+	-	26:08:76
2	GN	+	+	18:10:76
3	K	+	+	18:10:76
4		+	+	09:09:77
5		-	-	19:10:77
6	SN	+	+	03:11:77
7		-	+-	14:11:77
8		+	-	11:01:78
9		+	+	15:03:78
10	WW	+	+	28:08:78

DNA electrophoresing with a mobility characteristic of high molecular weight (MW) linear DNA was found in most urine pellet extracts, and was assumed to have originated from micro-organisms that contaminated the viral pellets at some stage prior to DNA extraction, or to have been present in the original urine, perhaps in desquamated cells from the urinary tract.

The first pictures of covalently closed circular (CCC) DNA molecules were obtained from early isolates on polyacrylamide gels, where bands were observed migrating in the so-called "forbidden region" slower than high MW linear DNA (Harley *et al.*, 1973). These were interpreted as containing circular nucleic acid species, and were assumed to be polyomavirus DNA (Fig. 5-2).

A plot of mobility against gel concentration produced curves characteristic of CCC and NOC species (Fig. 5-3). Gel concentration effects the mobility of various DNA species differently (Harley *et al.*, 1973). Thus supercoiled DNA species of different molecular weight produce a family of curves with

similar shape, whereas linear double stranded DNA or single stranded species produce curves with different slopes. The curve for HPV NOC DNA was similar to the SV40 NOC DNA curve.

Note that the mobility values for this HPV curve were obtained from electrophoreses performed at different times, so that correlation is not as close as would be expected if the gels had been run together.

5.3. RESTRICTION ANALYSIS

5.3.1. Handling of Restriction Enzymes

Restriction enzymes had not been used previously in our laboratory, and initial trial digestions using lambda DNA showed that careful technique was required to obtain reproducible results. The most recurrent problems encountered were loss of restriction enzyme activity and degradation of the DNA sample, possibly by contaminating DNases from hands, etc.. An attempt was made to minimize loss of enzyme activity by aliquotting the enzyme in Eppendorf tubes which were kept until required at -20C or in liquid nitrogen. As the use of enzymes increased, and stocks were used up within a few months of purchase, this practice was abandoned. DNase contamination was avoided by wearing sterile plastic or rubber surgical gloves when handling the enzyme and DNA samples.

5.3.2. Isolate SN

The first preparation to give useful yields of putative viral DNA was from a patient S.N. (Fig. 5-2), and this DNA was

analysed further to confirm its identity.

This preparation contained an estimated 2-5μg of predominantly circular DNA, which was sufficient for a preliminary characterization using restriction endonucleases. The DNA was not quantitated accurately by spectroscopy for fear of losing or contaminating such small quantities in cuvettes, etc.. A rough estimation of the quantities present was obtained by comparison of ethidium bromide fluorescent intensity between bands of polyomavirus DNA and a known quantity of lambda DNA on agarose gels. A Vitatron TLD Universal Densitometer was used to give relative quantitations of the DNA peaks. The intense incident UV beam caused quenching of the ethidium bromide fluorescence, however, making quantitation impossible unless each band was exposed to UV light for the same length of time. This made alignment of the gel to give maximum fluorescent signal difficult, as the band faded while it was being aligned.

The geometry of the detection system allowed only a limited section of each band to be recorded. Use could not be made of the lateral oscillator mechanism of the machine to integrate the signal from a whole band because unacceptable overlap would have occurred in the signals from neighbouring bands that lay very close together. This integration feature is more suitable for quantitating discrete "spots" on TLC plates, etc.. Small adjustments in gel alignment caused shifts in the relative intensities of the signals from different bands, and thus quantitation results from this procedure were not reliable. The trace did allow band mobilities to be accurately measured, and revealed "shoulders" and minor bands which aided the interpretation of coelectrophoresed samples.

The restriction endonucleases EcoRI, HindIII and BamHI were

used to digest aliquots of SN DNA, using conditions that had been shown previously to digest 1μg of lambda DNA to completion. The digestions were run on 1% agarose cylindrical gels, in parallel with lambda digests as controls (Fig. 5-4). Lanes 1-3 each contained 3μg of lambda DNA marker digested for 3h with HindIII, BamHI, or EcoRI. Lane 4 contained 1μg of undigested lambda DNA, and lane 5, undigested SN DNA (1μl). Lanes 6-8 each contained 3μl of the SN DNA preparation digested with the same quantity of HindIII, BamHI or EcoRI as the lambda controls.

EcoRI (lane 8) and BamHI (lane 7) both cut the circular SN DNA once, producing a linear full-length molecule with an electrophoretic mobility, under the conditions of this experiment, intermediate between those of the CCC and NOC forms. It should be noted that BamHI only achieved a partial digestion, so that traces of the uncut circular species were still present. Comparison with the lane containing undigested SN DNA (lane 5) showed the relative increase in intensity of the linear band, which was only just discernible in the undigested prep.

HindIII made three cuts. Two bands are clearly visible in lane 6, and a third, faster-moving band was observed at a position indicated by the arrow (Fig. 5-4). This third band was demonstrated more clearly on later runs using acrylamide gels.

The lambda digests shown in lanes 1,2 and 3 demonstrate that digestion was complete using this substrate. The incompleteness of the BamHI digestion of SN DNA may thus be due either to an inhibitory contaminant present in the SN DNA prep which inactivated the enzyme, or to a proportion of the SN DNA population being resistant to BamHI digestion. These possibilities were not investigated because of the limited quantities of DNA available. Martin _et al_. (1979) encountered

partial resistance to BamHI digestion in their analysis of JC virus DNA, which they attributed to the heterogeneity of the DNA. This heterogeneity was due to the effect of cell passage. Our DNA may have become partially modified chemically in the urine to make it resistant to digestion, but it cannot be ruled out that it may be heterogeneous in sequence, although this is unlikely. BamHI was in general less reliable than the other enzymes used at this time, even using lambda as substrate.

Only very approximate MW estimates may be obtained from this experiment because:

(i) There were no lambda digest fragments of suitable size to cover the range of MW's of the HindIII SN digest (the smallest lambda-HindIII fragment was not visible on this gel).

(ii) Fragment mobilities of preparations run on different gels, even simultaneously in the same apparatus, tend to be variable.

More accurate MW assessments were postponed until a slab gel system had been established, and more suitable markers obtained.

Nevertheless, the digestion pattern and MW estimates obtained here indicated that our SN isolate was closely related to BK virus. The presence of only three HindIII fragments suggested at first that our isolate was more closely related to the BK(MM) strain, which gave a similar pattern of three fragments with HindIII.

5.3.3. Isolate WW

DNA extracted from a virus-enriched urine preparation from this patient was sufficiently pure and homogeneous to allow more detailed characterization. Two preparations were received from

Dr. Lecatsas. One was a crude pellet that had undergone limited differential centrifugation. No DNA was obtained from this prep. The other had been further purified by filtration through Millipore filters, and yielded approximately 10μg of DNA. A preliminary digestion with HindIII gave a pattern very similar to SN on electrophoresis. In order to obtain a more accurate estimate of the MW's of these fragments, the digested DNA was compared with a set of markers by co-electrophoresis through an acrylamide slab gel. The markers used were HindIII digests of lambda and PM2 DNA. (Fig. 5-5). Lane 2 contained a mixture of HindIII digests of WW, lambda and PM2 DNAs, that were run separately in lanes 1, 3 and 4. The top band in lane 1 contained contaminating high MW linear DNA resistant to HindIII digestion. Band mobilities were measured from photographs of the gel, and MW estimates obtained by comparison with the marker fragments (Fig. 5-6; Table 5-2).

5.4. Molecular Weight Markers

Values of the MW's of marker DNAs are being periodically updated in the literature. Values for different sets of markers obtained by different authors using a variety of methods tend to be more or less at variance when the markers are compared by co-electrophoresis. Thus a standard curve based on the mobility and MW values of one set of standards does not necessarily overlap that based on another. This problem was encountered with a number of sets of values given in earlier literature for lambda and PM2 fragments (Thomas and Davis, 1975; McParland *et al*., 1976; Brack *et al*., 1976; Streek *et al*., 1974; Philippsen *et al*., 1978). However, more up-to-date values are more

compatible (Daniels et al., 1980) (Table 5-2). Absolute values will only be obtained once the markers have been fully sequenced. The problem of anomalous mobilities of some DNA species in acrylamide gels will be discussed elsewhere. Suffice it to say here that varying the conditions employed for a particular electrophoresis, such as gel concentration, composition, temperature and voltage, affects the mobility of fragments and often the resultant MW values. Thus, although acrylamide gel electrophoresis can resolve very minor differences between two DNA species, MW's derived from such gels must be interpreted with reservation (Gressel et al., 1975).

Mobility of DNA fragments through agarose gels is arguably less affected by base composition etc., and thus gives truer MW values, but this type of gel is less suitable for the analysis of small DNA fragments below 800bp (about 0.5Md). Here again, the absolute electrophoretic mobility of DNA in agarose gels is very dependent on the type, make, and batch of agarose used, and the conditions employed for preparing the gel (Peacock and Dingman, 1978; McMaster and Carmichael, 1977). Thus, standard markers were always used for MW estimations.

5.4.1. BK Virus Prototype

An ideal marker for characterizing our HPV isolates would have been the prototype BK(WT) itself (Gardner et al., 1971). Professor Prozesky's group at the National Institute for Virology, Sandringham, possessed a stock of this strain, and kindly sent us some frozen HEF cells and medium containing propagated virus. DNA was extracted from this culture by a modification of Hirt's method (Hirt, 1967), and aliquots

analysed by electrophoresis through 2.4% and 1.2% agarose cylindrical gels.

The results indicated that, although the majority of the DNA in the preparation appeared to be circular, it was not homogeneous. The picture obtained was very similar to the early results reported by Howley et al. (1975b), and showed that this preparation contained a high proportion of defective genomes, presumably deletion mutants. One aliquot was electrophoresed on a 1.2% agarose slab gel, which achieved a separation of the mutant DNAs into at least 5 species, the NOC pattern matching closely the CCC pattern. A similar result was obtained on cylindrical agarose gels. Fig. 5-7 shows a comparison of the two (Lanes 2 and 4). The lower family of bands were CCC molecules, with the corresponding NOC species "duplicated" above. The smeared band visible in between appeared to be linear BK DNA, with a MW around 5,000bp as expected. Interestingly, the defective species in linear form did not separate in these gels to the same extent as the circular forms. "a" and "b" were presumably the HindIII-A and -B fragments of BK (Howley et al., 1975a), although the excess of "a" over "b" is unexplained. "c" and "d" indicate faint blurred bands that may correspond to the HindIII-C and -D fragments.

This preparation was thus unsuitable as a control. It was decided that attempting to select for the original full length prototype genome would be impractical. Selection would have involved either repeated passage of virus at very low MOI, and/or repeated plaque purification, or transformation of cells with non-defective DNA which had been separated from deletion mutants by recovery from bands on electrophoresed gels (Howley et al., 1975b). Even though these methods result in BK DNA

which appears homogeneous, defectives can soon accumulate again after a few passages. At this stage we did not have the facilities to culture BK virus ourselves. To save time, and to ensure that we were working with a reliably characterized preparation, a stock of plaque-purified BK(MM) virus was obtained from Dr. P. Howley upon request. The MM strain was chosen because our strains also gave three fragments upon HindIII digestion.

5.4.2. BK(MM) DNA Extraction

MM DNA was extracted by us from HEF cultures infected with MM virus in a similar manner to the BK prototype described above. In order to minimize contamination with cellular DNA, virus harvested from the culture medium was resuspended in DNase buffer and incubated with 20μg/ml DNase at 37C for 30min. However, this treatment had no effect compared with the portion of the virus preparation that was not DNase treated. A small amount of high MW linear DNA contaminated both preparations. Thus the DNase was not active under the conditions used, or else the linear DNA observed was still contained within cellular material which had not been fully removed by the clearing spin. This DNA was not apparent after restriction with HindIII, so did not affect coelectrophoresis results.

This preparation was sufficiently pure for digestion with restriction endonuclease HindIII, and was used for comparative electrophoresis with HPV isolates SN and WW.

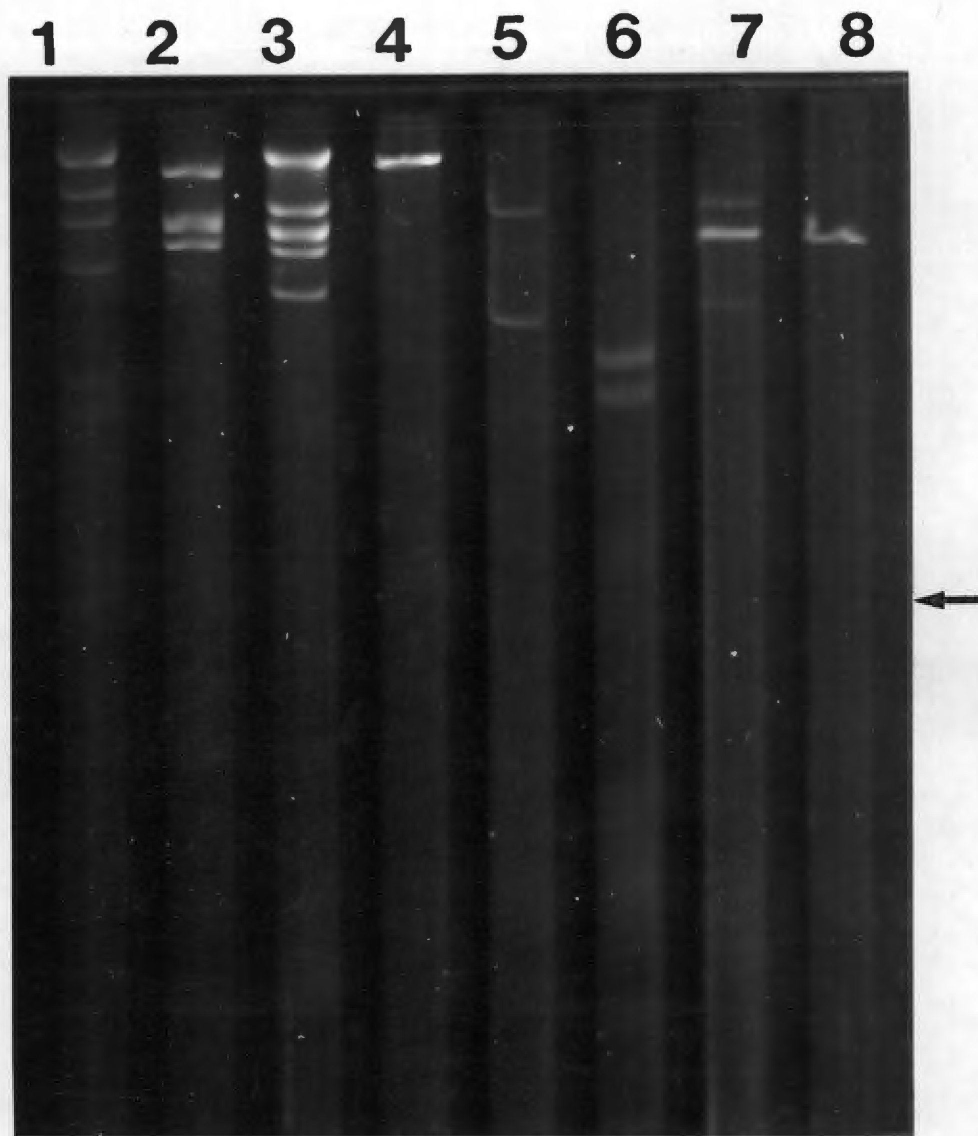


Figure 5-4. Restriction of SN DNA

Electrophoresis was through 1% cylindrical agarose gels in Tris-borate buffer for 3h at 50V.

Gels 1-3: 3μg lambda DNA digested with HindIII, BamHI or EcoRI
Gel 4: 1μg of lambda DNA
Gel 5: 1μl of SN DNA
Gels 6-8: 3μl of SN DNA digested with the same quantity of HindIII, BamHI or EcoRI as the lambda controls.

Arrow indicates the position of the SN HindIII-C fragment (Gel 6).

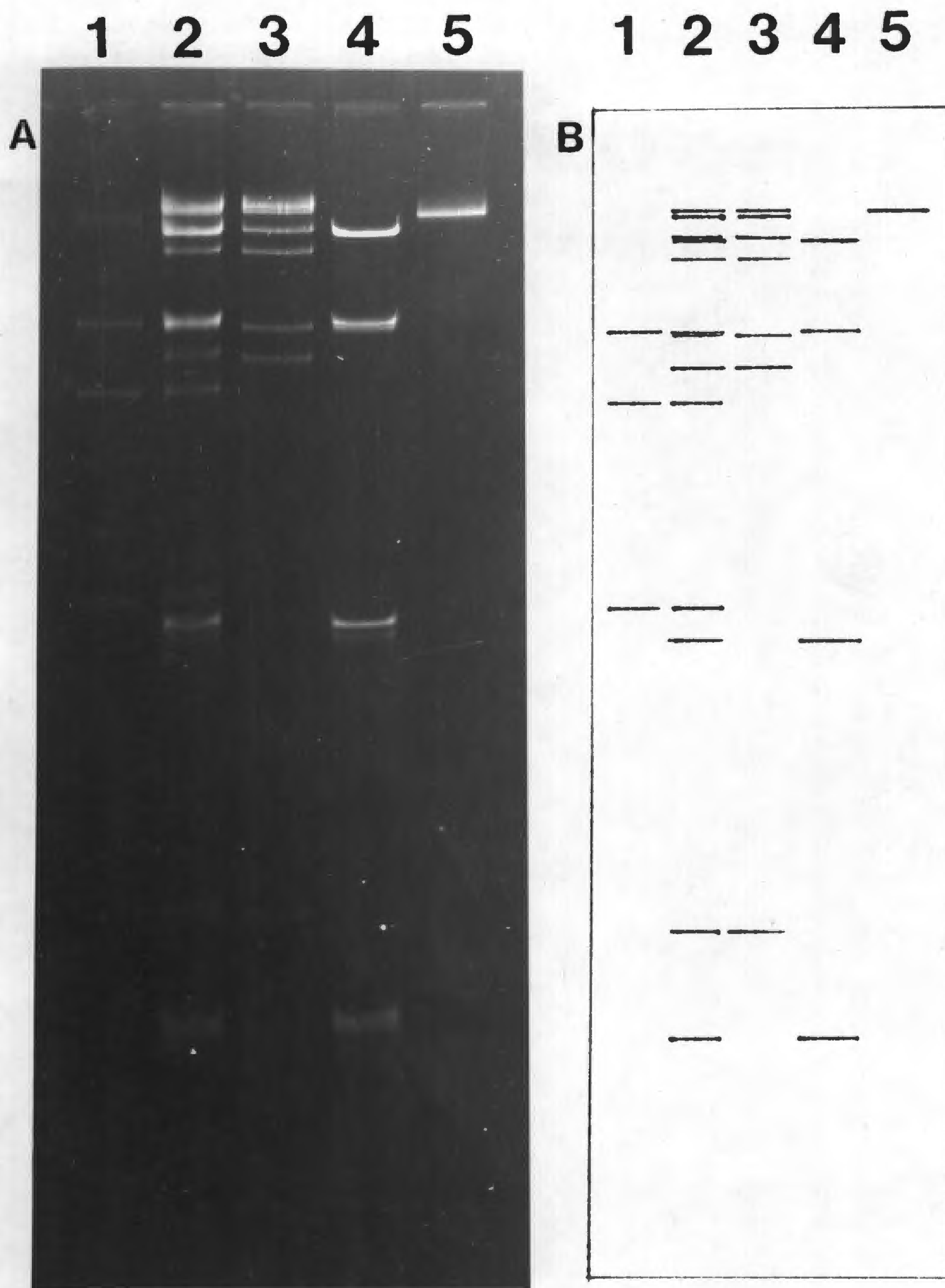


Figure 5-5. Coelectrophoresis of WW HindIII Fragments with Markers

A. Photograph of gel stained with EtdBr and illuminated with UV light.
B. Diagrammatic illustration of the bands obtained.
Lane 1: WW/HindIII Lanes 3, 4: lambda/HindIII + PM2/HindIII
Lane 5: 0.5μg lambda DNA

Electrophoresis was through a 3% polyacrylamide slab gel set and run at 4C, 100V for 18h.

Table 5-2. Comparison of DNA MW Marker Values

A. HindIII Fragments of Lambda DNA

Band No.	a	b	c	d
1	23,513	23,000 (98)	22,440 (95)	23,600 (100)
2	9,593	9,800 (102)	9,400 (98)	9,640 (101)
3	6,764	6,600 (98)	6,240 (92)	6,640 (98)
4	4,447	4,500 (101)	4,340 (98)	4,340 (98)
5	2,286	2,500 (109)	2,200 (97)	2,260 (99)
6	1,945	2,200 (113)	1,930 (99)	1,980 (102)
7	585	490 (84)	660 (113)	560 (96)
	------	------ ---	------ ---	------ ---
Total	48,580	49,000 (101)	47,240 (97)	49,000 (101)

B. HindIII Fragments of PM2 DNA

Band No.	e	f
1	5,840	4,300 (74)
2	2,240	2,150 (96)
3	940	1,210 (127)
4,5	470	540 (113)
6	240	210 (93)
7	80	60 (80)
	-----	----- ---
Total	10,250	9,000 (88)

Molecular weights are given in base pairs (bp). Percentages of the most up-to-date values (Column a for lambda and Column e for PM2) are given in parentheses. A conversion factor of 652d per bp was used throughout.

References:

a. (Daniels _et al._, 1980). Exhaustive assessment of fragment sizes for lambda digested with 12 different endonucleases. Calibrated against sequenced marker fragments. These values were used in the construction of the standard curves in Fig. 5-5.

b. (Ter Schegget _et al._, 1980). Figures quoted from Murray and Murray (1975).

c. Values for "DNA MW Markers II" from 1978 Boehringer Mannheim Biochemicals catalogue.

d. (Philippsen _et al._, 1978). Calibrated against ØX174am3RF sequenced fragments. Values used in 1980/81 Boehringer catalogue for their "DNA MW Marker II" fragments.

e. (Brack _et al._, 1976). Values given as % genome length obtained from EM measurements of contour length. Converted to MWs using a value of 6.68Md for the whole PM2 genome (Johnson and Grossman, 1977), and 652d per bp. These values were used for the standard curves in Figs. 5-5 and 5-8.

f. (Streek _et al._, 1974). MW values obtained from electrophoretic mobility measurements.

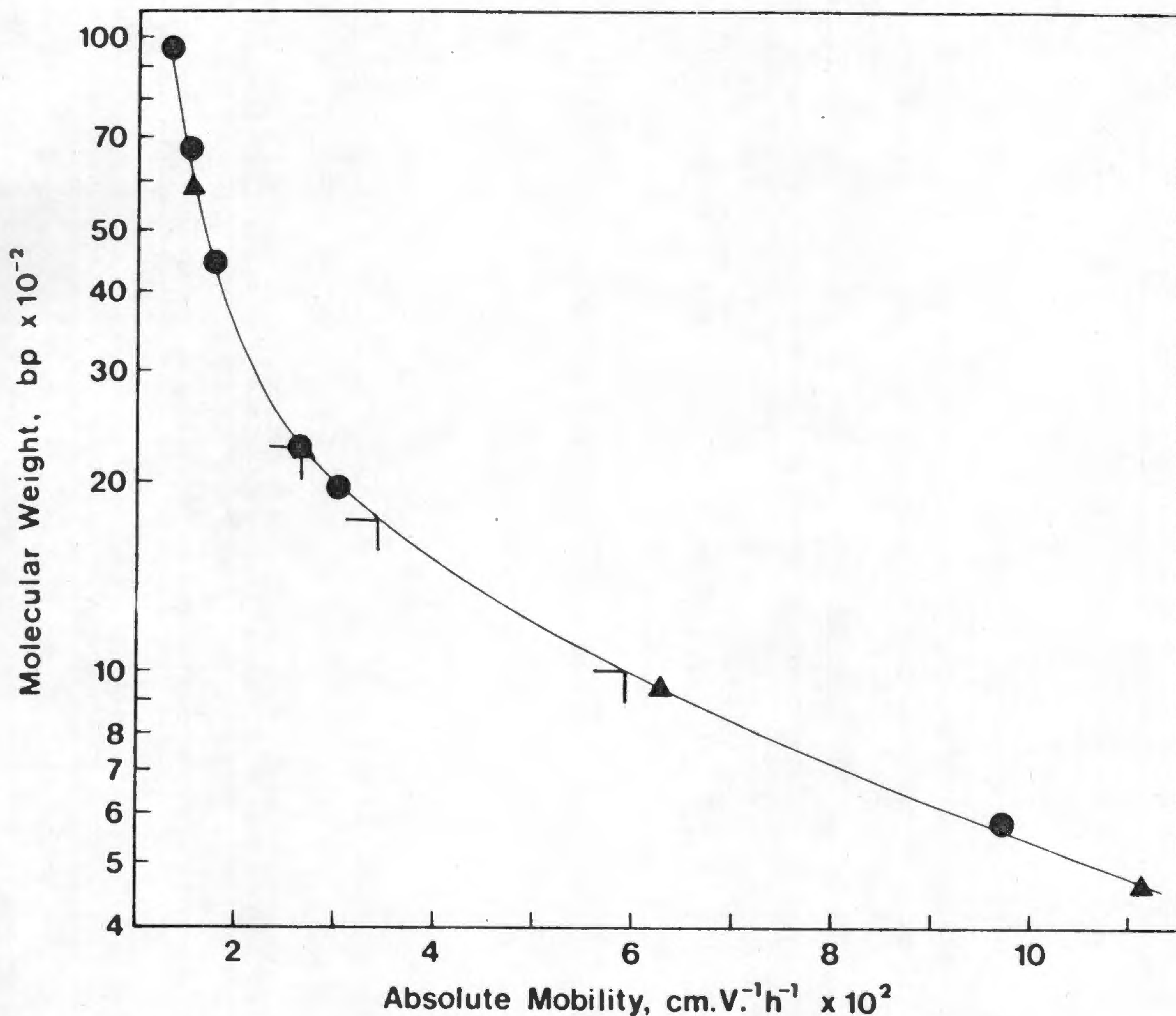


Figure 5-6. Molecular Weights of WW HindIII Fragments. (Experiment I)

● Lambda HindIII fragments
▲ PM2 " "
┐ WW " "

Electrophoretic mobilities are from Fig. 5-5. Molecular weights for the marker fragments are from Table 5-2, Columns a and e. Resultant MWs for the WW fragments are listed in Table 5-3.

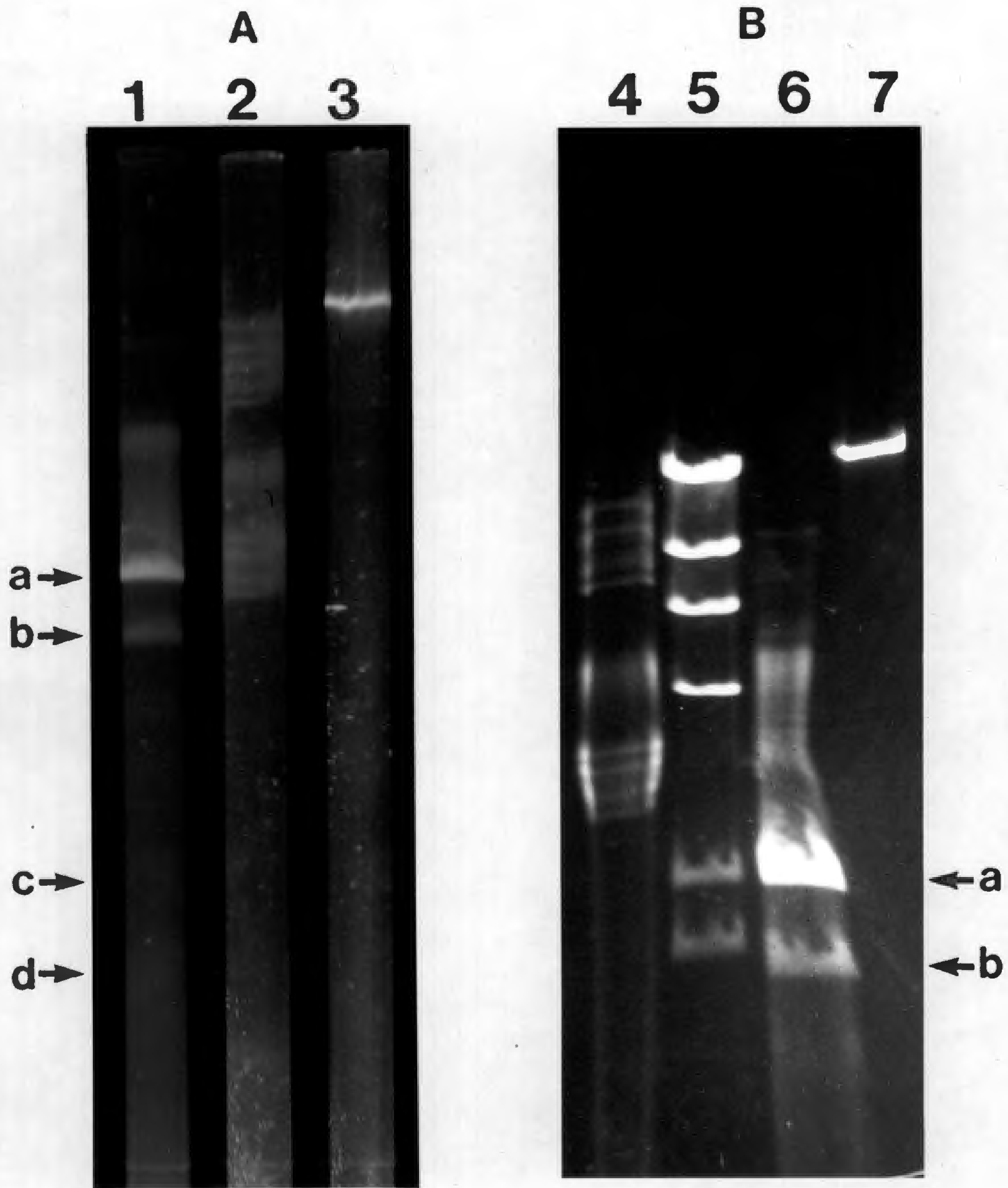


Figure 5-7. Defective Genome of BK(WT) DNA

Lanes 1 and 6: BK(WT)/HindIII
Lanes 2 and 4: BK(WT) DNA
Lanes 3 and 7: Lambda DNA marker
Lane 5: Lambda/HindIII
A. 1.2% cylindrical agarose gels run at 50V for 4h.
B. 1.2% vertical agarose slab gel, run at 100V for unspecified time.
Tris-borate buffer was used.

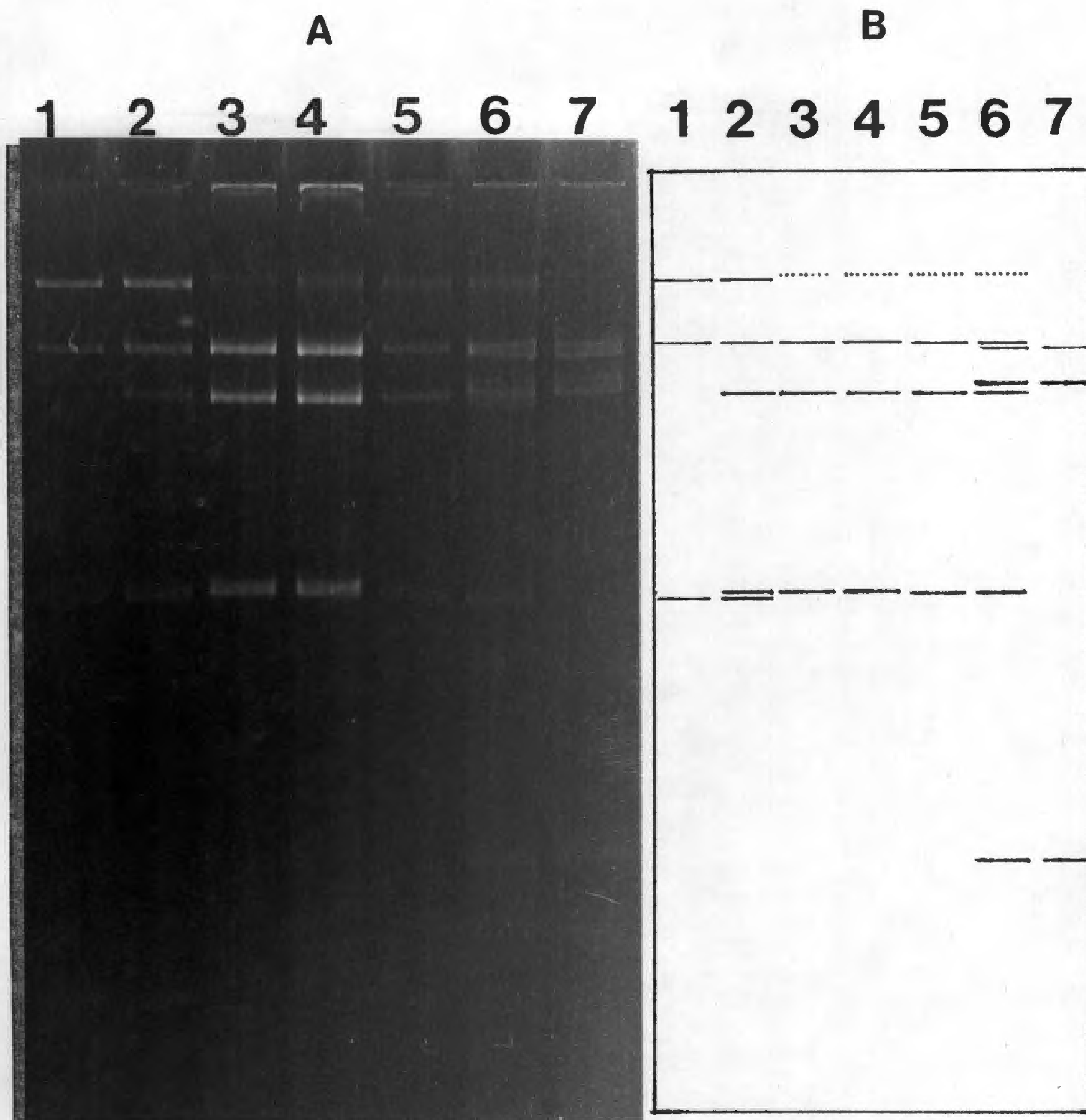


Figure 5-8. Coelectrophoresis of WW, SN, MM and PM2 HindIII Fragments.

HindIII digests were electrophoresed through a 2.7% polyacrylamide slab gel for 15h at 100V and 4C.

Lane 1:	PM2	Lane 2:	PM2 + WW
Lane 3:	WW + SN	Lane 4:	SN
Lane 5:	WW	Lane 6:	WW + MM
Lane 7:	MM		

A. Photograph of EtdBr-stained gel.
B. Diagrammatic representation of the bands.

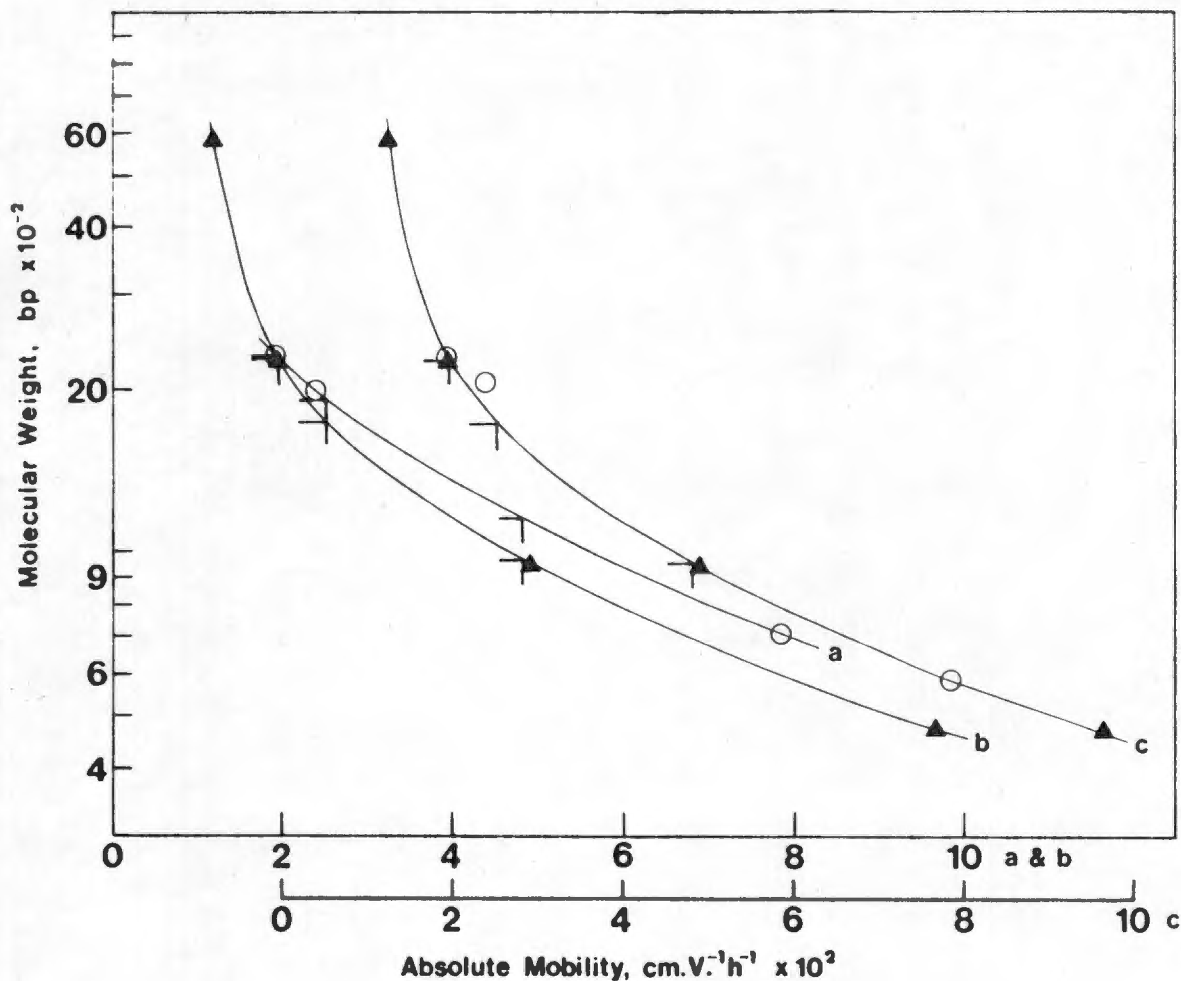


Figure 5-9. Molecular Weights of WW HindIII Fragments

Experiment II

▲ PM2/HindIII
○ MM/HindIII
┐ WW/HindIII

Electrophoretic mobilities are from Fig. 5-8. PM2 molecular weights are the same as in Fig. 5-6.
a. MM HindIII MWs taken from Howley et al., (1975a).
c. MM HindIII MWs taken from the nucleotide sequence (Yang and Wu, 1979c). Values for WW HindIII fragments taken from curve c are listed in Table 5-3.

5.5. MOLECULAR WEIGHTS OF SN AND WW HINDIII FRAGMENTS

Table 5-3. Molecular Weights of Human Polyomavirus (HPV) HindIII Fragments

Fragment	BK(WT)	BK(Dun)	BK(MM)
A	2,300	2,300	2,300
B	1,925	1,924	2,076
C	555	512	587
D	416	416	
Total	5,196	5,153	4,963

Direct urine isolate WW (=SN)

Fragment	Experiment I	Experiment II	Average	
A	2,290	2,240	2,270	(1.48Md)
B	1,750	1,730	1,740	(1.14Md)
C	1,010	960	990	(0.65Md)
Total	5,050	4,930	4,990	(3.25Md)

MW values given in base pairs (bp), except for equivalent values in daltons given in parentheses. BK(WT) and BK(Dun) values are from Yang and Wu (1979c). BK(MM) values are from Seif et al., (1979). Experiment I is from Fig. 5-6. Experiment II is from Fig. 5-9.

DNA preparations of SN, WW, MM and PM2 were digested with HindIII. All the remaining stock of SN DNA was digested. Completeness of digestion was checked by running one fifth of each prep (except SN) on agarose gels. It was initially thought that only a trace of SN was left, so no check was made of this prep.

Aliquots of each digest were subjected to coelectrophoresis on a 2.7% polyacrylamide slab gel at 4C, 100V for 15h (Fig. 5-7). HindIII digests of WW and MM were run together in lane 6; WW and SN were run together in lane 3, and WW and PM2 were run together in lane 2. Other lanes contained individual digests.

Although some bands were not sharp, the leading boundaries of the bands were clear enough to provide accurate mobility measurements. A low concentration gel was chosen to maximize the resolution of larger, slower migrating fragments, but experience has shown that "sloppy" slab gels give poorer band definition.

The difference in mobility between the HindIII-C fragments of WW and MM was immediately apparent. No detectable difference could be observed between the two independent isolates WW and SN. This result is of great interest, as it suggests that these isolates may represent a BK variant which is widespread, or else common locally.

The PM2 and MM mobility values were used to construct a standard curve (Fig. 5-9). MW values for the MM fragments obtained from Howley _et al_. (1975a) produced an anomaly. The HindIII-C fragment of MM appeared to have too high a mobility for its published MW, compared to the PM2 fragments. At this stage it appeared that the curve would have to be constructed using either the PM2 values or the MM values, because the two sets fell on curves which diverged at lower MWs (Fig. 5-9, a and b). MW estimates using the PM2 values agreed well with those obtained in the previous co-electrophoresis experiment (2,240, 1,740, and 965bp compared to 2,290, 1,750, and 1,010bp from the previous gel (Fig. 5-6). An MM standard curve, however, gave values for WW of 2,270, 1,910, and 1,160bp (Fig. 5-9, a). This discrepancy was puzzling, as we were assured that the MM MW values were accurate (Howley, 1979, personal communication). When the complete nucleotide sequence of BK(MM) was eventually published, the base pair values obtained formed a much smoother standard curve with the PM2 HindIII values. This curve (Fig.

5-9, c) gave MW values for SN and WW HindIII fragments of 2,240, 1,730, and 960bp, which are in good agreement with previous estimates using PM2 and lambda HindIII fragments as standards (Table 5-3).

A figure for the whole genome of 4,990bp corresponds to a MW of 3.25Md, taking 652 as the MW of one base pair (disodium salt). The GC base pair anion has a MW of 616 compared with 615 for AT, excluding associated water molecules. The theoretical average MW is hard to determine in the absence of precise knowledge of the ionic and hydrated state of the molecule. Values inferred from published data vary. Johnson and Grossman (1977) assumed a MW of 3.51Md for ØX174 RF DNA, which contains 5,372bp. This gives 653 for the MW of one base pair. Yang and Wu (1979c) gave a figure of 3.26Md for the MW of BK(MM). The sequence contains 4,963bp, which gives 657 per base pair, whereas the BK prototype was given as 3.45Md, and contains 5153bp, or 670 per bp (Seif _et al_., 1979). By contrast, the data of Daniels _et al_. (1980) for lambda DNA implied a figure of 627 per base pair (49,000bp in a MW of 30.8Md).

The modern trend amongst molecular biologists is to quote MWs as base pairs, but as can be seen, converting published values for restriction fragments from daltons does not necessarily give a true base pair value.

5.6. Cloning of BK DNA into pBR322

Recombinant DNA techniques were developed in conjunction with Louise Olliver. The transformation of bacterial cells under P2 containment conditions was performed in the Dept. of Medical Microbiology, Tygerberg Hospital, and in the Dept. of Bacteriology, UCT Medical School. Many of these techniques were new to South Africa.

DNA ligation, nick translation and Southern gel hybridization were carried out by Louise Olliver. Transformation procedures, selection of recombinants and plasmid extraction, purification, and restriction analysis were performed by myself at Tygerberg Hospital. Colony hybridization experiments were conducted in conjunction with Ms. Olliver.

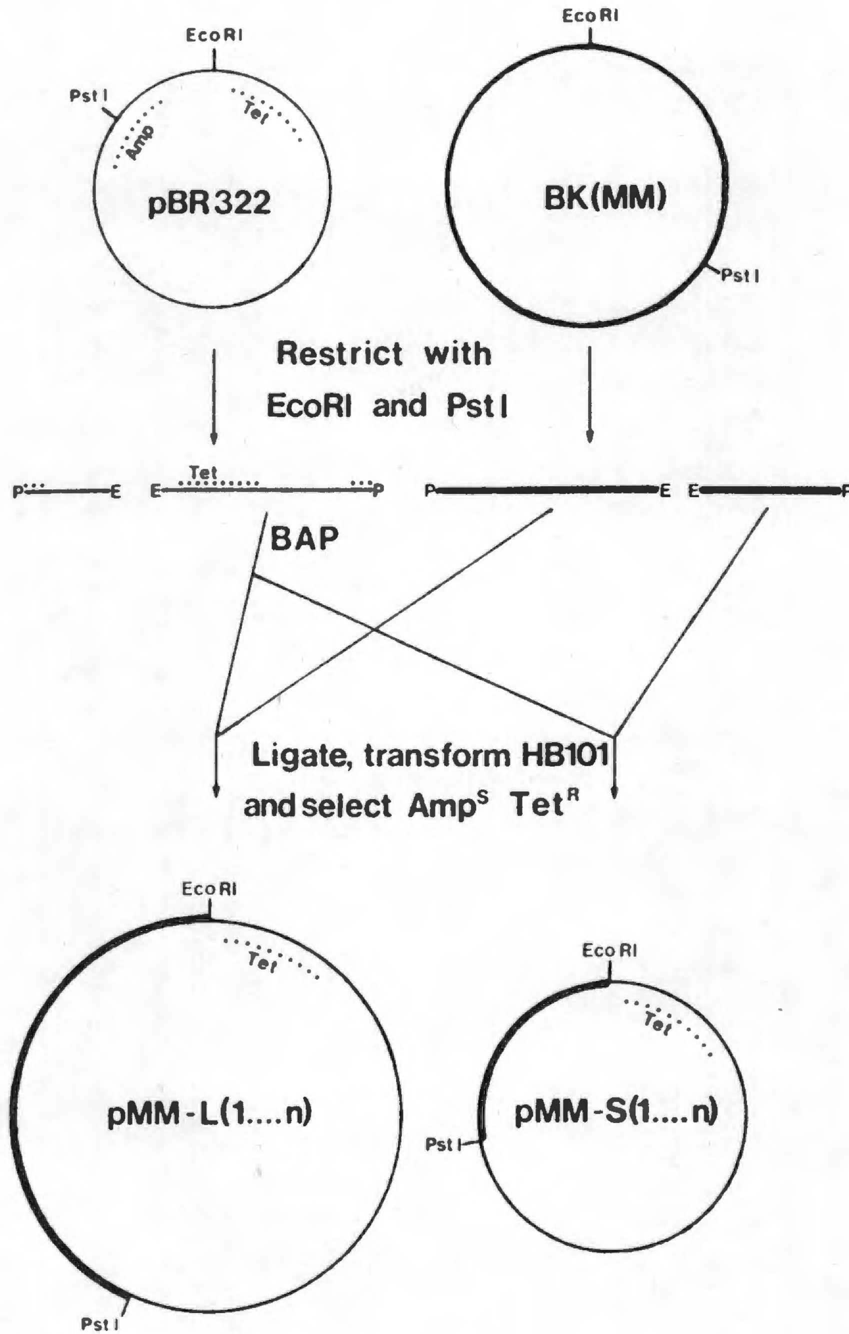


Figure 5-10. Scheme for Cloning BK(MM) DNA

Light lines: pBR322 DNA
Heavy lines: MM DNA
Dotted lines: Antibiotic resistance genes

Figures are not to scale. Tentative nomenclature for the resulting clones is given.

5.6.1. MM DNA

As a control experiment, attempts were made to clone MM DNA fragments generated by double digestion with PstI and EcoRI into similarly digested pBR322, using HB101 and C600 as host cells (Fig. 5-10). As mentioned in Section 4.2.2.2 this double digest approach lowered the containment requirement from P3, EK1 or P2, EK1(CV) to P1 EK1, as the transforming region of the MM DNA was disrupted. It also simplified the analysis of recombinants, thanks to the fewer potential orientations of restriction fragments during ligation.

The failure of an early transformation attempt in HB101 with PstI/EcoRI digested MM and WW DNAs ligated to similarly digested pBR322, was found to be due mainly to inadequate ligation. Very little recircularized recombinant DNA was detected by gel electrophoresis. The experiment was repeated with MM and pBR322 DNA using a fresh T4 ligase preparation. This experiment yielded recombinant transformants with HB101, as described below.

As an attempt to increase the proportion of recombinant plasmids, some of the pBR322 PstI/EcoRI digested DNA used for ligation was first treated with bacterial alkaline phosphatase (BAP), which removed the terminal 5' phosphoryl groups from the DNA fragments and prevented ligation of reannealed pBR322 fragments, because T4 DNA ligase requires the 5' phosphate. Ligation between pBR322 fragments and non BAP-treated BK fragments was still possible, although only at the 5' end of the BK strand with an intact phosphoryl group. The final products contained single-strand nicks, but NOC plasmids are still able to transform recipient cells efficiently, and are

probably covalently closed by cellular repair mechanisms. In theory, all transforming plasmids should have contained insert (non-pBR322) DNA (Bolivar and Backman, 1979).

Complete restriction and successful ligation were checked by running digested and ligated aliquots on agarose gels. Restriction with PstI and EcoRI produced two pBR322 fragments of 748 and 3614bp (0.46 and 2.14Md), and MM fragments of 3,308 and 1,655bp (1.08 and 2.16Md). After ligation, higher MW species were apparent. Equimolar amounts of PstI/EcoRI cleaved pBR322 (100ng) and MM (80ng) were ligated using 0.25 Weiss units of T4 ligase (BRL) in 20µl of ligase buffer. This corresponded to a j/i ratio of 2.7 with respect to a dimer of the large PstI/EcoRI fragments of pBR322 + MM (MW = 4.30Md), and a j/i ratio of 3.2 with respect to a dimer of the large pBR322 fragment and the small MM fragment (MW = 3.22Md). These j/i ratios lie within the range found to maximize circularization as opposed to continued concatemerization in ligation reactions (Dugaiczyk et al., 1975; Olliver, 1981, MSc Thesis, UCT).

C600 and HB101 cells were used as recipients. C600 cells were grown in 40ml LB to an OD600 of 0.45, and HB101 to an OD of 0.35. After induction of antibiotic resistance, HB101 cells were spread on LA plates containing 15µg/ml Tc (Tc15 plates), and C600 on plates containing 30µg/ml Tc (Tc30 plates). Plates were read after 24h incubation. The transformation frequency of uncut pBR322 was approximately 240/ng in HB101 and >600/ng in C600. The C600 cultures were contaminated with a low level of easily distinguishable non-coliform colonies. Coliform colonies were toothpicked onto fresh Tc30 and Ap50 (containing 50µg/ml Ap) plates after 24h incubation. HB101 plates were replica plated onto Ap50 plates after 44h incubation. HB101 colonies

that did not grow on Ap plates were checked by toothpicking onto fresh Tc15 and Ap50 plates. The majority were confirmed to be Tc(R)Ap(S). A total of 37 C600 and over 100 HB101 Tc(R)Ap(S) transformants were obtained. A selection of these were analysed further. Extraction and electrophoresis of plasmids showed that Ap(S)Tc(R) colonies contained plasmids larger than pBR322, indicating insertion of foreign DNA into the amp gene. Recombination had therefore been successful, and the insertion of MM DNA sequences was later confirmed (see Section 5.6.3).

5.6.2. WW DNA

The same procedure was followed immediately after confirmation of the success of the MM cloning experiment, using the same preparations of PstI/EcoRI digested pBR322, and a preparation of WW that, although initially resistant to restriction enzyme digestion, was successfully cleaved after the DNA had been repeatedly washed in 70% ethanol to remove possible traces of phenol or other impurities (Fig. 5-11). The ligation mixture was used to transform competent HB101 and C600 cells. HB101 was plated on Tc15 plates, and C600 on Tc25 plates. After 40h the C600 background was just detectable, indicating that the Tc was being inactivated, presumably by prolonged incubation at 37C. Larger colonies were picked off and tested on fresh Tc and Ap plates. In all, 71 C600 and 68 HB101 colonies were tested. The C600 colonies were found to be either Tc(S)Ap(S), growing very weakly on ageing Tc plates, or Tc(R)Ap(R). All HB101 colonies tested were Tc(R)Ap(R).

This unexpected result (confirmed by repeated testing) suggested that the transformants contained intact pBR322

molecules only. However, a selection of clones were tested further along with the Ap(S)Tc(R) MM/pBR322 clones. To date this transformation has not been repeated for fear of wasting the last remaining traces of WW DNA, before the ligation and transformation methodologies have been made more reliable.

5.6.3. Colony Hybridization with an MM DNA Probe

Circles of Millipore filter paper were cut to fit Falcon disposable filters, sterilized, and placed onto selective LA plates containing either 25 (Tc25) or 15 (Tc15) μg/ml tetracycline. A selection of putative C600 recombinants were toothpicked onto the membranes on Tc25, HB101 clones onto Tc15 plates. Colonies were grown on the membranes overnight, and then subjected to colony hybridization procedures with 32P labelled MM DNA probe. Circles were autoradiographed for 28h. A number of colonies produced dark spots, indicating specific hybridization, although a background radiation was observed in most colonies (Fig. 5-12). This could probably have been reduced by further washing steps. No putative WW/pBR322 transformants hybridized, confirming the failure to obtain fully Ap(S)Tc(R) colonies. A number of the MM/pBR322 clones hybridized, and some of these were further characterized.

5.6.4. Characterization of Recombinant Plasmids

A number of clones transformed with ligated MM/pBR322 DNA were subjected to rapid microscale plasmid extraction to obtain plasmid suitable for restriction. A PstI/EcoRI double digestion was performed to regenerate pBR322 and inserted fragments. A

compromise buffer was used, with a final concentration of 50mM Tris-HCl (pH7.5), 10mM $MgCl_2$, 50mM $(NH_3)_2 SO_4$ and 100µg/ml BSA. 1U each of PstI and EcoRI were added together, and incubated for several hours. The whole preps (40µl) were electrophoresed through a 1.2% 16-slot vertical agarose slab gel at 125mA for 1h40 (Fig. 5-13). In lane 10, the large PstI/EcoRI fragment (3614bp) of pBR322 was observed. The small fragment had been lost in the smeared material near the bottom of the gel. Lane 11 showed the CCC (lower) and NOC bands of undigested pBR322. Relative to these, it was observed that the two undigested MM/pBR322 recombinant preps (lanes 12, 14) contained a larger plasmid, indicative of an insert, whereas the WW/pBR322 clone (lane 13) contained a plasmid of the same mobility as pBR322, suggesting that this plasmid was not a recombinant. This is supported by the Ap(R)Tc(R) phenotype of this clone. Lanes 4, 12, 13, and 14 also contained a linear plasmid DNA band running between the CCC and NOC species. Most of the digested MM/pBR322 clone preps revealed the large pBR322 PstI/EcoRI fragment (lanes 2, 5, 6), with lanes 5 and 6 showing in addition the same 1650bp insert, later identified as the small PstI/EcoRI fragment of MM. The slow moving band in lane 6 was probably residual NOC DNA, or possibly an additional large insert. The DNA in lane 4 resisted digestion, and was the only prep with appreciable quantities of chromosomal DNA contamination. CCC, NOC and faint full-length linear plasmid DNA bands were also visible. The slow moving extra bands in lanes 1 and 3 remain unexplained. Two extractions of C600 WW/pBR322 colonies that grew weakly on Tc plates predictably failed to show plasmids (lanes 8, 9). The HB101 WW/pBR322 Ap(R)Tc(R) colony in lane 7 showed the large PstI/EcoRI pBR322 fragment and a faster-moving band that was

probably undigested CCC DNA, assuming the plasmid was intact pBR322 from microbiological evidence. Lane 15 contained marker fragments of HindIII digested lambda DNA.

A number of clones were identified from gels of PstI/EcoRI digests as having inserts of a size corresponding to one or other MM PstI/EcoRI fragment. Plasmids extracted from these clones by the gentle lysis method (see Section 8.7.2) were run, together with pBR322, a 1% horizontal agarose slab gel (Fig.5-14, A). Lanes 1-4 showed four plasmids of the same mobility, presumed to contain the large MM PstI/EcoRI fragment. The plasmids in lanes 6-9 were presumed to contain the small fragment. Lane 5 was a preparation of pBR322 which consisted predominantly of linear and NOC monomeric pBR322 DNA. However, it was adequate to show also the position of monomeric CCC pBR322 running faster than the putative recombinants as would be expected. The other faint bands in this prep represent various forms of dimeric pBR322 present in the commercial preparation. Fig. 5-14 (B) shows a 1.2% horizontal agarose slab of these plasmids digested with PstI/EcoRI, along with digests of pBR322 in lane 5 and MM DNA in lane 6. The sizes of the fragments produced corresponded as anticipated to the large fragment of pBR322 and either the large (lanes 1-4) or small (lanes 7-10) MM fragment. Lanes 8 and 9 also contained a large number of faint bands generated by restriction of contaminant chromosomal DNA in these preps.

5.6.4.1. Probing for Putative MM Inserts

The identities of the putative MM clones were confirmed by probing with 32P-labelled MM DNA (Fig. 5-15). Two

representative clones were digested with PstI and EcoRI to regenerate the vector and inserted fragments, which were separated by agarose gel electrophoresis (Fig. 5-15A). The fragments were transferred to nitrocellulose paper by the Southern technique and hybridized to MM probe. The resulting autoradiograph is shown in Fig. 5-15B. Lanes 1 and 4 are PstI/EcoRI digests of putative pBR322/MM recombinants. The upper band in each is pBR322 DNA, corresponding to the large PstI/EcoRI fragment of pBR322 shown in lane 2. None of these bands hybridized with MM probe (Fig. 5-15B). The lower band in lane 1 corresponded with the smaller PstI/EcoRI MM fragment in lane 3, and both hybridized to MM probe. Similarly, the lower band in lane 4 corresponded to the large PstI/EcoRI fragment in lane 3, and hybridized also. Because the large MM and pBR322 PstI/EcoRI fragments run close together and may have caused confusion on the autoradiograph, the putative MM large-fragment clone was digested with PstI, EcoRI and TaqI. TaqI cuts pBR322 into many small fragments, but MM DNA has no site for it. This is because the TaqI recognition sequence contains a 5'-CG-3' element which is very rare in eukaryotic DNA. TaqI recognises the sequence T↑CGA (BRL 1981 Catalogue). Thus in lane 5 the MM fragment is unaffected, whereas the large pBR322 fragment from lane 4 is reduced to small fragments (lanes 5 and 6).

These clones were used to prepare large quantites of plasmid DNA containing MM sequences, which were nick translated with 32P to make a probe suitable for screening DNA preparations from patient samples suspected of containing BK-type polyomavirus. Use of labelled probe increased the sensitivity and specificity of such screening procedures significantly, as described below.

5.6.5. Probing for BK DNA Sequences in Human Isolates

An opportunity to test the specificity of probing with labelled MM DNA occurred when a DNA preparation from another renal transplant patient (GR) was found by gel electrophoresis to contain a heavy contaminating smear of DNA, obscuring any polyomavirus DNA bands which may have been present (Fig. 5-16A). Southern gel blots of this DNA hybridized with BK probe revealed two bands containing BK-specific sequences, at roughly the expected positions for CCC and NOC (or linear) polyomavirus DNA (Fig. 5-16B). This result suggested that enough polyomavirus DNA was present to purify by CsCl/ethidium bromide density gradient centrifugation. Alternatively, all the DNA present in the prep could be cloned directly in a sort of "shotgun" experiment, and plasmids containing sequences homologous to BK selected for. Sufficient mapping information could probably be obtained by restricting the whole prep with selected endonucleases and revealing the polyomavirus bands by Southern gel blotting. This work is still in progress.

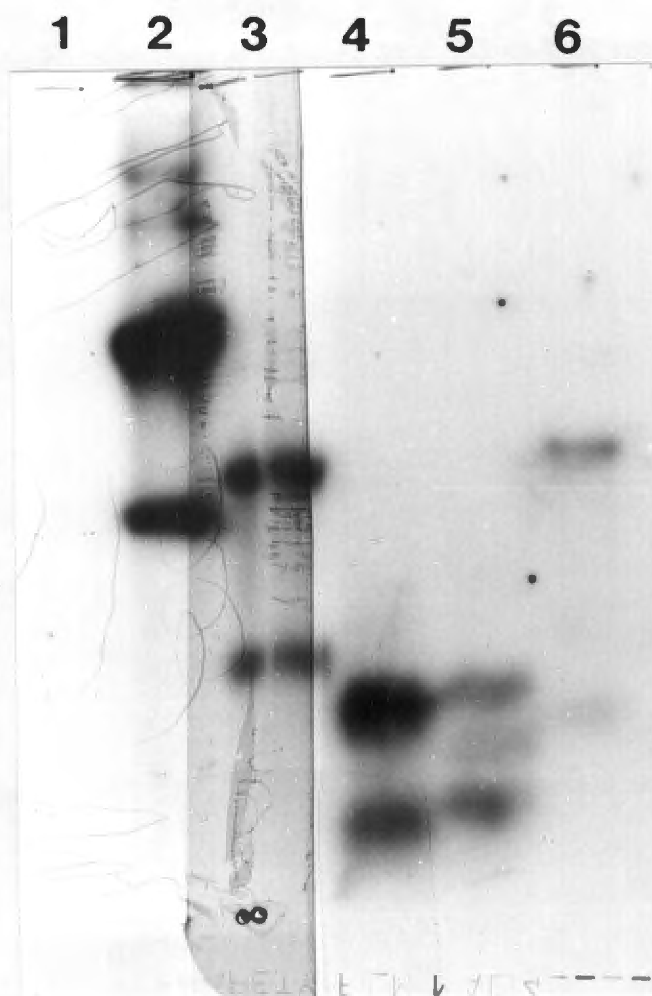


Figure 5-11. Detection of Polyomavirus Fragments on an Agarose Slab Gel Using 32P-BK(MM) Probe.

Lane 1: pBR322 DNA
Lane 2: BK(MM) DNA
Lane 3: PstI/EcoRI digested BK(MM) DNA
Lane 4: PstI/EcoRI/HindIII digested BK(MM) DNA
Lane 5: " " " " BK(WW) DNA
Lane 6: PstI/EcoRI digested BK(WW) DNA

The gel contained insufficient DNA to be detected by EtdBr staining. Polyomavirus-specific DNA was detected by Southern gel blotting and hybridization to MM DNA extracted from cell culture which was labelled with 32P by nick translation.

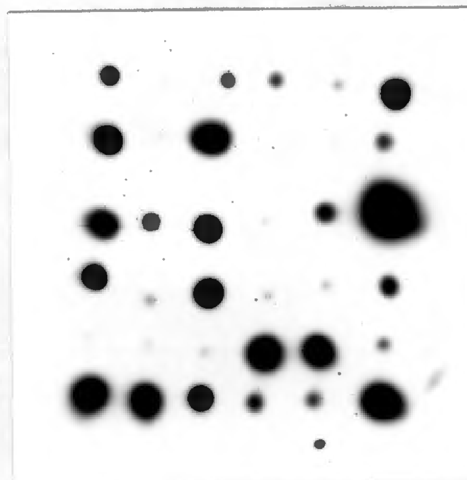


Figure 5-12. Colony Hybridization of Putative Recombinants

Ap(S)Tc(R) colonies transformed with BK(MM)/pBR322 recombinant DNA, probed with 32P-labelled MM DNA.

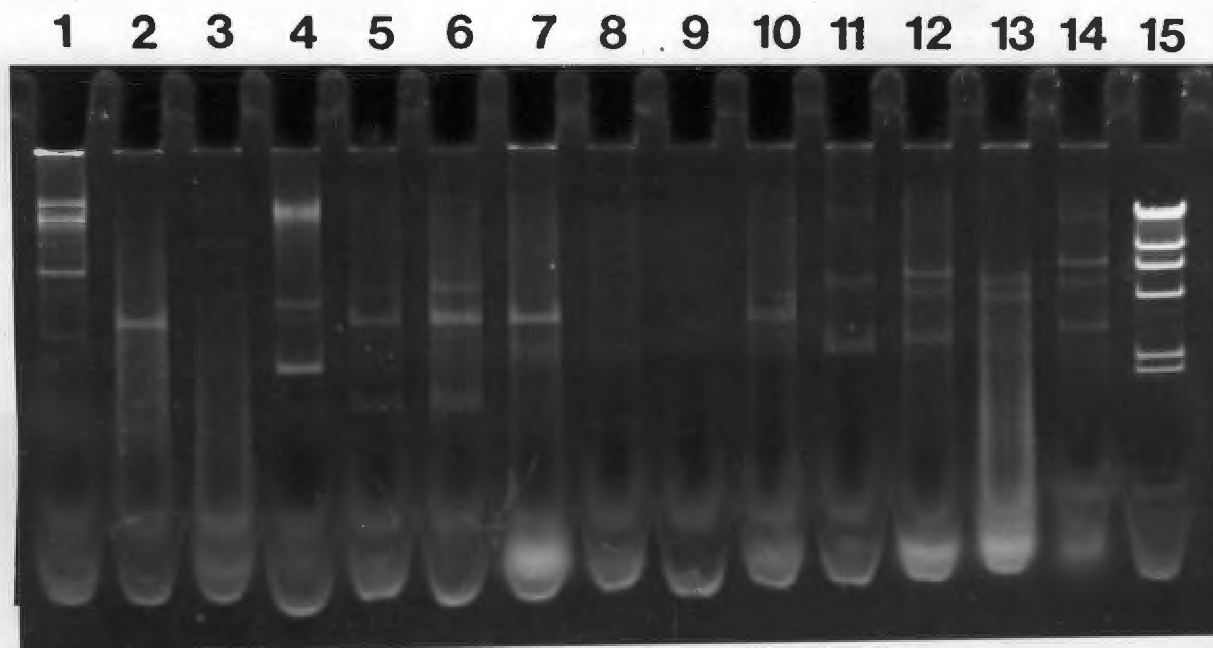


Figure 5-13. Preliminary Characterization of Recombinant Plasmids

1% vertical agarose gel electrophoresis of PstI/EcoRI digests of putative MM and WW recombinant clones.
Lanes 1, 2: pBR322/MM in C600. Lanes 3-6, 12, 14: pBR322/MM in HB101. Lanes 7, 13: pBR322/WW in HB101. Lanes 8, 9: pBR322/WW in C600. Lane 10: pBR322 in HB101. Lane 11: pBR322 in C600.
Lane 15: HindIII digested lambda DNA.
Lanes 1-10: PstI/EcoRI digests.

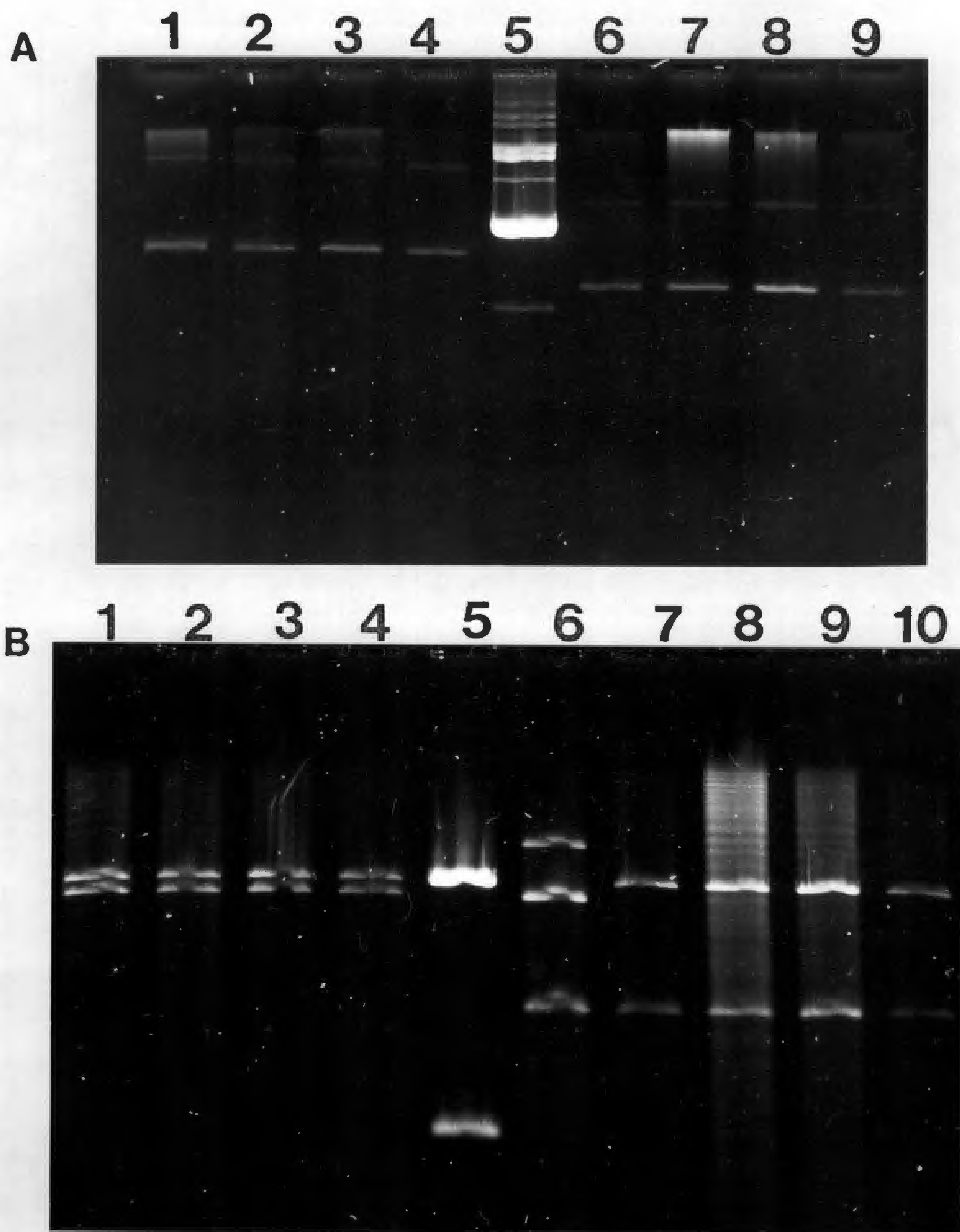


Figure 5-14. Analysis of Putative BK(MM) Clones

A. 1% horizontal agarose gel of plasmid extracts.
Lanes 1-4: Putative large MM PstI/EcoRI fragment clones.
Lane 5: pBR322 DNA (mostly NOC and dimers).
Lanes 6-9: Putative small MM PstI/EcoRI fragment clones.

B. 1.2% horizontal agarose gel of PstI/EcoRI digested plasmids.
Lanes 1-4: Same clones as lanes 1-4, Fig. A.
Lane 5: pBR322.
Lane 6: PstI/EcoRI digested MM DNA.
Lanes 7-10: Same clones as lanes 6-9, Fig. A.

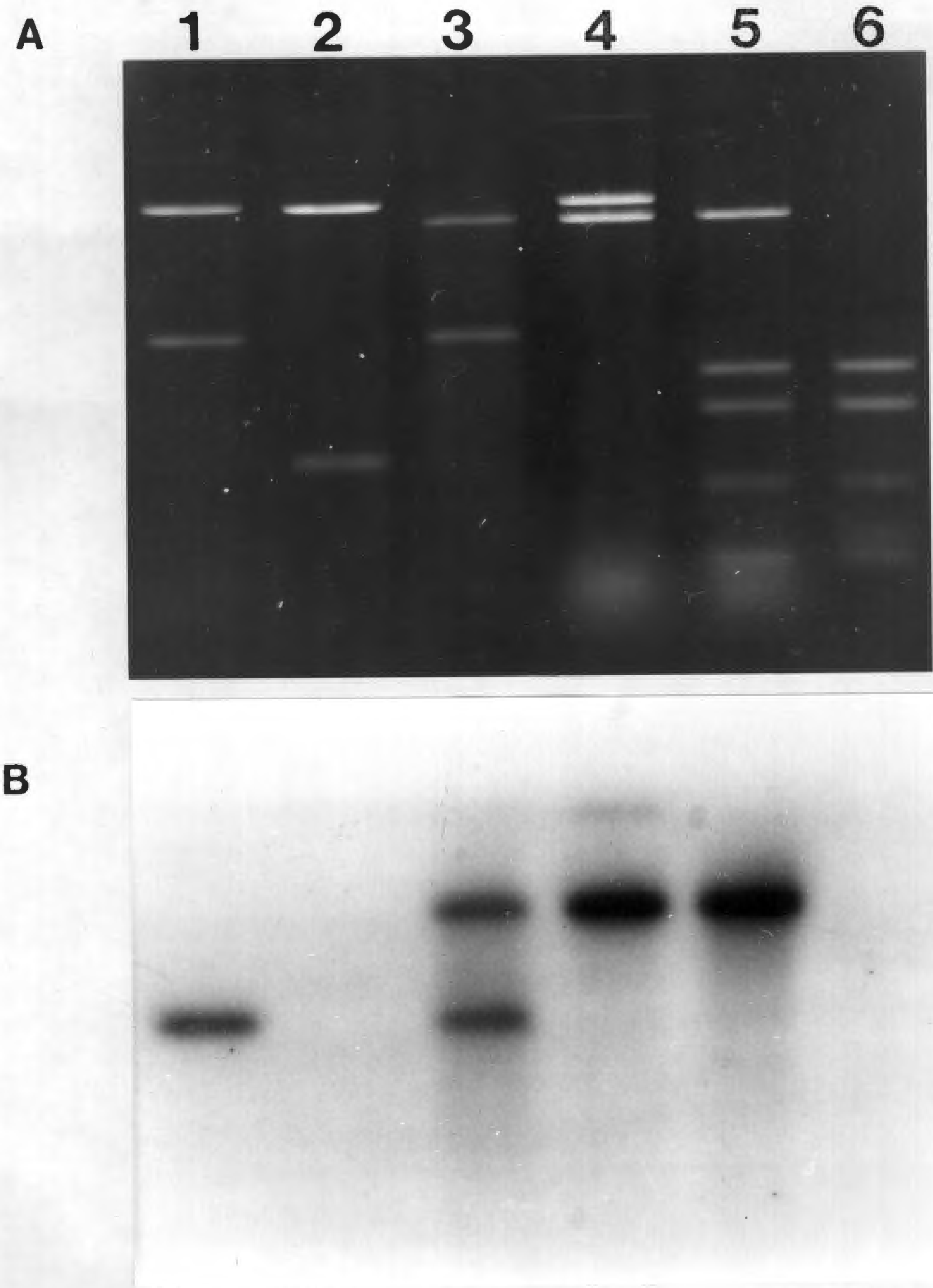


Figure 5-15. Confirmation of BK(MM) Clones

A. EtdBr-stained horizontal agarose gel.
B. Autoradiograph of Southern blot of above gel hybridized with MM DNA probe.

Lane 1: PstI/EcoRI digest of small MM fragment clone (pMM-S1).
Lane 2: " " " " pBR322.
Lane 3: " " " " MM.
Lane 4: " " " " large MM fragment clone (pMM-L1).
Lane 5: PstI/EcoRI/TaqI digest of large MM fragment clone.
Lane 6: " " " " " pBR322.

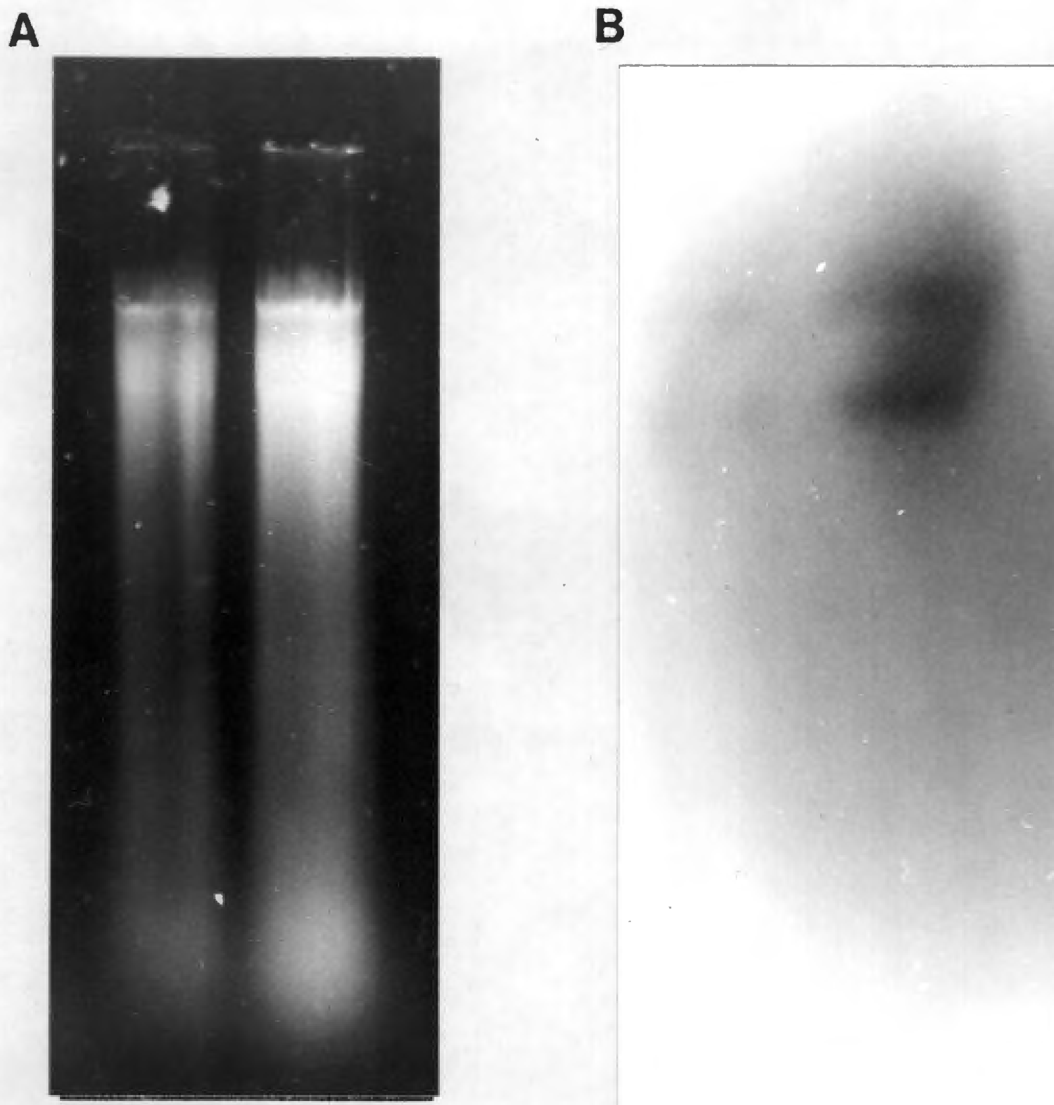


Figure 5-16. Screening DNA Extracted from Human Urine for BK DNA Sequences

A. EtdBr-stained gel of DNA extracted from urine of patient GR

B. Autoradiograph of a Southern blot of this gel hybridized with 32P-labelled probe from a mixture of large MM and small MM PstI/EcoRI fragment recombinant plasmid DNAs.

6. ADDENDUM - LATEST RESULTS

6.1. Restriction Mapping of New Isolates

Very recently, another urine isolate (P.R.) has been obtained containing predominantly polyomavirus-specific DNA. Restriction of this material with HindIII gave the same sized fragments as WW and SN (Fig. 6-1, lane 5). Lane 6 contained a HindIII digest of pMM-L1. The original MM HindIII-C fragment was thereby regenerated, and can be seen running faster than the PR HindIII-C fragment. The two larger fragments in lane 6 both contain portions of MM and pBR322 DNA. The HindIII-C fragment of GR DNA may also be discerned above the contaminating background running in the same position as the PR C-fragment. This implies that all four direct urine isolates tested in this fashion are the same.

In preparation for cloning experiments using the scheme adopted for MM (see Section 5.6) these preps were digested with PstI and EcoRI. An extra 300bp fragment was observed in a PR digest (Fig. 6-1, lane 2) running below the expected two fragments. Lane 1 contained a PstI/EcoRI digest of pMM-L1 which regenerated the pBR322 fragment (upper band) and the large MM insert (lower band). This insert ran slightly faster than the corresponding PR fragment in lane 2. The same result had been obtained with WW DNA (see below). A PstI digest of GR DNA also revealed an extra 300bp fragment when probed with MM DNA (Figs. 6-2 A and B, lane 1). The same fragment should have shown up in lane 4, a PstI/EcoRI digest of GR, but it must be assumed that the background hybridization obscured any BK-specific band in this region. A parallel channel of ØX174 digested with HincII

provided size markers, and confirmed the 300bp estimate of MW. The other channels in Fig. 6-2 contain various digests of MM and GR DNA, and show nicely the specificity of hybridization of the 32P-labelled MM DNA probe. The background in the GR lanes was due either to degraded BK-specific DNA or to insufficiently stringent post-hybridization washes to eliminate all non-specific binding.

It cannot be stated categorically that the WW (and SN) strains also have an extra PstI site. However, such an assumption is consistent with the observed anomalous band mobilities of fragments after PstI/EcoRI digestion of WW DNA (Fig. 5-11). PstI/EcoRI digestion of MM DNA produces fragments of 3308 and 1655bp (Lane 3). WW, having a larger HindIII-C fragment, would be expected to have a slower moving large PstI/EcoRI fragment (as observed, Fig. 5-11, Lane 6) and a smaller PstI/EcoRI fragment of the same mobility as the corresponding MM fragment (See Fig. 7-1). However, this fragment had a significantly greater mobility than expected. This could be due to the loss of the postulated extra ±300bp PstI fragment, and it would thus appear that an extra PstI site occurs between the common PstI and EcoRI sites in all four of our clinical isolates.

Examination of the published BK sequence reveals a potential PstI site involving a single base change 302 bases anticlockwise from the existing PstI site, at base number 1353 (Yang and Wu, 1979c). The BK sequence in this region is:

```
            1340        1350        1360        1370
      TTGTCTTACA AATCTAGCTT GCAGGGTTTT AGGGACAGGA
```

and either a single base change (T to C) at position 1349 or a

single base deletion at this site would generate a PstI site (CTGCA↑G).

Fine restriction mapping of this region is now in progress. The two PstI-generated fragments of PR DNA have been cloned into pBR322, and will provide viral DNA for future sequencing experiments.

6.2. Cloning of a Clinical Isolate

The first successful cloning of human polyomavirus DNA isolated directly from urine was achieved with material from patient P.R.. PstI-digested PR and pBR322 DNAs were ligated and used to transform competent HB101 cells. Many thousands of Tc(R) colonies were obtained, approximately 10% of which were Ap(S).

Forty putative recombinant colonies were screened by another rapid extraction method (Holmes and Quigley, 1981). Briefly, 5ml of an overnight culture was harvested, the cells resuspended in 0.35ml of STET buffer (8% sucrose, 5% Triton X-100, 50mM EDTA, 50mM Tris, pH8.0), and 25µl of fresh lysozyme (10mg/ml in TE) added. Without prior incubation, the specimens were boiled for 40s and centrifuged in a microfuge for 10min. In some experiments the resultant pellet was compact, and the supernatant was drawn off and transferred to a fresh tube. Sometimes the "pellet" formed a cohesive gelatinous clot which was fished out intact, leaving the supernatant behind. This supernatant was largely free of chromosomal DNA. An equal volume of isopropyl alcohol (2-propanol) was added to the supernatant, and the tubes placed at -18C for >10min. The tubes were centrifuged for 5min and the DNA pellets dissolved in 50µl

of sterile water.

Aliquots were electrophoresed on a 0.8% horizontal gel (Fig. 6-4). Further aliquots were digested with 4U/tube PstI for 2h30. Samples 1-15 had a further 4U added after 2h (Fig. 6-5A). After electrophoresis, a common band in all the successfully digested preps was observed. This was presumed to be regenerated linear pBR322. Lanes 1, 2, 15, 16, 18, 22, 25, 26, 30, 33, 36, 38 and 39 also had a band running slightly slower than this, which was presumed to be the large PstI fragment of PR. Lanes 1, 34 and 40 had bands of varying mobility representing unknown inserts. Lanes 3, 4, 6-9, 11-13, 21, 23, 24, 27-29, 31, 32 and 37 had only the presumed pBR322 band visible. These were likely to represent recombinants with the small 300bp PstI fragment of PR. This fragment would run in a position obscured by the RNA contaminating the preps.

Figure 6-3 shows a gel of the first 12 clones from Fig. 6-4 extracted by the same procedure, except that a loop of cells from a colony growing on a plate was used instead of a 5ml overnight culture.

Confirmation of these clones was achieved by probing with labelled BK DNA (Fig. 6-5B). Thus, clones 1, 2, 15, 16, 18, 22, 26, 33-36, 38 and 39 contain the large PstI fragment of PR. No hybridization was observed on this gel in lanes containing putative recombinants of the small PstI PR fragment. This was either because the gel had been electrophoresed too long and the fragment lost, or because the small fragment hybridized with insufficient probe to show up on this exposure (the probe was not fresh and had been used several times before), or because none of these clones actually contained this fragment. However, clones of this fragment were confirmed in other experiments.

Figure 6-6 shows a gel and autoradiograph of 8 putative MM clones. DNA in lanes 1, 4, 6 and 8 hybridized with the BK probe and represent the small PR PstI fragment. Lanes 2, 5 and 7 represent the large PR PstI fragment. DNA in lane 3 hybridized poorly, and probably represents a clone of non-BK-specific DNA.

These clones are in the process of being further characterized at present. They will be used to provide fragments for DNA sequencing of selected regions of the PR (= WW?) genomes, for minute comparison with the prototype BK.

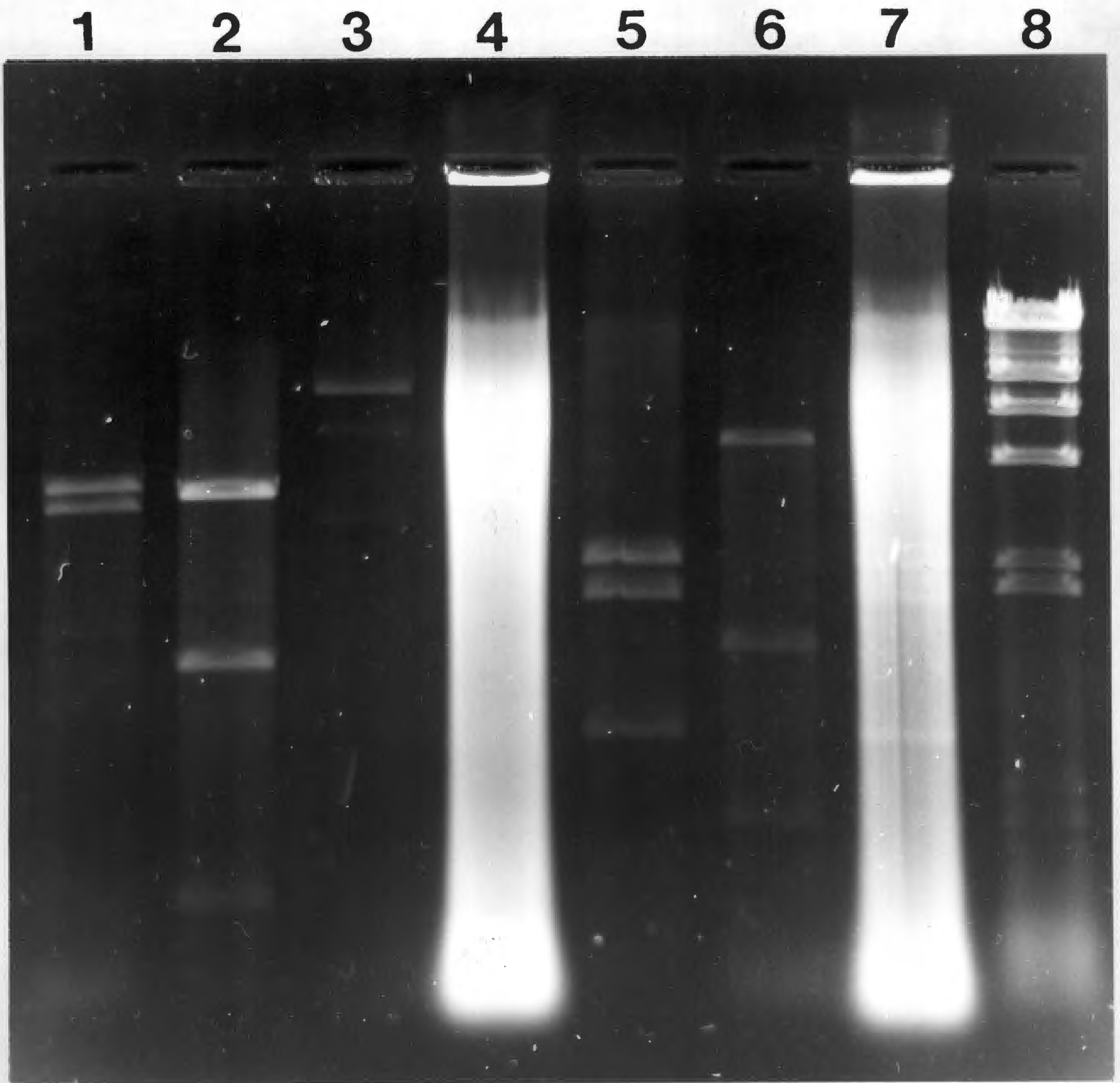


Figure 6-1. Characterization of New Isolate PR

Horizontal agarose gel of restricted DNA from GR, PR and the recombinant MM/pBR322 clones.

Lane 1:	PstI/EcoRI digested pMM-L1 (see Fig. 5-10)			
Lane 2:	" " "	PR DNA		
Lane 3:	" " "	pMM-S1	" "	"
Lane 4:	" " "	GR DNA		
Lane 5:	HindIII digested PR DNA			
Lane 6:	" "	pMM-L1		
Lane 7:	" "	GR DNA		
Lane 8:	" "	Lambda marker		

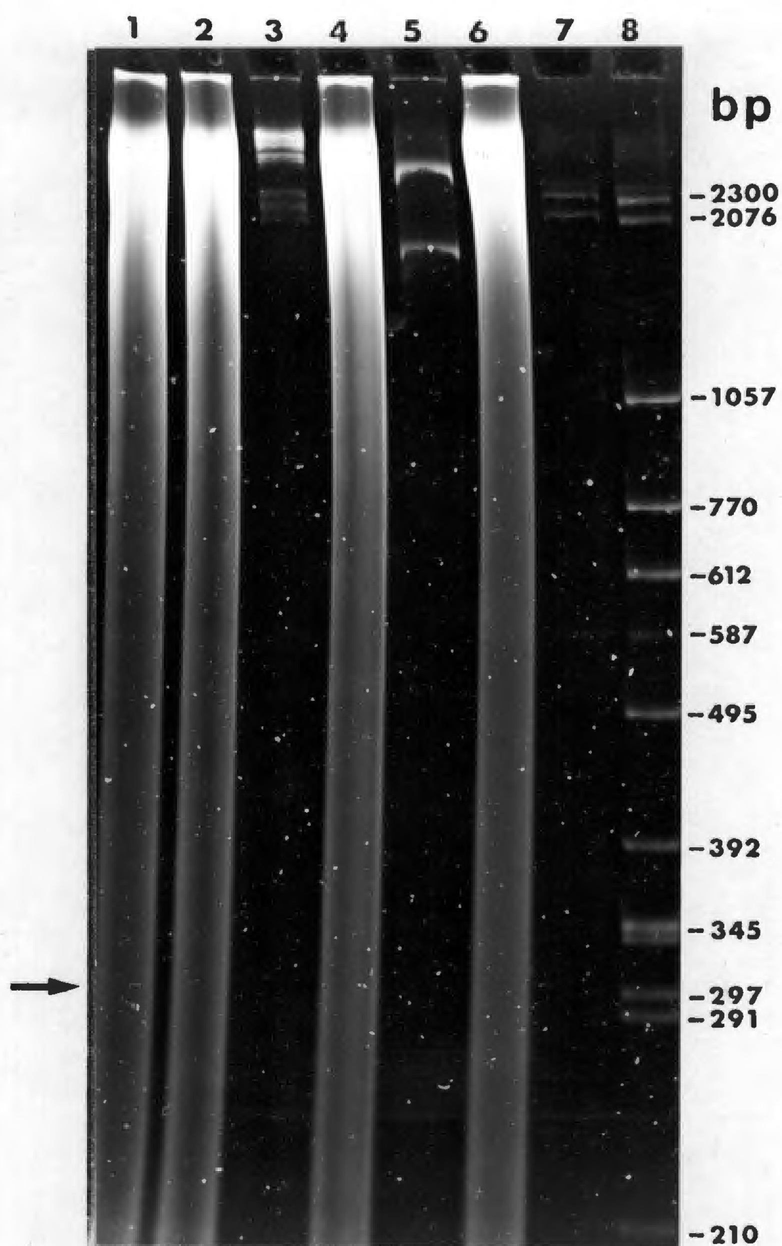


Figure 6-2. A. Confirmation of Extra PstI Site

Vertical acryLamide gel of restricted GR and MM DNA with MW markers. EtdBr-stained gel.

Lane 1:	GR + PstI	Lane 2:	GR + EcoRI
Lane 3:	Lambda + HindIII	Lane 4:	GR + PstI/EcoRI
Lane 5:	MM + PstI/EcoRI	Lane 6:	GR + HindIII
Lane 7:	MM + HindIII	Lane 8:	ØX174 + HincII + MM + HindIII

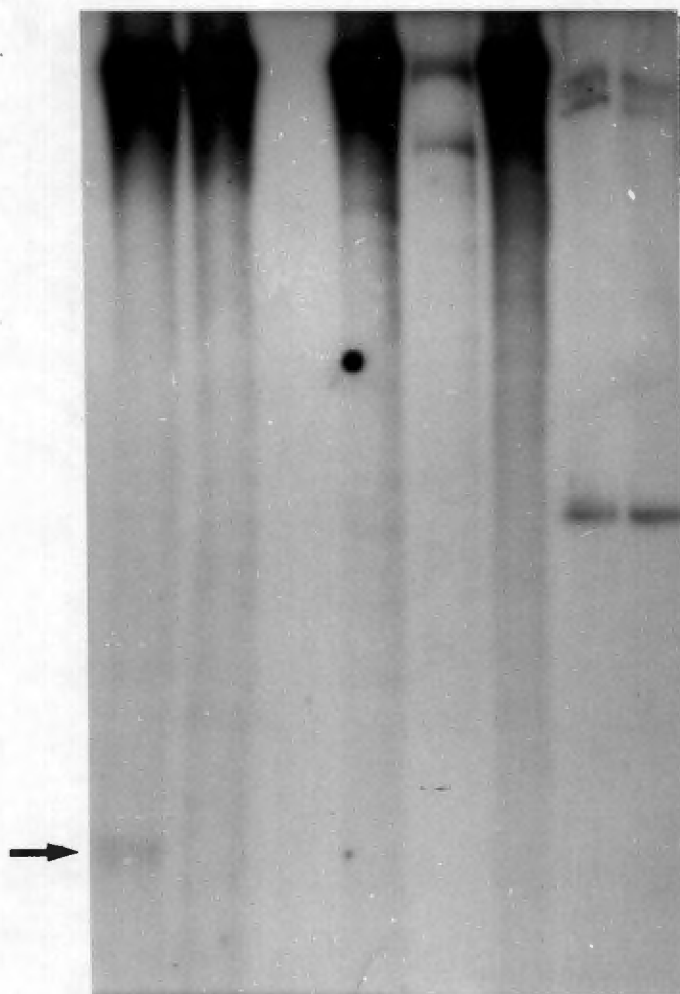


Figure 6-2. B. Autoradiograph of Southern Blot of Gel in Fig. 6-2A, Using MM Probe

The extra 300bp PstI fragment of GR is indicated by an arrow.

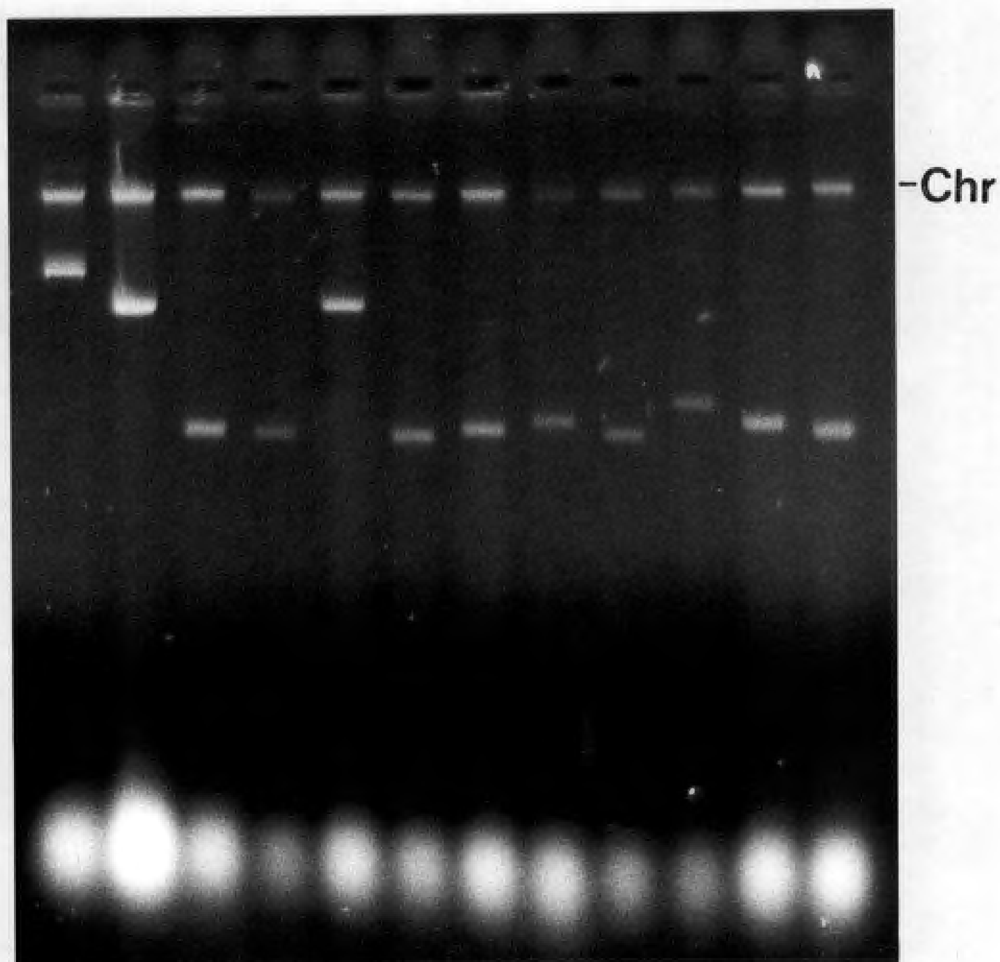


Figure 6-3. Putative PR Recombinant Plasmids
Loop of Cells Extracted by Rapid Boiling Method

1.2% horizontal gel in PB run for 2h30 at 70V of plasmids extracted by the method of Holmes and Quigley (1981).

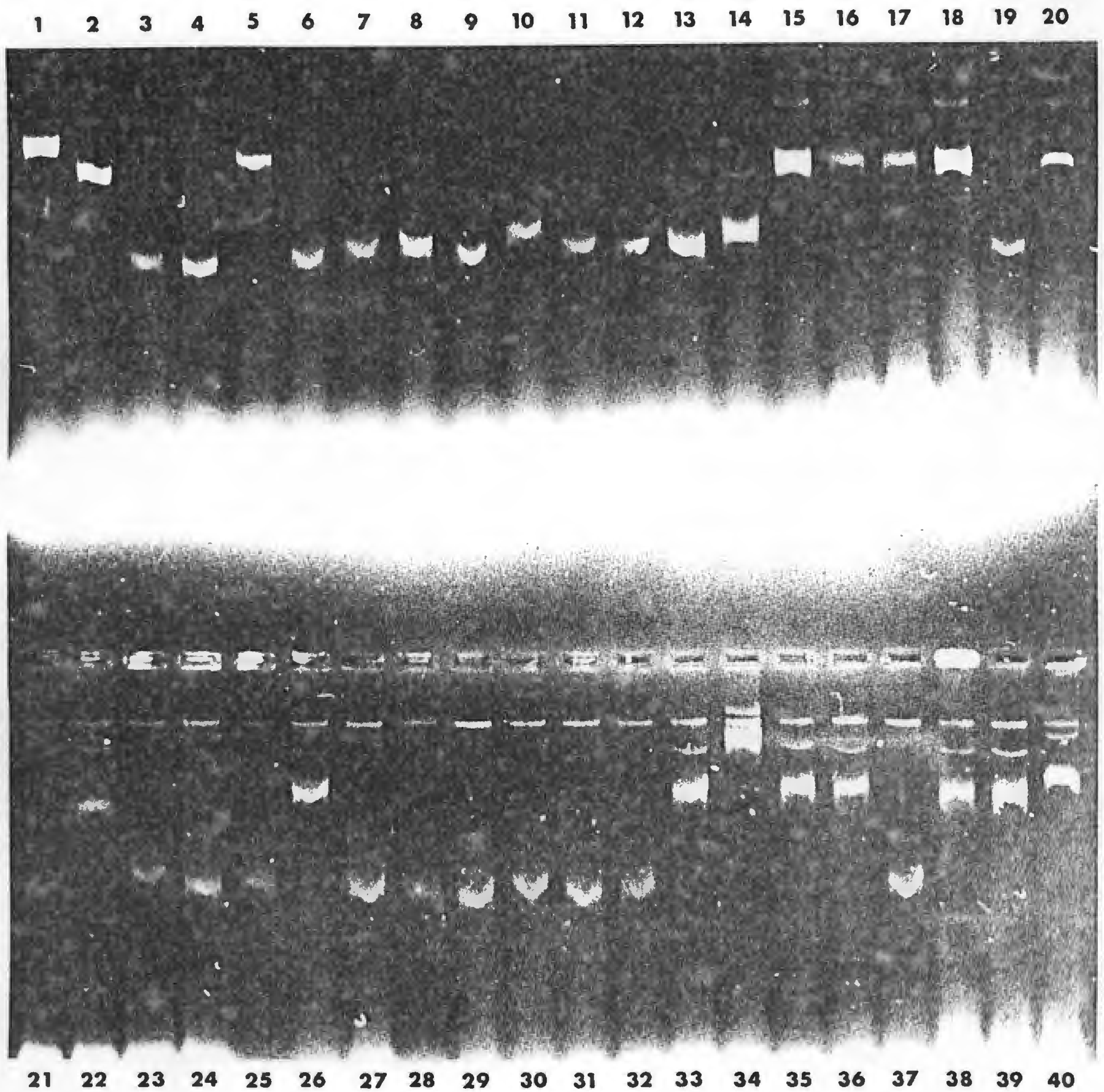


Figure 6-4. Putative PR Recombinant Plasmids
5ml Cultures Extracted by Rapid Boiling Method

0.8% horizontal gel in PB run for 4h at 70V (200mA) of plasmids extracted by the method of Holmes and Quigley (1981).

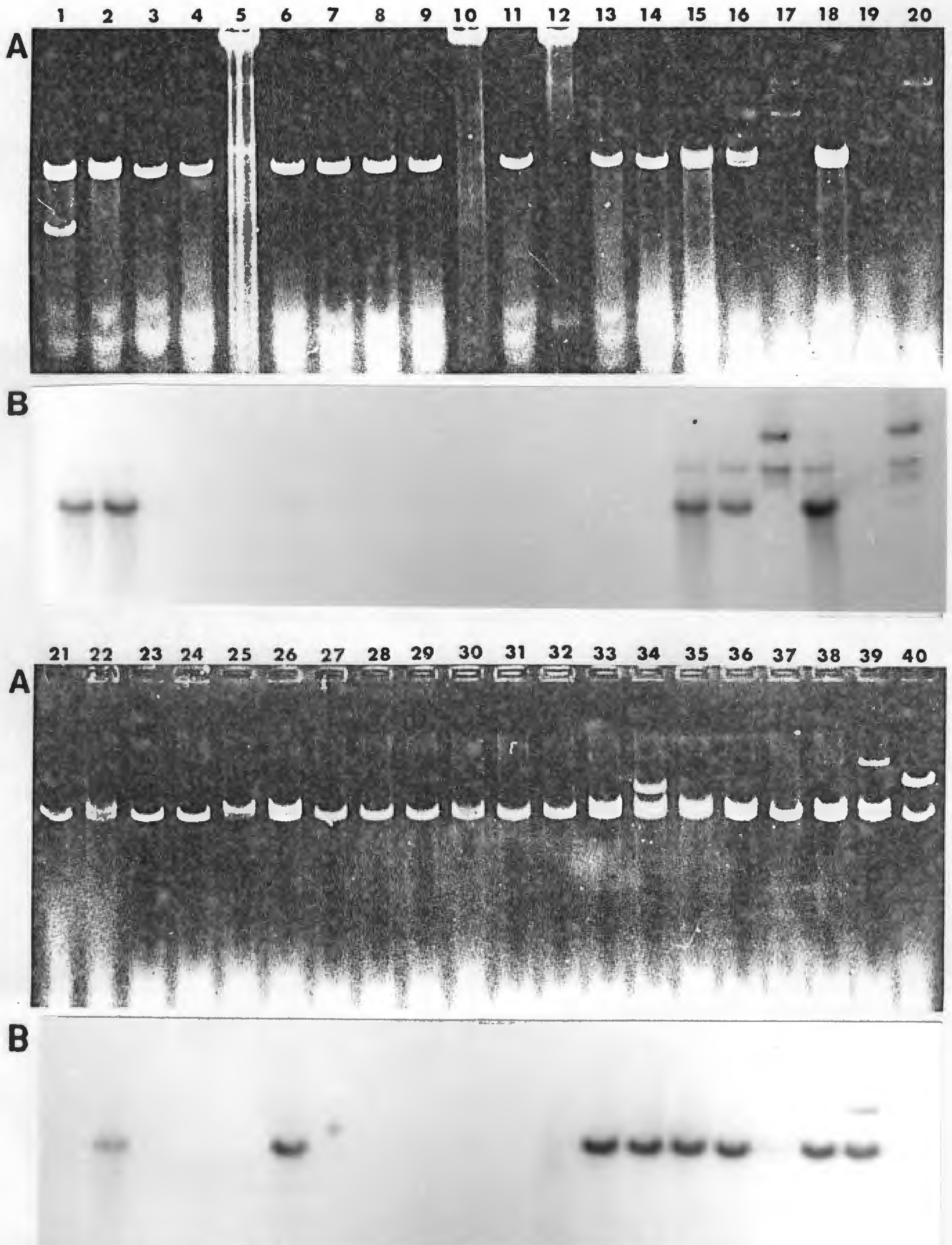
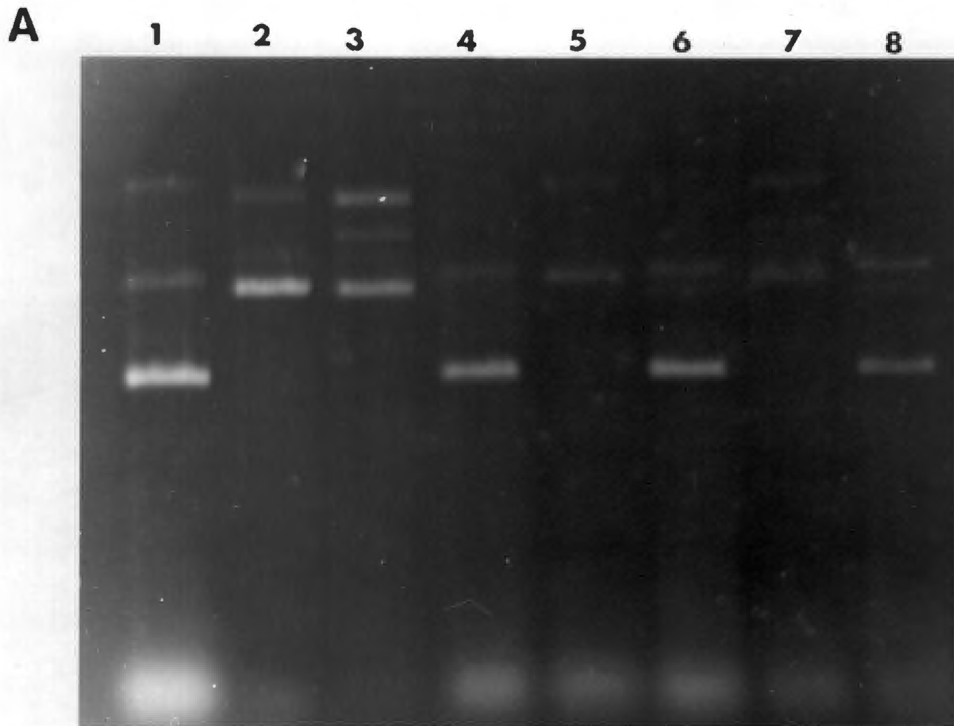


Figure 6-5. PstI Digests of Putative Recombinant Plasmids

A. 1% horizontal agarose gel in PB run at 75V (200mA) for 4h, stained in EtdBr.
B. Autoradiograph of Southern blot of the above gel probed with BK DNA.



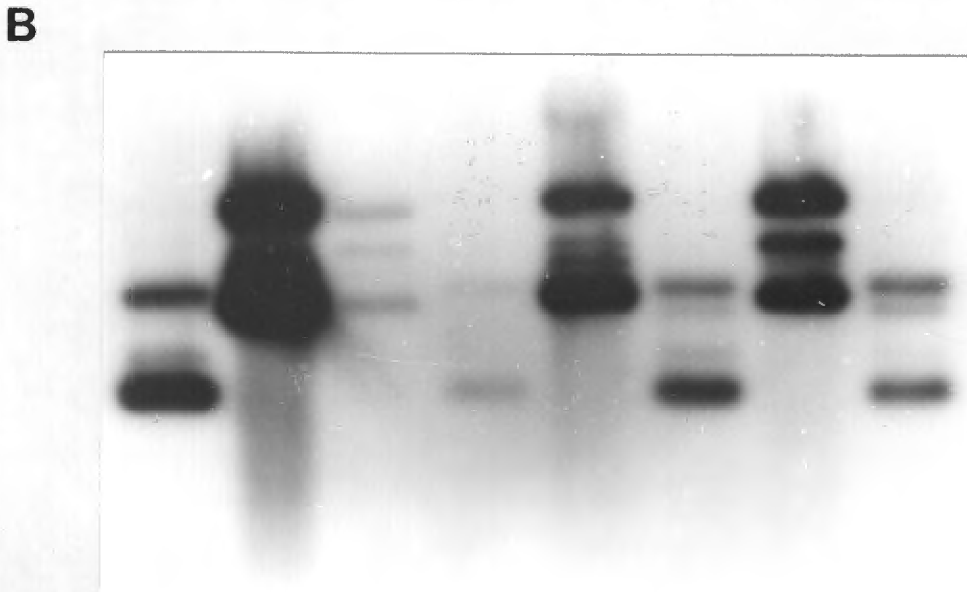


Figure 6-6. Confirmation of Small PstI PR Fragment Clones

A. Ethidium bromide stained gel.

B. Autoradiograph of Southern blot of the above gel probed with BK DNA.

7. DISCUSSION

In this work the identity of human polyomavirus isolates from the urine of renal transplant recipients has been investigated by the analysis of DNA extracted directly from those isolates. Initially, samples were selected in which the presence of polyomavirus-type particles had been established by electronmicroscopic examination (Lecatsas et al., 1973). Techniques have since been described that have been claimed to be sensitive enough to detect 10^{-13} g of DNA homologous to BK DNA (Botchan et al., 1976). This has been made possible by the use of DNA probes labelled to high specific activity with 32P which specifically hybridize with complementary sequences transferred from analytical agarose gels to nitrocellulose membranes (Southern, 1975). Previously, the most sensitive detection method for DNA extracts run in analytical gels was by ethidium bromide staining, which allowed detection of bands containing down to 10ng of DNA at best, and which could not directly distinguish virus-specific sequences from other DNA species. The nature of the bands obtained by ethidium bromide staining had to be determined by comparing their electrophoretic mobilities in different gel concentrations (Harley et al., 1973), and by relating restriction endonuclease digest patterns with those obtained with known strains of BK. Hybridisation with a BK probe, by comparison, establishes immediately whether a given band contains BK specific sequences. The technique can be refined to give information on the degree of homology of various portions of the genome under investigation by hybridizing restriction digests run on agarose gels with BK (or other) probes under various conditions of stringency (Howley et

Table 7-1. Human Polyomavirus Isolates Used for Restriction Analysis

Isolate	Date	3 Hind III sites demonstrated	Extra Pst I site demonstrated	Cloned into pBR322
SN	3.11.77	Yes	No	No
WW	28.8.78	Yes	Indirectly	No
GR	26.6.81	Yes	Yes	No
PR	24.11.81	Yes	Yes	Yes

All DNA was extracted from virus-enriched urine preparations from kidney transplant patients, supplied by Dr. G. Lecatsas, Dept. of Microbiology, University of Pretoria. GR and PR were isolated subsequent to the compilation of Table 5-1. SN and WW correspond to specimens 6 and 10 in Table 5-1.

al., 1979a).

Such techniques had not yet been developed by the time most of the viruses in this study were isolated, and characterization was limited to partial restriction analysis of those preparations with sufficient viral DNA for detection on gels by ethidium bromide staining. This approach sufficed to show that two preparations from different patients (SN and WW) were indistinguishable from each other by EcoRI and HindIII digestion, and were clearly different from, but related to, prototype BK and BK(MM). Until recently, these were the only two isolates from which sufficient DNA was obtained for this type of analysis. The HindIII pattern was very similar to that reported recently for GS virus (Pater et al., 1979). Very recent work has shown that GS in fact differs from our strains. GS was isolated from the urine of a kidney transplant patient by S.D. Gardner, and like our isolates and most other strains was serologically identical to BK virus (Wright et al., 1976). GS was, however, successfully propagated in culture. Recently, two further isolates (GR and PR) have produced the same restriction patterns as WW and SN.

It is highly significant that our four independent preparations gave the same unusual restriction pattern, and suggests that this BK variant might be widespread. The several years separating the isolation of these four strains bars the possibility that the specimens had been mixed accidentally after isolation. Although none of these strains had been successfully adapted to culture, whether this was for genetic reasons or because of other interfering factors was not established. All patients were native-born South Africans, and received kidneys from local donors, so the strain(s) could represent a Southern

African variant of BK.

When the early isolates SN and WW were shown to have the same variant pattern, it was obviously vital to further characterize the strains to prove whether they were in fact identical to each other and/or to GS virus. However, very small quantities of DNA remained, and the patients had stopped secreting virus particles. Two approaches were possible: (1) The new technique of nick translation could be used to label the remaining DNA with 32P, and thus increase the sensitivity of detection of restriction fragments. This would allow a more detailed map to be produced. (2) The viral genome could be cloned into a bacterial system. Then, as much viral DNA as desired could be obtained for further characterization. Detection of restriction fragment bands in gels by using 32P-labelled probe DNA from BK(WT) or another known strain ran the risk that some fragments would not be homologous to any of the probe sequences, and thus would not hybridize. Such an approach would however have given much useful information, and was used in the primary identification of human polyomavirus sequences in recent urine extracts (Fig. 5-16). It could form the basis of a useful screening procedure. It was decided that the recombinant DNA approach offered the best chances of further characterization of the virus genome, and would also allow selected regions to be sequenced if necessary.

Attempts were thus made to incorporate subgenomic fragments of WW DNA into pBR322. The two PstI/EcoRI generated fragments were cloned separately for the reasons given in Section 4.2.2.2. Success was achieved with MM DNA prepared from cell culture, but the first attempt to clone WW DNA in an identical experiment failed. A number of possible reasons can be given for this,

besides the fact that much less WW DNA than MM DNA was used. Transformation was successful, as Ap(R)Tc(R) colonies were obtained, seemingly containing pBR322 only. Restriction had also been successful, as shown by the Southern gel blot in Fig. 5-11. Ligation, therefore, appeared to be the culprit. It is possible that the 14-day delay between the restriction of WW and pBR322, and the ligation experiment allowed the terminal 5' phosphoryl groups on the WW and pBR322 molecules to degrade, which would prevent ligation. This risk could have been minimized by treatment of the WW DNA with T4 polynucleotide kinase to replenish the 5' phosphoryl groups, but this was not done. Very recently, one of the later isolates, PR, has been cloned successfully.

Some of the cloning results with MM were puzzling. Transformation with MM plus BAP-treated pBR322 fragments gave consistently higher numbers of Tc(R) transformants than mixtures with non-BAP-treated pBR322 DNA, even though the DNA concentrations in the ligation reaction were the same. Non-BAP-treated material would have been expected to contain a greater number of religated molecules, although a proportion of these would have been religated pBR322 molecules. The mixtures with BAP-treated vector should have contained only vector-donor hybrids, but there was no obvious reason why the absolute number of pBR322-containing plasmids should have been higher. One explanation is that the purification procedure to which BAP-treated pBR322 was subjected resulted in a DNA preparation containing fewer ligase-inhibiting contaminants, thus allowing more efficient ligation.

A number of preliminary conclusions can be drawn from the restriction analysis results obtained earlier. One is that published marker fragment molecular weights cannot be relied upon until backed up by sequence data. Since a large part of this work was devoted to obtaining accurate MW estimations, the variation in published MW values for various well-known markers was frustrating. [Due to updating of marker values, the final MW values in Table 5-3 differ slightly from those published by us recently (Mew et al., 1981)]. Fortunately, in future, an adequate range of sequenced markers should become available. Lambda, the most commonly available marker, has yet to be fully sequenced - not surprizing, perhaps, since it is about nine times the size of BK! Daniels et al. (1980) have performed exhaustive MW estimations on the lambda restriction map and provide the most reliable values available to date.

The MW of the WW HindIII-B fragment given in Table 5-3 is possibly on the low side. Examination of Fig. 5-9, curve c shows that the MM HindIII-B fragment coordinate is the only point to deviate significantly from the smooth curve through the other data points, having a mobility too great for its known MW (as determined from the base sequence). This anomaly may be due to an unusual tertiary structure adopted by sequences in this fragment. Assuming a high degree of sequence homology between the HindIII-B fragments of WW and MM, the real MW of the WW fragments may also be higher than that estimated from its mobility.

The MW value of the HindIII-C fragment of WW (990bp) is almost equal to the sum of the HindIII-C and -D fragments of BK(WT) (555 + 416 = 971bp) suggesting that WW is missing a

HindIII site at the junction of the C and D fragments in BK(WT). This is similar to findings with GS (Pater et al., 1979), but contrasts with the situation in BK(MM), which is missing a 262bp section of BK(WT), including the HindIII site between fragments B and D (Fig. 7-1). This region is known to be variable between different strains, and even MM has a 3bp deletion immediately to the left of the HindIII site apparently missing in WW ("d" in Fig. 7-1).

HindIII

BK(WT) 5'-ACTTCCTTA[AAT]AAGCTTTT 3220

(Note that the orientation of the WW fragments in Fig. 7-1 has not been proved, but is a reasonable assumption). Although the HindIII-B fragment of WW is smaller than the corresponding MM fragment, it cannot be said at this stage whether it is identical to the BK(WT) B fragment.

The extra PstI site discovered in our strains occurs in a region of the BK genome not known for its variability, lying within the coding sequence for late viral coat polypeptides (See Section 3.3). A putative site on the published BK sequence in the expected region which could generate a PstI site by a single point mutation has been identified. This region is in the process of being fully analysed to identify the differences between our strains and BK(WT).

Analysis of the published maps and sequences for various BK strains throws up some puzzling facts, but as yet sheds little light on differences in their gene expression, especially in regard to the oncogenic potential of these viruses. This

question is one of the main motivations for studying the BK viruses in such detail. It is amazing that all attempts to isolate BKV or BK DNA sequences from normal individuals (pregnant women excluded) have failed, despite the prevalence of BK antibodies in the population. Where does it hide?

One of the characteristics of the non-coding "variable region" between 0.66 and 0.72mu is the presence of tandem repeat sequences, which differ in arrangement between different strains (Fig. 7-2). BK(WT) possesses a 64-base sequence repeated twice, 40- and 44-base portions of which are repeated elsewhere. BK(MM) contains an 87-base repeat, a 61-base portion of which is repeated elsewhere. All these repeats contain sequences in common. In fact they may be arranged to overlap one another, thereby generating a "primordial" repeat sequence of 107bp which contains all of them (Fig. 7-2). It would appear that each strain of BK has suffered a number of deletion and rearrangement events in this region during its evolution. It is difficult to assign any functional role to these sequences, which seem to represent evolutionary "junk". They perhaps merely act as a buffer region between the origin of replication, which occurs nearby, and the initiating regions for RNA transcription. If it is true that the BK(Dun) strain is less susceptible to genomic modification in cell passage than BK(WT), then this region must influence the genetic stability of the virus, as the 43bp segment missing from the Dun strain occurs there (Fig. 7-2).

Within the course of this work, SV40, BK(WT) and BK(MM) have all been fully sequenced by various laboratories, providing a wealth of information on the genetic organisation of these closely related genomes (Fiers <u>et al</u>., 1978; Yang and Wu, 1979c; Seif <u>et al</u>., 1979). The spliced structure of many eukaryotic

mRNAs has been revealed (Hamer and Leder, 1979), including that of the polyomaviruses (Manaker et al., 1979). Proteins are predicted from DNA sequences before they are detected in nature (Jay et al., 1981), but the significance of many non-coding regions in the genome is still only to be guessed at. Why, for instance, has the human mitochondrial genome eliminated all "inessential" sequences, even to the extent of some translational "stop" codons (which are generated by polyadenylation of RNA transcripts), whereas the yeast mitochondrion harbours a much larger genome with extensive non-coding sequences and a more normal eukaryotic type of structure (Anderson et al., 1981)? These completely unanticipated discoveries and the questions which they give rise to are a result of investigative procedures nearly all developed within the past eight years. This has meant that many of the approaches adopted at the start of this work, for instance, became completely outmoded within a couple of years. Molecular genetics can truly be described as one of the most "breathless" fields in science today. A plethora of genetic structures have already been unravelled, but these have mostly served to undermine the great unifying theories of gene function developed in recent decades. Technology has outstripped basic understanding, resulting for instance in the unprecedented self-imposed moratorium on recombinant DNA experiments by scientists in the USA recently, thereby giving the government bureaucracy a wonderful opportunity to erect edifices of control rivalling in complexity the genetic elements with which they deal.

It is not difficult to fall into the same trap as the physicists at the end of the last century, who saw themselves as

having a mere tidying-up operation to perform before all knowledge of the physics of matter was complete. It is easy to ask "what happens when gene expression and evolution are completely understood?". Just as modern physicists delving into the new world of subatomics have become increasingly mystical and imprecise, so the fate of future biologists will probably be to go through repeated cycles of rediscovery and re-rejection of God and the Divine Plan.

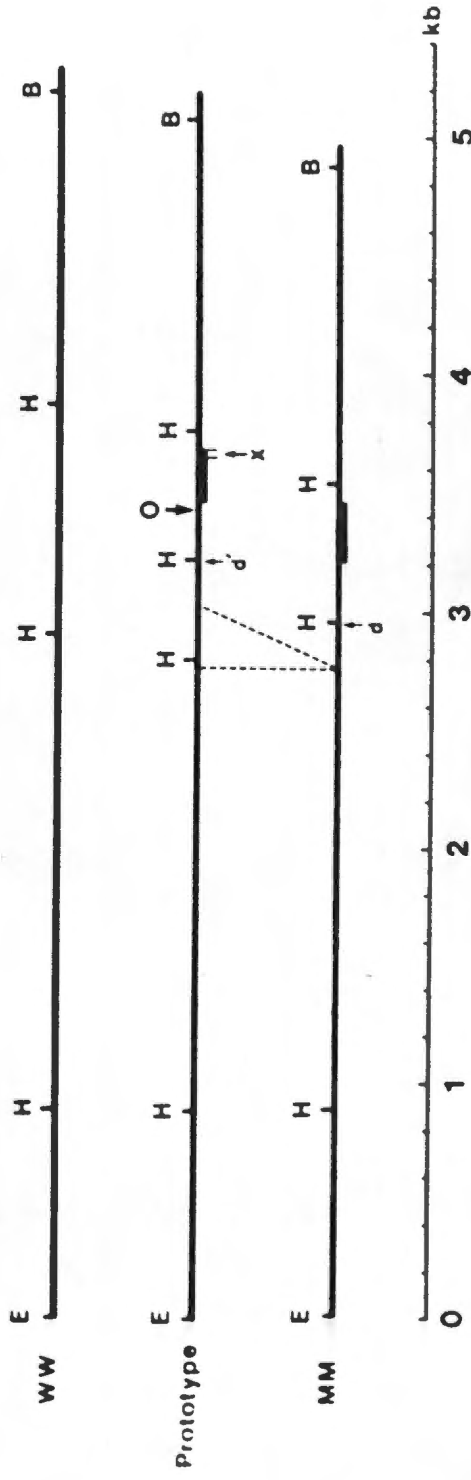


Figure 7-1. Comparison of the Restriction Maps of BK(WW), BK(WT) and BK(MM)

The maps have been opened at the unique EcoRI sites in each molecule.
E = EcoRI; H = HindIII; B = BamHI; O = origin of replication;
x = 43bp region deleted in the BK(Dun) strain;
d' = 3bp segment deleted in the BK(MM) strain;
d = corresponding position in BK(MM).

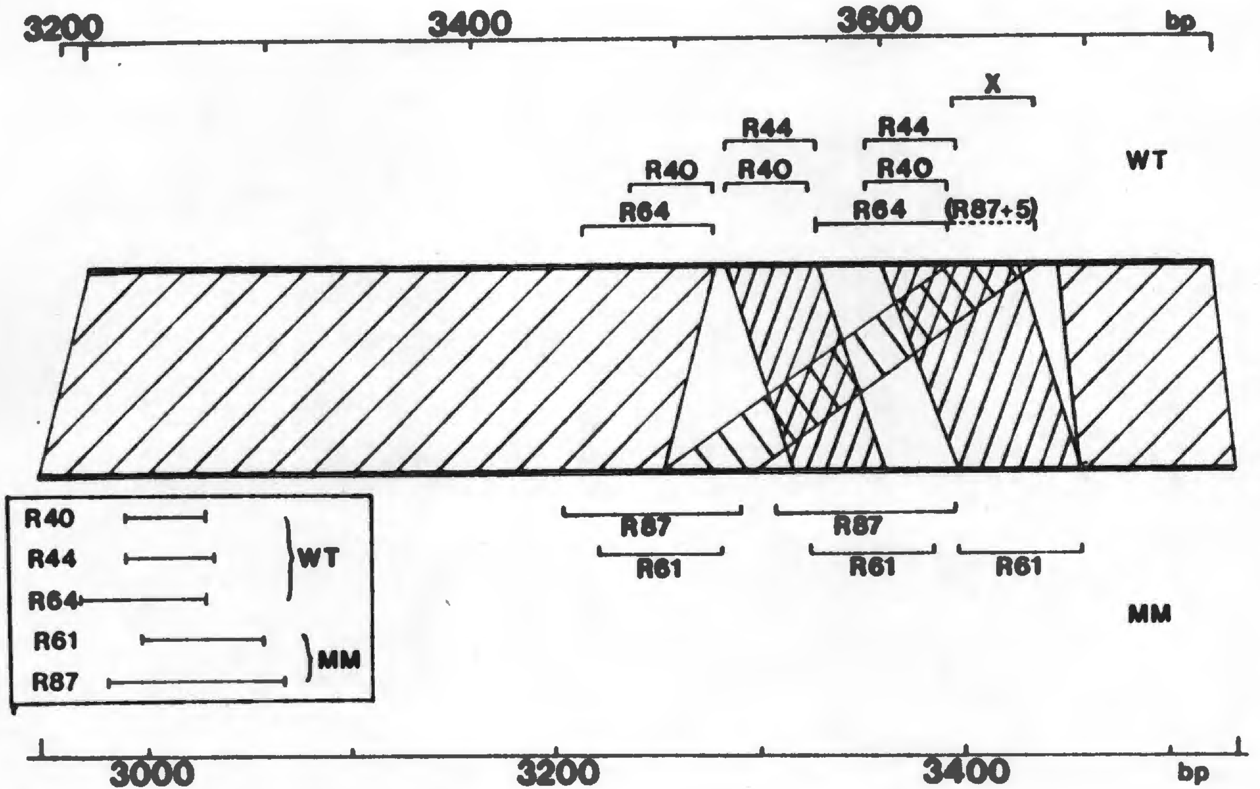
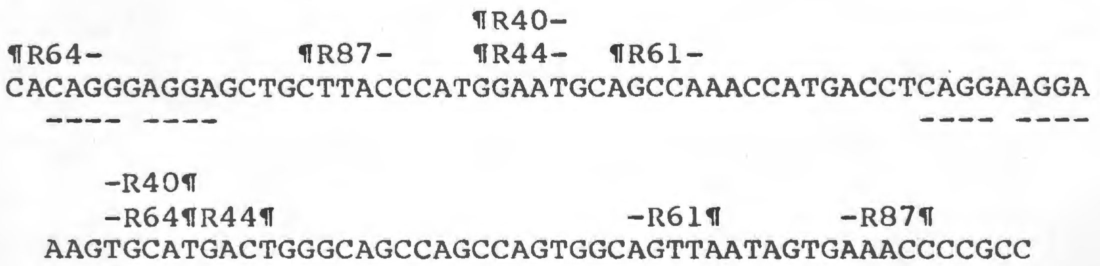


Figure 7-2. Comparison of the Tandem Repeat Sequences of BK(WT) and BK(MM)

The HindIII-C fragments of BK(WT) (above) and BK(MM) (below) have been aligned. Homologous sections are indicated by hatches. Repeat sequences are indicated by bars with lengths in bp. X = 43bp segment missing from BK(Dun). R87+5 indicates a segment in BK(WT) identical to the R87 sequence in BK(MM) except for an additional 5 bases. The repeat sequences of both strains have been aligned in the inset box. Sequence information is from Yang and Wu (1979c) and Seif et al. (1979).

The "complete" repeat sequence containing all those shown is:



8. METHODS

Media and solutions used frequently are listed in section 15.

8.1. Cell Culture Propagation of MM Virus

MM virus was propagated in human embryonic fibroblasts (HEF) by Professor Prozesky's laboratory (Institute of Virology, Sandringham, Transvaal), and later in human glioma cells by Professor Naudé in the Department of Virology, UCT Medical School (Lecatsas _et al_., 1974; Takemoto _et al_., 1974; Seehafer _et al_., 1978). These laboratories had facilities for propagating potentially hazardous viruses which we then lacked.

8.2. Purification of DNA from Urine Pellets

DNA was extracted from suspensions of virus enriched urine pellets received from Dr. Lecatsas at the University of Pretoria. Dr. Lecatsas obtained these pellets from the urine of kidney transplant patients by differential centrifugation (Lecatsas and Van Wyk, 1978). Some samples were further purified by Millipore filtration. Samples were received by post in 200-500µl suspensions containing antibiotics.

A modified version of Hirt's procedure was used to extract DNA from these pellets (Hirt, 1967).

Procedure

Four ml of extraction buffer (0.6% w/v SDS, 0.01M EDTA, pH7.5) was added to the pellet, and left at room temperature for 10-20min. Crude pellets appearing to contain a large amount of impurities were incubated for 15min at 37C. 5M NaCl was added to give a final concentration of 1M, mixed gently by inversion to minimize shearing the DNA, and left at 4C overnight. The precipitate containing most of the SDS, proteins, and hopefully the high MW linear DNA, was removed by centrifugation at 17,000xg and 4C for 30min.

The supernatant was decanted, and extracted three times with chloroform reagent. The phases were mixed by inversion or gentle vortexing for 1min. The BK genome is small enough to resist shearing by gentle vortex treatment. The phases were separated by centrifugation at 2,500xg for several minutes, and the aqueous phase saved. Some samples contained brown-coloured impurities which were not removed by this treatment. Extraction with phenol reagent left a colourless aqueous phase. In some cases a 1:1 mixture of phenol and chloroform reagents was employed.

One or more phenol extraction steps would probably have been advisable in all cases. Subsequent experience gained in the extraction of plasmids from bacteria suggested that extraction with phenol was more effective than with chloroform, but that the phenol must ultimately be completely removed by repeated

chloroform or ether extractions. After a final ether extraction, remaining ether was removed by blowing air or nitrogen over the surface until the odour could no longer be detected. Precautions were taken against handling ether in the vicinity of a naked flame.

The SDS/protein pellet was further extracted for trapped viral DNA by resuspending in a stronger extraction buffer (1% w/v SDS, 1M Sodium perchlorate, pH 8.0) and performing repeated chloroform, or phenol and chloroform, extractions.

After extraction, the clear aqueous phases were precipitated with ethanol.

After spinning down at 6-10,000xg the pellets were dried in vacuo, or in a stream of nitrogen, and resuspended in a small volume of Tris buffer - usually EcoRI or HindIII digestion buffer. The samples were tested for the presence of circular DNA species by gel electrophoresis at this stage. Positive samples were purified further, if necessary, by repeating the alcohol precipitation cycle once or twice.

Note: Caesium chloride ethidium bromide buoyant density centrifugation was avoided during the purification for fear of losing the very small quantities of viral DNA these samples were expected to contain.

8.3. Purification of BK DNA from Cell Culture

The procedure followed was similar to that for extracting DNA from urine pellets (Hirt, 1967). Efforts were made to avoid contamination of the laboratory with virus-containing material, especially prior to DNA extraction. This became difficult when loading capped Beckman angle-rotor centrifuge tubes. Swing-bucket rotor tubes are easier to handle without spillage, but no swing-bucket rotor with adequate volume capacity for spinning down approximately 300ml of medium was available. Beckman now have available a "quick-seal" tube system for use in angle and vertical rotors that is suitable for most containment work.

Procedure

Falcon flasks with any cells still attached were decanted of medium, which was extracted as described below. 1ml of 0.6% w/v SDS in 0.01M EDTA (pH7.5) was added per flask at 25C. Cells detached within 30min and were transferred to Corex centrifuge tubes, where 5M NaCl was added to 1M. After centrifugation, extraction proceeded as described for urine extracts (see above), except that an RNase digestion with 10μg/ml RNase at 25C for 1h was sometimes performed, prior to chloroform or phenol extractions.

The medium was cleared at 2,500rpm for 20min in a benchtop centrifuge. Free virus was pelletted at 110,000xg (38,000rpm) using a 60Ti rotor in a Beckman L2-65B ultracentrifuge at 4C for 1h. The pellet was resuspended in 1ml of DNase buffer per centrifuge tube and treated with 20μg/ml DNase at 37C for 30min to destroy any cellular DNA, while viral DNA remained safely packaged within the virion. An equal volume of 2x Hirt lysis

buffer (i.e. 1.2% w/v SDS, 0.02M EDTA, pH7.5) was added, and incubated at 37C for 15min. The procedure was then the same as for a urine extract (see Section 8.2).

The cell debris pellet from the clearing spin was resuspended in Hirt lysis buffer (1ml per culture flask equivalent) and incubated at 37C for 1h. After sodium chloride precipitation, purification of viral DNA proceeded as for a urine extraction (see section 8.2).

8.4. DNA Restriction

Because most restriction endonucleases are labile enzymes, certain precautions needed to be taken in their use. Stocks were always kept cold, and were kept out of the freezer for as short a period as possible when in use. An attempt was made early on to aliquot the enzymes into separate tubes sufficient for one digestion, but since the volume of enzyme involved was about 1ul it was difficult to ensure that all aliquots retained the same activity, and did not become desiccated. This procedure was thus abandoned. For reliable results, the enzyme was first checked by observing its digestion of a pure substrate such as commercially obtained lambda DNA, giving a known pattern after gel electrophoresis, followed soon after by restriction of the desired DNA sample.

Restrictions were carried out in Eppendorf tubes, which were sometimes siliconized to reduce DNA binding, although this made the tubes difficult to mark with a marker pen. Buffers were Millipore filtered, and enzyme was pipetted using micropipettes with disposable teflon tube tips, to avoid cross-contamination.

Sterile rubber or plastic gloves were usually worn to avoid contamination of the sample with DNases or bacteria, etc., from the skin, and also to reduce exposure to potentially hazardous DNA.

The amount of enzyme used depended on the amount of DNA to be digested, and the residual activity of the enzyme as estimated from preliminary digests of lambda DNA. If enough sample DNA was available, an aliquot was removed after digestion and tested for complete digestion by electrophoresis, prior to molecular weight determination experiments or recombinant manipulations. Digestion was limited to 2-3h, so that extra enzyme was added rather than extending the incubation time, in order to avoid non-specific cleavages, degradation, or enzyme deterioration. Reactions were stopped with one tenth volume of stopping buffer, and the tubes heated to 65C for 5-10min immediately prior to electrophoresis.

The buffers and volumes used were those recommended by the manufacturers. Suggested buffers for the same enzyme varied slightly between manufacturers, but in fact the same buffer may be used for a number of different enzymes (e.g. 10mM Tris-HCl, pH7.5, 7mM 2-mercaptoethanol, 7mM $MgCl_2$; Yang and Wu, 1978c). Early experiments were performed with enzymes from Miles Laboratories, but it was found that Boehringer provided a better service, and BRL a more comprehensive selection.

8.5. GEL ELECTROPHORESIS

8.5.1. Agarose Cylindrical Gels

Gels were cast in perspex tubes with an internal diameter of 6mm (external diameter 8mm) and length 12cm. Gel length was thus about 10cm. The bottoms were sealed with parafilm, supported by gauze held in place with an elastic band.

Gels containing from 0.7 to 2.5% agarose could be made. The appropriate weight of agarose was boiled in buffer under reflux or in a steam bath for at least 30min. Alternatively, the agarose was boiled in 90% final volume of water, and 10x stock buffer was added later to the required concentration (Dugaiczyk et al., 1975). The molten solution was cooled to 45-50C, and poured into the tubes. The gel was allowed to set for several hours before use. Two methods for forming flat tops to the gels were tried:
(1) The gel was extruded partly, and the top few millimetres sliced off perpendicularly with a razor blade. The gel was returned to the tube and the bottom gauze replaced.
(2) The bottom of the gel was formed against flat parafilm, and this surface used as the top after extruding the other end of the gel a short way and cutting it roughly square to prevent the possibility of air bubbles being trapped during electrophoresis.

Eight gels could be run simultaneously in a homemade linear apparatus. Samples, up to 100µl, were layered on as soon as the buffer tanks had been filled. 5-10% glycerol was first added to the samples, and sometimes 0.003% bromophenol blue as a tracking dye. Electrophoresis was continued at constant voltage (usually 50V) for a time that experience had shown to give the desired separation of bands of interest. (Note: The top buffer compartment was connected to the negative terminal of the power supply.)

Thereafter, the gels were extruded into test-tubes containing electrophoresis buffer with 0.5-1.0µg/ml ethidium bromide, and stained for >15min. Fast-moving bands tended to diffuse after prolonged staining. Bands were visualized by placing the gels in a perspex tray, covered with ethidium bromide-containing buffer, under a Camag universal ultra-violet light in a darkened room. Either 254nm or 375nm lamps were used depending on conditions. The shorter wavelength gave better contrast, but was less suitable when the DNA was to be subsequently extracted from the gel, as it caused more damage to the molecule. Black paper was placed under the tray to give a neutral background.

8.5.1.1. Gel Photography

Photographs were taken with a 35mm camera clamped directly over the gels, with the lamp positioned to one side. An orange (Kodak gelatin Wratten filter no. 22) and a UV (no. 2B) filter were used to eliminate light of undesired wavelengths, selecting for the orange fluorescence of ethidium bromide associated with double-stranded nucleic acids under UV.

This lamp arrangement produced rather low-intensity fluorescence of bands, requiring extended photographic exposures of up to 90min to record faint bands. The much more satisfactory transilluminator (Ultraviolet Products, Inc.) was only obtained when this work was largely completed, and cylindrical agarose gels had been superceded by slab gels in most applications.

(Note: It was necessary to place the orange filter above the UV filter to avoid fluorescence of the orange filter caused by UV light.)

It is advisable to wear protective UV screening goggles while using UV apparatus, especially the transilluminator which produces very intense radiation.

Ethidium bromide is mutagenic, so contact with the skin was avoided, especially when handling the pure dust or concentrated stock solutions. Surgical rubber or plastic gloves were worn for this purpose.

8.5.2. Vertical Agarose Slab Gels

Several models of home-made apparatus were used, all requiring one plate with a section cut out from the top to fit around the upper buffer compartment, and a backing plate of the same size without a cut-out. A backing plate made from sandblasted glass helped prevent gel slippage or collapse during electrophoresis. The gel thickness was determined by perspex spacers placed down the sides of the gel between the clamped plates. The bottom was sealed with masking tape, and the side spacers sealed by pouring molten 2% agarose along their outside edges.

After the mold had been completely filled with molten agarose prepared as for cylindrical gels, a perspex slot-former was positioned at the top, avoiding bubbles, to create slots of the desired depth. Slots in agarose gels were kept as shallow as possible to avoid breakage of inter-slot agarose "walls" during slot former removal. To avoid disturbance of the gel after setting, the mold was clamped to the buffer tank before pouring. The masking tape used to form the bottom of the gels prevented leakage of molten agarose and supported the gel in the mold during electrophoresis. It allowed the passage of current so did not need to be perforated.

After setting for 2h the slot former was carefully removed, the complete apparatus assembled, buffer added, and samples transferred to the slots. Electrophoresis was continued until the bromophenol blue marker in the samples had migrated a suitable distance. The plates were then unclamped, and the notched plate removed carefully. The backing plate plus gel were immersed in buffer containing 0.5-1.0μg/ml ethidium bromide for 30min. Band visualization was achieved by placing the plate on a sheet of black paper and illuminating with UV light, or preferably, using a transilluminator. With the latter the gel was placed directly onto the apparatus' filter. Photographic exposures using a transilluminator were in the region of seconds compared to the extended times required using the Camag lamp.

8.5.3. Horizontal Agarose Slab Gels

These gels are very easy to prepare and run, and can give a resolution almost as sharp as the best vertical slabs. Their disadvantage is that sample volume capacity is limited by the thickness of the gel and the dimensions of the well former.

Apparatus was purchased from BRL (Models HO and H3) but was not available until the final stages of this study.

Agarose was prepared as before, and poured onto the levelled base tray which had been prepared by making retaining "walls" from waterproof sticky tape. The desired thickness was controlled by the volume added. The well former was placed in position, ensuring a small gap between the bottom of the comb teeth and the base plate. The gel was covered and allowed to set for 2h. After removal of the well former and tape "sides", the gel was placed in the electrophoresis apparatus and completely submerged in buffer to a depth of several millimetres. Samples, prepared as before, were placed in the wells, taking care to avoid overflowing. The apparatus was covered, and electrophoresis performed. Staining and photography were carried out as before.

The wells may be loaded before the gel is completely submerged. Buffer was added to the level of the gel without covering its surface, and electrophoresis commenced until all sample dye had entered the gel. The gel was then submerged and the run completed. The quality of the bands obtained was sometimes impaired, however, as streaking of the bands back towards the wells tended to occur. Likewise, covering the gel with a layer of cling-film (Saran Wrap) instead of submerging in buffer also produced inferior results.

8.5.4. Polyacrylamide Cylindrical Gels

The same apparatus was used as for agarose gels. Certain types of plastic inhibited the polymerization of the acrylamide mixture, and tubes of such material had to be avoided. Polymerization of low concentration gels (<2.5%) required specially purified acrylamide. Certain commercial suppliers provide recrystallized acrylamide and bis (e.g. Bio-Rad) but we purified our own stocks (see Section 15). Stock solutions were made up in water, containing 20% acrylamide of which 2.5% was the cross-linking reagent bis. This was suitable for gel concentrations >3.5%. For lower concentrations, 5% cross-linker was used. Solutions were kept at 4C. Other ingredients were made up fresh each day. Quantities required for 40ml of mixture for gels of 2-4% are given in Table 15-1.

Acrylamide monomer is highly toxic, and the same precautions are advisable when handling it as with ethidium bromide i.e. the wearing of surgical gloves.

Procedure

A 10% w/v solution of sodium persulphate and a 10% v/v solution of TEMED (N,N,N',N'-tetramethylethylenediamine) were prepared. The volumes of all the reagents (except the sodium persulphate) required for the desired gel concentration (see Table 15-1) were mixed in a conical flask. This solution, the persulphate solution, and about 10ml of water were thoroughly degassed in a vacuum chamber until bubbling had virtually ceased. Oxygen inhibits polymerization of acrylamide and needs to be excluded. The correct volume of persulphate was added to the acrylamide mixture, mixed gently to avoid aeration, and pipetted into the gel tubes which were held in a vertical position. Immediately, about 100µl of degassed water was carefully layered over the top to prevent reoxygenation of the gel, and to allow a flat top to form when the gel set. The interface tended to move down several millimetres during polymerization. The gel should set within 20min, and may conveniently be made the day before use. If kept cold and protected from evaporation, gels may be stored a week or more before use.

The parafilm covering the bottom of the gel was perforated or removed to allow passage of current, and the tubes installed in the electrophoresis apparatus. The gels were "cleared" at 125V for 1h, to remove catalyst and other impurities which could affect subsequent electrophoresis. Samples were loaded and electrophoresed as for agarose gels, although higher voltages and longer runs were required to achieve adequate band migration.

Gels were removed from the tubes by carefully "blowing" them out with a syringe into tubes containing buffer with ethidium bromide. Visualization and photography was performed as for agarose gels, although sloppy, low concentration acrylamide gels had to be handled much more carefully to avoid damage.

8.5.5. Vertical Polyacrylamide Slab Gels

Gel molds were made up as for agarose slabs, except that the bottoms were sealed with a perspex spacer, and the backing plate was clear glass. The molds used produced gels 200x150x1.5mm. Plates were thoroughly cleaned with Decon 75 or chromic acid and distilled water. To aid separation of the plates after a run, the notched plate was treated with Photoflo 600 (Kodak) which caused the gel to stick preferentially to the backing plate. Prevention of leaks was essential. Other methods of sealing the spacers, such as greasing them, were tried, but found to be unsatisfactory compared to agarose.

Degassed monomer and catalyst solutions were prepared as before. After equilibrating the solutions to the temperature required, and mixing, the solution was poured into the mold and the slot former positioned, ensuring that it denied access of air to the top of the gel. Gels were left overnight before use.

The slabs were "cleared" at 150-200V for about 1h before use.

Modifications

(1) Most polyacrylamide gels were set at 4C. Polymerization was slower, but a firmer gel resulted. Low concentration gels required this low temperature to set at all.

(2) Many slabs were run at 4C in the cold room, initially to counteract the heating effect of electrophoresis in the gel, as the apparatus had no cooling system. Temperature also affected band mobility (see Discussion).

(3) During long overnight runs, the buffer was recycled slowly through a peristaltic pump to prevent buffer exhaustion. Such exhaustion caused excessive pH changes in the buffer compartments and adversely affected banding patterns.

(4) Ethidium bromide was included in the gel mixture and running buffer of some gels, to avoid post-run staining. However, samples containing SDS or large amounts of DNA or RNA tended to pick up the ethidium bromide as they migrated, causing uneven staining of slower-moving bands in the same channel.

8.6. RECOMBINANT DNA TECHNIQUES

8.6.1. Transformation

Transformation of bacteria with plasmid DNA had not previously been performed in our laboratory, and we had no prior experience of the technique. Several published methods were compared, and also a protocol obtained from Dr. F. Robb at the Dept. of Microbiology, UCT. The UCT procedure was a modification of the method of Cohen et al. (1973), and was the most straightforward and rapid, so the other methods were compared with this.

None of them gave, in our hands, substantially higher transformation frequencies of E. coli K12 strains with plasmid pBR322 than this quick method, so were not adopted for the recombinant plasmid cloning experiments.

The other procedures tried were those of:-
(1) Norgaard et al. (1978), developed specifically for use with Chi1776 as recipient.
(2) Dagert and Ehrlich (1979), who investigated the effect of prolonged incubation of the cells in calcium chloride solution on the number of competent cells.
(3) Molholt and Doskočil (1978), who claimed a six-fold increase in transformation frequency over that obtained by Cohen et al. (1972), by growing the recipients in medium containing 0.5M sucrose, and adding 1μg/ml lysozyme with the transforming DNA. I found that this procedure increased the frequency of HB101 transformation with pBR322 by about three-fold, but did not significantly affect the rate using C600.

Several factors appeared to be of general significance:-
(1) Cells grown to an OD600 of 0.2-0.3, instead of the 0.6 or

higher recommended by the UCT protocol, transformed at higher frequency, although this effect was not investigated in detail (Norgard et al., 1978).
(2) Great care had to be taken in preparing plates containing antibiotics to ensure optimum selection of transformants. This was especially the case with tetracycline (Tc). This antibiotic was rapidly inactivated by heat, and was thus added to molten agar which had been cooled to 45C prior to pouring plates.

The intrinsic resistance of different coli strains to Tc was found to vary. HB101 was sensitive to concentrations down to 12.5µg/ml of chlortetracycline (Sigma) whereas C600 was resistant to up to 20µg/ml. Different strains thus had to be selected on plates containing different concentrations, as pBR322 does not confer very high resistance to Tc compared with wild type R-plasmids (Rodriguez et al., 1977). Prolonged incubation of plates at 37C also caused the Tc (both oxy- and chlortetracyline) to go off, and background growth developed after several days, depending on the initial concentration and coli strain. This was because Tc is bacteriostatic only.
(3) Washing the cells in calcium chloride or buffer before making them competent did not affect the transformation frequency using the UCT procedure, although most published methods suggest at least one washing step.

Maximizing transformation frequency perhaps becomes academic when an excess of plasmid DNA is used, but in some experiments very little donor DNA was available for the construction of recombinants, so high frequencies and a reliable procedure were important. The most critical step in the experiment was the generation of properly ligated recombinant molecules from restriction fragments of donor and vector DNA.

8.6.1.1. Transformation procedure

This method is based on the UCT Dept. of Microbiology protocol.

An overnight culture was set up, preferably from a single colony of the recipient strain. This was diluted 1:100ml in LB and grown to the required OD. [The protocol recommended an OD600 of 0.6, but an OD of 0.2-0.3 gave a higher proportion of competent cells (Norgard et al., 1978). A larger initial culture volume may be desireable to give the same total number of cells.] The cells were harvested at 4C and 2,500xg for 10min, resuspended in 2ml cold 50mM $CaCl_2$ solution, and kept on ice. The cells could be left in this condition for some time without adverse affect (Dagert and Ehrlich, 1979). 0.1ml was added to each DNA sample in sterile Eppendorf or small Falcon tubes and left on ice for 30min. 10-50ng of transforming DNA was recommended per tube. An excess of DNA, or contamination with a high proportion of linear DNA, may inhibit transformation (Norgard et al., 1978). The cells were then heat shocked at 45C for 2min, chilled on ice, diluted with 3ml LB (antibiotic-free), and shaken at 37C for 1-2h to allow expression of the plasmid phenotype. Up to 100µl aliquots were then plated on selective plates containing the relevant antibiotic(s).

Norgaard et al. (1978) and Dagert and Ehrlich (1979) observed that plating 100µl per plate produced fewer transformant

colonies per cell than 5 or 10µl per plate, and thus recommended plating a smaller inoculum. However, the number of plates required multiplies accordingly unless transformation volumes are reduced.

Untransformed competent cells were plated as controls, and sometimes cells from the original log phase culture. The sterility of the transforming DNA was checked on a non-selective plate.

Omitting the heat shock step decreased the frequency of transformation of C600 with pBR322 by up to 10-fold (results not shown). However, in recent discussions with workers at the UCT Dept. of Microbiology, this step was considered by them to be unnecessary. The procedure should perhaps be tested for each strain of recipient to be used. The mechanism of transformation is still obscure, and the in vitro methods are rather empirical.

8.6.2. Characterization of Transformed Colonies

Once resistant colonies had been obtained on selective plates there was a need to distinguish between contaminants, spontaneously resistant mutants, transformants containing intact vector plasmid only, and those containing recombinant plasmids. Thereafter, the recombinant needed to be analysed for the presence of the desired "donor" DNA, and the arrangement of the DNA in the plasmid had to be determined.

8.6.2.1. Contaminants

These could usually be readily identified by their different morphology, but when doubt arose colonies were tested using routine microbiological techniques.

8.6.2.2. Spontaneous Mutants

These were very rare, and usually not as resistant as transformants. They were found, of course, not to contain any plasmids. Problems occurred if the antibiotic concentration in the selective plates was not high enough to suppress all background growth. Growing cells from a single colony helped to prevent heterogeneity of intrinsic resistance from developing.

8.6.2.3. Vector Plasmid Transformants

Only pBR322 will be considered. This has resistance genes for ampicillin (Ap) and tetracycline (Tc), and single restriction sites for the enzymes HindIII, EcoRI, BamHI, SalI, and PstI (Sutcliffe, 1978). Insertions at the PstI site, for instance, give recombinant plasmids which are Tc(R)Ap(S). These were distinguished from re-ligated pBR322 transformants that were Tc(R)Ap(R) by transferring Tc(R) colonies to Ap plates. Ap(R) colonies were rejected. Colonies were transferred by one

of two methods:

8.6.3. Replica Plating

If there were a large number of Tc(R) colonies to be tested this method was followed. It was first described by Elek and Hilson (1954),

Requirements

(1) A wooden cylindrical block to fit into a Petri dish.

(2) Circles or squares of high quality furnishing velvet about 5cm larger than the block, sterilized by autoclaving.

(3) A wooden or metal ring to hold the velvet tightly over the end of the block.

Procedure

The culture plate was pressed carefully onto the velvet pad avoiding any smudging action. A fresh plate was pressed likewise over the pad. Both plates were marked so that they could be orientated after incubation. By "looking through" both plates held in the correct orientation colonies that did not grow on the second selective plate were identified as recombinants.

8.6.4. Toothpick Transfer

If relatively few transformants were to be tested, they were transferred to fresh selective plates using sterile wooden toothpicks. The plates were marked with a numbered grid drawn either directly onto the base of the plate, or onto a sheet of graph paper attached thereon.

Each colony was touched with a toothpick, and marked onto an assigned grid position, perhaps on several selective plates containing relevant antibiotics. Positive colonies were streaked on selective plates to obtain single colonies prior to further analysis.

This procedure was also used to confirm the nature of colonies selected by replica plating.

8.6.5. Colony Hybridization

When a suitable probe of the donor DNA was available, this method was used directly after primary selection of transformants to identify positive recombinants. The probe was DNA which was able to hybridize specifically to the desired DNA insert. It was labelled to high specific activity with 32P (or 3H) by nick translation (see section 8.6.6). In our experiments the probe was BK(MM) DNA which had been purified from infected HEK cells in culture (Section 8.3).

The method was based on that of Grunstein and Hogness (1975).

Procedure

Circles of nitrocellulose paper were cut to fit Petri dishes (Millipore HA, 0.45µm pore size), orientated with pencil marks, autoclaved at 15lb/sq.in. for 10min between sheets of filter paper, and dried. Thereafter the membranes were handled only with sterile forceps.

The following schedule refers specifically to the selection of BK DNA-containing recombinants from Ap(S)Tc(R) transformants.

The circles were placed onto LA plates containing 15-25µg/ml tetracycline, and transformed Ap(S)Tc(R) colonies transferred to the paper with toothpicks. An alternative would have been to select directly for transformants by spreading the transformed suspension on plates with Millipore circles already in place. Plates were incubated at 37C for about 18h, until colonies were approximately 1mm diameter. Circles were then removed from the plates, carefully blotted on absorbent paper to remove excess medium, and then subjected to colony lysis procedure, ensuring the colonies were not smudged or directly touched.

Colonies were lysed at room temperature by floating the circles on 0.5N NaOH, with the colonies face-up, for 7min. They were then blotted, and placed on absorbent paper soaked in 1M Tris-HCl, pH7.4, for 1min. Full neutralization was achieved by transferring the circles to fresh pads soaked in the same Tris buffer for 5min, blot drying, and placing on pads containing 0.5M Tris-HCl, 1.5M NaCl, pH7.4, for 5min.

Colony residues were fixed onto the membranes and dried by application of a strong vacuum under the circles for 30min. (Dismantled Falcon disposable filters (cat. no. 7102) may be used for this purpose if circles of appropriate size are used.) The circles were then dipped upside down in a minimum volume of fresh Proteinase K solution (2mg/ml in 1xSSC) in a Petri dish for 20min at room temperature. They were then blotted dry, transferred to 10ml 1xSSC for 2min, and completely dried by vacuum. Dry membranes were floated in 10ml chloroform for 2min, air dried, and washed in 10ml 2xSSC for 2min to remove loose cellular debris. Circles were again dried for 30min by vacuum, followed by baking in an 80C oven for 2h, between sheets of filter paper.

Hybridization with 32P-labelled BK(MM) DNA

Refs: Wensick et al., (1974); Grunstein and Wallis, (1979).

SET Buffer

Tris-HCl	0.12M	pH 7.1
NaCl	0.6M	
EDTA	8mM	

Denhardt's Solution (Denhardt, 1966)

Ficoll	0.2% w/v
BSA Fraction V	0.2% w/v
Polyvinylpyrridone	0.2% w/v

In: 3xSSC

Prehybridization Solution

4 x SET Buffer
10 x Denhardt's Solution
50µg/ml sonicated herring sperm DNA
0.1% w/v sodium pyrophosphate (NaPPi)
0.1% w/v SDS

Procedure

Dried nitrocellulose membranes were washed with gentle shaking for 1h at 68C in 25ml prehybridization solution. The Denhardt's solution and herring sperm DNA prevent nonspecific binding of single-stranded radioactive probe DNA to the filters, and NaPPi reduces nonspecific binding of 32P-nucleotides. This reduces background radioactivity in the filters.

10^7 cpm of 32P-labelled BK(MM) DNA at a specific activity of 2.5×10^7 cpm/µg (8.3×10^7 dpm/µg) in 525µl of TES buffer was denatured by boiling for 5min. Prehybridization buffer was added to a volume of 5ml. Membranes were transferred to a plastic bag, probe solution added, all air bubbles excluded, and the bag heat sealed. The bag was placed in a water-tight box and incubated at 68C for 64h in a gently shaking water bath to allow hybridization to take place.

Membranes were then removed from the bag and two post-hybridization washes performed. The first was in 50ml of prehybridization solution (minus herring sperm DNA) at 68C for 1h with shaking, and the second in 200ml 0.66xSET at 20C for 1h with shaking. Membranes were blotted dry on 3MM filter papers, wrapped in cling film and autoradiographed for 28h.

If background radioactivity was still high, a further wash in 0.1xSET at room temperature for 1h was performed.

8.6.6. Nick Translation

Ref: Rigby et al., (1977).

Nick Translation Buffer (NT)

Tris-HCl	60mM	pH7.5
$MgCl_2$	6mM	
NaCl	50mM	

Kept as a 10x stock, filter-sterilized, and stored at 4C. Fresh DTT was added to 6mM to diluted buffer on day of use.

Nucleotide Triphosphates

Labelled and unlabelled deoxyribonucleotide triphosphates were tested by high pressure liquid chromatography (HPLC) for their state of phosphorylation. A 3-99% gradient of filtered 0.8M $Na_2H_2PO_4$, pH4.3, was used, on an anion exchange column, type G12 D1S5 CS6, with a flow rate of 4ml/min, monitoring at 260nm. Fractions were collected at 30sec intervals and counted in a β-scintillation counter.

DNA may be either 3H-labelled with dTTP, [methyl,1',2'-3H]-, or 32P-labelled with dCTP, [alpha-32P]-. 32P-labelling allows for more rapid and sensitive autoradiography due to its higher activity, but such material has to be used within a few weeks because of the short (14 day) half-life of 32P.

32P must be handled with great care. Radiation can be harmful to the retina, so work was performed behind a thick perspex screen. Routine disposal procedures for radioactive materials were followed.

Lyophilization

Tracer was supplied in 50% ethanol which had to be removed prior to use. It was dried under vacuum for 30-60min. An amount of tracer determined to be sufficient for one labelling experiment was dried in a siliconized Eppendorf tube, and the other ingredients added subsequently.

32P-Labelling of MM DNA

Approximately 1μg of MM DNA in ±25μl of NT buffer was added to 15-50μCi of dried [alpha-32P]-dCTP, plus a 2-fold excess of unlabelled triphosphates (dATP, dGTP and dTTP). The mixture was equilibrated to 15C, and 6-10U of E. coli DNA polymerase-1 added. (1 unit = amount of enzyme that incorporates 10nmol of total nucleotide into acid precipitable form in 30min at 37C using a poly(dA-dT) template primer). Acid-precipitable counts were determined at 30min intervals by transferring 0.5μl of reaction mixture onto a piece of filter paper, placing in a scintillation vial with ±10ml of 6% TCA, and counting in a β-scintillation counter. The filter was then washed 3x with TCA and recounted, to give the proportion of acid-precipitable

counts. Ideally, the reaction slowed down after 1-2h. The reaction was stopped by addition of 10mM EDTA, 0.6% w/v SDS (final concentration), pH7.2. Unincorporated triphosphates were separated from the DNA by passage through a 1ml column of Sephadex G-50 in a sterile Pasteur pipette. Beads were swollen by heating at 37C for 1h in water, and then equilibrated with 3 column volumes of TES buffer. The column was allowed to run dry, the sample layered on top, and eluted with TES buffer. 150µl fractions were collected, counted, and aliqots electrophoresed through a 1% agarose slab gel that was autoradiographed to determine the sizes of labelled products. Probe specificity was tested by hybridization to a Southern gel blot of a gel containing pBR322, E. coli DNA, human chromosomal DNA, BK(MM) and WW DNAs.

8.6.7. Southern Gel Transfer

Ref: Southern (1975)

This method was used to transfer DNA fragments from agarose slab gels to nitrocellulose filters, maintaining the banding pattern obtained on the gel, for hybridization with a radioactively-labelled DNA probe.

The gel (usually a 1% horizontal slab) was stained with ethidium bromide and photographed, for future comparison with autoradiographs of hybridized filters. DNA was denatured by soaking the gel in 250ml 0.5M NaOH, 1.5M NaCl for 3-5h with gentle rotatory shaking. Gels were rinsed in distilled water and neutralized in 250ml 0.5M Tris-HCl, 20xSSC (pH5.5) for 1-2h.

A thick piece of filter paper (e.g. Whatman 3MM) soaked in 20xSSC (pH7.0) was placed flat onto a glass plate bridging across a tray containing 20xSSC, with the ends of the paper hanging into the buffer to act as wicks. The treated gel was placed onto the paper, avoiding bubbles of air. The paper around the gel was covered with cling film. A sheet of Millipore HA 0.45µm nitrocellulose paper was floated on 2xSSC, and then positioned on the gel, again avoiding bubbles. Two pieces of thick filter paper cut to the size of the nitrocellulose sheet were soaked in 2xSSC and placed on top, ensuring that the membrane did not shift relative to the gel, because it would already have taken up some of the DNA from the gel. A thick wad of absorbent paper sheets was piled on top with similar care, over which was laid a glass plate weighed down evenly with lead weights. DNA transfer was carried out at 4C for 48-62h, with occasional replenishment of the bottom sheet of filter paper with 20xSSC to prevent drying.

Thereafter, gel lane positions were marked onto the membrane, which was then cut into orientated pieces of convenient size, soaked in 2xSSC for >10min, and baked at 80C for 2h or until dry. Membranes could be stored at this stage between sheets of filter paper, sealed in plastic, at 4C.

Hybridization of 32P-Labelled probe DNA to Southern Gel Transfer Membranes

Ref: Southern (1975)

This protocol is very similar to that for colony hybridization, and in fact the same procedure could be followed for all hybridizations to membrane-bound DNA.

Hybridization Solution

3xSSC
1x Denhardt's Solution
0.1% w/v SDS
50µg/ml sonicated herring sperm DNA
0.1% w/v NaPPi

Posthybridization Solution

3xSSC
10x Denhardt's solution
0.1% w/v SDS

Procedure

Nitrocellulose membranes were soaked at 65C in 50-150ml of fresh, preheated solutions of 3xSSC for 60min; then in hybridization solution for 1h. 0.25-0.60µg 32P-labelled DNA probe (6-12x10^6 cpm) was heat denatured by boiling for 5min, made up to 5ml with hybridization solution, and added to the membranes contained in a plastic bag that was then sealed, excluding air bubbles. A number of filters could be hybridized in the same bag, separated by washed, blank nitrocellulose sheets. The bag was placed in a plastic box and shaken gently at 65C for 48-62h.

Membranes were washed 6x for 2min in 50-150ml of post-hybridization solution, then twice for 30min with shaking. All handling was performed with sterile forceps, and sterile gloves were worn. Background radiation in the filter was monitored using a Geiger counter, and further washes performed if necessary. Three stringent washes in 0.1xSSC, 0.1% w/v SDS were performed at 65C for 30 min. Membranes were blotted, dried at 37C, and stuck onto a sheet of cardboard in the correct orientation, wrapped in cling film, and autoradiographed.

8.6.8. Autoradiography

32P-labelled gels or filters, wrapped in cling film, were autoradiographed with Kodak X-Omat MA film, using Kodak intensifying screens, in X-ray type cassettes. Exposure was at -20C (or -70C if possible) for 3-62h, depending on the 32P signal intensity. Films were developed with Kodak Liquid X-ray developer and fixer (FX40).

8.7. PLASMID EXTRACTION

A number of published methods were tried, and modified, in an attempt to achieve consistent results. Different approaches were adopted for primary screening of recombinants and for preparation of larger quantities of pure plasmid for detailed characterization.

8.7.1. Microscale Screening of Recombinant Colonies

Ref: Birnboim and Doly, 1979

This method worked reasonably well without practice or modification. Plasmids extracted from 0.5ml of culture or less could be identified on agarose gels, with or without prior restriction digestion.

Reagents

Solution I (Lysozyme mixture)

Lysozyme	2 mg/ml	
Glucose (dextrose)	50mM	
EDTA	10mM	
Tris-HCl	25mM	pH8.0

Used freshly prepared. CDTA (Cyclohexane diamine tetraacetate) is supposed to work even better than EDTA, being more soluble in alcohol and forming stronger complexes with metal ions.

Solution II (Lysis mixture)

NaOH	0.2M
SDS	1% w/v

Stable for about 1 week at room temperature.

Solution III

Sodium acetate	3M
pH 4.8	

Dissolved in a minimum of distilled water, pH adjusted with glacial acetic acid, and made up to final volume. Stored at room temperature.

Solution IV

Sodium acetate	0.1M
Tris-HCl	0.05M
pH 8.0	

RNase A	1mg/ml

Made up in 0.05M Tris-HCl, pH8.0, heated to 100C for 10min, and stored frozen.

The method is geared to the use of a benchtop centrifuge able to accommodate 1.5ml Eppendorf-style tubes, and capable of 8-10,000xg.

Procedure

(1) To make plasmid suitable for electrophoresis.

Operations were carried out at room temperature unless otherwise stated.

Cells were grown in 2.5ml LB + antibiotic selecting for the plasmid in 6ml Falcon tubes for 18h. 0.5ml was transferred to a 1.5ml Eppendorf tube. (The remainder was stored, if required, at -20C in 40% glycerol.) The tubes were centrifuged for 15sec (longer if the cell concentration was low), and the supernatant removed with a fine-tipped aspirator or pasteur pipette. The cells were resuspended in 100µl of Solution I and incubated for 30min at 0C. 200µl of Solution II were added and the mixture gently vortexed. It should go clear and slightly viscous. The tubes were left at 0C for 5min. 150µl of Solution III were added, and mixed gently by inversion for a few seconds. The tubes were left at 0C for 60min, then centrifuged for 5min. 0.4ml of supernatant were transferred to a second tube. 1ml of cold ethanol was added, and the tubes kept at -20C for 30min. (If the cell number was low, 25µg of tRNA was added to aid plasmid precipitation.) The tubes were spun for 2 minutes, and the supernatant aspirated. The pellet was dissolved in 100µl of Solution IV, reprecipitated with 200µl of cold ethanol, and kept at -20C for 10min. The tubes were again spun and aspirated. The pellet was then dissolved in 40µl distilled water, and 10µl of 5x electrophoresis buffer added. 25-50µl were applied to agarose gels for analysis.

(2) Modifications to prepare plasmid DNA for restriction enzyme digestion and transformation.

Procedure (1) was followed, except that the resuspension/reprecipitation step was repeated. The pellet was finally resuspended in 36µl distilled water, and 4µl of RNase solution were added. Incubation was for 30min at 37C. An appropriate volume of concentrated enzyme buffer was added, and 1 unit of restriction enzyme. Digestion at 37C was continued for 60min. The sample was then mixed with concentrated

electrophoresis buffer, and aliquots applied to a gel for analysis.

For transformation, the 2x resuspended/precipitated DNA was resuspended in 40µl distilled water or dilute buffer, and a few microlitres added to competent cells.

8.7.2. Gentle Lysis Procedure

This method is based on that of Meyers et al. (1976), modified by Dr. J. Thompson (Dept. of Genetics, University of the Witwatersrand).

Culture volumes from 50ml to 1l or more were used. For bulk isolation of plasmids exhibiting relaxed replication, such as pBR322 and its derivatives, the plasmid was amplified by treatment with chloramphenicol.

Growth

A single colony was selected from a streak of bacteria grown on a selective plate, dispersed into 1-10ml of broth in a test tube, and incubated overnight at 37C, preferably with shaking. This saturated culture was inoculated 1:100 into the required volume of broth in Ehrlenmeyer flasks, and grown in a rotary shaker or shaking water bath at 37C. The number of copies per cell of relaxed plasmids was increased to 1,000-3,000 by addition of chloramphenicol to the broth when the cells reached an OD600 of 1.5. This stopped the replication of the bacterial chromosome but did not affect plasmid DNA synthesis directly. Chloramphenicol (Boehringer) was added at a concentration of 150µg/ml, and the cultures were further shaken at 37C for 12 hours (or overnight). (Some commercial brands of chloramphenicol are not effective in plasmid amplification if they have been chemically modified to increase the solubility of the drug, as they are inactive until injected into an animal.)

Lysis

Volumes were suitable for 500ml to 1l cultures, but were scaled down when necessary.

Detergent Solution

1M Tris pH8.0	5ml
0.5M EDTA pH8.5	12.5ml
10% v/v Triton X-100	1ml
Water	81.5ml

Made up fresh on day of use.

The cells were pelletted by centrifugation at 6,000rpm for 10min, resuspended in half the original volume of TE (pH8.0) and pelletted again. The pellet was resuspended in 12.5ml of cold 25% sucrose in 0.05M Tris (pH8.0). 2.5ml of fresh lysozyme

solution (10mg/ml in TE) was added, and swirled gently on ice for 5min (minimum). 2.5ml of 0.5M EDTA (pH8.5) was added and swirled occasionally on ice for 5min. 20ml of cold detergent solution was added to lyse the cells, and swirled gently on ice for 10min until the solution cleared partially, and became highly viscous. The lysate was centrifuged at 20,000rpm in the SS34 head of the Sorvall RC2B refrigerated centrifuge for 30min at 4C. The supernatant was carefully removed, the volume measured, and 0.95g CsCl and 0.1ml 10mg/ml ethidium bromide solution added per ml. This solution should have a refractive index of 1.394 +- 0.001.

The mixture was spun at between 35,000 and 45,000rpm in a Beckman 50Ti rotor for at least 48h. The higher the speed, the steeper the gradient produced, and the closer the plasmid band(s) run to the chromosomal DNA. The DNA bands were visualized under long wavelength UV illumination in a darkroom. The lower band, containing plasmid DNA, was removed by one of two methods:

(1) The side of the tube was punctured using a 16-18 gauge needle, and the band carefully removed into a syringe.

(2) A 16-18 gauge needle on a 1ml syringe barrel connected to a peristaltic pump was lowered carefully into the tube while air was slowly pumped though to prevent solution rising up the needle before the lower band was reached. When the top of the needle orifice was positioned below the band, the pump was reversed, and the band slowly removed. The syringe was replaced for every sample to prevent cross contamination. Sometimes, the chromosomal (or more strictly speaking "non-supercoiled DNA") band was first removed to minimize contamination of the plasmid with chromosomal DNA.

If no lower band was visible, a 1ml volume of solution was removed from a level below the chromosomal band corresponding to the expected position of a plasmid band, and further purified. Useful quantities of plasmid DNA could be present without being visible under these conditions.

The ethidium bromide was removed by repeated extractions with isopropanol (2-propanol) saturated with CsCl solution of density 1.57 +- 0.02g/cc, or with isoamyl alcohol. Extraction was continued until no further purple colouration of the solvent resulted.

The caesium chloride was removed by dialysis.

8.7.2.1. Preparation of Dialysis Tubing

The tubing was boiled for about 10min in distilled water containing small amounts of EDTA and sodium carbonate to remove heavy metal contaminants. The tubing was then transferred to fresh, boiling distilled water for 10min, and washed twice in cold distilled water. Membranes were then ready for use, or could be stored either in water in the cold, or in 66% ethanol. Ethanol was removed prior to use by washing again in water.

8.7.2.2. Dialysis

Sealed and labelled dialysis bags were placed in 1 or 2 litres of buffer (10mM Tris-HCl, pH8.0, 1mM EDTA) and stirred at 4C for several hours. The bags were transferred to several litres of fresh buffer and dialysis continued overnight.

The plasmid solution was then precipitated at -20C overnight with 1/10th volume of 3M sodium acetate and 2 volumes of ethanol. The resuspended preparation could be tested by gel electrophoresis, and further purified, if necessary, for restriction analysis or repeated transformation.

9. REFERENCES FOR PART I

Anderson, S., Bankier, A.T., Barrell, B.G., de Bruyn, M.H.L., Coulson, A.R., Drouin, J., Esperon, I.C., Nierlich, D.P., Roe, B.A., Sanger, F., Schreier, P.H., Smith, A.J.H., Staden, R., and Young, J.G. (1981). Sequence and organization of the human mitochrondrial genome. Nature 290: 457-465.

Birnboim, H.C., and Doly, J. (1979). A rapid alkaline extraction procedure for screening recombinant plasmid DNA. Nucleic Acids Res. 7: 1513-1523.

Blaese, R.M., Strober, W., Brown, R.S., and Waldman, T.A. (1968). The Wiskott-Aldrich Syndrome. A disorder with a possible defect in antigen processing or recognition. Lancet i: 1056-1061.

Bolivar, F., Rodriguez, R.L., Greene, P.J., Betlach, M.C., Heyneker, H.L., and Boyer, H.W. (1977). Construction and characterization of new cloning vehicles. II. A multipurpose cloning system. Gene 2: 95-113.

Bolivar, F., and Backman, K. (1979). Plasmids of E. coli as cloning vectors. In: Methods in Enzymology, Vol. 68; Academic Press, Ed. R. Wu.

Botchan, M., Ozanne, B., Sudgen, B., Sharp, P.A., and Sambrook, J. (1974). Viral DNA in transformed cells. III. The amounts of different regions of the SV40 genome present in a line of transformed mouse cells. Proc. Natl. Acad. Sci. USA. 71: 4183-4187.

Botchan, M., Topp, W., and Sambrook, J. (1976). The arrangement of simian virus 40 sequences in the DNA of transformed cells. Cell 9: 269-287.

Bouck, N., Beales, N., Shenk, T., Berg, P., and di Mayorca, G. (1978). New region of the simian virus 40 genome required for efficient viral transformation. Proc. Natl. Acad. Sci. USA. 75: 2473-2477.

Brack, C., Eberle, H., Bickle, T.A., and Yuan, R. (1976). A map of the sites on bacteriophage PM2 DNA for the restriction enzymes HindIII and HpaII. J. Mol. Biol. 104: 305-309.

Bradley, M.K., and Dougherty, R.M. (1978). Transformation of African Green Monkey cells with the RF strain of human papovavirus BKV. Virology 85: 231-240.

Cathala, F., Hauw, J.-J., and Escourolle, R. (1973). Isolement et étude ultrastructurale d'un virus de type papova non neutralisé par le sérum de référence anti-SV40 au cours de la leucoencéphalopathie multifocale progressive. C.R. Acad. Sci. [D] (Paris). 276: 1081-1084.

Cohen, S.N., Chang, A.G.Y., Boyer, H.W., and Helling, R. (1973). Construction of biologically functional bacterial plasmids in

vitro. Proc. Natl. Acad. Sci. USA. 70: 3240-3244.

Cohen, S.N., Chang, A.G.Y., and Hsu, L. (1972). Nonchromosomal antibiotic resistance in bacteria. Genetic transformation of Escherichia coli by R-factor DNA. Proc. Natl. Acad. Sci. USA. 69: 2110-2114.

Coleman, D.V., Daniel, R.A., Gardner, S.D., Field, A.M., and Gibson, P.E. (1977). Polyoma virus in urine during pregnancy. Lancet ii: 709-710.

Coleman, D.V., Field, A.M., Gardner, S.D., Porter, K.A., and Starzl, T.E. (1973). Virus induced obstruction of the uretic and cystic duct in allograft recipients. Transplant Proc. 5: 95-98.

Coleman, D.V., Gardner, S.D., and Field, A.M. (1973). Human polyomavirus infection in renal allograft recipients. Brit. Med. J. 3: 371-375.

Crawford, L.V., Cole, C.N., Smith, A.E., Paucha, E., Tegtmeyer, P., Rundell, K., and Berg, P. (1978). Organization and expression of early genes of simian virus 40. Proc. Natl. Acad. Sci. USA. 75: 117-121.

Dagert, M., and Ehrlich, S.D. (1979). Prolonged incubation in calcium chloride improves the competence of Escherichia coli cells. Gene 6: 23-28.

Daniels, D.L., De Wet, J.R., and Blattner, F.R. (1980). New map of bacteriophage lambda DNA. J. Virol. 33: 390-400.

Danna, K.J., Sack, G.H., Jr., and Nathans, D. (1973). Studies of simian virus 40 DNA. VII. A cleavage map of the SV40 genome. J. Mol. Biol. 78: 363-376.

Dhar, R., Lai, C.-J., and Khoury, G. (1978). Nucleotide sequence of the DNA replication origin for human papovavirus BKV: Sequence and structural homology with SV40. Cell 13: 345-358.

Dhar, R., Seif, I., and Khoury, G. (1979). Nucleotide sequence of the BK virus DNA segment encoding small t antigen. Proc. Natl. Acad. Sci. USA. 76: 565-569.

Dougherty, R.M. (1976). Induction of tumors in Syrian hamsters by a human renal papovavirus, RF strain. J. Natl. Cancer Inst. 57: 395-400.

Dougherty, R.M., and DiStephano, H.S. (1974). Isolation and characterization of a papovavirus from human urine. Proc. Soc. Exp. Biol. Med. 146: 481-487.

Dugaiczyk, A., Boyer, H.W., and Goodman, H.M. (1975). Ligation of EcoRI endonuclease-generated DNA fragments into linear and circular structures. J. Mol. Biol. 96: 171-184.

Elek, S.D., and Hilson, G.R.F. (1954). Combined agar diffusion and replica plating techniques in the study of antibacterial

substances. J. Clin. Pathol. 7: 37-44.

Editorial, (1978). Has BK virus found a disease? Lancet ii: 876-877.

Farrell, M.P., Mântyjârvi, R.A., and Pagano, J.S. (1978). T antigen of BK papovavirus in infected and transformed cells. J. Virol. 25: 871-877.

Field, A.M., Gardner, S.D., Goodbody, R.A., and Woodhouse, M.A. (1974). Identity of a newly isolated human polyomavirus from a patient with progressive multifocal leucoencephalopathy. J. Clin. Pathol. 27: 341-347.

Fiers, W., Contreras, R., Haegeman, G., Rogiers, R., Van de Voorde, A., Van Heuverswyn, H., Van Herreweghe, J., Volgaert, G., and Ysebaert, M. (1978). Complete nucleotide sequence of SV40 DNA. Nature 273: 113-120.

Fiori, M., and di Mayorca, G. (1976). Occurence of BK virus DNA in DNA obtained from certain human tumors. Proc. Natl. Acad. Sci. USA. 73: 4662-4666.

Freund, J., di Mayorca, G., and Subramanian, K.N. (1979). Mapping and ordering of fragments of BK virus DNA produced by restriction endonucleases. J. Virol. 29: 915-925.

Fried, M., Klein, B., Murray, K., Greenaway, P., Tooze, J., Boll, W., and Weissman, C. (1979). Infectivity in mouse fibroblasts of polyoma DNA integrated into plasmid pBR322 or lambdoid phage DNA. Nature 279: 811-816.

Gardner, S.D. (1973). Prevalence in England of antibody to human polyomavirus (B.K.). Brit. Med. J. 1: 77-78.

Gardner, S.D. (1977a). Implication of papovaviruses in human disease. In: Comparative Diagnosis of Viral Diseases, Vol. I; Human and Related Viruses, Part A; edited by E. Kurstak and C. Kurstak, Academic Press, New York.

Gardner, S.D. (1977b). The new human papovaviruses: Their nature and significance. In: Recent Advances in Clinical Virology, edited by A.P. Waterson, pp.93-115. Churchill-Livingstone, New York.

Gardner, S.D. (1981). Personal communication.

Gardner, S.D., Field, A.M.. Coleman, D.V., and Hulme, B. (1971). New human papovavirus (B.K.) isolated from urine after renal transplantation. Lancet i: 1253-1257.

Gerber, M.A., Shah, K.V., Thung, S.N., and Zu Rhein, G.M. (1980). Immunohistochemical demonstration of common antigen of polyomaviruses in routine histologic tissue sections of animals and man. Amer. J. Clin. Pathol. 73: 794-797.

Graessmann, A., Graessmann, M., Tjian, R., and Topp, W.C. (1980). Simian virus 40 small-t protein is required for loss of actin cable networks in rat cells. J. Virol. 33: 1182-1191.

Gressel, J., Rosner, A., and Cohen, N. (1975). Temperature of acrylamide polymerization and electrophoretic mobilities of nucleic acids. Anal. Biochem. 69: 84-91.

Griffin, B.E., Fried, M., and Cowie, A. (1974). Polyoma DNA: A Physical Map. Proc. Nat. Acad. Sci. USA. 71: 2077-2081.

Grunstein, M., and Wallis, J. (1979). Colony hybridization. In: Methods in Enzymology, Vol. 68; Academic Press; ed. R. Wu.

Grunstein, M., and Hogness, D.S. (1975). Colony hybridization: A method for the isolation of cloned DNAs that contain a specific gene. Proc. Natl. Acad. Sci. USA. 72: 3961-3965.

Gruss, P., Lai, C.-J., Dhar, R., and Khoury, G. (1979). Splicing as a requirement for biogenesis of functional 16S mRNA of simian virus 40. Proc. Natl. Acad. Sci. USA. 76: 4317-4321.

Hamer, D.H., and Leder, P. (1979). Splicing and the formation of stable RNA. Cell 18: 1299-1302.

Harley, E.H., White, J.S., and Rees, K.R. (1973). The identification of different structural classes of nucleic acids by electrophoresis in polyacrylamide gels of different concentration. Biochim. Biophys. Acta 299: 253-263.

Hirt, B. (1967). Selective extraction of polyoma DNA from infected mouse cell cultures. J. Mol. Biol. 26: 365-369.

Holmes, D.S., and Quigley, M. (1981). A rapid boiling method for the preparation of bacterial plasmids. Anal. Biochem. 114: 193-197.

Howley, P.M. (1979). Personal communication.

Howley, P.M. (1980). Molecular biology of SV40 and the human polyomaviruses BK and JC. In: Viral Oncology, edited by G. Klein, pp.489-550; Raven Press, New York.

Howley, P.M., and Martin, M.A. (1977). Uniform representation of the human papovavirus BK genome in transformed hamster cells. J. Virol. 23: 205-208.

Howley, P.M., Israel, M.A., Law, M.-F., and Martin, M.A. (1979a). A rapid method for detecting and mapping homology between heterologous DNAs. J. Biol. Chem. 254: 4876-4883.

Howley, P.M., Khoury, G., Byrne, J.C., Takemoto, K.K., and Martin, M.A. (1975a). Physical map of the BK virus genome. J. Virol. 16: 959-973.

Howley, P.M., Mullarkey, M.F., Takemoto, K.K., and Martin, M.A. (1975b). Characterization of human papovavirus BK DNA. J. Virol. 15: 173-181.

Howley, P.M., Newell, N., Shah, K.V., Law, M.-F., Gruss, P., Sauer, G., and Kelly, T.J., Jr. (1979b). Identification of the primate papovavirus HD as the stump-tailed macaque virus. J. Virol. 30: 400-403.

Israel, M.A., Martin, M.A., Takemoto, K.K., Howley, P.M., Aaronson, S.A., Solomon, D., and Khoury, G. (1978). Evaluation of normal and neoplastic human tissue for BK virus. Virology 90: 187-196.

Israel, M.A., Simmons, D.T., Hourihan, S.L., Rowe, W.P., and Martin, M.A. (1979). Interrupting the early region of polyoma virus DNA enhances tumorigenicity. Proc. Natl. Acad. Sci. USA. 76: 3713-3716.

Jay, G., Nomura, S., Anderson, C.W., and Khoury, G. (1981). Identification of the SV40 agnogene product: A DNA binding protein. Nature 291: 346-349.

Johnson, P.H., and Grossman, L.I. (1977). Electrophoresis of DNA in agarose gels. Optimizing separations of conformational isomers of double- and single-stranded DNAs. Biochemistry 16: 4217-4225.

Jung, M., Krech, U., Price, P.C., and Pyndiah, M.N. (1975). Evidence of chronic persistent infections with polyomaviruses (BK type) in renal transplant recipients. Arch. Virol. 47: 39-46.

Lai, C.-J., Goldman, N.D., and Khoury, G. (1979). Functional similarity between the early antigens of simian virus 40 and human papovavirus BK. J. Virol. 30: 141-147.

Lane, D.P., and Hoeffler, W.K. (1980). SV40 large T shares an antigenic determinant with a cellular protein of molecular weight 68,000. Nature 288: 167-170.

Law, M.-F., Takemoto, K.K., and Howley, P.M. (1979). Characterization of the genome of the murine papovavirus K. J. Virol. 30: 90-97.

Lebowitz, P., and Weissmann S.M. (1980). Organisation and transcription of the simian virus 40 genome. Current Topics Microbiol. Immunol. 87.

Lecatsas, G., and Prozesky, O.W. (1974). E.m. studies on the development and cytopathology of a human polyoma virus (B.K.). Proc. S. Afr. Soc. Path. Congr., Johannesburg. p37.

Lecatsas, G., and Prozesky, O.W. (1975). Excretion of morphological variants of human polyoma virus. Arch. Virol. 47: 393-397.

Lecatsas, G., and van Wyk, J.A.L. (1978). DNA viruses in urine after renal transplantation. S.A. Med. J. 53: 787-788.

Lecatsas, G., Boes, E.G.M., and Horsthemke, E. (1981). Intermediate size papovavirus particles in pregnancy urine. J. gen. Virol. 52: 359-362.

Lecatsas, G., Prozesky, O.W., and Scheepers, F. (1974). The cytopathology and development of a human polyoma virus (B.K.). Archiv ges. Virusforsch. 44: 319-327.

Lecatsas, G., Prozesky, O.W., van Wyk, J., and Els, H.J. (1973). Papova virus in urine after renal transplantation. Nature 241: 343-344.

Lecatsas, G., Schoub, B.D., and Prozesky, O.W. (1976a). Development of a new human polyoma virus strain (MG). Arch. Virol. 51: 327-333.

Lecatsas, G., Schoub, B.D., Prozesky, O.W., Pretorius, F., and de Beer, F.C. (1976b). Polyoma virus in urine in aplastic anaemia. Lancet i: 259-260.

Lewis, A.M., Jr., and Martin, R.G. (1979). Oncogenicity of simian virus 40 deletion mutants that induce altered 17-kilodalton t-proteins. Proc. Natl. Acad. Sci. USA. 76: 4299-4302.

Lowy, D.R., Dvoretzky, I., Shober, R., Ming-Fan, L., Engel, L., and Howley, P.M. (1980). In vitro tumorigenic transformation by a defined sub-genomic fragment of bovine papilloma virus DNA. Nature 287: 72-74.

Major, E.O., and DiMayorca, G. (1973). Maligant transformation of BHK21 clone 13 cells by BK virus - a human papovavirus. Proc. Natl. Acad. Sci. USA. 70: 3210-3212.

Manaker, R.A., Khoury, G., and Lai, C.-J. (1979). The spliced structure of BK virus mRNAs in lytically infected and transformed cells. Virology 97: 112-121.

Manz, H.J., Dinsdale, H.B., and Morrin, P.A.F. (1971). Progressive multifocal leucoencephalopathy after renal transplantation. Demonstration of papova-like virions. Annal. Int. Med. 75: 77-81.

Martin, R.G., and Chou, J.Y. (1975). Simian virus 40 functions required for the establishment and maintenance of malignant transformation. J. Virol. 15: 599-612.

Martin, J.D., Frisque, R.J., Padgett, B.L., and Walker, D.L. (1979). Restriction endonuclease cleavage map of the DNA of JC virus. J. Virol. 29: 846-855.

Mason, D.H., Jr., and Takemoto, K.K. (1976). Complementation between BK human papovavirus and a simian virus 40 tsA mutant. J. Virol. 17: 1060-1062.

McCutchan, J.H., and Pagano, J.S. (1968). Enhancement of the infectivity of simian virus 40 deoxyribonucleic acid with diethylaminoethyl dextran. J. Natl. Cancer Inst. 41: 351-357.

McMaster, G.K., and Carmichael, G.G. (1977). Analysis of single and double-stranded nucleic acids on polyacrylamide and agarose gels by using glyoxal and acridine orange. Proc. Natl. Acad. Sci. USA. 74: 4835-4838.

McParland, R.H., Brown, L.R., and Pearson, G.D. (1976). Cleavage of lambda DNA by a site-specific endonuclease from Serratia marcescens. J. Virol. 19: 1006-1011.

Meneguzzi, G., Barbanti-Brodano, G., and Milanesi, G. (1978a). Transcription of BK virus DNA by Escherichia coli RNA polymerase: Size and sequence analysis of RNA. J. Virol. 25: 940-943.

Meneguzzi, G., Pignatti, P.F., Barbanti-Brodano, G., and Milanesi, G. (1978b). Minichromosome from BK virus as a template for transcription in vitro. Proc. Natl. Acad. Sci. USA. 75: 1126-1130.

Mew, R.T., Lecatsas, G., Prozesky, O.W., and Harley, E.H. (1981). Characteristics of BK papovavirus DNA prepared directly from human urine. Intervirology 16: 14-19.

Meyers, J.A., Sanchez, D., Elwell, L.P., and Falcow, S. (1976). Simple agarose gel electrophoretic method for the identification and characterization of plasmid DNA. J. Bact. 127: 1529-1537.

Miao, R., and Dougherty, R.M. (1977). Characterization of human papovavirus RFV: Comparison with SV40 and BKV. J. Gen. Virol. 35: 67-75.

Miyamura, T., and Takemoto, K.K. (1979). Virology 98: 279-282.

Miyamura, T., Yoshiike, K., and Takemoto, K.K. (1980). Characterization of JC papovavirus adapted to growth in human embryonic kidney cells. J. Virol. 35: 498-504.

Molholt, B., and Doskočil, J. (1978). Increased transformation frequency in E. coli. Biochim. Biophys. Res. Commun. 82: 477-483.

Murray, K., and Murray, N.E. (1975). Phage lambda receptor chromosomes for DNA fragments made with restriction endonuclease III of Haemophilus influenzae and restriction endonuclease I of Eschericia coli. J. Mol. Biol. 98: 551-564.

Newell, N., Lai, C.-J., Khoury, G., and Kelly, T.J. Jr. (1978). Electron microscope study of the base sequence homology between simian virus 40 and human papovavirus BK. J. Virol. 25: 193-201.

Norgard, M.V., Keem, K., and Monahan, J.J. (1978). Factors affecting the transformation frequency of Escherichia coli strain Chi1776 by pBR322 plasmid DNA. Gene 3: 279-292.

Osborn, J.E., Robertson, S.M., Padgett, B.L., Walker, D.L., and Weisblum, B. (1976). Comparison of JC and BK human papovaviruses with simian virus 40: DNA homology studies. J. Virol. 19: 675-684.

Osborn, J.E., Robertson, S.M., Padgett, B.L., Zu Rhein, G.M., Walker, D.L., and Weisblum B., (1974). Comparison of JC and BK human papovaviruses with simian virus 40: Restriction endonuclease digestion and gel electrophoresis of resultant fragments. J. Virol. 13: 614-622.

Padgett, B.L., Walker, D.L., Zu Rhein, G.M., and Eckroade, R.J.

(1971). Cultivation of papova-like virus from human brain with progressive multifocal leucoencephalopathy. Lancet i: 1257-1260.

Padgett, B.L., Walker, D.L., Zu Rhein, G.M., Hodach, A.E., and Chou, S.M. (1976). JC papovavirus in progressive multifocal leucoencephalopathy. J. Infect. Dis. 133: 686-690.

Pater, A., Pater, M.M., and di Mayorca, G. (1980). Arrangement of the genome of the human papovavirus RF virus. J. Virol. 36: 480-487.

Pater, M.M., Pater, A., and di Mayorca, G. (1981). Genome analysis of MG virus, a human papovavirus. J. Virol. 39: 968-972.

Pauw, W., and Choufoer, J. (1978). Isolation of a variant of BK virus with altered restriction endonuclease pattern. Arch. Virol. 57: 35-42.

Peacock, A.C., and Dingman, C.W. (1978). Molecular weight estimation and separation of ribonucleic acid by electrophoresis in agarose-acrylamide composite gels. Biochemistry 7: 668-674.

Philippsen, P., Kramer, R.A., and Davis, R.W. (1978). Cloning of the yeast ribosomal DNA repeat unit in SstI and HindIII lambda vectors using genetic and physical size selections. J. Mol. Biol. 123: 371-386.

Purchio, A.F., and Fareed, G.C. (1979). Transformation of human embryonic kidney cells by human papovavirus BK. J. Virol. 29: 763-769.

Reddy, V.B., Thimmappaya, B., Dhar, R., Subramanian, B., Zain, S., Pan, J., Ghosh, P.K., Celma, M.L., and Weissman, S.M. (1978). The genome of simian virus 40. Science 200: 494-502.

Reese, J.M., Reissig, M., Daniel, R.W., and Shah, K.V. (1975). Occurence of BK virus and BK virus-specific antibodies in the urine of patients receiving chemotherapy for malignancy. Infect. Immun. 11: 1375-1381.

Rodriguez, R.L., Tait, R., Shine, J., Bolivar, F., Heyneker, H., Betlach, M., and Boyer, H.W. (1977). Characterization of tetracycline and ampicillin resistant plasmid cloning vehicles. In: Molecular Cloning of Recombinant DNA. Miami Winter Symposia 13: 73-84; Academic Press, New York.

Sack, G.H. Jr., Narayan, O., Danna, K.J., Weiner, L.P., and Nathans, D. (1973). The nucleic acid of an SV40-like virus isolated from a patient with progressive multifocal leucoencephalopathy. Virology 51: 345-350.

Seehafer, J., Carpenter, P., Downer, D.N., and Colter, J.S. (1978). Observations on the growth and plaque assay of BK

virus in cultured human and monkey cells. J. Gen. Virol. 38: 383-387.

Seehafer, J., Downer, D.N., Salmi, A., and Colter, J. (1979). Isolation and characterization of BK virus-transformed rat and mouse cells. J. gen. Virol. 42: 567-578.

Seif, I., Khoury, G., and Dhar, R. (1979). The genome of human papovavirus BKV. Cell 18: 963-977.

Shah, K.V., Daniel, R.W., and Warszawski, R.M. (1973). High prevalence of antibodies to BK virus, an SV40-related papovavirus, in residents of Maryland. J. Infect. Dis. 128: 784-787.

Simmons, D.T., and Martin, M.A. (1978). Common methionine tryptic peptides near the amino-terminal end of primate papovavirus tumor antigens. Proc. Natl. Acad. Sci. USA. 75: 1131-1135.

Sleigh, M.J., Topp, W.C., Hanich, R., and Sambrook, J.F. (1978). Mutants of SV40 with an altered small t protein are reduced in their ability to transform cells. Cell 14: 79-88.

So, M., Gill, R., and Falcow, S. (1976). Mol. Gen. Genet. 142: 239-249.

Steffen, M., Krieg, P., Pernfuss M., Sauer, E., Eisinger, V., and Sauer, G. (1980). Growth dynamics of a latent primate papovavirus. J. Virol. 35: 865-875.

Streek, R.E., Philippsen, P., and Zachau, H.G. (1974). Cleavage of small bacteriophage and plasmid DNAs by restriction endonucleases. Eur. J. Biochem. 45: 489-499.

Sutcliffe, J.G. (1978). pBR322 restriction map derived from the DNA sequence: accurate DNA size markers up to 4361 nucleotide pairs long. Nuc. Acids Res. 5: 2721-2728.

Sutcliffe, J.G. (1979). Cold Spring Harbor Symposium on Quantitative Biology 43: 77.

Taguchi, F., Hara, K., Kajioka, J., and Nagaki, D. (1980). BK virus haemagglutinin in urine of a patient with BK virus infection. Acta Virol. 24: 154.

Takemoto, K.K., and Mullarkey, M.F. (1973). Human papovaviruses, BK strain: Biological studies including antigenic relationships to simian virus 40. J. Virol. 12: 625-631.

Takemoto, K.K., Howley, P.M., and Miyamura, T. (1979a). JC human papovavirus replication in human amnion cells. J. Virol. 30: 384-389.

Takemoto, K.K., Linke, H., Miyamura, T., and Fareed, G.C. (1979b). Persistent BK papovavirus infection of transformed human fetal brain cells. I. Episomal viral DNA in cloned lines deficient in T-antigen expression. J. Virol. 29: 1177-1185.

Takemoto, K.K., Rabson, A.S., Mullarkey, M.F., Blaese, R.M., Garon, C.F., and Nelson,D. (1974). Isolation of papovavirus from brain tumor and urine of a patient with Wiskott-Aldrich syndrome. J. Natl. Cancer Inst. 53: 1205-1207.

Ter Schegget, J., Voves, J., Van Strien, A., and Van der Noordaa, J. (1980). Free viral DNA in BK virus-induced hamster tumor cells. J. Virol. 35: 331-339.

Thimmappaya, B., and Shenk, T. (1979). Nucleotide sequence analysis of viable deletion mutants lacking segments of the simian virus 40 genome coding for small t antigen. J. Virol. 30: 668-673.

Thomas, M., and Davis, R.W. (1975). Studies on the cleavage of bacteriophage lambda DNA with EcoRI restriction endonuclease. J. Mol. Biol. 91: 315-328.

Uchida, S., Watanabe, S., Aizawa, T., Furuno, A., and Muto, T. (1979). Polyoncogenicity and insulinoma-inducing ability of BK virus, a human papovavirus, in Syrian golden hamsters. J. Natl. Cancer Inst. 63: 119-124.

Van der Noordaa, J. (1976). Infectivity, oncogenicity and transforming ability of BK virus and BK virus DNA. J. gen. Virol. 30: 371-373.

Van Heuverswyn, H., Cole, C., Berg, P., and Fiers, W. (1979). Nucleotide sequence analysis of two simian virus 40 mutants with deletions in the region coding for the carboxyl terminus of the T antigen. J. Virol. 30: 936-941.

Volckaert, G., Feunteun, J., Crawford, L.V., Berg, P., and Fiers, W. (1979). Nucleotide sequence deletions within the coding region for small-t antigen of simian virus 40. J. Virol. 30: 674-682.

Watanabe, S., Yoshike, K., Nozawa, A., Yuasa, Y., and Uchida, S. (1979). Viable deletion mutant of human papovavirus BK that induces insulinomas in hamsters. J. Virol. 32: 934-942.

Wensink, P., Finnegan, D., Donelson, J., and Hogness, D. (1974). A system for mapping DNA sequences in the chromosomes of *Drosophila melanogaster*. Cell 3: 315-325.

Weiner, L.P., Narayan, O., Penney, J.B., Jr., Herndon, R.M., Feringa, E.R., Tourtellotte, W.W., and Johnson, R.T. (1973). Papovavirus of JC type in progressive multifocal leucoencephalopathy. Arch. Neurol. 29: 1-3.

Wold, W.S., Green, M., Mackey, J.K., Martin, J.D., Padgett, B.L., and Walker, D.L. (1980). Integration pattern of human JC virus sequences in two clones of a cell line established from a JC virus-induced hamster brain tumor. J. Virol. 33: 1225-1228.

Wold, W.S.M., Mackey, J.K., Brackmann, K.H., Takemori, N., Rigden, P., and Green, M. (1978). Analysis of human tumors and human malignant cell lines for BK virus-specific DNA sequences. Proc. Natl. Acad. Sci. USA. 75: 454-458.

Wright, P.J., Bernhardt, G., Major, E.O., and di Mayorca, G. (1976). Comparison of the serology, transforming ability, and polypeptide composition of human papovaviruses isolated from urine. J. Virol. 17: 762-775.

Yang, R.C.A., and Wu, R. (1978a). BK virus DNA: Cleavage map and sequence analysis. Proc. Natl. Acad. Sci. USA. 75: 2150-2154.

Yang, R.C.A., and Wu, R. (1978b). Cleavage map of BK virus DNA with restriction endonucleases MboI and HaeIII. J. Virol. 27: 700-712.

Yang, R.C.A., and Wu, R. (1978c). Physical mapping of BK virus DNA with SacI, MboII, and AluI restriction endonucleases. J. Virol. 28: 851-864.

Yang, R.C.A., and Wu, R. (1979a). BK virus DNA sequence coding for the amino-terminus of the T-antigen. Virology 92: 340-352.

Yang, R.C.A., and Wu, R. (1979b). BK virus DNA sequence: Extent of homology with simian virus 40 DNA. Proc. Natl. Acad. Sci. USA. 76: 1179-1183.

Yang, R.C.A., and Wu, R. (1979c). BK virus DNA: Complete nucleotide sequence of a human tumor virus. Science 206: 456-462.

Yang, R.C.A., and Wu, R. (1979d). Comparative study of papovavirus DNA: BKV(MM), BKV(WT) and SV40. Nucleic Acids Res. 7: 651-668.

Yang, R.C.A., Van de Voorde, A., and Fiers, W. (1975). Location of the small restriction fragments, Hind-L, Hind-M, and Hpa-E on the simian virus 40 genome. Virology 68:260-265.

Yang, R.C.A., Young, A., and Wu, R. (1980). BK virus DNA sequence coding for the t and T antigens and evaluation of methods for determining sequence homology. J. Virol. 34: 416-430.

Zu Rhein, G. (1969). Association of papova-virions with a human demyelinating disease (progressive multifocal leucoencephalopathy). Progr. Med. Virol. 11: 185-247.

Zu Rhein, G.M., and Varakis, J. (1974). Papovavirus in urothelium of treated lymphoma patient. Lancet ii: 783-784.

10. PART II

CHARACTERIZATION OF ROTAVIRUS RNA

11. ROTAVIRUS LITERATURE REVIEW

11.1. Discovery of Rotaviruses

11.1.1. EDIM

Only comparatively recently have outbreaks of acute diarrhoea in mammals been associated with the viruses now generally referred to as "rotaviruses". The first reports of such outbreaks in suckling mice, wrongly ascribed at first to Salmonella infection, came from several places in the United States before 1944 (Flewett and Woode, 1978; Syverton and Olitsky, 1934). An outbreak in the Harvard Medical School in 1944 was associated with cytoplasmic inclusions in the epithelium of the small intestine (Pappenheimer and Cheever, 1948).

The disease occurred mainly in autumn and early winter, and collodion membrane filtrates were infectious, indicating a viral agent. The mice produced a yellowish watery diarrhoea, but usually recovered after a few days. The viral agent was characterized as a highly infectious, rather heat-resistant virus, ether resistant, and 65-75nm in diameter from its filtration endpoint (Kraft, 1957). It differed from LIVIM (lethal intestinal virus of infant mice), which was ether sensitive and more heat sensitive, and from all known reovirus types. Adams and Kraft (1967) showed by EM that (1) EDIM virus replication was cytoplasmic, (2) virus particles aggregated into distended endoplasmic vesicles in large numbers, (3) infected cells were shed, or burst into, the intestinal lumen, liberating

very large numbers of virus particles, and (4) only the cells on the sides and tips of the intestinal villi were affected, and not those in the crypts.

No-one has yet succeeded in propagating EDIM virus in cell culture, although since recent work has concentrated on the human and calf rotaviruses, newly developed techniques for culturing "difficult" rotaviruses may not yet have been applied to this virus (See Section 11.6.1). Rubenstein et al. (1971) took the virus through three passages in organ cultures of mouse ileum and two passages in organ cultures of caecum, but failed with mouse fibroblast cultures.

11.1.2. Neonatal Calf Diarrhoea Virus

Epizootic diarrhoea of newborn calves has plagued stockbreeders for centuries (Flewett and Woode, 1978). Up to 30% losses are not rare. Mebus et al. (1969) in Nebraska first showed that a 65nm virus (NCDV) was the etiologic agent. A similar virus was isolated in tissue culture in England (Woode et al., 1974) and Northern Ireland (McNulty et al., 1976a).

11.1.3. SA11

SA11 was one of a series of "simian agents" isolated from monkeys by Malherbe and his colleagues in South Africa. It was isolated in 1958 from a rectal swab from a vervet monkey (Malherbe and Strickland-Cholmley, 1967). SA11 was one of the few rotaviruses which adapted readily to cell culture, and produced characteristic eosinophilic inclusions in primary vervet monkey kidney cells. SA11 has not been associated with

disease in monkeys, but has induced diarrhoea in young calves and gnotobiotic piglets (Rodger et al., 1977).

11.1.4. "O" Agent (OA)

Malherbe and Strickland-Cholmley (1967) also isolated a rotavirus from pooled filtered washings of apparently healthy sheep and cattle intestines from the municipal abbatoir in Johannesburg. It grew more slowly than SA11 in vervet monkey kidney cells, and has never been isolated directly from calves or lambs, so its original host has not been identified.

11.1.5. Infantile Gastroenteritis Virus (IGV,HGV)

Early attempts to prove the viral etiology of non-bacterial acute infectious diarrhoea in infants had varying results. Light and Hodes (1943) found that filtrates of human faeces caused diarrhoea in calves, but nobody could substantiate these results until 1976 (Mebus et al., 1976).

Unknown to each other, at least three independent groups began detecting virus particles in duodenal biopsies and/or faecal extracts (Bishop et al., 1973; Flewett et al., 1973; Middleton et al., 1974). Since then it has been established that a large proportion of cases of acute gastroenteritis in young children are due to rotaviruses (Flewett and Woode, 1978). Older children and adults may occasionally be affected, but have normally developed immunity at an early stage (Hara et al., 1976; Von Bonsdorff et al., 1978).

11.1.6. Other Host Species

Rotaviruses have been isolated from piglets (Lecce *et al.*, 1976; McNulty *et al.*, 1976c; Woode *et al.*, 1976a), foals (Flewett *et al.*, 1975), lambs (Snodgrass *et al.*, 1976), rabbits (Bryden *et al*, 1976), deer (Tzipori *et al.*, 1976), pronghorn antelope (Reed *et al.*, 1976), birds (Todd *et al.*, 1980) and cats (Hoshino *et al.*, 1981).

These viruses are all morphologically indistinguishable and antigenically related (Flewett and Woode, 1978).

A small 28nm virus known as the "Norwalk Agent" was associated with outbreaks of "winter vomiting disease", but did not appear to be the causative agent in most acute gastroenteritis cases in young children (Flewett and Woode, 1978; Schoub, 1979; Cheever and Muller, 1947). The role of this and other "parvoviruses" in human and animal disease will not be discussed here (Storz and Bates, 1973; Wyatt *et al.*, 1978).

11.2. Epidemiology (Human Rotavirus)

Acute infantile gastroenteritis is the leading cause of death among infants in Africa, Asia, and Central and South America. In South African Coloured and Asiatic populations, and probably in Black populations also, it causes the highest infant mortality, and ranks second to pneumonia in the White population (Schoub, 1979). Rotavirus has been implicated in infantile gastroenteritis outbreaks throughout the world under very different ecological, socio-economic and climatic conditions (Editorial, Lancet, 1975). In temperate climates it is more prevalent in autumn and winter, being detected in up to 78% of

cases in peak periods, with little or no infection in the summer (Schoub, 1979).

Some authors found that the 6 to 24 month age group is the most critically affected by rotavirus enteritis (Schoub, 1979). Flewett and Woode (1978) found that rotavirus accounted for 60% of enteritis cases in the 1 to 3 year age group, for only 25% of cases in children under 1 year old, and for 20 to 40% between the ages of 4 and 6 (Flewett and Woode, 1978). Outbreaks in older children and adults are rarer (Hara et al., 1976; Von Bonsdorff et al., 1978).

11.2.1. Clinical Features

The first characteristic symptom of rotavirus infection is prolonged vomiting and fever, followed by diarrhoea. This results in isotonic dehydration with raised blood urea, frequently coupled with compensated metabolic acidosis (Schoub, 1979). Large amounts of rotavirus particles are excreted, up to 10^{11} particles/ml having been detected, (Flewett and Woode, 1978).

11.2.2. Diarrhoeal Mechanism

Virus infection destroys the disaccharidase-synthesizing cells in the brush-bordered epithelium, causing an accumulation of lactose or other disaccharides in the lumen of the bowel. Only monosaccharides are absorbed by the healthy bowel. Thus, osmotic draining of body fluid into the lumen occurs, and hence the watery stools and body dehydration (Flewett and Woode, 1978). Reduction of lactose levels by stopping milk or other

disaccharide intake, and giving water or dilute electrolyte solution, usually stops diarrhoea rapidly.

11.3. Gastroenteritis Epidemics in Livestock

Rotaviruses have been isolated in a number of enteritis outbreaks in many species of mammal (See Section 11.1.6), and are a major cause of concern because of the stability of the virus in the open. Calf rotavirus has been shown to be stable for 7 months at room temperature (Woode and Bridger, 1975).

Much difficulty has been encountered in developing a vaccine against rotavirus infection, partly because of problems in adapting strains to cell culture. Also, vaccines from one strain have had limited effect in protecting against infections from other rotavirus strains (Thouless et al., 1977). Attenuated vaccines have been of limited efficacy, perhaps because of the overwhelming dose of virus likely to be received by an animal exposed to contamination (Flewett and Woode, 1978).

11.4. Morphology of Rotaviruses

All rotaviruses so far studied by negative stain electronmicroscopy have had virtually the same, if not an indistinguishable, morphology (Rodger et al., 1975; Woode et al., 1976b; Esparza and Gil, 1978; Rodger et al., 1977; Flewett et al., 1974; Els and Lecatsas, 1972; Holmes et al., 1975; Martin et al., 1975). The intact virus is spherical, consisting of a double capsid structure. Two main structures are usually obtained by differential centrifugation of virus isolates. The double capsid structure has a buoyant density in caesium

chloride of between 1.33 and 1.36 g/ml (Newman et al., 1975; Rodger et al., 1975; Obijeski et al., 1977; Bridger and Woode, 1976; Tam et al., 1976; Todd and McNulty, 1977; Elias, 1977), and a single capsid form is always obtained, with a density of about 1.37 g/ml. Less dense bands contain mainly empty particles. Tubular structures have also been observed in the EM (Woode et al., 1976b; Esparza and Gil, 1978; Holmes et al., 1975) but these are of unknown function. Bacteriophages have also been seen in faecal extracts, and can be mistaken for isometric viruses. They are, however, morphologically distinguishable (Bishop et al., 1974; Editorial, Lancet, 1975).

The rotavirus inner capsid primary structure has 32 capsomeres arranged in T=3 symmetry. There is disagreement about the number of subunits making up these capsomeres. Martin et al. (1975) proposed a model consisting of 180 units, each a trimer, giving 540 wedge-shaped structural units in all, arranged in the T=9 symmetry of an icosadeltahedron. The unstable inner core of the virus exposed by enzymatic digestion exhibited clear icosahedral symmetry (Palmer et al., 1977). The orbiviruses were supposed to be the same. Hexagonal cores were observed inside virus particles viewed by EM of infected cells (Chasey, 1977); this shape may be generated by an icosahedral particle. Stannard and Schoub (1977) and Esparza and Gil (1978) proposed models in which the 32 capsomeres contained 960 subunits arranged in T=16 symmetry. The two models differed only in the number of holes in the mesh-structure of the capsids, Stannard and Schoub giving 92, Esparza and Gil 162.

The outer shell is very thin (4-5nm) and has the same symmetry as the inner capsid layer (Esparza and Gil, 1978). It provides the "rim" of the "wheel" which gave rotaviruses their

name, the "spokes" being a result of staining of projections on the inner capsid.

11.4.1. Morphogenesis

Several particle types have been observed in cells infected with rotavirus. Infection of piglets with pig rotavirus resulted in changes in the epithelial cells of the small intestine, and a calf rotavirus caused sporadic infections in cells from the ileum and jejunum, but not the duodenum, of colostrum-deprived calves (Chasey, 1977). Similar changes have been observed in cultured cells (McNulty *et al.*, 1976b). Infected cells appeared less dense than normal, with swollen nuclei, and particles were associated with distended rough endoplasmic reticulum.

The normal course of events appears to be, in brief, as follows: Electron-dense 25-35nm cores are formed within the cytoplasm in dense inclusions, or "viroplasm", which may or may not be membrane-associated. The cores pass into the cytoplasm and acquire a less dense shell, either 50-70nm or 68-72nm in diameter, depending on the observer. Chasey (1977) postulated that a larger, 70-80nm particle consisting of a membranous envelope around a dense core, is a precursor, the membrane being lost eventually. The single capsid particle then acquires an outer capsid shell, forming the typical "double-capsid structure", which is supposedly the mature particle. There is some confusion as to whether the double capsid form is the same as the "precursor" form observed by Chasey (1977), or whether his 50-70nm form represents the mature particle, and a rarer 50-65nm form the single capsid particle. Chasey considered that

this particle represented a non-infectious, possibly abortive form, found predominantly in damaged cells, where they occurred in great numbers within large vacuoles, often forming crystalline arrays. Such cells never contained other particle types, however. Correlation of the observations from different authors, especially when a variety of forms are in evidence, is thus difficult, reflecting perhaps the problem of artefacts generated by the staining (etc.) techniques used in electronmicroscopy. The precise manifestations of rotavirus infection depend also on the strain and cell type used (Hoshino et al., 1981).

Another morphological variety observed by Chasey (1977) in calf intestines, by others in mice (Adams and Kraft, 1967; Banfield et al., 1968), consisted of clusters of 50nm particles connected by thin filaments. These filaments may represent dsRNA leaking out of degraded particles, or condensations of capsid material around incomplete nucleoprotein cores (Chasey, 1977). Similar phenomena were observed by Lecatsas with bluetongue virus and reovirus (Lecatsas, 1968a and b).

Esparza et al. (1980) studied the multiplication of human rotavirus in LLC-MK2 cells. They also observed the budding of particles through cellular membranes, but could not eliminate the possibility of this being an EM artifact. The outer capsid appeared to be acquired either during budding through the rough endoplasmic reticulum or in the so-called "viral factories" (="viroplasm"?). Only single-shelled particles were observed inside cytoplasmic vesicles, supporting Chasey's findings. It must be concluded that the process of acquisition of the outer shell has still not been elucidated fully.

11.5. Antigenic Properties: Detection of Rotaviruses

There are antigenic determinants on the inner capsid layer common to all rotaviruses, and the main serological differences are associated with the outer capsid (Editorial, Lancet, 1975; Woode et al., 1976b).

A number of serological tests have been applied to the detection and differentiation of rotaviruses, with variable results. Methods were usually compared to EM detection as the standard. The criticism of EM as a routine screening procedure used to be its cost in time and equipment (Flewett and Woode, 1978). Recently, however, EM has been considered to be a rapid diagnostic aid, its sensitivity relative to other techniques coming into question (Brandt et al., 1981).

Counter current immunoelectroosmophoresis (IEOP) has been described as more (Grauballe et al., 1977), or less (Middleton et al., 1976), or equally sensitive (Tufvesson and Johnsson, 1976) as EM, depending on the avidity of the serum used.

Ellens and de Leeuw (1977b) found the enzyme-linked immunosorbent assay (ELISA) much more sensitive than either EM or IEOP in the diagnosis of calf rotavirus, and used the technique to detect 1 ng/ml of viral protein of human rotavirus (Ellens and de Leeuw, 1977a).

A very recent paper by Brandt et al. (1981) made a detailed comparison of direct EM, immune EM (IEM), and ELISA techniques for detecting gastroenteritis viruses in children, and found direct EM the best for rapid diagnosis, IEM more sensitive in visualizing viruses from rectal swabs, and ELISA the most sensitive of all, although subject to false-positive reactions unless confirmed by a modified ELISA using different antibody

dilutions.

11.5.1. Cell Culture as a Diagnostic Tool

Diagnosis by propagation in cell culture and plaque assay proved very unreliable for most strains of rotavirus until very recently, when the requirement for trypsin for efficient viral propagation was discovered by a number of groups at about the same time (Matsuno et al., 1977; Theil et al., 1977; Babiuk et al., 1977; Almeida et al., 1978). Previously, only SA11, OA and a few strains of calf rotavirus had been successfully adapted to cell culture (Malherbe and Strickland-Cholmley, 1967; McNulty et al., 1976b; McNulty et al., 1977). Sonication of viral preparations (McNulty et al., 1977) and centrifugation of virus onto cell monolayers (Banatlava et al., 1975) have both been used successfully for plaque assay and in vitro propagation of a number of rotaviruses, notably some human strains (Theil et al., 1977; Matsuno et al., 1977; Smith et al., 1979; Ramia and Sattar, 1979; Wyatt et al., 1980; Mason et al., 1980; Esparza et al., 1980; Chasey, 1977). Considering that trypsin was demonstrated to enhance reovirus infectivity in 1965 (Spendlove and Schaffer, 1965), it is perhaps surprizing that it was not tried with rotavirus earlier.

A distinction must be drawn between virus passage through cell culture and the demonstration of abortive viral infection as demonstrated by EM and fluorescent antibody studies (Banatvala et al., 1975; Elias, 1977).

Wyatt et al. managed to adapt poorly cultivable strains of rotavirus to cell propagation by repeated passage through cell culture (Wyatt et al., 1976) and/or gnobiotic animals (Wyatt et

al., 1980). Greenberg et al. (1981) resorted to "rescuing" non-cultivable human rotavirus by obtaining "reassortants" from mixed infections with ts mutants of a cultivable bovine rotavirus, containing a mixture of RNA segments from the two viruses.

11.6. Cell Culture Characteristics of Rotaviruses

The short history of in vitro propagation of rotaviruses in cell culture may be divided into two parts - the "Pre-trypsin" and "Post-trypsin" eras. Prior to the discovery of the positive effect of trypsin, culture attempts with most strains produced variable yields, poorly defined cytopathic effects (CPE), or they failed completely. Much effort was put into finding a permissive system for culture of human strains, with very limited success (Kalica et al., 1978b; Cameron et al., 1977; Wyatt et al., 1980).

The first report of successful rotavirus cultivation was probably Malherbe and Strickland-Cholmley (1967), who grew SA11 and "O" Agent (OA) to titres of $10^{4.5}$ -$10^{5.5}$ TCID50 in primary vervet monkey kidney cells (VMK). At that time, these viruses had not been formally grouped as "rotaviruses", as they had not yet been differentiated from orbiviruses or reoviruses.

After many attempts a few strains of calf rotavirus were adapted to serial tissue culture in bovine embryo kidney by 1971 (Mebus et al., 1971).

In general, the presence of serum in the culture medium had to be avoided, as it contains a heat-resistant virus inhibitory factor (Smith et al., 1979). Not all cells in a culture are susceptible, and the host cell range is limited (McNulty et al.,

1977). The CPE is difficult to identify conclusively using phase contrast microscopy. After staining, it is characterized by eosinophilic inclusions in the cytoplasm (Malherbe and Strickland-Cholmley, 1967). A detailed report of the culture of bovine rotavirus was made by McNulty et al. (1977). Infected cells appeared granular, often spindle-shaped, and eventually detached, forming rafts joined by elongated strands of cytoplasm. Rolled cultures were more severely affected than stationary ones, although always less than 100% of cells were affected. Infectivity was increased 10x by sonication of viral preparations, which dispersed aggregates.

11.6.1. Plaque Assay and the Effect of Trypsin

As mentioned above the effect of trypsin was reported by a number of groups in 1977 and 1978. This led to the development of plaque assays for rotaviruses. Matsuno et al. (1977) developed a plaque assay for NCDV using MA104 cells from rhesus monkey kidney. Interestingly, their motivation was that the CPE of NCDV infection was not easily recognizable. Many factors were found to be critical, including the make of agar used in the overlay, and the cell line used. They found that overlay containing 2.2 mg/5ml of DEAE (a cationic polymer) alone produced small plaques, trypsin (2μg/5ml) alone produced fewer, larger plaques, and a combination of the two the best, most reproducible results. Babiuk et al. (1977) reported similar results with calf rotavirus in BSC-1 cells. They were prompted (somewhat belatedly!) by the reports of enzymatic enhancement of infectivity of reovirus (Spendlove and Schaffer, 1965). They found that pretreatment of faecal samples with trypsin increased

the level of detection. Theil *et al.* (1977) found that pretreatment of porcine rotavirus with trypsin or pancreatin was necessary for propagation in primary porcine kidney cells. (Pancreatin contains mainly trypsin, amylase and lipase). Almeida *et al.* (1978) found that trypsin was required throughout the replicative cycle of the virus, in contrast to Theil (1977). The enzyme caused the cells to detach after 12h, but this did not interfere with viral replication.

Since then, the development of a plaque assay has become almost routine in studies of many rotaviruses. Human strains have finally been induced to propagate to high titre (Esparza *et al.*, 1980), and plaque assays have been developed for SA11 (Smith *et al.*, 1979; Ramia and Sattar, 1979) and feline rotavirus (Hoshino *et al.*, 1981).

Whether trypsin enhances the growth of readily cultivable rotaviruses has given rise to controversy. It has been reported that growth of SA11 is not enhanced by trypsin (Schoub, B.D., and Bertram, D.M., Proc. 4th Int. Congr. Virol. The Hague, 1978, Abstr. no. W35/10), nor is trypsin required for plaque formation (Wyatt et al., ibid, Abstr. no. W35/8). Other authors disagree (Ramia and Sattar, 1979). In a very recent paper, Hoshino *et al.* (1981) remarked that ease of culture was inversely proportional to the ability of a rotavirus to induce diarrhoea. Hence their feline rotavirus, which was less dependent on the presence of trypsin than many pathogenic rotaviruses, failed to induce symptoms when administered to specific pathogen-free colony cats.

The mechanism of action of trypsin was the subject of two very recent papers. Clark *et al.* (1981) found that trypsin enhancement was not due to the break-up of viral aggregates, or

to greater attachment of virus particles to the cell wall, or to the digestion of viral inhibitors, such as interferon. Trypsin treatment of virions was found to facilitate subsequent uncoating of the particles after they had infected a cell. This activated the viral RNA polymerase present in the inner core, and permitted replication. This paper, and that of Estes et al. (1981), reported that trypsin cleaves an outer shell component ("V3" or "VP3") into two fragments, one of which is the "V5" (or "VP5") found on gels of virion polypeptides. The other fragment was considered by Estes et al. to be "V8" (or "VP8"), but Clark et al. thought that it was slightly smaller than V8. Treated double-shelled particles remained intact despite this cleavage, but were more infectious. Single-shelled species were not formed.

11.7. THE ROTAVIRUS GENOME

The rotavirus genome consists of segmented, double-stranded RNA (Newman et al., 1975; Rodger et al., 1975). This unusual arrangement also occurs in reovirus, wound tumour virus, rice dwarf virus, and cytoplasmic polyhedrosis virus (Kalmakoff et al., 1969; Rodger et al., 1975; Wood, 1973). The dsRNA molecule is very stable, being resistant to all common nucleases. It is thus easy to extract from virus-containing material or purified particles.

11.7.1. Molecular Weight Determination

Molecular weight determinations of rotavirus genome segments have nearly always involved coelectrophoretic comparison with reovirus dsRNA on polyacrylamide gels. Reovirus has been the only well-characterized dsRNA standard available, although its use suffers from some severe drawbacks, mainly because the sizes of some of the rotavirus segments extend beyond the range of reovirus. Also, the mobility versus log MW plot for dsRNA has been assumed erroneously to be linear (Bozarth and Harley, 1976). These points will be expanded in the Discussion. The rotavirus segments are too dispersed in size to be grouped into convenient size classes that lend themselves to measurement under the EM. Thus they cannot be sized by average contour length measurement, as was done for reovirus (Bellamy *et al.*, 1967; Shatkin *et al.*, 1968; Kalica *et al.*, 1976).

Gel electrophoresis allows the differentiation of segments varying only slightly in MW, but the relationship between mobility and MW is not as straightforward as was initially assumed (Harley *et al.*, 1977). A standard curve needs to be constructed using standard markers, and more suitable ones than reovirus have not been used.

Nevertheless, much useful information has been gained from comparisons between different rotaviruses by coelectrophoresis. Early results showed that the genome consisted of at least 11 segments ranging in MW from 0.24 to 2.2Md, with a total of about 14Md (Rodger *et al.*, 1975; Newman *et al.*, 1975). The most prominent characteristic was the overall similarity in the pattern of different rotaviruses, the bands falling into 4 groups. Although there was considerable variation in the exact

mobilities of the bands obtained with different species, the overall pattern remained clearly recognizable (Kalica et al., 1976; Kalica et al., 1978b). This is in contrast to the CPVs which all have 10 dsRNA segments (Wood, 1973) but exhibit a greater variety of gel patterns. These results confirmed the placing of the rotaviruses in the Reoviridae, although it has been debated whether they are more closely related to the reoviruses or to the orbiviruses. The similarity in patterns may also reflect the serological cross-reactivity between different rotaviruses.

Gel patterns have been published for rotaviruses from humans (Rodger et al., 1975; Kalica et al., 1976; Schnagl and Holmes, 1976; Obijeski et al., 1977; Espejo et al., 1977; Kalica et al., 1978b; Kalica et al., 1978c; Espejo et al., 1979; Rodger and Holmes, 1979; Smith and Tzipori, 1979; Espejo et al., 1980c; Espejo et al., 1980a; Schnagl et al., 1981; Rodger et al., 1981), calves (Rodger et al., 1975; Kalica et al., 1976; Verly and Cohen, 1977; Schnagl and Holmes, 1976; Kalica et al., 1978b; Rodger and Holmes, 1979; Smith and Tzipori, 1979), Pigs (Todd and McNulty, 1976; Todd and McNulty, 1977; Kalica et al., 1978c; Smith and Tzipori, 1979), lambs (Todd and McNulty, 1977), poultry (Todd et al., 1980), mice, deer and foals (Smith and Tzipori, 1979), SA11 (Kalica et al., 1976; Kalica et al., 1978b; Kalica et al., 1978c; Rodger and Holmes, 1979), and "O" Agent (OA) (Kalica et al., 1976; Kalica et al., 1978b; Kalica et al., 1978c). Different strains from the same host species often exhibit minor variations in mobility of one or more segments, although the patterns usually fall into one of several "types". These differences often correlate with serological differences. Then again, rotaviruses from pig and calf were found by Todd and

McNulty (1976) to be indistinguishable, with only one segment differing from a lamb isolate. However, the gels in this paper were not of the finest resolution. Nevertheless, a slight difference was detected between the culture-adapted calf strains from Northern Ireland (McNulty _et al_., 1976a) and Nebraska (NCDV) (Mebus _et al_., 1969). These strains were very similar to OA (Kalica _et al_., 1978b). Verly and Cohen (1977) reported considerable variation between NCDV and fresh isolates in France, and smaller differences between the various fresh isolates themselves. Kalica _et al_. (1978b) found no variation in NCDV itself after extensive passage. Variation was reported between two pig rotavirus isolates (Kalica _et al_., 1978c).

Different isolates of human infantile gastroenteritis virus have been subjected to the minutest comparison in a number of papers by Espejo _et al_. in New Mexico, by Rodgers, Schnagl _et al_. in Australia, and by Kalica _et al_. in Washington D.C. (Kalica _et al_., 1978b). Progressive refinements in electrophoretic techniques revealed greater fine variation in the "electropherograms" of different isolates. Each group studied the changes in RNA patterns in isolates from epidemics in the same area over a number of years, and found a different type predominating in each epidemic (Espejo _et al_., 1980c; Espejo _et al_., 1980a; Schnagl _et al_., 1981; Rodger _et al_., 1981). Some types persisted for a number of years in the same area, emphasizing the stability of individual patterns. Surprizingly, no identical patterns common to both regions were found in 19 varieties isolated from Melbourne (Rodger _et al_., 1981) and 12 varieties from Central Australia (Schnagl _et al_., 1981). Schnagl _et al_. (1981) and Espejo _et al_. (1977) suggested that the reassortment of genome segments between human, and possibly

animal, strains could be a natural evolutionary mechanism. Such "reassortants" have been obtained *in vitro* (Matsuno *et al*., 1980; Greenberg *et al*., 1981).

Most authors found the rotavirus genome to contain 11 segments in equimolar proportion, compared to 12 in orbiviruses and reovirus, and 10 in CPVs. Obijeski *et al*. (1977) reported 15 segments in a human strain studied. The discrepancy arose in the estimation of the molar ratio in a gel band containing more than one segment. Most authors found this band to contain 3 segments, but Obijeski *et al*. estimated it to contain 6 segments by scanning the methylene blue stained gels in a spectrophotometer at 610nm. These results have been questioned (Kalica *et al*., 1978b). Kalica *et al*. offered the explanation that Obijeski *et al*. were working with a mixed culture of rotaviruses that were heterogeneous in the segments in dispute. This explanation would only be valid if each heterogeneous segment had been observed on the gel as a separate band. Assuming that the segments are in eqimolar proportion a mixed culture with heterogeneity in one segment only, say, would give a gel picture in which all the segments were still in equimolar proportion except for the heterogeneous segments, which would be in reduced concentration. This was not observed. A more accurate method to quantitate the RNA in each band than that used by Obijeski *et al*. (1977) is to label with 32P and count thin gel slices (Harley *et al*., 1977), or to end-label the segments using 3H-borohydride (Millward and Graham, 1970). Todd and McNulty (1977) scanned methylene blue stained gels of lamb rotavirus, and obtained an ambiguous molar ratio of 3.7 for the multiple band. This further illustrates that quantitations from optical scans of nucleic acids in gels must be interpreted with

caution.

11.8. Analysis of Rotavirus Polypeptides

The number of virus-specific polypeptides identified by PAGE has varied between 5 and 11 for different authors. A maximum of 10 have been observed from analysis of virions (Obijeski et al., 1977), but 11 polypeptide products were obtained using in vitro transcription and translation of rotavirus RNA in rabbit reticulocyte lysates (Mason et al., 1980). This variation reflects the fact that not all gene products are translated in the same amount, so some may be below the level of detectability of some systems.

Newman et al. (1975) obtained 5 polypeptides on 7.5% PAGE, with MWs ($x10^3$) of 135, 122, 96, 36, and 30. Rodger et al. (1975) obtained a clearer separation with clean virion preparations on 7.5% gels, showing 8 bands with human rotavirus and 9 with calf virus. Obijeski et al. (1977) resolved 10 proteins in all, recovered from the phenol phase of an RNA extraction from human rotavirus virions. They calculated that there was insufficient RNA to code for all the proteins on a one-to-one basis. This indicated that some of the bands observed resulted from post-translational modification of primary gene products. As in most other papers, two major proteins were obtained accounting for 85% of the total mass, with MWs of 135,000 and 40,000. Both continuous and discontinuous SDS polyacrylamide gel systems gave the same number of bands, but greater clarity was obtained with the discontinuous system. They used a protein coding ratio of 18:1 (Martin and Zweerink, 1972) to conclude that even their estimate

of 15 RNA segments had slightly insufficient coding capacity.

Todd and McNulty (1977) compared the polypeptide profiles of single capsid and double capsid lamb rotavirus separated by buoyant density CsCl centrifugation, and obtained 10 polypeptides with double-capsid virions, and 6 with single. From this they concluded that 4 minor polypeptides constituted the outer shell, with MWs of 72,000, 45,000, 37,000 and 30,000. This agreed with the results of Rodger et al. (1975), but Bridger and Woode (1976) associated only one 72,000 dalton protein with the outer capsid. The main problem encountered by all authors was the removal of all faecal and/or other non-viral contaminants from viral preparations.

Rodger et al. (1977) obtained 8 polypeptide components, which migrated in a very similar manner, from SA11, human and calf rotaviruses. The calf and SA11 preps had an additional minor component. They showed that the outer capsid components are glycoproteins. Three proteins were associated with the outer shell of human rotavirus, and 4 with calf and SA11. Kalica and Theodore (1979) obtained 8 polypeptides from double-shelled SA11 using continuous PAGE, but only 5 from single-shelled virions. The single-shelled particles were RIA-positive and HA-negative, confirming that the haemagglutinin (HA) of SA11 (Kalica et al., 1978a) resides on an outer capsid component.

11.9. Coding Assignments for Rotavirus Genome Segment

The allocation of rotavirus polypeptides to their respective genome segments by in vitro translation techniques, as has been done for reovirus (McCrae and Joklik, 1978), has been accomplished only very recently.

A start was made by Cohen and Dobos (1979) who synthesized 11 ssRNA species in vitro from calf rotavirus dsRNA templates using endogenous RNA polymerase from rotavirus cores. This ssRNA was active as message in rabbit reticulocyte lysate, but poor resolution of in vitro synthesized polypeptides was achieved on gels.

Two papers appearing in the same issue of Journal of Virology arrived at coding assignments for SA11 using slightly different approaches. Smith et al. (1980) used denatured dsRNA directly as a template for protein synthesis in a wheat germ system. Mason et al. (1980) used the same approach as Cohen and Dobos (1979), taking it a stage further by first fractionating the synthesized ssRNA by velocity sedimentation centrifugation. Smith et al. adopted the most rigorous approach, eluting single segments of dsRNA from a preparative gel and translating each denatured segment individually. The close triplet of bands 7,8 and 9 could unfortunately not be separated from one another. No product was obtained that co-migrated with three of the outer capsid polypeptides (at least one of which is known to be glycosylated). A very recent paper by Dyall-Smith and Holmes (1981) describes the assignment of the two smallest genome segments (10 and 11) of SA11 to the smallest outer capsid protein (O4) and another small polypeptide (NS3). Interestingly, the eleventh segment of both SA11 and the human strain with a "long" electropherotype both coded for O4, whereas another human strain with a "short" electropherotype, in which the eleventh band has the same mobility as the tenth band of the "long" strain, has the coding assignments reversed. However, the gene products are of a similar size.

Espejo et al. (1980b) reported substantial heterogeneity in

mobility of the polypeptides of the inner layer as well as the outer layer of human rotaviruses with a differing genomic composition. This is surprizing considering the antigenic cross-reactivity of the inner layers.

12. RESULTS AND DISCUSSION

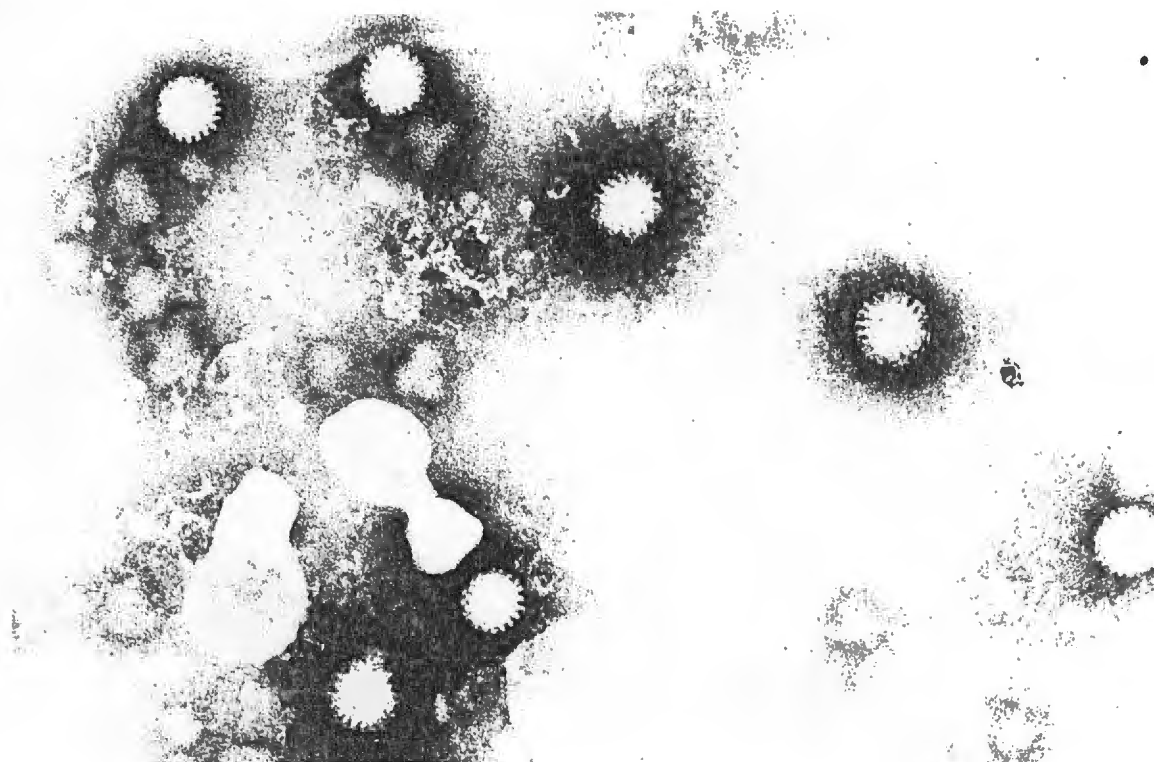


Figure 12-1. Electronmicrograph of SA11 Rotavirus
Magnification: 120,000. Courtesy of Dr. L. Stannard.

12.1. Cell Culture Propagation of SA11 and OA

The main objective of in vitro propagation of these rotaviruses was to obtain stocks of genomic RNA for physico-chemical analysis - not to perform an in-depth study of their cell culture properties. Nevertheless, the culture conditions that would support the reproducible propagation of rotavirus to high titres underwent extensive investigation.

CV1 cells, a well-known line of established green monkey kidney cells, were used. They were known to support SA11

replication, and they had the merit of being readily available and easy to grow. Other cell lines were not tried.

Samples of medium containing SA11 or OA were kindly sent by Dr. Schoub from the National Institute for Virology, Sandringham, Johannesburg. The concentration of virus in the medium was unknown, but aliquots of each were extracted directly, and gave clearly identifiable rotavirus bands (Fig. 12-11A).

First attempts at viral propagation were made using MEM from which serum had been omitted (Malherbe and Strickland-Cholmley, 1967). The cells were poorly maintained under these conditions, and it was difficult to distinguish between cell deterioration due to virus infection and that due to the poor medium. Some authors have added low concentrations of foetal calf serum (Newman et al., 1975; McNulty et al., 1976b). Serum was avoided in my experiments as it was thought, perhaps erroneously, that any serum present would inhibit the propagation of these viruses.

Yields of SA11 were obtained from these cultures, as demonstrated by the extraction of virus-specific dsRNA from some cultures, but results were very variable. High inocula were required to cause observable differences under phase contrast microscopy between infected and control cultures (Fig. 12-2). High inocula were also required to obtain appreciable virus multiplication. This ran counter to the virologist's maxim that high inocula induce interference effects. Rotaviruses may be a special case. McNulty et al. (1977) found CPE difficult to detect in cell cultures infected with high dilutions of NCDV from Nebraska, although the Northern Ireland strain of calf rotavirus gave uneqivocal CPE under similar conditions. I found

that low inocula of SA11 gave no clear virus-specific CPE even after several weeks incubation. At any rate, the presence of high concentrations of virus particles - possibly defective or non-infectious - did not seem to inhibit replication or give rise to genomic variants. It is possible that low inocula were inactivated by antiviral agents either from the medium (e.g. from residual serum) or produced by the cells themselves (c.f. interferon).

It did not prove possible during the course of this study to quantitate the PFU or TCID50 of virus stocks because of the difficulty of achieving reproducible propagation, but the amount of rotavirus RNA extracted from cell cultures gave a good indication of the success of replication. The original stock bottle of SA11 obtained from Dr. Schoub retained viability for several years at 4C.

The main cause of poor reproducibility was thought to be either residual serum carried over from the cell growth medium, or that confluent cells in a state of contact inhibition may be less susceptible to infection. No viral propagation at all was observed when inoculated flasks were grown in normal serum-containing medium. The first possibility was tested initially by washing the cells several times with serumless medium. This did not give rise to a great improvement, and increased the chances of contamination. The second possibility was tested by inoculating pre-confluent cultures, but no clear-cut results were obtained. CPE seemed to be associated more with cells growing at the edges of confluent patches, but similar phenomena were sometimes observed in control flasks, and may have been due to the serumless medium (Fig. 12-2).

12.1.1. Newman Tytell Medium

A partial solution was found in the use of Newman Tytell Serumless Medium (NTM) as maintenance medium during virus replication. This enriched medium was an improvement on the MEM without serum (SFM) used previously. Sub-confluent cells remained healthy in appearance for several weeks after substitution of normal growth medium, and were even observed to replicate and spread. Yields of virus were still variable, however, and the identification of viral-specific CPE under phase contrast microscopy was still a frustrating process (Fig. 12-3).

In an effort to eliminate serum altogether, CV1 cells were subcultured directly into NTM. Cells attached to the flasks successfully, especially if residual versene-trypsin (VT) was minimized by changing the medium 2-6h after subculture. Surprizingly, a new batch of NTM did not allow 100% reattachment of cells to the flasks. As the medium was obtained from the same source (Gibco) this result was hard to explain. Using this new batch, it was observed that cells appeared spindle-shaped, and attached to the flask at only a few points (Fig. 12-3). After several days cells tended to lift off completely.

Cell cultures that had been inoculated with SA11, but which were not exhibiting much apparent sign of infection, were subcultured directly into fresh NTM. Medium from the older batch which supported better cell reattachment was used. After further incubation for 2 days many cells in the control (uninfected) flask were spindle-shaped but still attached, whereas flasks previously inoculated with varying amounts of stock SA11 medium exhibited dramatic changes.

Extraction of these cultures showed exceptionally high yields of SA11 RNA, especially from those cultures originally inoculated with a larger quantity of virus stock. Figure 12-16 4, shows the dsRNA extracted from the cell debris from a single flask into which infected cells had been subcultured in NTM. Various dilutions of the original stock SA11 medium were added to flasks containing cells which had already been subcultured in NTM, no serum being present (Fig. 12-3). Considerable CPE was observed for all dilutions down to 10^{-4} . When these cultures were subbed during the infectious phase little cell reattachment took place, but a great increase in free virus in the medium was observed (Fig. 12-16, lanes 1,10). Fig. 12-16, lane 1, represents 20% of the RNA prep from free virus harvested from 5 culture flasks, and lane 2 represents 10% of the RNA extracted from the cell debris from the same 5 flasks. From the approximately equal fluorescence intensity in these two lanes it may be estimated that about one third of the progeny virus in this experiment were released into the medium. Surprizingly, a flask inoculated with a 10^{-5} dilution of SA11 stock medium failed to produce any observable virus (Fig. 12-16, lane 3). This may indicate that a minimum inoculation exists to cause active virus propagation, but this was not further investigated. The SA11 samples on this gel were all DNase treated, and no trace of contaminating nucleic acids may be seen.

At this stage the reasons for this dramatic increase in yield were not known. One idea was that the disruption of the extracellular matrix with VT during subculture enabled the rotavirus to gain access to a greater proportion of cells. This would have helped to explain why stationary confluent cultures, which produce a substantial matrix, seemed to be less

susceptible to infection. Alternatively, freshly subbed cells usually undergo a rapid increase in growth and replication, which may affect viral propagation. However, even control cells subbed directly in NTM did not thrive as well as in serum containing medium, and certainly did not replicate extensively. Another explanation was that subculturing the cells, and the presence of proteases in the VT, removed virus inhibitors from the medium (However, see Section 11.6.1).

12.1.2. Effect of Trypsin on Rotavirus Propagation

Soon after this discovery, the effect of trypsin on the in vitro propagation of rotaviruses was reported by Almeida at the International Virology Congress at the Hague (Almeida, J.D. Proc. 4th Int. Congr. Virol. The Hague, 1978, Abstr. No. P35/1), which was attended by E.H.H.. Our observations supported her findings. The use of trypsin in rotavirus culture has been reviewed in Section 11.6.1.

12.1.2.1. Addition of VT to Culture Medium

Table 12-1. Versene-Trypsin (VT) Added to Culture Medium

VT Added	Final Trypsin Concentration
0	0
0.5ml of 1x VT	16μg/ml
1.0ml of 1x VT	31μg/ml
0.2ml of 10x VT	65μg/ml
0.5ml of 10x VT	160μg/ml

CV1 cells were grown in MEM, washed, and the medium replaced with 15ml NTM per flask. Flasks were inoculated with 0.1ml stock SA11 medium, and different amounts of VT solution added

(Table 12-1). No antibiotics were used. After 22h incubation, varying degrees of cell detachment and clumping were observed (Fig. 12-4), the flasks with higher VT concentrations being more affected. Some cells exhibited SA11-specific CPE. Continued incubation increased the proportion of detached cells in each flask containing VT. After 4 days, cells in flasks with only 15µg/ml trypsin were mostly detached, but the few remaining cells were surprizingly normal in appearance. Any remaining attached cells in flasks with higher trypsin concentrations were greatly elongated and spindle-shaped. Some detached cells had the appearance of inflated balloons. Flasks with no trypsin eventually developed CPE in some cells, but few cells detached.

RNA extracted from free virus and cellular debris was subjected to PAGE (Fig. 12-6). From the flasks to which no VT was added, detectable SA11 RNA was extracted from cell debris but not from free virus (Fig. 12-6, lanes 1 and 9). Varying amounts of SA11 RNA were found in trypsin-containing flasks, in both free virus and cell debris fractions (Fig. 12-6). Each lane contains the RNA extracted from one flask. It may be concluded that addition of VT to the culture medium during SA11 propagation greatly increased the yield of SA11 progeny. This effect was presumed to be due to the trypsin component of VT. In the absence of VT, little free virus was released into the medium, although SA11 RNA was found associated with cell debris. A much higher proportion of the progeny SA11 was released into the medium when VT was present, although this proportion varied in different flasks. Increased yields of SA11 were produced at all concentrations of VT used in this experiment. VT was used in several subsequent propagation experiments, and greatly increased yields of SA11 RNA were obtained.

The optimal concentration of trypsin was investigated further using pure recrystallized trypsin, as the activity of the trypsin component of the VT solutions used was not known. The possible effects of the other VT components on rotavirus replication were not investigated.

12.1.2.2. Use of Pure Trypsin

A stock solution of trypsin (Miles Cat. # 36-555-1, "3260 NFU/mg"), 10mg/ml in 1mM HCl, was filter sterilized. To determine the effect of this trypsin stock on cell cultures, flasks with confluent growths of CV1 cells were drained, washed with NTM, and incubated further in NTM containing trypsin. Concentrations of 5μg/ml and 10μg/ml were used (Almeida et al., 1978). After 4h, cells incubated in the presence of 10μg/ml trypsin were almost 100% detached, and cells in the presence of 5μg/ml showed significant rounding-up and partial detachment (Fig. 12-5).

In a subsequent experiment with SA11-infected cultures, 2μg/ml trypsin was used. Cells were washed as above, and NTM containing 2μg/ml trypsin added. Flasks were inoculated with 50μl of SA11 stock medium. After 5h, cells had started to clump (Fig. 12-5). After 22h, most cells had become rounded due to the retraction of cell processes, and many had detached. Little difference was observed between control and SA11-infected flasks. Incubation was continued for 6 days, by which time all cells had lost any characteristic morphology. The flasks were then extracted, and good yields of SA11 RNA were obtained (Fig. 12-7).

It is clear from these results that this trypsin preparation

was far more active, judging by its effect on cell attachment, than the crude VT solution used before. The activity of the trypsin preparation was not assayed.

Enough RNA had been obtained by this time to complete the analysis experiments described in this work, so no further investigations into the effects of trypsin were performed.

Similar results were obtained with the OA stock, but the dsRNA extracted from these cultures proved anomalous (see Section 12.5).

12.1.3. Infections

Infection of cell cultures with bacteria, yeasts and fungi was a persistent problem. Routine sub-cultures in MEM containing donor calf serum were rarely affected, but when NTM or serumless MEM were used, especially when antibiotics were omitted, infections were frequent. Strangely, incubation of the media bottles concerned never revealed the presence of contaminants in the remaining medium. These were thus either present in the cell cultures all the time, but suppressed by the presence of serum, or were picked up during the handling of the flasks. When infections occurred, usually only a proportion of flasks were affected, suggesting that the starting cultures were not infected, or else at very low levels. Since infection seemed to be associated with some batches of media more than others, the outside surface of the media bottles was suspected as being the source of contaminants. Washing all bottles with 66% ethanol before use reduced the incidence of contamination, and was subsequently adopted as standard procedure. It still remained puzzling, however, why the medium in the media bottles

rarely if ever became contaminated. This was never satisfactorily resolved.

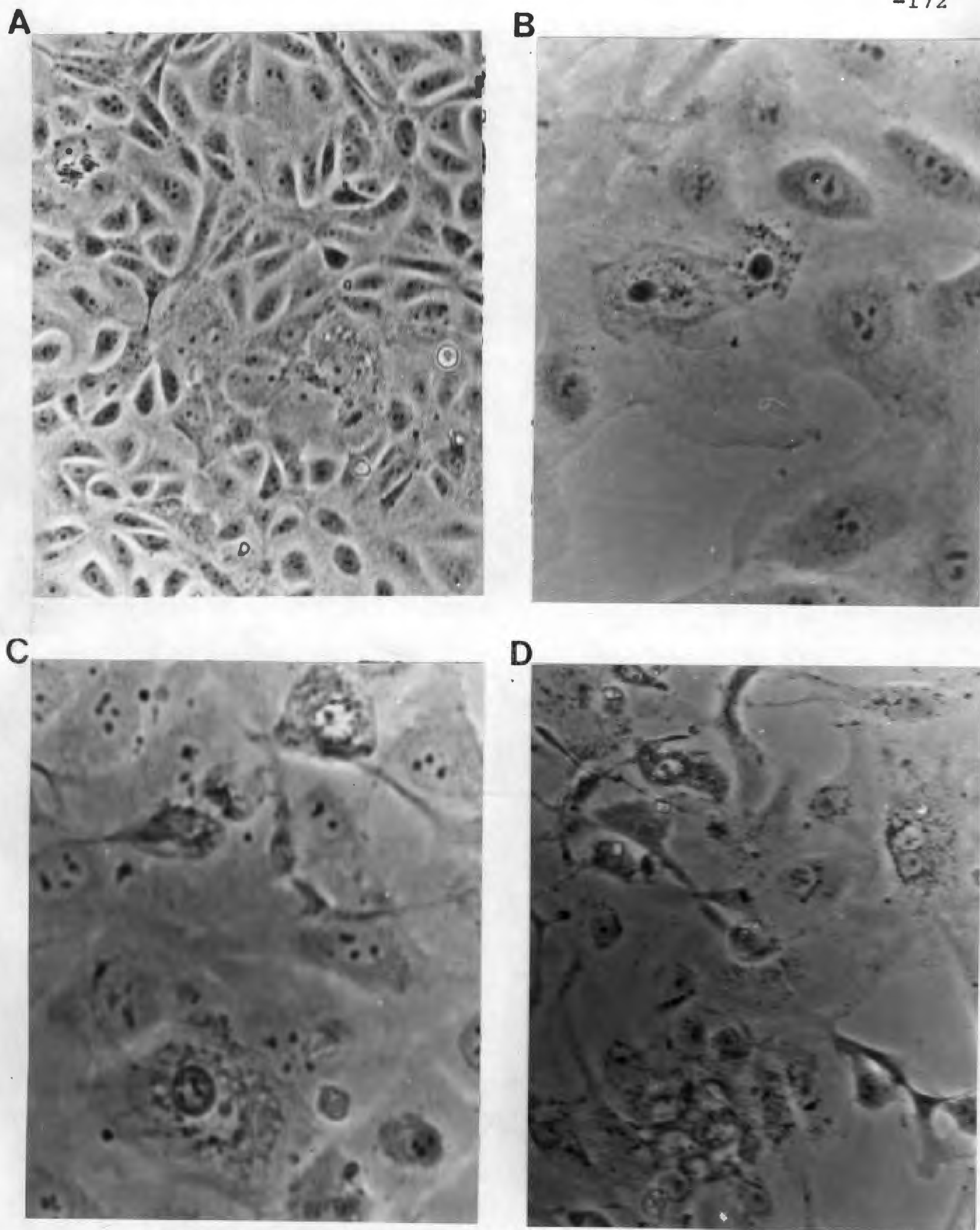


Figure 12-2. Cultivation of SA11 Rotavirus in Serum-Free Medium

A. Uninfected CV1 cells in serum-free medium (SFM) (x100).
B. "Pseudo-CPE" in uninfected cell culture in SFM (x200).
C. CV1 cells in SFM infected with stock SA11 virus (x200).
D. " " " " " " " OA (x100).

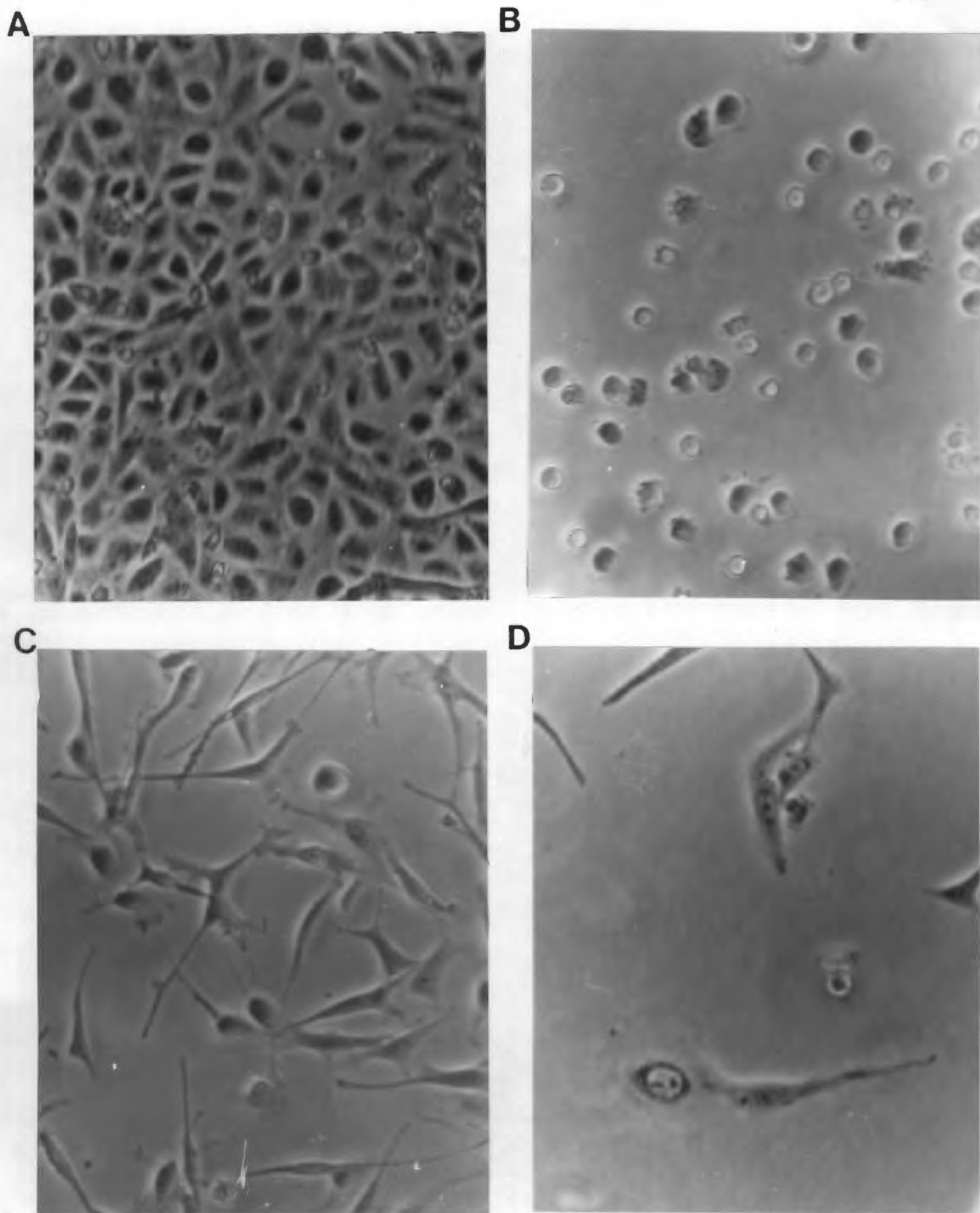


Figure 12-3. Cultivation of SA11 Rotavirus in Newman Tytell Medium

A. CV1 cells in NTM infected with SA11, not subcultured.
B. " " " " " " " 6h after subculture in NTM, before medium change.
C. As B, 19h after medium change.
D. As C, using different batch of NTM.

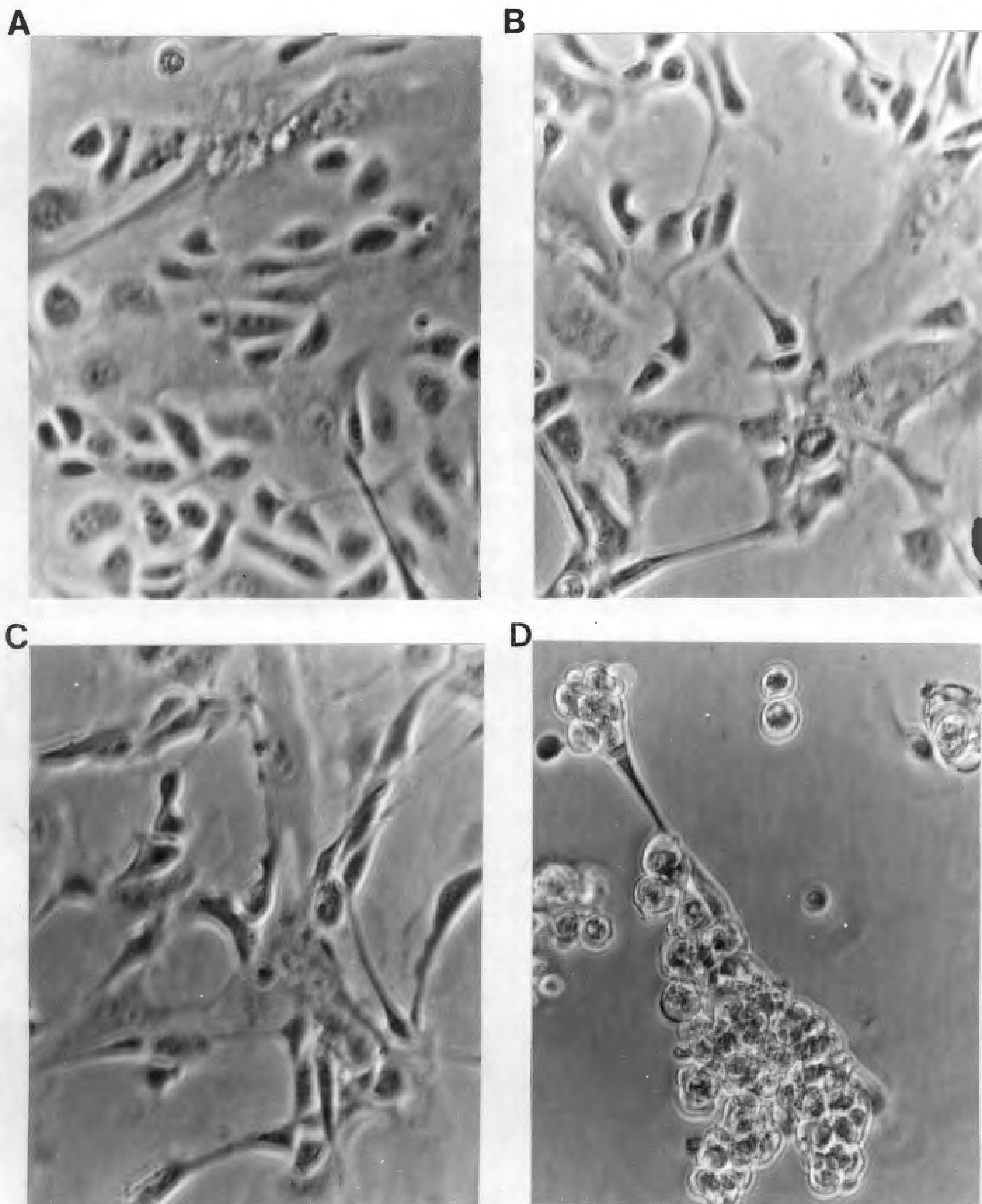


Figure 12-4. CV1 Cells Infected with SA11 in the Presence of Versene-Trypsin

SA11-infected CV1 cells in NTM 22h after the addition of the following amounts of trypsin:
A. 0. B. 15μg/ml. C. 30μg/ml. D. 60μg/ml.

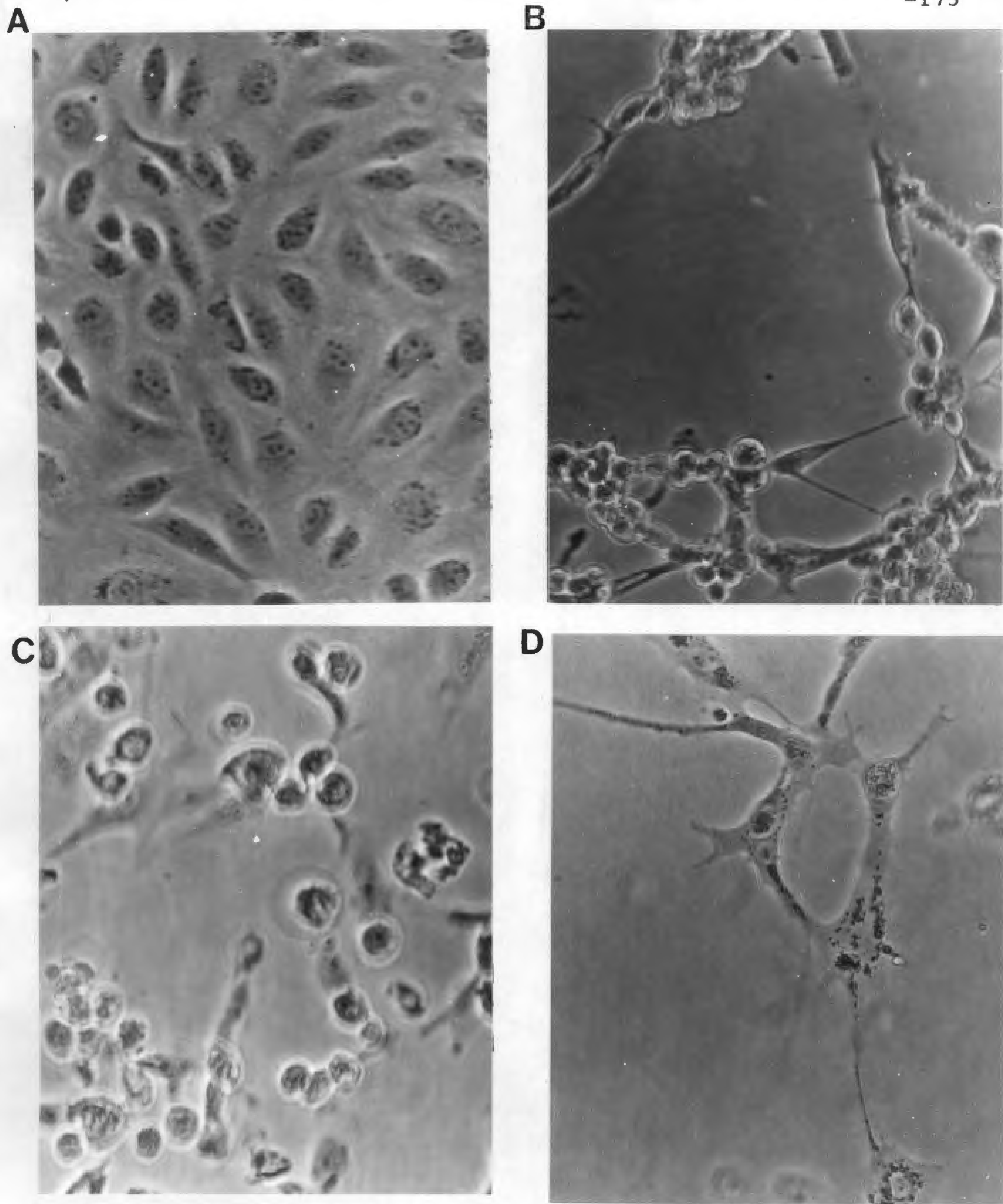


Figure 12-5. Propagation of SAll in the Presence of Pure Trypsin

A. Infected cells without trypsin.
B. Infected cells 4h after addition of 5μg/ml pure trypsin.
C. " " 5h " " " 2μg/ml " "
D. " " 22h " " " 2μg/ml " "

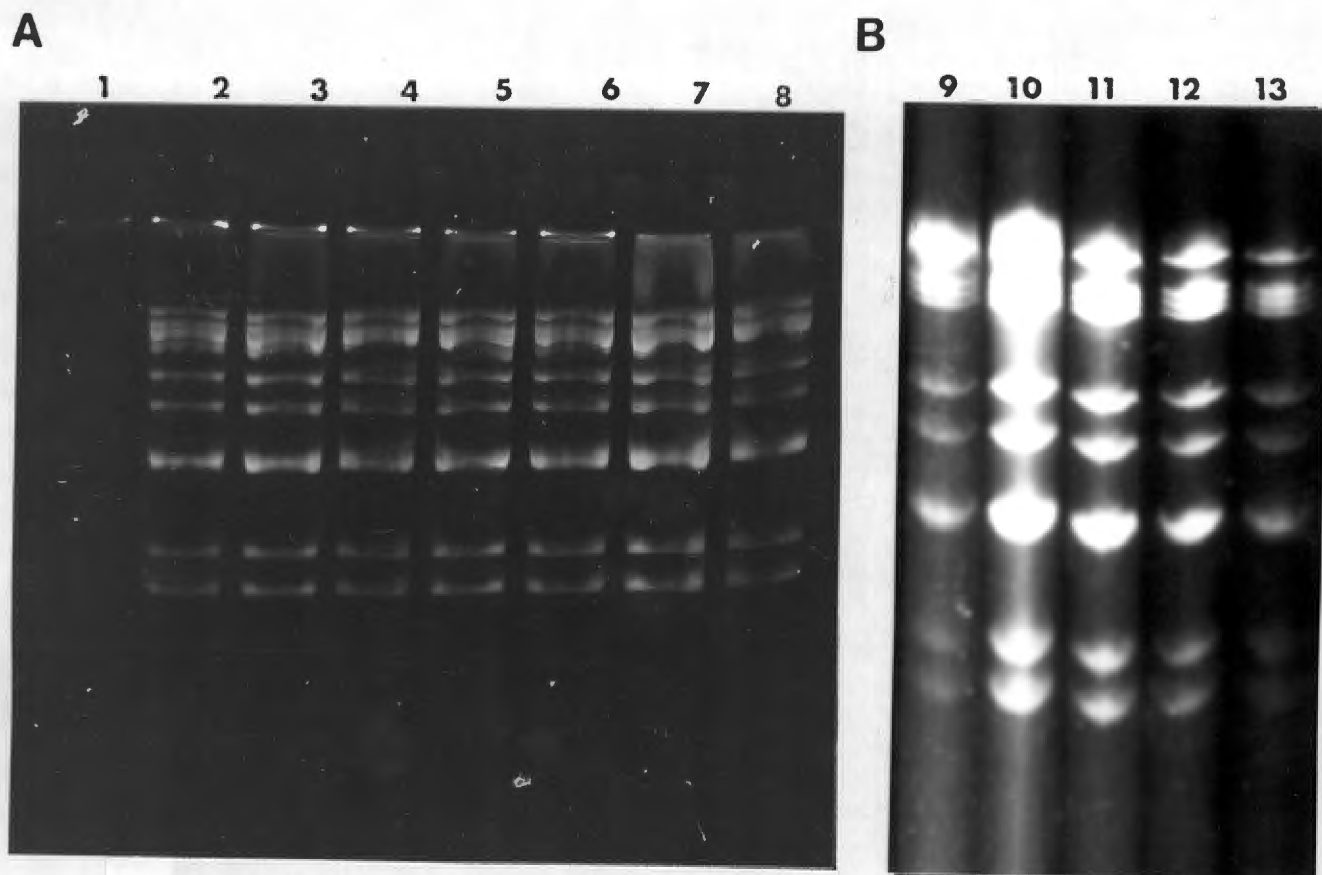


Figure 12-6. Effect of VT on SA11 Propagation - Analysis of RNA

A. 3.5% PAGE (slab).
B. 2.5% PAGE (cylindrical).
Lanes 1-6: RNA extracted from free virus in medium.
Lanes 7-13: RNA extracted from cell debris.
Lanes 1, 9: No VT added.
Lanes 2, 7, 10: 16μg/ml trypsin present
Lanes 3, 4, 8, 11: 31μg/ml trypsin present.
Lanes 5, 12: 65μg/ml trypsin present.
Lanes 6, 13: 160μg/ml trypsin present.

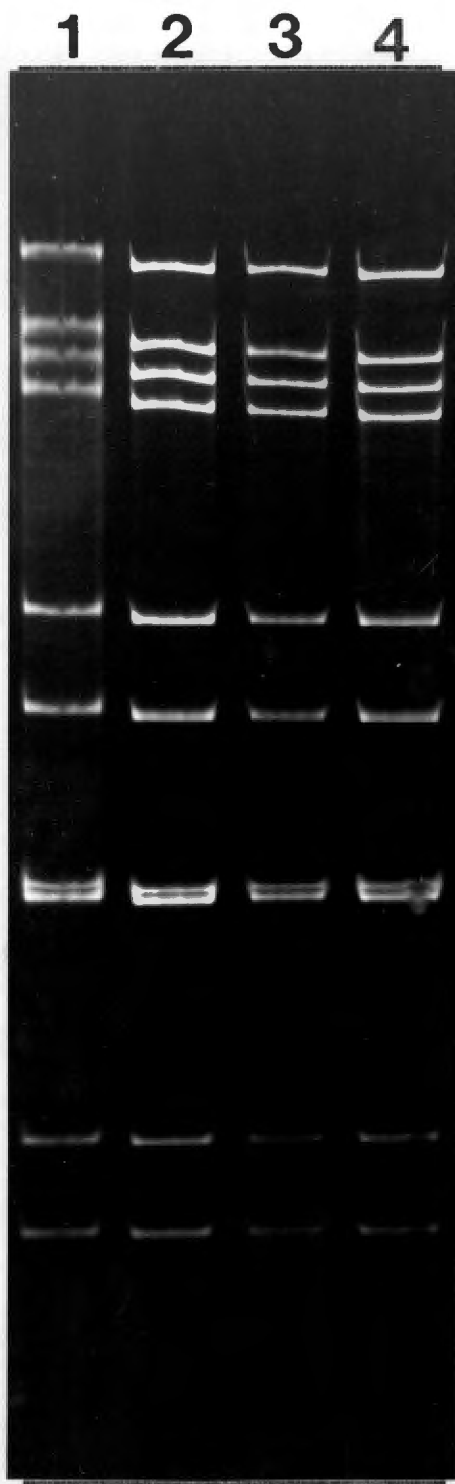


Figure 12-7. Effect of Pure Trypsin on SA11 Propagation

4% PAGE (slab) set and run at room temperature, 20h at 75V.
Lanes 1, 3: RNA extracted from free virus (DNase treated).
Lanes 2, 4: " " " cell debris " "

12.2. Buoyant Density of SA11 Virus

A number of peaks were obtained in a UV trace of a CsCl density gradient centrifugation of SA11 virus propagated in CV1 cells (Fig. 12-8). Procedure II (see Section 13.3) was followed. The main peak had a density of 1.39, and a smaller peak occurred at 1.36g/ml. A "rippled" peak also occurred at around 1.43g/ml. Fractions under these peaks were pooled and particles harvested by diluting with 10 vol of 10mM Tris-HCl, pH7.5, and centrifuging at 100,000xg for 2h. Pellets were extracted for dsRNA and electrophoresed on a 3.3% polacrylamide slab gel (Fig. 12-10). The amount of viral RNA recovered from these peaks was in the order 1.43>1.39>1.36. Rodger _et al_. (1975) obtained peaks at buoyant densities of 1.30, 1.36 and 1.37 with human and calf rotaviruses. Tam _et al_. (1976) reported peaks at 1.38, 1.36, 1.30 and 1.28g/ml. Similar results were obtained with various rotaviruses by other authors (Newman _et al_., 1975; Obijeski _et al_., 1977; Todd and McNulty, 1977). They all found from EM examination that bands at densities of 1.30g/ml and less contained empty particles devoid of RNA. Bands around 1.36 and 1.38 contained double and single shelled particles containing RNA. These bands probably correspond to our peaks at 1.36 and 1.39g/ml. The relative peak heights, and quantities of viral RNA extracted, indicate that the majority of the free SA11 virus particles from cultures of CV1 cells were single shelled (1.39g/ml peak) rather than double shelled (1.36g/ml peak). The peak at 1.43g/ml probably contained aggregated viral particles, or particles associated with cellular debris.

The preps obtained from this gradient were free from

contaminating DNA by gel analysis. However, the more simple procedure of digesting direct RNA extractions with DNase gave equally-clear gel pictures. Thus CsCl DGC was not used routinely to obtain dsRNA preparations.

12.3. Buoyant Density of SA11 RNA

Caesium sulphate equilibrium density gradient centrifugation of 32P-labelled SA11 RNA resulted in a UV peak at a density of 1.595g/ml, with a shoulder or minor peak at 1.620g/ml. The main 32P activity peak also occurred at 1.595 g/ml (Fig. 12-9). Fractions within this peak were pooled, and examined for rotavirus RNA by gel electrophoresis. The presence of characteristic bands, with no contaminating DNA (Fig. 12-10), confirmed that the peak at 1.595g/ml contained SA11 RNA. However, SA11 RNA was recovered from all fractions from 19-29. 29 corresponded with the bottom of the tube. The dsRNA peak occurred near the bottom of the tube because the gradient was fairly shallow. The starting density was 1.52g/ml, about mid-way between the expected densities for dsDNA (1.41) and dsRNA (1.59).

This density figure agrees well with published figures for dsRNA from other rotaviruses (Obijeski _et al_., 1977 - 1.59g/ml; Petric _et al_., 1976 - 1.57g/ml; both for human rotaviruses) and distinguishes the nucleic acid from ssRNA (1.65g/ml) and dsDNA (1.41g/ml) (Obijeski _et al_., 1977).

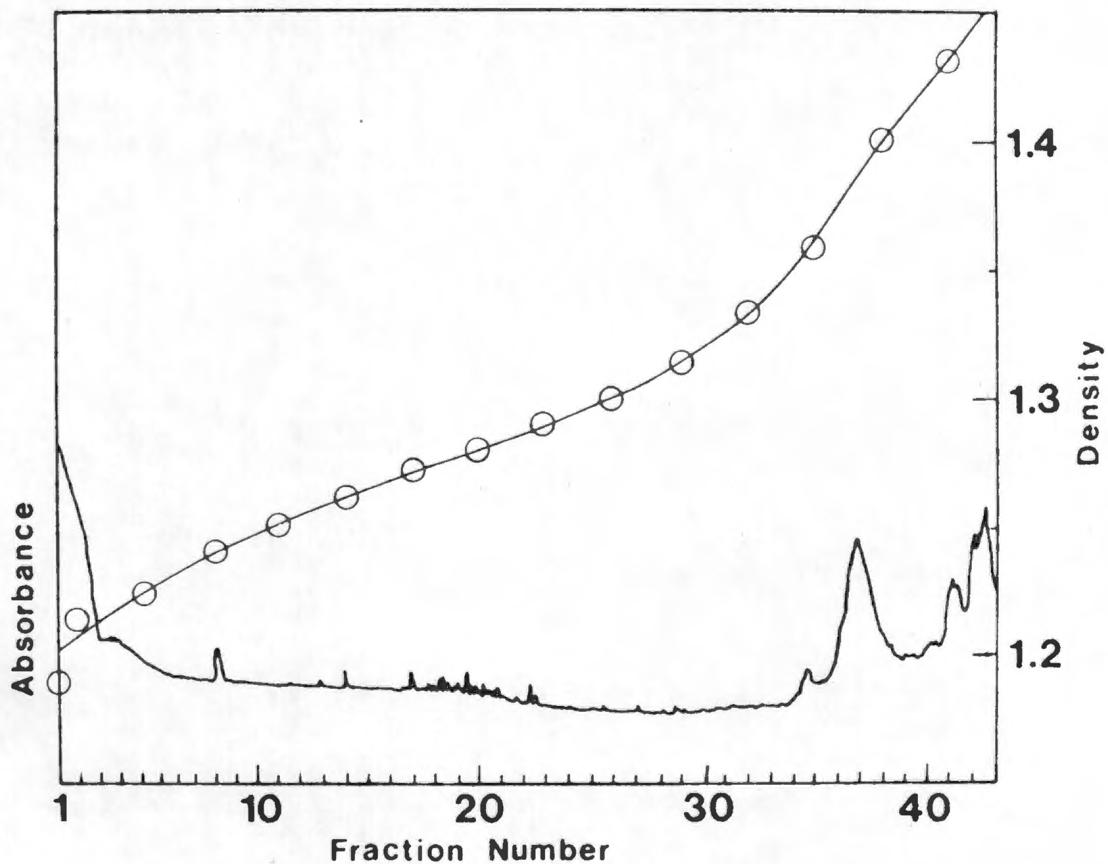


Figure 12-8. Caesium Chloride Density Gradient Centrifugation of SA11 Rotavirus

Trace: UV absorption at 260nm.
O Density profile.

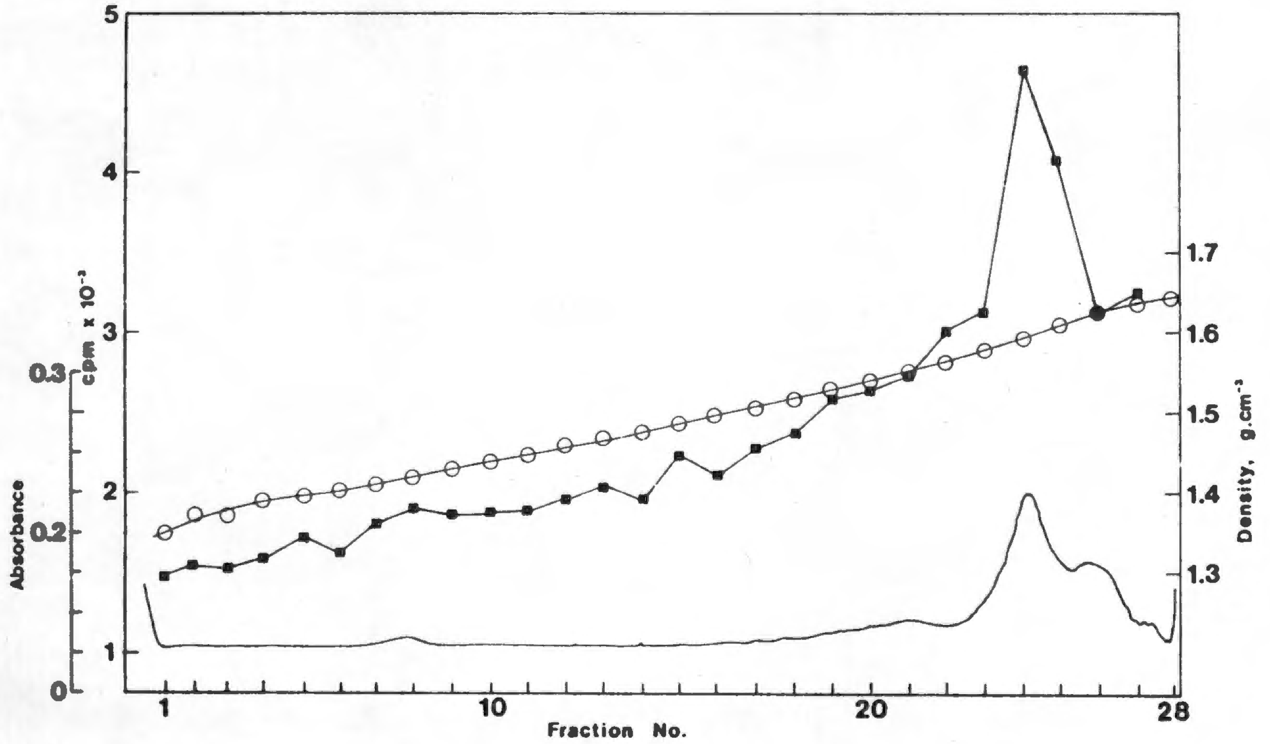


Figure 12-9. Caesium Sulphate Equilibrium Density Gradient Analysis of the SA11 Genome

Trace: UV absorption at 260nm.
○ Density profile.
■ 32P activity profile.

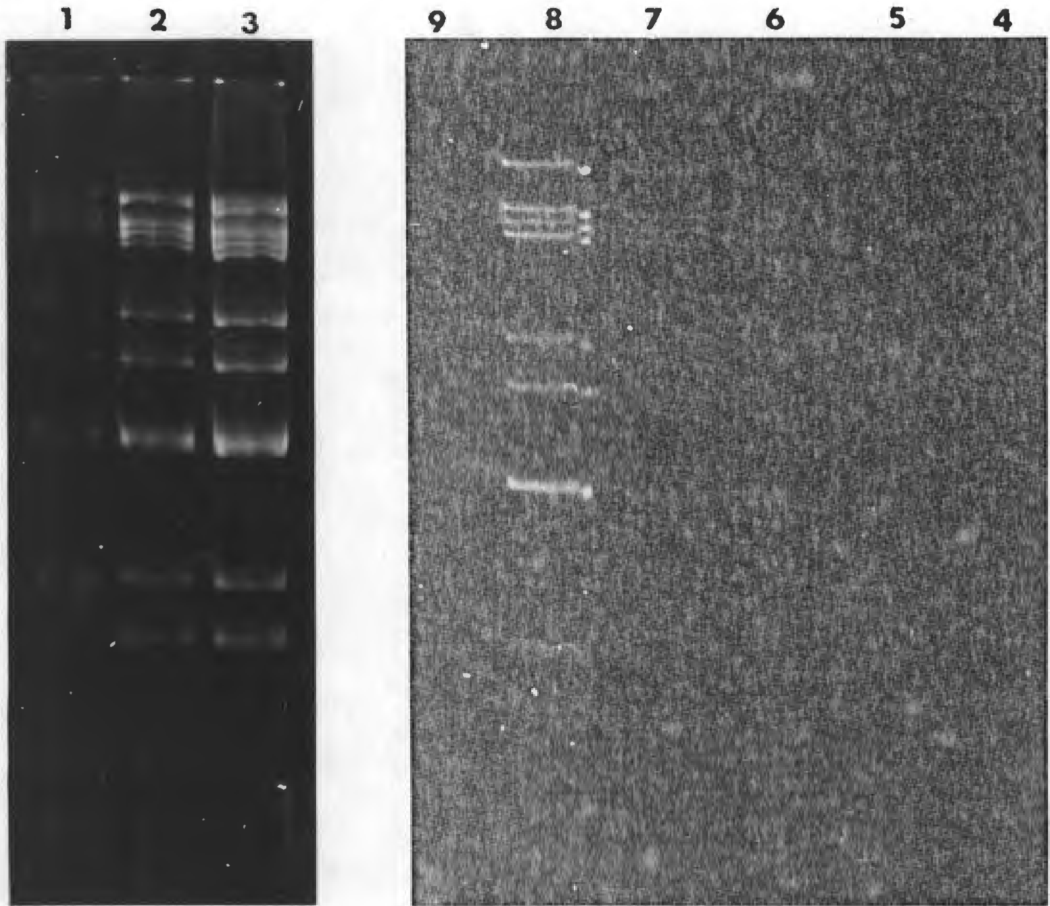


Figure 12-10. Gel analysis of RNA from CsCl and Cs_2SO_4 Gradient Fractions

A. RNA extracted from CsCl peaks in Fig. 12-8.
   Lane 1: Fractions 33-35 (1.36g/cc peak).
   Lane 2: Fractions 36-38 (1.39g/cc peak).
   Lane 3: Fractions 39-42 (1.43g/cc peak).

B. RNA dialysed from Cs_2SO_4 density gradient fractions (Fig. 12-9).

Lane							
Lane 4:	100%	of	material	from	fractions	1-10.
" 5:	"	"	"	"	"	11-20.
" 6:	"	"	"	"	"	20-23.
" 7:	20%	"	"	"	"	24-25.
" 8:	50%	"	"	"	"	26-27.
" 9:	"	"	"	"	"	28.

12.4. Extraction of EDIM and IGV

Approximately 5ml of a diarrhoeal stool specimen from an infant with gastroenteritis, and 20ml of a watery stool specimen from a mouse infected with EDIM virus were obtained from Dr. Schoub. RNA was extracted from these specimens as described in the Methods, Section 13.4.4. The EDIM sample resulted in a partially insoluble pellet after ethanol precipitation, but the insoluble fraction was removed by centrifugation. The supernatant was examined for viral RNA.

Analysis of one fifth of each prep on polyacrylamide gels showed characteristic rotavirus RNA bands in both preps. The EDIM bands were faint but clear (Fig. 12-11A). The IGV prep, which contained more RNA, also produced a band in a position characteristic of chromosomal DNA. No attempt was made to quantitate the preps spectroscopically. They were used directly in coelectrophoresis experiments with SA11, OA, and a CPV from <u>Heliothis armigera</u> as described below.

12.5. Molecular Weights of Rotavirus Genome Segments

Experiment I

SA11 RNA extracted from virions purified by CsCl DGC was coelectrophoresed with marker CPVa2 dsRNA kindly provided by E.H.H. (Fig. 12-11B). The CPV RNA bands unfortunately obscured the fainter SA11 bands in the central gel containing the mixed preps, but MW estimates were obtained by comparison with the gel containing SA11 alone (Bozarth and Harley, 1976). The known CPVa2 MWs were used as standards (Harley <u>et al</u>., 1977), and the values obtained (Fig. 12-12) are listed in Table 12-2.

Experiment II

A multiple coelectrophoresis experiment was performed comparing SA11 genome segments with those from CPV and an IGV sample extracted from faecal specimens (Fig. 12-11C). Differences were observed between SA11 and IGV in all but 3 genome segments. The coelectrophoresis between SA11 and CPVa2 allowed calibration of the band mobilities (Fig. 12-13), and the resultant MW values for SA11 and IGV are listed in Table 12-2. Mobility measurements were facilitated by performing UV fluorodensitometric scans of each gel (Fig. 12-14).

OA RNA from material passaged through CV1 cells was found unexpectedly to be indistinguishable from SA11 (Fig. 12-15). This contradicted earlier gel pictures of an extract of the original OA stock medium obtained from Dr. Schoub (Fig. 12-11A). Pictures published since then also show clear differences between the two genomes (Kalica _et al_., 1978b and c). Thus, in the final coelectrophoresis experiment, material extracted from the original stock medium was used.

Experiment III.

A final coelectrophoresis experiment was performed on a 4% polyacrylamide slab gel containing 0.5μg/ml EtdBr (Fig. 12-16). All the remaining stocks of EDIM, and OA extracted from the original stock medium, were used. Despite the unfortunate curvature of the bands in some of the lanes, this gel provided much interesting information. The fine resolution of the bands in lanes 8 and 9 containing OA showed that the original stock of OA appeared to have been a mixed culture. Lane 9 contained this prep alone, and it may be seen that a number of the bands are

split. The coelectrophoresis with SA11 RNA in lane 10 showed that some of these bands were reinforced, suggesting that the original OA was mixed with SA11. The only other explanation for this gel result is that some SA11 material spilled over from a neighbouring slot. However, assuming that the original culture was mixed explains our cell propagation results, from which only SA11-type RNA was obtained. It suggests that CV1 cells are more permissive for SA11 than for OA, and that SA11 was thus preferentially propagated in mixed culture. Another picture of OA RNA extracted from stock medium (Fig. 12-15, lane 4) shows a fuzziness of the bands which supports the idea that this original stock was mixed.

The risk of contamination by different virus strains being grown in the same laboratory is well-known (Wyatt et al., 1980). The OA stock medium was used on many occasions to inoculate CV1 cultures, but the RNA extracted from these cultures was invariably of the SA11 pattern (Fig. 12-15).

This coelectrophoresis experiment permitted MW estimates to be made for the EDIM and OA genome segments using SA11 as a standard (Fig. 12-17). It was possible to distinguish the OA bands from the SA11 bands in lanes 8 and 9 and thus obtain mobility measurements for OA. This information is compiled, together with previous results, in Table 12-2.

It is impossible, without direct comparison, to say whether the IGV isolate analysed here is identical in electropherotype to any already described. It falls, however, into the "Type 21" group of Espejo et al. (various papers), and resembles the pattern obtained by Rodger and Holmes (1979) for a human isolate. As stated in the literature review, new electropherotypes of human rotavirus are identified from nearly

every outbreak examined.

Little emphasis has been placed on absolute MW estimations of dsRNA genome segments by PAGE in the literature to date. The commonest marker has been reovirus Type III. This has 10 segments which fall into 3 distinct size classes, the average MWs of which have been precisely determined by contour length measurement in the EM. This provides 3 marker values only. It has been conveniently assumed that the relationship between log MW and electrophoretic mobility is linear for dsRNA, in contrast to the well-established exponential curve obtained with dsDNA. In other words, dsRNA has been gratuitously assumed to behave like ssRNA or ssDNA on PAGE. This has been shown to be erroneous (Bozarth and Harley, 1976), and thus all MW values given in the literature for rotavirus dsRNA genome segments are invalid, especially for the higher MW species in the region where the MW curve deviates most strongly from linearity. The perpetuation of this error is a result of the rarity of well-characterized high MW dsRNA molecules, and of the difficulty of measuring their MWs accurately by alternative methods.

12.5.1. Molecular Weight Estimations by 32P-Labelling

Adding 32Pi to the cell culture medium results in the incorporation of 32P into the phosphate backbone of newly synthesized nucleic acid molecules. Assuming the available concentration of 32Pi at the site(s) of synthesis in the cell remains constant, the amount of label incorporated should be proportional to the length of each molecule, irrespective of

base sequence. Hence the amount of label is proportional to the MW of each molecule. Assuming also that the genome segments of rotavirus are present in equimolar proportion, the relative MWs of each segment may be estimated from a quantitation of the 32P activity of dsRNA bands obtained by gel electrophoresis (Harley et al., 1977).

CV1 cells were washed twice with SFM and inoculated with a 10 dilution of stock SA11 in SFM containing 32Pi at an activity of 10µCi/ml. Incubation was continued for 5 days. By this time marked cell death was apparent. Two days later all the cells had detached. RNA was extracted and purified as normal, taking standard precautions with the handling of 32P.

RNA was run on 10 and 20cm cylindrical 4% polyacrylamide gels containing 10% glycerol. The EtdBr-stained gel was photographed, (Fig. 12-18), and then sliced in 1mm slices as described in Section 13.6.1. Radioactivity profiles are shown in Fig. 12-19 and 12-20 The prep of RNA extracted from cell debris contained a small peak corresponding to DNA which had resisted DNase digestion (Fig. 12-18, lane 1). This was absent in the free virus extract (Fig. 12-18, lane 2). Much more RNA was extracted from the cell debris than from the free virus fractions, hence the difference in intensity and activity. The 32P activity in each peak was quantitated, and plotted against peak mobility (Fig. 12-21). The molar ratio in each peak was estimated from the difference between the actual activity and the projection onto a smooth curve through the points (ignoring points from presumed overlapping peaks) (Table 12-3). The validity of this procedure is based on the assumption that the rotavirus genome segments are present in the virus in equimolar

proportion (Harley et al., 1977). It only really serves to indicate the number of segments present in the multiple band 7, and to produce adjusted activity values more consistent with one another. Values varying from 3.34 to 2.93 were obtained for the molar ratio of band 7, which is consistent with the accepted number of 3 segments in this band.

Molecular weight estimates may be made directly from the 32P activity in each segment once a conversion factor of activity into MW is found. If 32P activity in each molecule is proportional to its length, and the molecules are present in equimolar proportion, then 32P activity in each discrete band (as adjusted to the curve in Fig. 12-21) is proportional to the MW of each segment also. The curve was calibrated from relative mobility measurements of CPV marker segments from the experiment shown in Fig. 12-11 (C). Resultant MWs for SA11 segments using a selection of CPV segments for calibration purposes are listed in Table 12-4. It may be seen that they are both internally inconsistent, and diverge from the MW values obtained by coelectrophoresis with CPV segments (Table 12-2). Basically, the curves in Fig. 12-21 are too flat, so that MW values for segments further away from the calibration points are increasingly divergent from values in Table 12-2.

It may be seen by observation that the curves for RNA extracted from purified virus and from cell debris are not overlapping. On a logarithmic plot this should be so if the bands were present in equimolar proportion and were uniformly labelled. A possible explanation is that the efficiency of counting of different slices was not uniform. This may arise if hydrolysis of the RNA in the gel slices was not complete, and/or incomplete elution of 32P from the slices had occurred.

It is possible that RNA extracted from cell debris rather than from purified virions is not in equimolar proportion, and that the higher MW species are less well represented. This would make sense if the efficiency of replication of larger fragments was lower, although I have no evidence of this. It would explain why the cell debris material produced a flatter curve than the virus-extracted material. If the virus-extracted curve is true, then the divergent results obtained with different CPV marker calibrations may be due to the MW values for the CPV markers being in error. However, these values were based on a large number of independent determinations (Harley *et al.*, 1977). The problems with using other MW markers have been discussed (Section 11.7.1).

Thus, despite the excellent 32P activity profiles obtained, and the small deviation of the points from a smooth curve (Fig. 12-21), quantitation results had to be interpreted cautiously.

Similar profiles were obtained in other labelling experiments (data not shown). Occasionally, the intensity of segment 3 was reduced relative to the other bands. A similar phenomenon was observed in some EtdBr-stained gels of material extracted from cell debris. RNA extracted from purified virions seemed to contain segments in closer equimolar proportion, but insufficient data was accumulated to draw meaningful conclusions on this point. Quantitation of EtdBr-stained bands by UV fluorometric scanning caused quenching of bands subjected to prolonged UV exposure, as occurred when the gel was being aligned and the machine settings adjusted. This tended to invalidate quantitation results using this method. Possibly, however, genomic segments are not synthesized with equal

efficiency in the cell, so that unpackaged material may not be in equimolar proportion. Hence material extracted from cell debris may not reflect the relative proportions found in virus particles. To investigate this point, pulse labelling studies of nascent viral RNA strands could be performed in order to measure their relative abundance in the cell at various stages after infection. This was not done.

A method has been described for denaturing nucleic acids with glyoxal (McMaster and Carmichael, 1977) which claims to result in overlapping log MW vs mobility curves for RNA and DNA after gel electrophoresis. If this is true, it should be possible to use sequenced DNA markers as standards in a coelectrophoresis with glyoxal-denatured rotavirus RNA. Preliminary experiments to test this technique were not successful (data not shown).

12.6. Voltage Effects on RNA Electrophoresis

SA11 RNA was run on 3% polyacrylamide cylindrical gels at voltages varying from 2.5 to 15 V/cm. A comparison was also made between acrylamide recrystallized as described in Section 15 and commercial material of "especial purity" (Bio-Rad). Gels were poured at 4C.

Aliquots of the same DNase-treated SA11 RNA prep were run on each gel. Electrophoresis was continued at each voltage until the bromophenol blue dye in each sample had migrated the same distance (see Fig. 12-22). EtdBr-stained gels were photographed (Fig. 12-22), and mobility values obtained from fluorodensitometric scans. Relative mobility values were calculated, and are represented in Fig. 12-23.

These results show that reducing the voltage reduced the

mobility of dsRNA species of MW>0.75Md to a greater extent than species smaller than this. This had the effect of increasing the separation of higher MW species (see bands 1-4, Fig. 12-23).

A similar result was obtained for dsDNA in agarose-acrylamide composite gels by Lishanskaya and Mosevitsky (1973). They used fragments of much higher MW (8-120Md), however, and found that low voltage gradients drastically reduced the relative mobility of high MW species (>31Md).

A lesser variation was observed in band mobility using different sources of acryamide. Since this experiment was not repeated, this minor variation cannot be considered significant. The sharpness of the bands was greater in gels using our recrystallized acrylamide, emphasizing the importance of using high purity ingredients for acrylamide gels.

As has been found in most results with cylindrical gels, simultaneous electrophoresis does not ensure constant band mobility for the same material. In other words, it is very difficult to make a batch of cylindrical gels identical in electrophoretic characteristics. Slab gels are an improvement in this respect, but are prone to uneven heating effects during runs, which can also affect band mobility.

The effects of different gel concentrations, setting temperatures, and buffer systems have been reported (Harley *et al*., 1973; Gressel *et al*., 1975; Ramig *et al*., 1977).

12.7. Digestion of SA11 RNA with RNase and DNase

RNA was extracted from 12 flasks infected with SA11 in the presence of trypsin. Fig. 12-24A shows a comparison of free virus and cell debris extracts before and after treatment with

DNase. Each gel represents 5% of each preparation, equivalent to 60% of one culture flask. The heavy band running above the dsRNA rotavirus bands in the undigested preps was completely removed by DNase treatment. Note that approximately equal amounts of RNA were extracted from free virus and cell debris fractions - a result of trypsin treatment.

DNase-treated material was resuspended in SSC of different concentrations and treated with RNase. SA11 RNA in low ionic strength solution was completely digested by 1μg/ml of RNase in 90min at 37C, whereas a similar sample in high ionic strength solution remained unaffected compared to a control (Fig. 12-24B). The only difference observed between the gels of the control with no RNase and the digested sample in 1xSSC was that the control (gel 3) contained a smear of material around the higher MW SA11 RNA bands which was not present in the RNase treated sample (gel 2). This smear was not present in the original DNase treated prep (Fig 12-24A). It may represent a small amount of denatured viral RNA resulting from the sample being redissolved initially in 0.01xSSC. This low ionic strength solution may have caused partial denaturation which was not reversed when the sample was made up to 1xSSC. Such single-stranded material would be susceptible to RNase at high ionic strength, explaining its absence from gel 2 (Fig. 12-24B).

This result confirms that the bands of SA11-specific nucleic acid were double-stranded RNA, being resistant to DNase digestion, and RNase at high ionic strength. Low ionic strength conditions destabilizes the paired strands, and local denaturation allows degradation by single-strand specific RNase I to occur.

1 2 3 4 5 6 7 8 9 10 11 12

Figure 12-11. Gel Electrophoresis of Four Rotavirus RNAs.

A. Cylindrical PAGE of newly extracted samples (4% gel).
1: 20% of human gastroenteritis (IGV) sample extract.
2: Crude extract of cell-cultured SA11 RNA.
3: 20% of EDIM extract from mouse faeces.
4: 20% of OA extracted from original stock medium.

B. Cylindrical PAGE of coelectrophoresis between SA11 and CPV RNAs.
5: SA11. 6: SA11 + CPV. 7: CPV.

C. Cylindrical PAGE of coelectrophoresis between CPV, SA11 and IGV RNAs.
8: IGV. 9: IGV + SA11. 10: SA11.
11: CPV + SA11. 12: CPV.

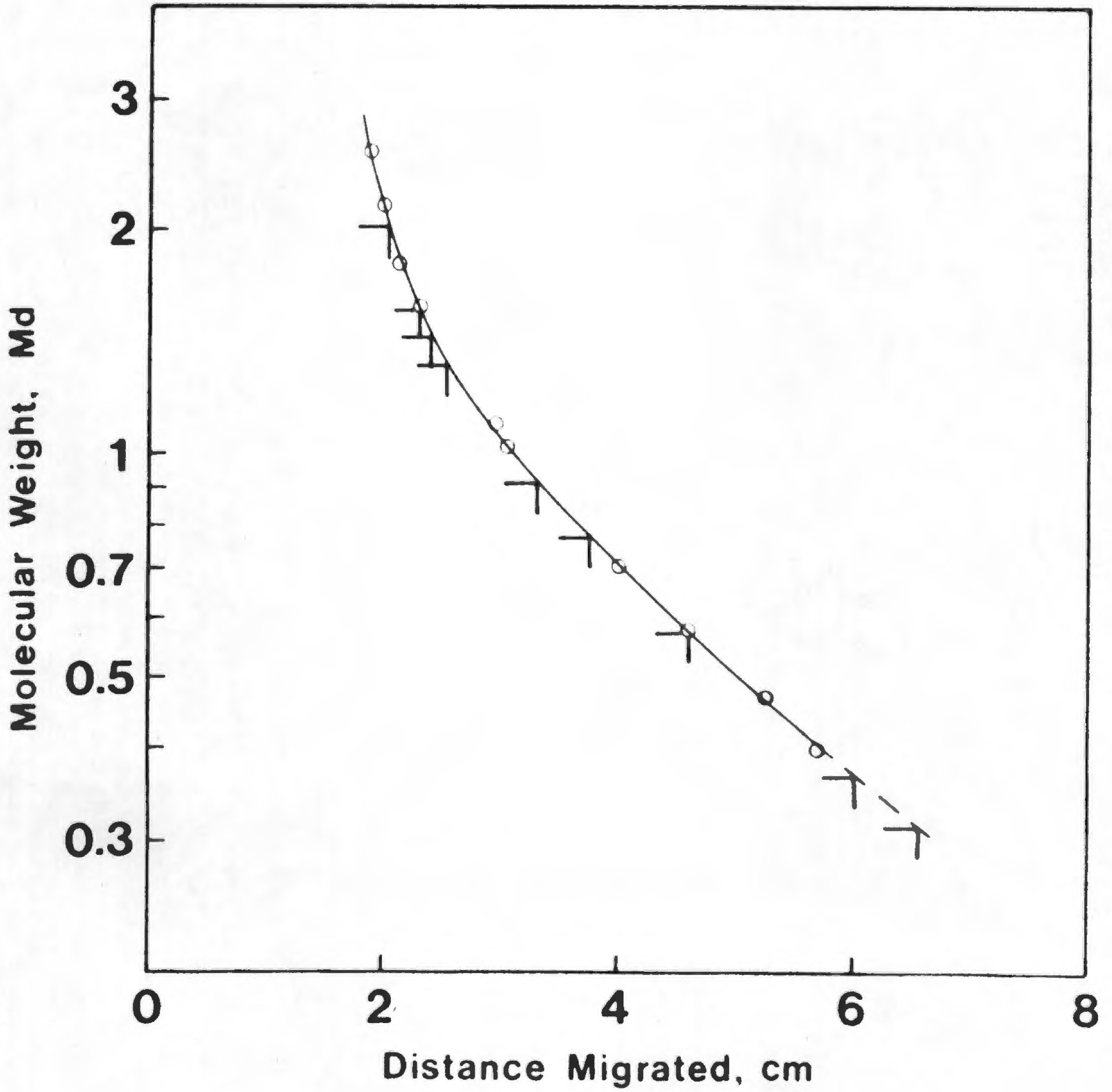


Figure 12-12. Determination of the Molecular Weights of SA11 Genome Segments

Plot of log MW versus mobility of CPV marker fragments (see Fig. 12-11 (B)).

○ CPV. ┐ SA11.

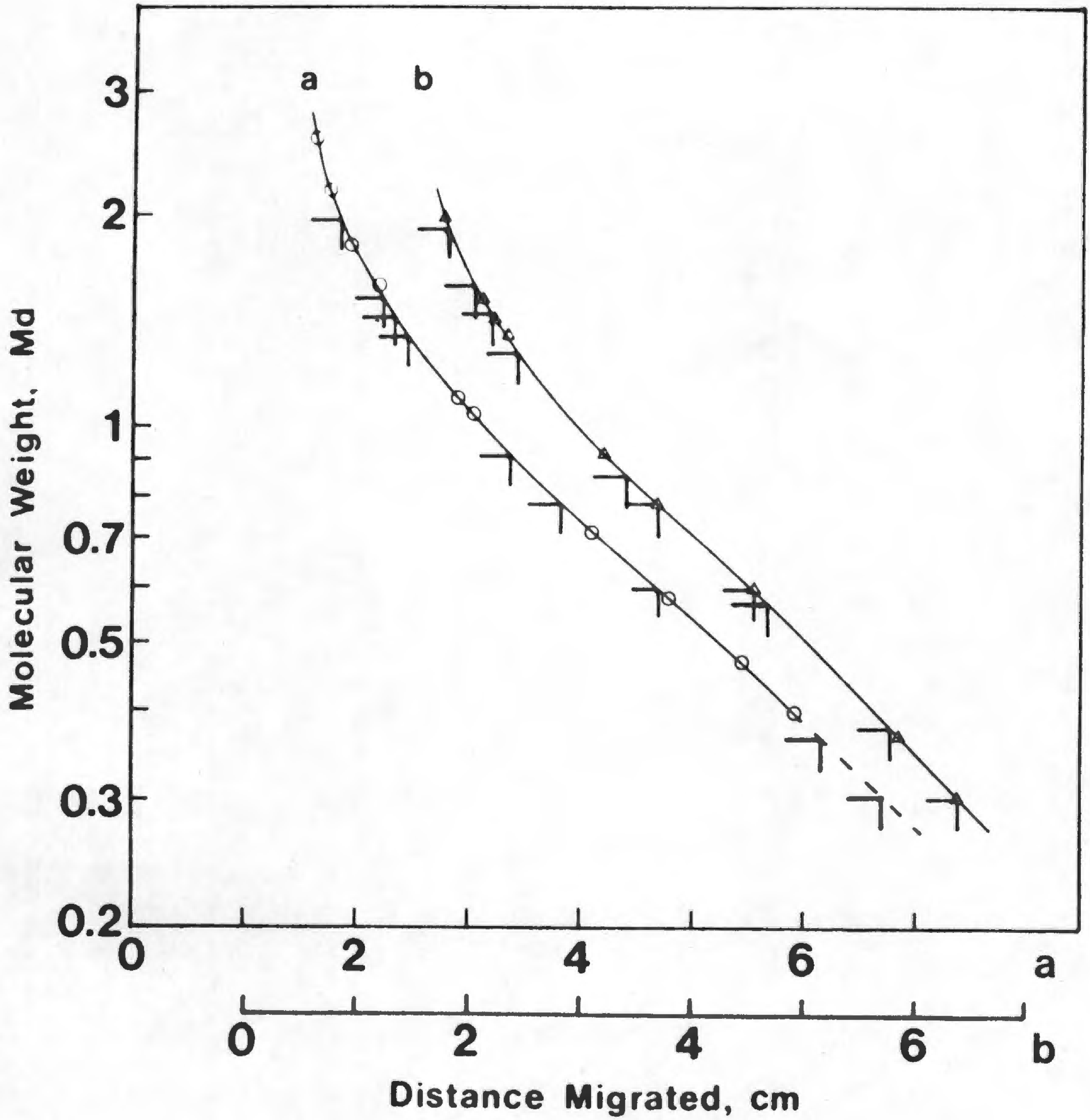


Figure 12-13. Determination of Molecular Weights of SA11 and IGV Genome Segments

a. Plot of log MW versus mobility of CPV marker segments (see Fig. 12-11 (C)).

○ CPV ┐ SA11

b. Similar plot using SA11 MWs as standards to determine IGV genome segment MWs.

△ SA11 ┐ IGV

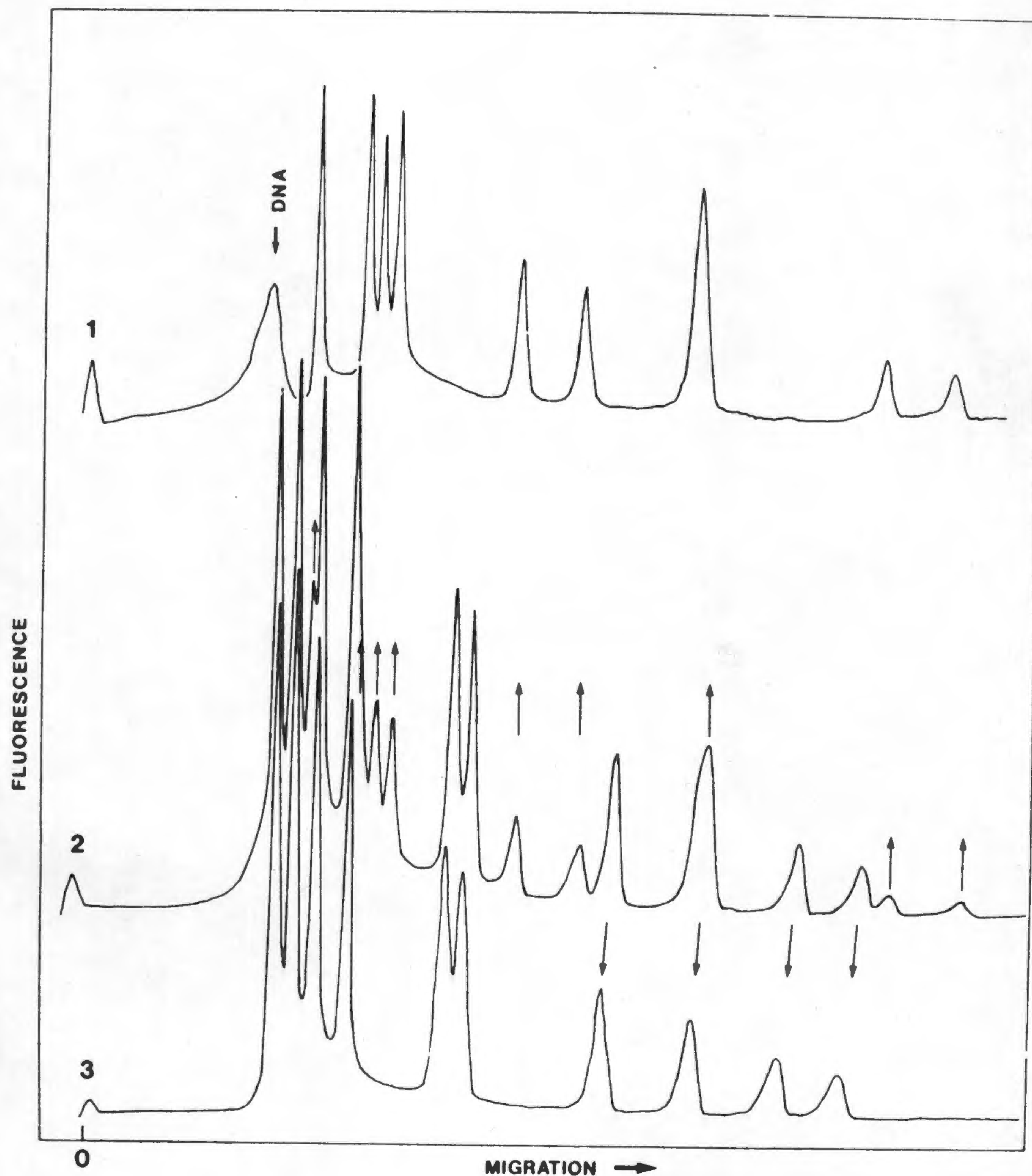


Figure 12-14. UV fluorometric scans of EtdBr-stained Gels Used for Mobility Measurements

Examples of traces obtained from scans of cylindrical gels.

1: SA11 (Fig. 12-11 (C), gel 10).
2: SA11 + CPV (" " " gel 11).
3: CPV (" " " gel 12).

Arrows indicate the assignment of peaks from the coelectrophoresis gel.

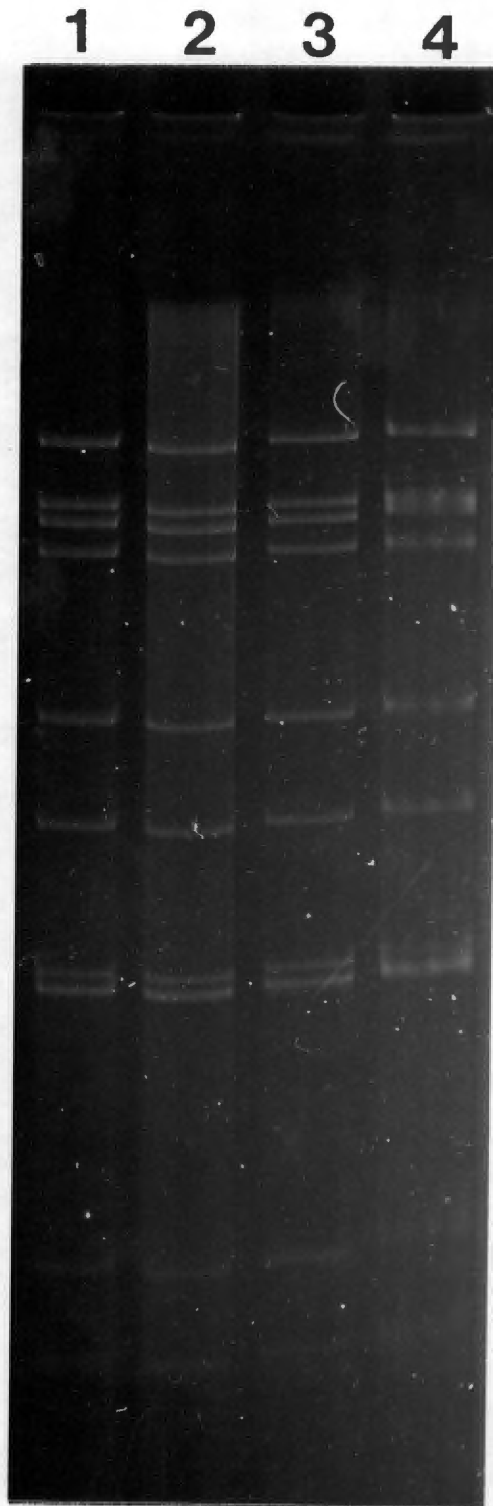


Figure 12-15. Comparison of OA and SA11 RNA from passaged material

Lanes 1,2: SA11; Lane 3: OA passaged through CV1 cells; Lane 4: RNA extracted from original stock medium

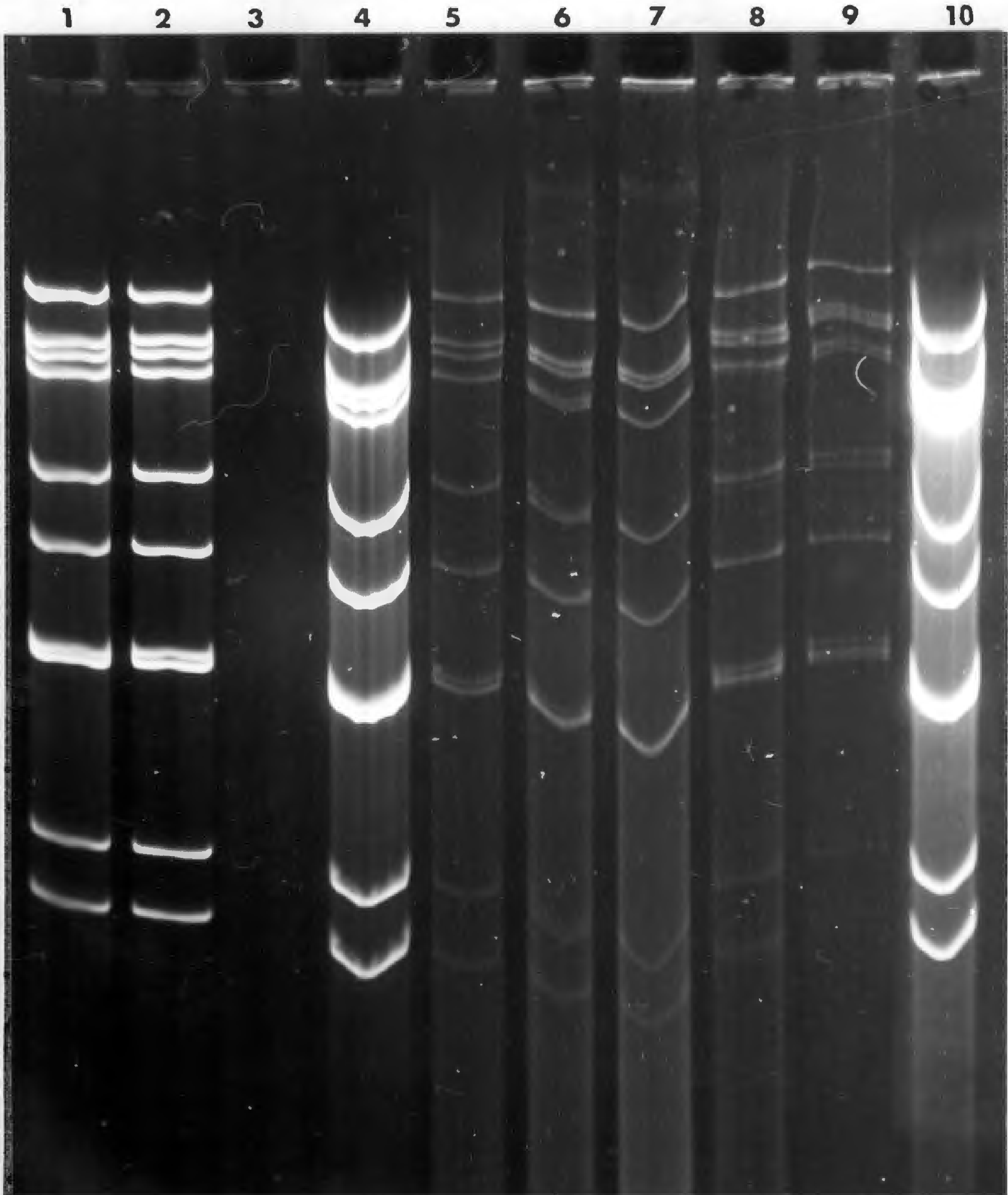


Figure 12-16. Polyacrylamide Slab Gel of Rotavirus RNA

Lanes 1-4, 10: Effect of subculture in NTM of CV1 cells infected with SA11.
Lanes 5-9: Coelectrophoresis (Experiment III) of SA11 with EDIM and OA.
Lane 1: 20% of RNA from virus from 5 flasks.
Lane 2: 10% of RNA from cell debris from 5 flasks.
Lane 3: 100% of RNA from cell debris from 1 flask originally inoculated with 10^{-5} dilution of stock SA11.
Lane 4: 100% of RNA from cell debris from 1 flask originally inoculated with 10^{-4} dilution of stock SA11.
Lane 10: As lane 1, but twice as much sample.

Lane 5: SA11. Lane 6: SA11 + EDIM. Lane 7: EDIM.
Lane 8: SA11 + OA (ex stock). Lane 9: OA (ex stock).

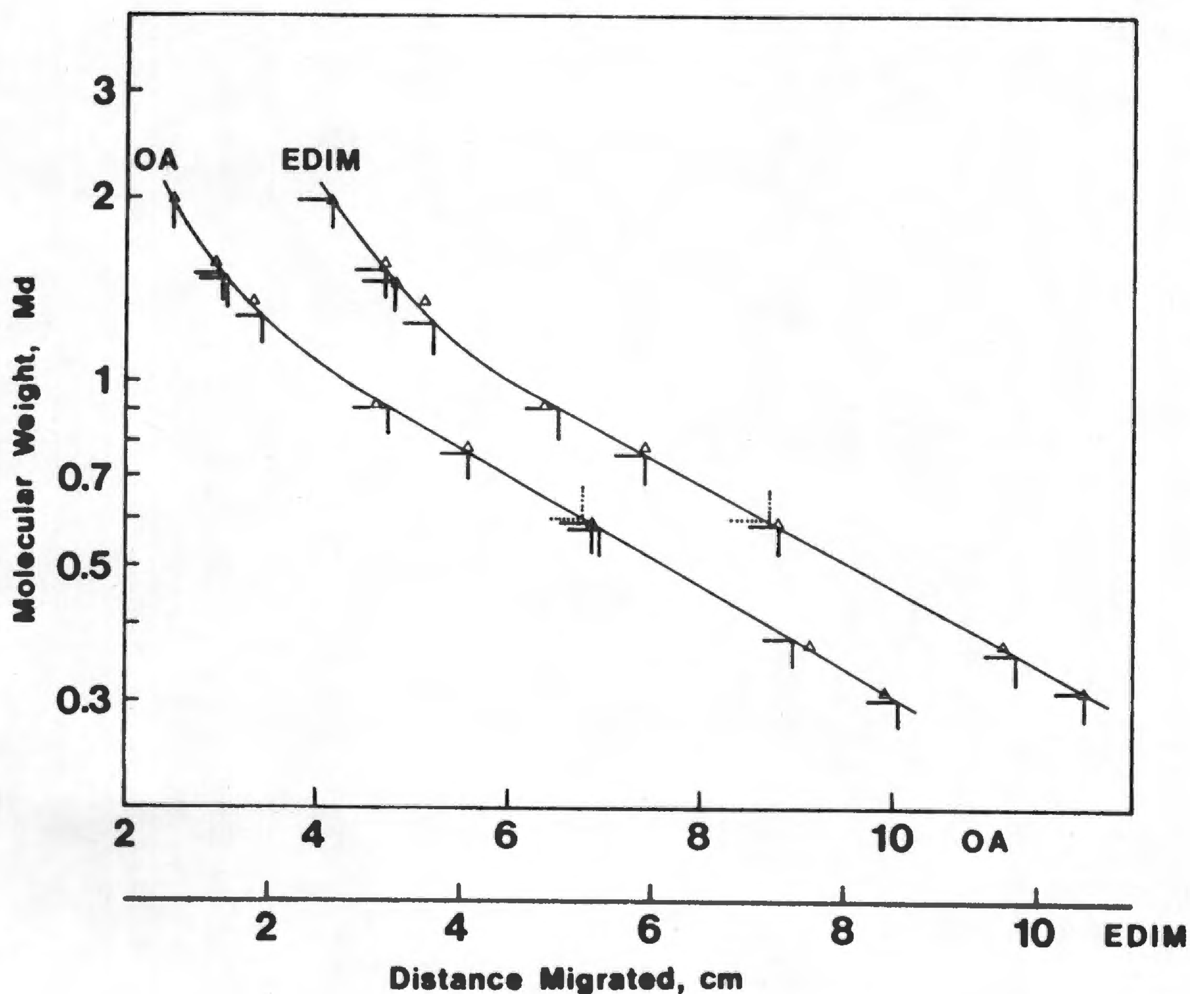


Figure 12-17. Determination of the MWs of OA and EDIM Genome Segments

Plot of log MW versus mobility of SA11 marker segments (see Fig. 12-16).

△ SA11 ⌉ OA or EDIM

⋯⋮ Position of SA11 band 7, previously unresolved.

Table 12-2. Molecular Weights of Rotavirus Genome Segments from Coelectrophoresis Results

Segment No.	CPV a	SA11 b	SA11 c	SA11 d	IGV e	OA f	EDIM g
1	2.55	2.00	1.97	1.97	1.90	1.97	1.97
2	2.15	1.57	1.51	1.54	1.58	1.50	1.51
3	1.80	1.45	1.41	1.43	1.43	1.47	1.46
4	1.58	1.32	1.35	1.34	1.27	1.29	1.25
5	1.10	0.92	0.91	0.91	0.85	0.90	0.90
6	1.03	0.77	0.78	0.78	0.78	0.76	0.76
7	0.71					0.59	
8	0.58	0.58	0.60	0.59	0.60		0.58
9	0.47				0.57	0.58	
10	0.40	0.37	0.37	0.37	0.38	0.38	0.36
11		0.32	0.30	0.31	0.30	0.30	0.31
Total		10.66	10.40	10.53	10.26	10.32	10.26

Values are given in megadaltons (Md).

a. Harley et al. (1976).
b. Experiment I (Figs. 12-11 (B), 12-12).
c, e. Experiment II (Figs. 12-11 (C), 12-13).
d. Average of b and c.
f, g. Experiment III (Figs. 12-16, 12-17).

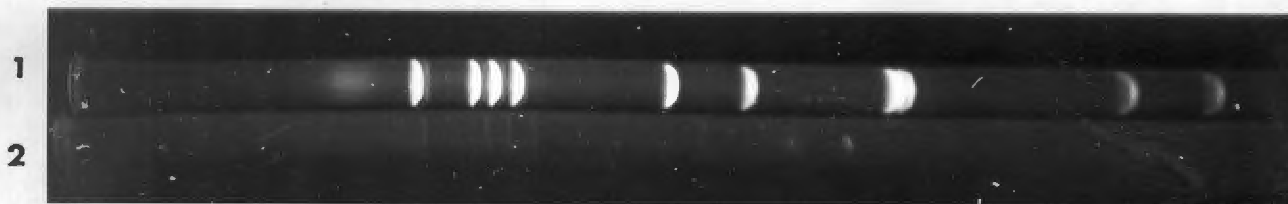


Figure 12-18. Electrophoresis of 32P-Labelled SA11 RNA through 20cm Polyacrylamide Gels

Gel 1: RNA from cell debris, DNase treated.
Gel 2: RNA from virus purified by CsCl DGC.

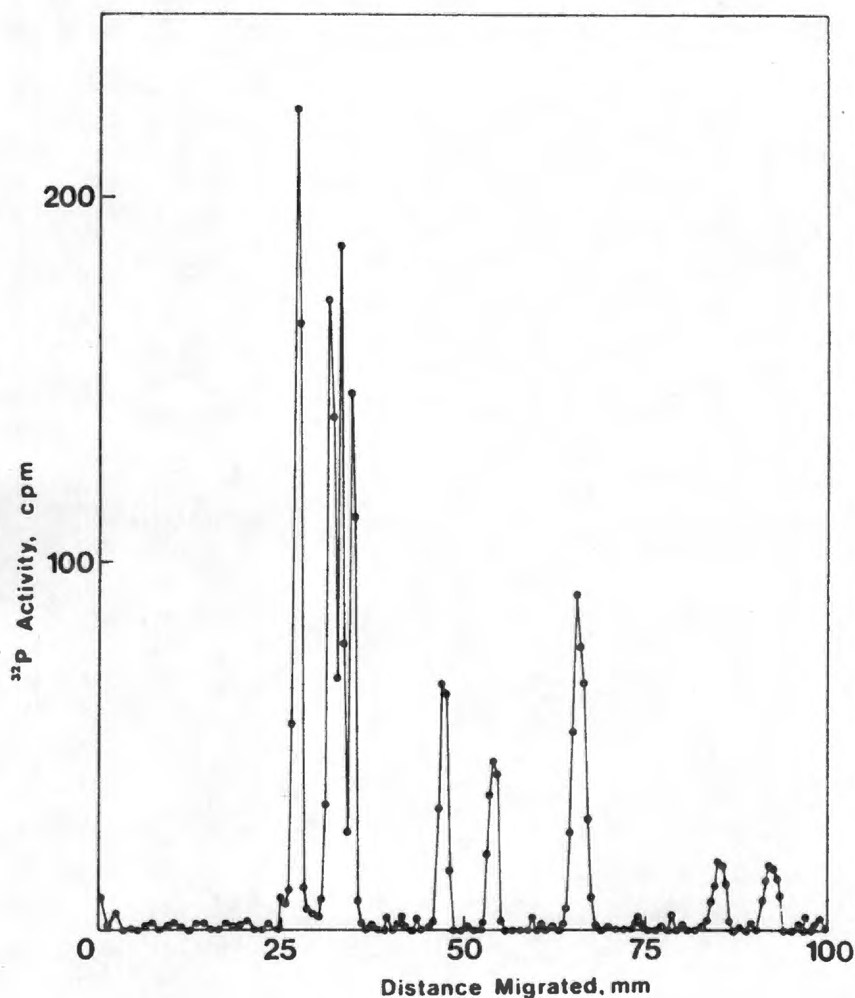


Figure 12-19. Radioactivity Profile of 32P-Labelled SA11 RNA after PAGE

Profile of Gel 2, Fig. 12-18. RNA extracted from purified virus.

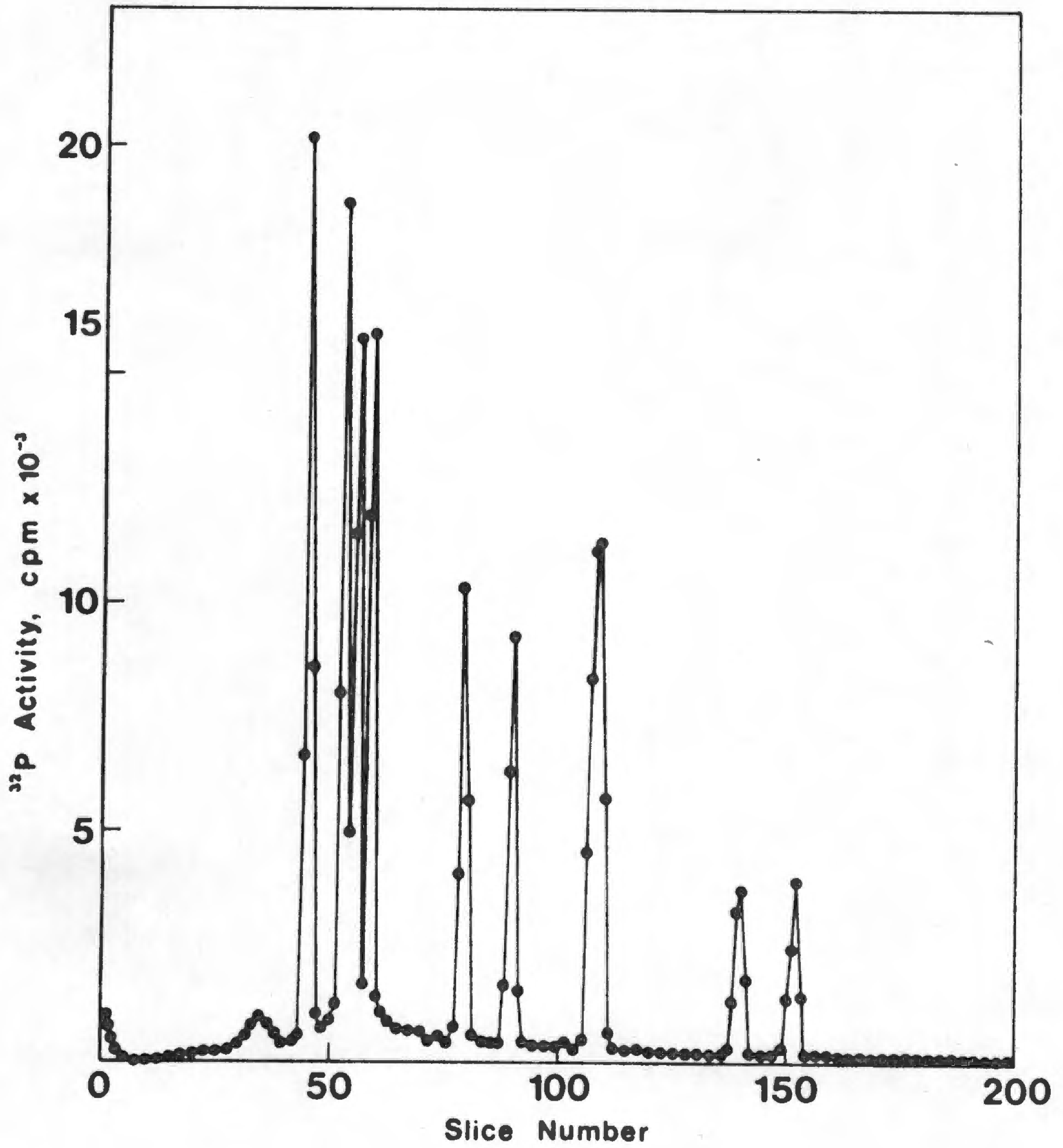


Figure 12-20. Radioactivity Profile of 32P-Labelled SA11 RNA after PAGE

Profile of Gel 1, Fig. 12-18. RNA extracted from cell debris.

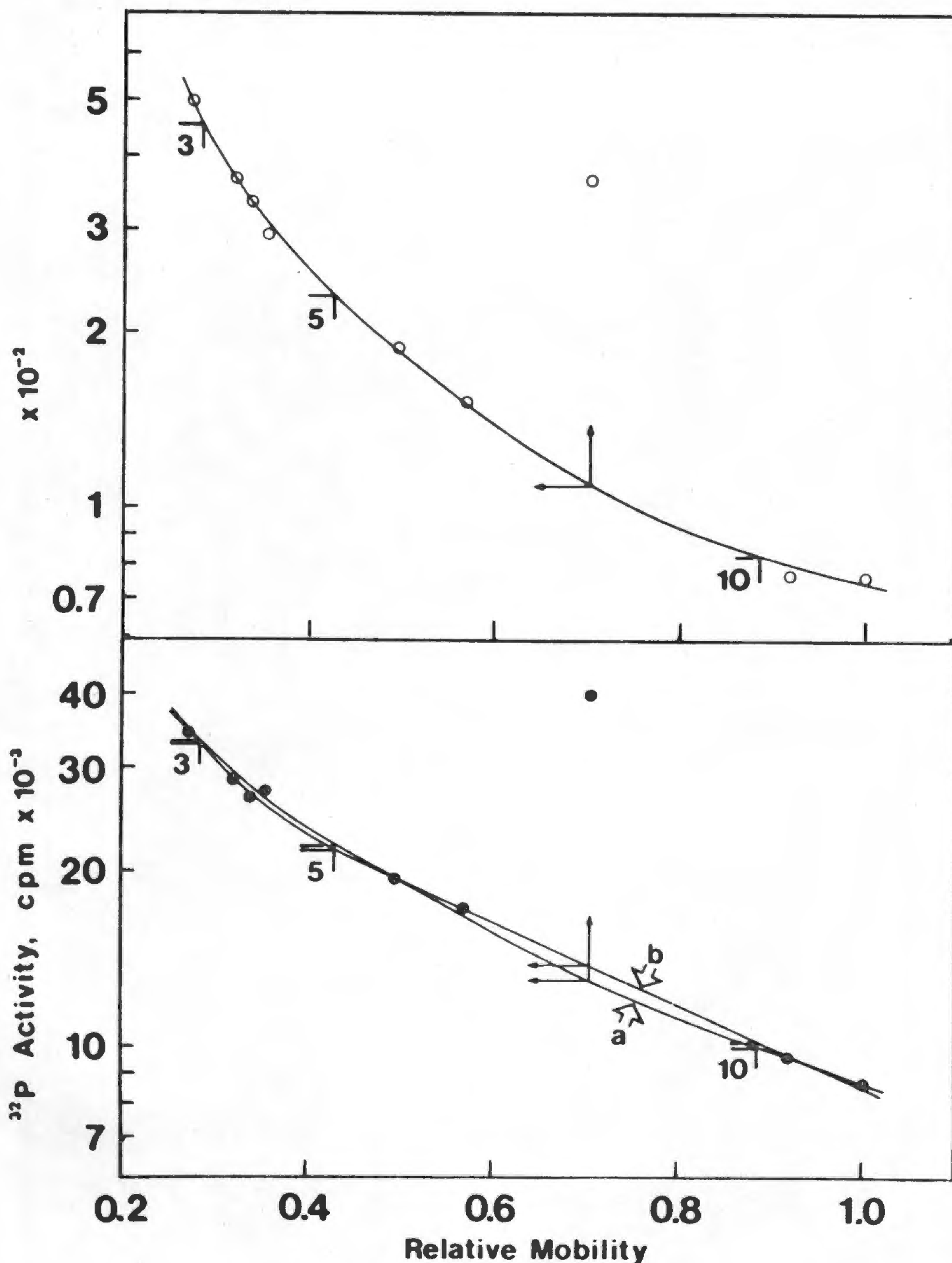


Figure 12-21. Plots of 32P Activity per Peak Versus Relative Electrophoretic Mobility for SA11 RNA Segments

○ Activities from 32P profile of Gel 2 (Fig. 12-19).
● " " " " " Gel 1 (Fig. 12-20).
↲ Projection of point for multiple band onto smooth curve drawn through the other points.
⌉ Calibration points of CPV segments from coelectrophoresis experiment (Fig. 12-11 (C)).
a. Smooth hand-drawn curve through points.
b. Curve drawn with bias towards linearity for smaller RNA segments.

Table 12-3. Molar Ratios of SA11 Genome Segments

A. From Fig. 12-20.

Band No.	Peak Activity (cpm)	Adjusted Activity (cpm)	Molar Ratio
1	495	495	1.00
2	367	365	1.00
3	334	335	1.00
4	293	308	0.95
5	188	186	1.01
7	364	109	3.34
8	77.2	80.1	0.96
9	76.5	74.8	1.02

			11.29

B. From Fig. 12-19.

Band No.	Peak Activity (cpm x 10^{-3})	Adjusted Activity (cpm x 10^{-3})		Molar Ratio	
		Curve a	Curve b	Curve a	Curve b
1	34.5	35.0	34.5	0.99	1.00
2	28.8	29.8	28.8	0.97	1.00
3	26.8	28.0	27.1	0.96	0.99
4	27.2	26.7	25.9	1.02	1.05
5	19.3	19.3	19.3	1.00	1.00
6	17.2	16.5	17.1	1.04	1.01
7	40.1	13.0	13.7	3.09	2.93
8	9.64	9.59	9.80	1.01	0.98
9	8.65	8.65	8.55	1.00	1.01
				-----	-----
				11.08	10.97

Table 12-4. Molecular Weights of SA11 Genome Segments from 32P Quantitation

A. Data from Figs. 12-20 and 12-21.
B. Data from Figs. 12-19 and 12-21.

A.

Band No.	CPV Segment Used for Calibration 3	5	10
1	1.95 Md	2.36 Md	2.39 Md
2	1.44 "	1.74 "	1.76 "
3	1.32 "	1.60 "	1.61 "
4	1.22 "	1.47 "	1.48 "
5	0.73 "	0.88 "	0.90 "
6	0.59 "	0.71 "	0.72 "
7,8,9	0.43 "	0.52 "	0.53 "
10	0.32 "	0.38 "	0.39 "
11	0.30 "	0.36 "	0.36 "

B.

Band No.	CPV Segment Used for Calibration 3	5	10
1	1.88 Md	1.75 Md	1.39 Md
2	1.57 "	1.49 "	1.18 "
3	1.48 "	1.40 "	1.11 "
4	1.41 "	1.33 "	1.06 "
5	1.05 "	0.96 "	0.76 "
6	0.93 "	0.82 "	0.65 "
7,8,9	0.75 "	0.65 "	0.52 "
10	0.53 "	0.48 "	0.38 "
11	0.47 "	0.43 "	0.34 "

Figure 12-22. Effect of Voltage on SA11 RNA Migration by PAGE

Gel	Voltage (V/cm)	Current (mA)	Time (min)	Power (watt.h)
1	15	22.5	90	5.0
2	15	22.5	90	5.0
3	10	12.5	195	4.0
4	10	12.5	195	4.0
5	5	5.0	360	1.5
6	5	5.0	360	1.5
7	2.5	2.5	720	0.75
8	2.5	2.5	720	0.75

Power values are approximate. Gels 1, 3, 5, and 7 were made from acrylamide and bis recrystallized in the lab. The rest were made from commercially purified material (Bio-Rad). Gels were run in pairs at each voltage.

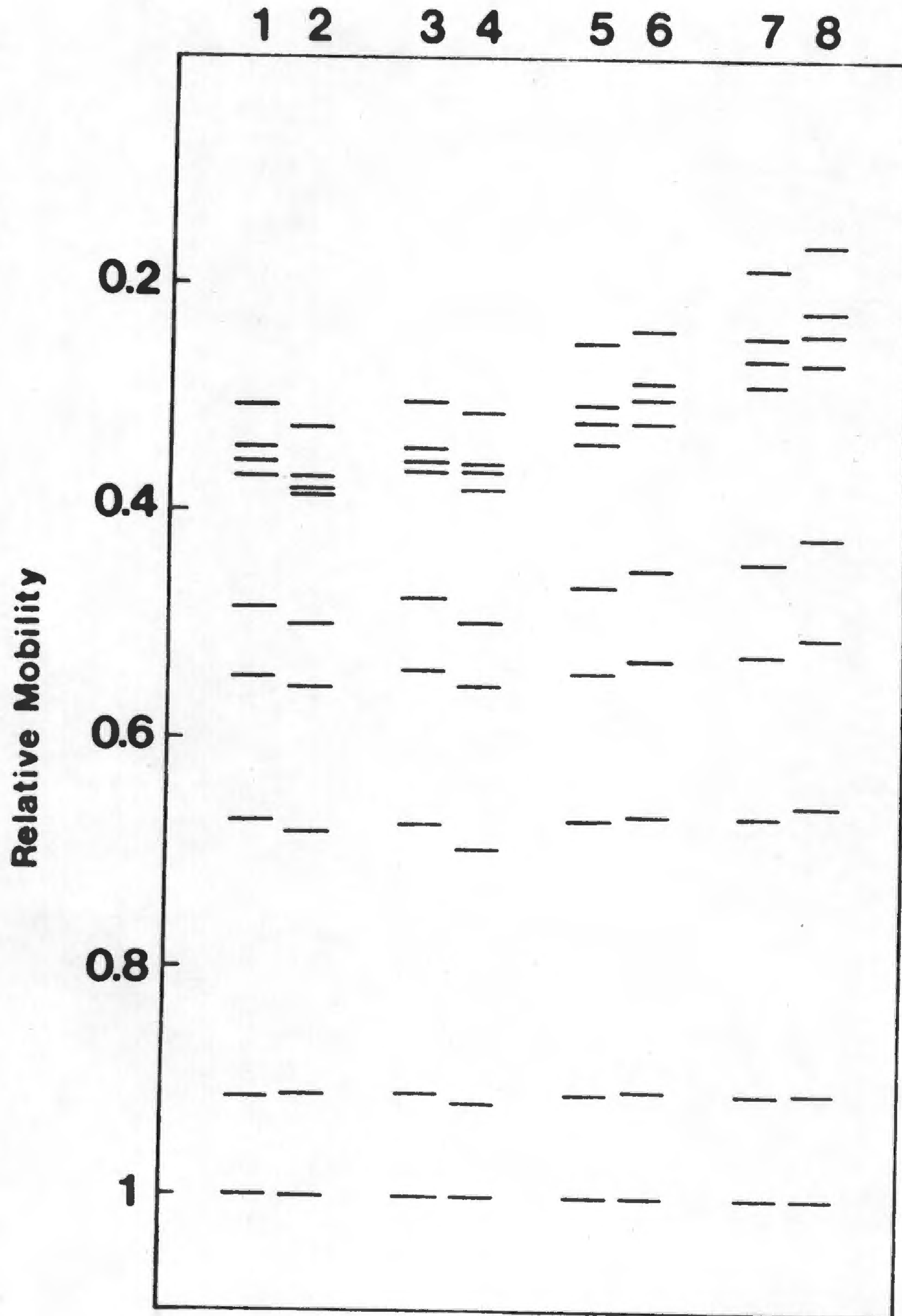


Figure 12-23. Diagrammatic Representation of the Relative Mobilities of SA11 RNA at Different Voltages

1, 2: 15V/cm. 3, 4: 10V/cm. 5, 6: 5V/cm. 7, 8: 2.5V/cm.

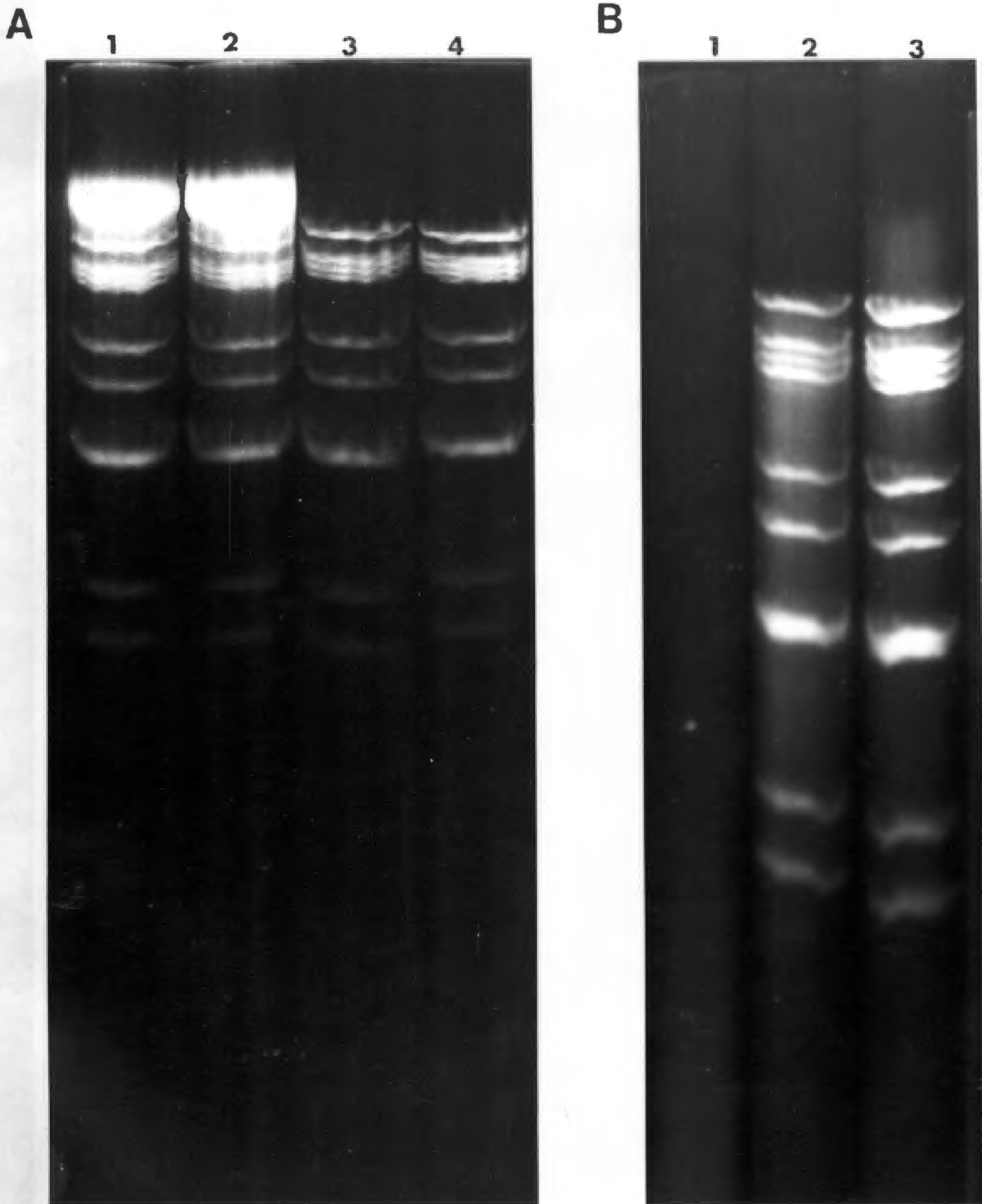


Figure 12-24. Treatment of SA11 RNA with DNase and RNase

A. Effect of DNase
Gel 1: Free virus extract. Gel 2: Cell debris extract.
Gel 3: Free virus extract + DNase.
Gel 4: Cell debris extract + DNase.

B. Effect of RNase
Gel 1: SA11 RNA in 0.01x SSC + RNase (1μg/ml).
Gel 2: " " " 1x " " " "
Gel 3: " " control.

Table 13-1. Rotaviruses Used in this Study

VIRUS	SOURCE OF MATERIAL STUDIED	REFERENCE
SA11 (Simian Agent-11)	Cell culture medium	Malherbe and Strickland-Cholmley (1967)
OA ('O'-Agent)	Cell culture medium	
EDIM (Epizootic Diarrhoea of Infant Mice Virus)	Mouse Stool	Kraft (1957)
IGV (Infantile Gastroenteritis Virus)	Human Stool	Bishop *et al.* (1973)

All speciments were obtained originally from Dr. B. Schoub, National Institute for Virology, Sandringham, Transvaal.

13. METHODS

13.1. Propagation of Rotavirus

Cell type - CV1 cells, an established line of African green monkey kidney cells, were used.

13.1.1. Routine Subculture

Cells were grown in sterile plastic Falcon 75ml flasks at 37C in a humidified incubator maintaining 5% CO_2 levels. Cells were observed and photographed under phase contrast microscopy.

Confluent monolayers were drained, and dispersed with 2ml versene-trypsin solution (VT), and distributed to 6 new flasks containing 15-25ml bicarbonate-buffered Eagle's modified minimal essential medium (MEM) containing 5% donor calf serum. Antibiotics were sometimes added to the medium (PSN - see Buffers and Solutions).

13.1.2. Infection with Rotavirus

A number of procedures were followed in an attempt to obtain high virus yields. Some of these are described here.

Procedure I. Medium was decanted, and the cells washed once with 5ml of MEM without serum (SFM). 10ml of SFM was added, and inoculated with virus-containing medium. PSN was sometimes added.

Procedure II. To remove traces of serum-containing medium, the cells were drained, aspirated with a sterile pipette, washed once with 5ml SFM, and aspirated again. 10-15ml SFM were added, with or without antibiotics, and inoculated with virus.

Procedure III. Same as procedure II, except that Newman Tytell serumless medium (NTM) was substituted for SFM.

Procedure IV. Medium was decanted, aspirated, and cells washed once with 5ml NTM. 15ml NTM were added, plus an appropriate quantity of VT solution, or a solution of pure trypsin in 0.1M HCl. Flasks were inoculated with virus as before.

In all cases incubation was continued, sometimes with medium changes, until a desired proportion of the cells showed CPE, or had detached from the flask surface, whereupon the flask contents were extracted for virus or viral dsRNA.

The relative merits of these procedures are discussed in the Results (Section 12.1).

13.2. 32P-Labelling of Rotavirus Nucleic Acids

To label rotavirus nucleic acids during replication in cell culture, 1-25µCi/ml of 32Pi (orthophosphate) was added to infected flasks approximately 2h after inoculation with virus. Incubation was continued as before, and nucleic acid extraction performed, observing the normal precautions when working with 32P (See Section 8.6.6).

13.3. Purification of Rotavirus from Cell Culture

Several variations on the CsCl buoyant density gradient theme were employed.

Procedure I - Preformed CsCl Gradient.

Ref: Kalica et al., 1976

A stepwise linear gradient of optical grade CsCl (BDH) in 0.2M Tris-HCl at pH8.0 was prepared in SW27.1 rotor tubes (Beckman), with densities ranging from 1.1-1.6g/ml in 0.05g/ml steps of 1ml each. Virus was pelletted from growth medium (first cleared by a low-speed spin) by ultracentrifugation at 100,000xg for 1h, resuspended in 0.2M Tris buffer and layered onto the gradient. The gradients were centrifuged for 6h at 27,000rpm at 15C using a Beckman L2-65B ultracentrifuge.

Procedure II - Equilibrium Density Gradient

Ref: Todd and McNulty, 1976

Virus pelletted from medium as described above was resuspended in 1-2ml of buffer (20mM Tris-HCl, pH7.5, 100mM NaCl, 2.5mM $MgSO_4$) and treated with 20µg/ml fresh DNase and 10µg/ml RNase at 37C for 1h. The virus suspension was then incorporated in 17ml of CsCl solution of density 1.30g/ml (refractive index = 1.36) in 20mM Tris-HCl, pH7.5, 2.5mM $MgSO_4$, and centrifuged for 40h at 27,000rpm in an SW27.1 rotor at 15C.

Procedure III

This was the same as procedure II except that a preformed CsCl gradient with a density range from 1.1 to 1.45g/ml in a SW50.1 rotor tube was used, and centrifuged for 3-5h at 110,000xg.

13.3.1. Analysis of CsCl Gradients

An ISCO gradient analyser was used. The bottoms of the tubes were punctured and their contents pumped, via a UV photometer coupled to a chart recorder, to an automatic sample collector. 0.3ml fractions were normally collected at a rate of 1ml/min.

The density of each fraction was measured using an Abbé refractometer. A linear relation ship exists between density and refractive index for caesium chloride solutions.

For viral preparations labelled with 32P, a 10µl aliquot of each fraction was added to 1ml water in a scintillation vial and counted by Cherenkov radiation in a β-scintillation counter.

Pooled fractions corresponding to peaks observed on the photometer trace and/or the radioactivity profile of the gradients were either dialysed against 20mM Tris-HCl, pH8.0 for 24h with several buffer changes, or diluted with ±10 vol of 10mM Tris-HCl, pH7.5 and centrifuged at ±100,000xg at 15C for 1h.

Viral dsRNA was extracted from the pellets by the methods described for virus harvested directly from culture medium (See Section 13.4.3).

13.4. Extraction of dsRNA from Infected Cell Cultures

A number of extraction methods were employed, and the most satisfactory one is described. The basic procedure was initially developed by Marmur (1961) for the purification of DNA from mammalian cells in tissue culture. It was modified as described below.

Quantities are appropriate for a single 75ml Falcon flask. In experiments to develop the optimal propagation conditions for virus three fractions were routinely extracted separately: (1) Cells remaining attached to the flask. (2) Cell debris floating in the medium. (3) Free viral particles released into the medium.

All operations were performed at room temperature unless otherwise stated.

13.4.1. Extraction of Cells Attached to Flask

The medium was drained, the remaining cells washed once with physiological saline, and then lysed with 5ml perchlorate-SDS reagent at 37C for 15min. The viscous lysate was transferred to a boiling tube and 5ml of a 1:1 mixture of phenol reagent and chloroform reagent added. The tube was vigorously vortex mixed for 1min. The emulsion was broken by centrifuging in conical tubes at 3000rpm in a benchtop centrifuge for 10min. The aqueous phase plus interphase were re-extracted with an equal volume of chloroform reagent by vortexing for 1min, and the phases separated as before, leaving the interface behind. The aqueous phase was chloroform extracted once more. Two volumes of ethanol were added and the mixture kept at -20C overnight, followed by centrifugation at 6,000-10,000xg at 4C for 30min. the pellet was drained, and dried in vacuuo or in a stream of nitrogen.

When further purification was required, the pellet was dissolved in 1ml of DNase buffer and treated with 20-50μg/ml DNase I solution at 37C for 30-60min. Occasionally, a digestion with 1μg/ml of RNase was also performed to remove residual SSRNA. The nucleic acid preparation was reprecipitated with alcohol, and the pellet resuspended in a suitable buffer. Treatment with proteinases was sometimes included.
Samples were stored in 1.5ml Eppendorf tubes at -20C.

A 1:1 phenol:chloroform mixture was used in the extraction initially because phenol reagent alone did not have a density sufficiently different from the aqueous phase (containing SDS etc.) to separate cleanly into two phases. The addition of chloroform ensured that the organic phase separated below the aqueous phase, from where it could be removed easily. It was discovered that this approach also produced on the whole a cleaner final nucleic acid preparation. A similar mixture has sometimes been adopted by other workers (e.g. Kalica et al., 1976).

13.4.2. Extraction of Cell Debris

Medium from infected cell cultures was spun at 3000rpm in a benchtop centrifuge for 10min. The supernatant was kept for free virus extraction (see below). The pellet was resuspended in 1-5ml perchlorate-SDS reagent and extraction performed as for cells still attached to the flask (see above).

13.4.3. Extraction of Free Virus

Medium cleared of cellular debris was subjected to ultracentrifugation at ±100,000xg for 1h. The pellet was resuspended in perchlorate-SDS reagent and extracted as above. Alternatively, the lysis procedure adopted for virus extracted from faecal material, using Buffer A and Buffer B, was used (see Section 13.4.4).

13.4.4. Extraction of Faecal Samples

Refs: Bishop et al., 1974; Obijeski et al., 1977.

The diarrhoeal specimen (5-10ml) was mixed with 10ml of distilled water and homogenized for 2-5min. 10ml of Arklone X (ICI) were added and homogenization continued for 10min. The emulsion was centrifuged at 10,000xg for 30min at 4C. The yellow-brown aqueous supernatant was removed, the volume measured, and PEG 6000 added to 8%, then left at 4C overnight. The precipitate was then harvested at 4000xg, 4C, for 30min, and resuspended in 2-4ml distilled water. Two ml of this suspension was layered onto 3ml of a 45% sucrose solution in 2mM Tris-HCl, pH7.0, and spun at ±100,000xg, 15C, for 150min in an SW50.1 rotor (32,500rpm). The pellet was resuspended in 2ml of Buffer A and homogenized if necessary. The virus particles were

disrupted with 2ml Buffer B, and incubated at 37C for 15min. A phenol extraction was performed by vortex mixing with an equal volume for 1min, and separating the phases by benchtop centrifugation at 3000rpm for 10min. The aqueous phase and solid interphase were extracted twice with chloroform reagent, and the aqueous phase alone a third time. Nucleic acid was precipitated with 2 vol ethanol.

13.5. Caesium Sulphate DGC of Nucleic Acids

A solution of optical grade caesium sulphate was prepared in 2mM Tris-HCl, pH7.5, with a density of 1.52g/ml. This corresponded to a RI of 1.3755. The 32P-labelled rotavirus nucleic acid sample was resuspended in a small volume of 2mM Tris and mixed with sufficient caesium sulphate to fill an angle-head rotor centrifuge tube (e.g. a 40 or 50 Beckman rotor). Tubes were centrifuged at 100,000xg for 64h at 15C.

The gradients were analysed in a similar manner to CsCl gradients containing intact virions (see Section 13.3.1). Fractions from UV absorbance or 32P activity peaks were pooled and dialysed as for DNA (see Section 8.7.2.2). The dialysed preps were precipitated with ethanol and resuspended in a small quantity of a suitable buffer (or water).

13.6. Gel Electrophoresis of dsRNA

Polyacrylamide gels were prepared and run as described in Sections 8.5.4 and 8.5.5.

13.6.1. Analysis of Gels Containing 32P-Labelled RNA

32P-labelled material was electrophoresed through cylindrical polyacrylamide gels containing 10% glycerol. The gels were transferred to aluminium foil troughs, cooled to -20C and frozen on dry ice. A Mickle gel slicer was used to fractionate the gel. The mounting block was cooled on dry ice and the frozen gel placed onto it. The gel was kept in place by running a small quantity of water underneath the gel, where it instantly froze. A thin "strap" of damp tissue paper was frozen over the bottom end of the gel to attach the gel more firmly during subsequent slicing. The block was replaced in the slicer and covered in crushed dry ice. The top few centimetres of the gel were allowed to almost thaw. Cutting was performed when slices could be obtained without splintering (too cold) and without sticking (too warm). 0.5-1.0mm slices were transferred to scintillation vials, the RNA hydrolysed and eluted with a volume of 1M KOH to give a final concentration (taking account of the slice volume) of 0.4M. The sealed vials were incubated at 37C for 2h. 10ml of scintillation fluid with an adequate aqueous uptake ability was added, and the vials shaken at intervals until the slices were dehydrated. 32P activity was then measured in a β-scintillation counter. Alternatively, the slices were incubated in 5ml water at 37C for >3h and counted in

a β-counter by Cerenkov radiation (Harley et al., 1977).

13.6.2. Fluorometric Scanning of EtdBr Stained Gels

Direct scanning at 260nm to measure the absorbance of nucleic acids in gels is not very sensitive. Gels stained with methylene blue may be scanned at longer wavelengths to give a "microdensitometer" trace, but the dye tends to stain nonspecifically. Obtaining a good background is thus difficult. Photographic negatives of stained gels have also been successfully scanned in a microdensitometer, although quantitation of bands by such methods cannot be considered accurate, as photographic film grain density is not necessarily proportional to exposure.

The fluorescence under UV of ethidium bromide (EtdBr) intercalated with double-stranded nucleic acids in gels may be scanned using a fluorometric scanner. Detection sensitivities comparable with careful photography may be obtained, and bands may be quantitated.

A Vitatron spectrofluorometric scanner was used. The mounting table was adapted to the scanning of cylindrical gels by placing the gel in a trough lined with black non-fluorescent and (ideally) non-reflecting material. Black light-proof plastic sheet was used. The object was to get a minimum background reading, and to allow the gel to be mounted at a height corresponding to the intersection of the 45-degree incident beam and the optical path of the photomultiplier. The machine table could unfortunately not be raised or lowered. This hampered scanning of slab gels, which sat too high when supported on their glass backing plates, creating alignment problems. This problem would not have arisen if gels of higher polyacrylamide concentration had been used, as such gels could have been removed from the backing plate without breaking.

The incident UV beam was passed through a Vitatron UVB filter, and light to the photomultiplier passed through a 0.25mm pinhole (for optimum band resolution) and a U4 orange filter. The Mode switch was set to lin II+, damping set at minimum level, and the sensitivity and background level of the output to the recorder adjusted for the most strongly fluorescent band in each gel or lane. Alignment of the gel in the beam was critical. The gel was scanned automatically.

13.7. Sensitivity of Rotavirus Nucleic Acid to RNase

Approximately 5µg of rotavirus nucleic acid in 1ml of DNase buffer was treated with 50µg/ml DNase at 37C for 30min. It was then precipitated with alcohol, resuspended in 0.1xSSC, reprecipitated, and finally resuspended in 90µl of 0.01xSSC. This was divided into 3x30µl aliquots. A fresh 1mg/ml stock solution of RNase I was prepared in 0.1xSSC. A 1:1 dilution of this was made in 0.01xSSC.

Low Ionic Strength Treatment

3µl (= 30µg) of diluted RNase was added to a 30µl aliquot and incubated at 37C for 90min.

High Ionic Strength Treatment

3.5µl 10xSSC was added to a second 30µl aliquot, followed by 30µg RNase. Incubation was as above.

The third aliquot was not treated. The preps were compared by PAGE.

14. REFERENCES FOR PART II

Adams, W.R., and Kraft, L.M. (1963). Epizootic diarrhea of infant mice: identification of the etiologic agent. Science 141: 359-360.

Adams, W.R., and Kraft, L.M. (1967). Electron-microscopic study of the intestinal epithelium of mice infected with the agent of epizootic diarrhea of infant mice (EDIM virus). Amer. J. Pathol. 51: 39-60.

Almeida, J.D., Hall, T., Banatvala, J.E., Totterdell, B.M., and Chrystie, I.L. (1978). The effect of trypsin on the growth of rotavirus. J. Gen. Virol. 40: 213-218.

Babiuk, L.A., Mohammed, K., Spence, L., Fauvel, M., and Petro, R. (1977). Rotavirus isolation and cultivation in the presence of trypsin. J. Clin. Microbiol. 6: 610-617.

Banatvala, J.E., Totterdell, B., Chrystie, I.L., and Woode, G.N. (1975). In vitro detection of human rotaviruses. Lancet ii: 821.

Banfield, W.G., Kasnic, G., and Blackwell, J.H. (1968). Further observations on the virus of epizootic diarrhoea of infant mice. Virology 36: 411-421.

Bellamy, A.R., Shapiro, L., August, J.T., and Joklik, W.K. (1967). Studies on reovirus RNA. I. Characterization of reovirus genome RNA. J. Mol. Biol. 29: 1-17.

Bishop, R.F., Cameron, D.J.J., Veenstra, A.A., and Barnes, G.L. (1979). Diarrhea and rotavirus infection associated with differing regimens for postnatal care of newborn babies. J. Clin. Microbiol. 9: 525-529.

Bishop, R.F., Davidson, G.P., Holmes, I.H., and Ruck, B.J. (1973). Virus particles in epithelial cells of duodenal mucosa from children with acute non-bacterial gastroenteritis. Lancet ii: 1281-1283.

Bishop, R.F., Davidson, G.P., Holmes, I.H., and Ruck, B.J. (1974). Detection of a new virus by electron microscopy of faecal extracts from children with acute gastroenteritis. Lancet i: 149-151.

Bozarth, R.F., and Harley, E.H. (1976). The electrophoretic mobility of double-stranded RNA in polyacrylamide gels as a function of molecular weight. Biochim. Biophys. Acta 432: 329-335.

Brandt, C.D., Kim, H.W., Rodriguez, W.J., Thomas, L., Yolken, R.H., Arrobio, J.O., Kapikian, A.Z., Parrott, R.H., and Chanock, R.M. (1981). Comparison of direct electron microscopy, immune electron microscopy, and rotavirus enzyme-linked immunosorbent assay for detection of gastroenteritis viruses in children. J. Clin. Microbiol. 13: 976-981.

Bridger, J.C., and Woode, G.N. (1976). Characterization of two particle types of calf rotavirus. J. Gen. Virol. 31: 245-250.

Bryden, A.S., Thouless, M.E., and Flewett, T.H. (1976). A rabbit rotavirus. Vet. Rec. 99: 323.

Cameron, D.J.S., Bishop, R.F., Veenstra, A.A., Barnes, G.L., Holmes, I.H., and Ruck, B.J. (1977). Pattern of shedding of two noncultivable viruses in stools of newborn babies. J. Med. Virol. 2: 7-13.

Chasey, D. (1977). Different particle types in tissue culture and intestinal epithelium infected with rotavirus. J. Gen. Virol. 37: 443-451.

Cheever, F.S., and Muller, J.H. (1947). Epidemic diarrheal disease of suckling mice. I. Manifestations, epidemiology, and attempts to transmit the disease. J. Exp. Med. 85: 405-416.

Clark, S.M., Roth, J.R., Clark, M.L., Barnett, B.B., and Spendlove, R.S. (1981). Trypsin enhancement of rotavirus infectivity: Mechanism of enhancement. J. Virol. 39: 816-822.

Cohen, J. (1977). Ribonucleic acid polymerase activity associated with purified calf rotavirus. J. Gen. Virol. 36: 395-402.

Cohen, J., and Dobos, P. (1979). Cell free transcription and translation of rotavirus RNA. Biochem. Biophys. Res. Commun. 88: 791-796.

Davidson, G.P., Bishop, R.T., Townsley, R.R.W., Holmes, I.H., and Ruck, B.J. (1975). Importance of a new virus in acute sporadic enteritis in children. Lancet i: 242-246.

Dyall-Smith, M.L., and Holmes, I.H. (1981). Gene-coding assignments of rotavirus double-stranded RNA segments 10 and 11. J. Virol. 38: 1099-1103.

Editorial. (1975). Rotaviruses of man and animals. Lancet i: 257-259.

Elias, M.M. (1977). Separation and infectivity of two particle types of human rotavirus. J. Gen. Virol. 37: 191-194.

Ellens, D.J., and de Leeuw, P.W. (1977a). Detection of infantile gastroenteritis virus (rotavirus) by ELISA. Lancet i: 1363.

Ellens, D.J., and de Leeuw, P.W. (1977b). Enzyme-linked immunosorbent assay for diagnosis of rotavirus infections in calves. J. Clin. Microbiol. 6: 530-532.

Els, H.J., and Lecatsas, G. (1972). Morphological studies on simian virus SA11 and the related "O" agent. J. Gen. Virol. 17: 129-132.

Esparza, J., Gorziglia, M., Gil, F., and Romer, H. (1980). Multiplication of human rotavirus in cultured cells: an

electron microscopic study. J. Gen. Virol. 47: 461-472.

Esparza, J., and Gil, F. (1978). A study on the ultrastructure of human rotavirus. Virology 91: 141-150.

Espejo, R.T., Avendano, L.F., Munoz, O., Romero, P., Eternod, J.G., Lopez, S., and Moncaya, J. (1980a). Comparison of human rotaviruses isolated in Mexico City and in Santiago, Chile, by electrophoretic migration of their double-stranded ribonucleic acid genome segments. Infect. Immun. 30: 342-348.

Espejo, R.T., Calderon, E., Gonzalez, N., Salomon, A., Martuscelli, A., and Romero, P. (1979). Presence of two distinct types of rotavirus in infants and young children hospitalized with acute gastroenteritis in Mexico City, 1977. J. Infect. Dis. 139: 474-477.

Espejo, R.T., Calderon, E., and Gonzalez, N. (1977). Distinct reovirus-like agnts associated with acute infantile gastroenteritis. J. Clin. Microbiol. 6: 502-506.

Espejo, R.T., Martinez, E., Lopez, S., and Munoz, O. (1980b). Different polypeptide composition of two human rotavirus types. Infect. Immun. 28: 230-237.

Espejo, R.T., Munoz, O., Serafin, F., and Romero, P. (1980c). Shift in prevalent human rotavirus detected by ribonucleic acid segment differences. Infect. Immun. 27: 351-354.

Flewett, T.H., Bryden, A.S., and Davies, H.A. (1973). Virus particles in gastroenteritis. Lancet ii: 1497.

Estes, M.K., Graham, D.Y., and Mason, B.B. (1981). Proteolytic enhancement of rotavirus infectivity: Molecular mechanisms. J. Virol. 39: 879-888.

Flewett, T.H., Bryden, A.S., and Davies, H.A. (1975). Virus diarrhoea in foals and other animals. Vet. Rec. 96: 477.

Flewett, T.H., Bryden, A.S., Davies, H., Woode, G.N., Bridger, J.C., and Derrick, J.M. (1974). Relation between viruses from acute gastroenteritis of children and newborn calves. Lancet ii: 61-63.

Flewett, T.H., and Woode, G.N. (1978). The rotaviruses. Brief review. Arch. Virol. 57: 1-23.

Grauballe, P.C., Genner, J., Meyling, A., and Hornsleth, A. (1977). Rapid diagnosis of rotavirus infections: comparison of electron microscopy and immunoosmophoresis for the detection of rotavirus in human infantile gastroenteritis. J. Gen. Virol. 35: 203-218.

Greenberg, H.B., Kalica, A.R., Wyatt, R.G., Jones, R.W., Kapikian, A.Z., and Chanock, R.M. (1981). Rescue of noncultivable human rotavirus by gene reassortment during infection with ts mutants of a cultivable bovine rotavirus. Proc. Natl. Acad. Sci. (USA). 78: 420-424.

Gressel, J., Rosner, A., and Cohen, N. (1975). Temperature of acrylamide polymerization and electrophoretic mobilities of nucleic acids. Anal. Biochem. 69: 84-91.

Hara, M., Mukoyama, J., Tsuruhara, T., Saito, Y., and Tagaya, I. (1976). Duovirus in schoolchildren with gastroenteritis. Lancet i: 311.

Harley, E.H., Rubenstein, R., Losman, M., and Lutton, D. (1977). Molecular weights of the RNA genome segments of a cytoplasmic polyhedrosis virus determined by a new comparative approach. Virology 76: 210-216.

Harley, E.H., White, J.S., and Rees, K.R. (1973). The identification of different structural classes of nucleic acids by electrophoresis in polyacrylamide gels of different concentration. Biochim. Biophys. Acta 299: 253-263.

Holmes, I.H., Ruck, B.J., Bishop, R.F., and Davidson, G.P. (1975). Infantile enteritis viruses: morphogenesis and morphology. J. Virol. 16: 937-943.

Hoshino, Y., Baldwin, C.A., and Scott, F.W. (1981). Isolation and characterization of feline rotavirus. J. Gen. Virol. 54: 313-323.

Kalica, A.R., Garon, C.F., Wyatt, R.G., Mebus, C.A., Van Kirk, D.H., Chanock, R.M., and Kapikian, A.Z. (1976). Differentiation of human and calf reoviruslike agents associated with diarrhea using polyacrylamide gel electrophoresis of RNA. Virology 74: 86-92.

Kalica, A.R., James, H.D., Jr., and Kapikian, A.Z. (1978a). Haemagglutination by simian rotavirus. J. Clin. Microbiol. 7: 314-315.

Kalica, A.R., Sereno, M.M., Wyatt, R.G., Mebus, C.A., Chanock, R.M., and Kapikian, A.Z. (1978b). Comparison of human and animal rotavirus strains by gel electrophoresis of viral RNA. Virology 87: 247-255.

Kalica, A.R., Wyatt, R.G., and Kapikian, A.Z. (1978c). Detection of differences among human and animal rotaviruses, using analysis of viral RNA. J. Amer. Vet. Med. Assoc. 173: 531-537.

Kalica, A.R., and Theodore, T.S. (1979). Polypeptides of simian rotavirus (SA-11) determined by a continuous polyacrylamide gel electrophoresis method. J. Gen. Virol. 43: 463-466.

Kalmakoff, J., Lewandowski, L.J., and Black, D.R. (1969). Comparison of the ribonucleic acid subunits of reovirus, cytoplasmic polyhedrosis virus, and wound tumor virus. J. Virol. 4: 851-856.

Kraft, L.M. (1957). Studies on the etiology and transmission of epidemic diarrhoea of infant mice. J. Exp. Med. 106: 743-755.

Kraft, L.M. (1966). Epizootic diarrhea of infant mice and

lethal intestinal infection of infant mice. In: Viruses of Laboratory Rodents. Nat. Cancer. Inst. Monogr. 20: 55-61.

Lecatsas, G. (1968a). Electron microscope study of the formation of bluetongue virus. Onderstepoort J. Vet. Res. 35: 139-150.

Lecatsas, G. (1968b). Electron microscope studies of a reovirus Type 1 in BHK 21 cells. Onderstepoort J. Vet. Res. 35: 151-158.

Lecce, J.G., King, M.W., and Mock, R. (1976). Reovirus-like agent associated with fatal diarrhoea in neonatal pigs. Infect. Immun. 14: 816-825.

Light, J.S., and Hodes, H.L. (1943). Studies on epidemic diarrhoea in the newborn: isolation of filterable agent causing diarrhoea in calves. Amer. J. Publ. Hlth. 33: 1451-1454.

Lishanskaya, A.I., and Mosevitsky, M.I. (1973). Size dependent separation of high molecular weight double-stranded DNA by means of gel electrophoresis. Biochem. Biophys. Res. Commun. 52: 1213-1220.

Malherbe, H.H., and Strickland-Cholmley, M. (1967). Simian virus SA11 and the related O agent. Arch. Gesamte Virusforsch. 22: 235-245.

Marmur, J. (1961). J. Mol. Biol. 3: 208

Martin, S.A., and Zweerink, H.J. (1972). Isolation and characterization of two types of bluetongue virus particles. Virology 50: 495-506.

Martin, M.L., Palmer, E.L., and Middleton, P.J. (1975). Ultrastructure of infantile gastroenteritis virus. Virology 68: 146-153.

Mason, B.B., Graham, D.Y., and Estes, M.K. (1980). In vitro transcription and translation of simian rotavirus SA11 gene products. J. Virol. 33: 1111-1121.

Matsuno, S., Hasegawa, A., Kalica, A.R., and Kono, R. (1980). Isolation of a recombinant between simian and bovine rotaviruses. J.Gen. Virol. 48: 253-256.

Matsuno, S., Inouye, S., and Kono, R. (1977). Plaque assay of neonatal calf diarrhea virus and the neutralizing antibody in human sera. J. Clin. Microbiol. 5: 1-4.

McCrae, M.A., and Joklik, W.K. (1978). The nature of the polypeptide encoded by each of the 10 double-stranded RNA segments of reovirus Type 3. Virology 89: 578-593.

McMaster, G.K., and Carmichael, G.G. (1977). An analysis of single-and double-stranded nucleic acids on polyacrylamide and agarose gels using glyoxal and acridine orange. Proc. Natl. Acad. Sci. USA. 77: 4835-4838.

McNulty, M.S., Allan, G.M., and McFerran, J.B. (1976a). Isolation of a calf rotavirus. Res. Vet. Sci. 21: 114-115.

McNulty, M.S., Allan, G.M., and McFerran, J.B. (1977). Cell culture studies with a cytopathic bovine rotavirus. Arch. Virol. 54: 201-209.

McNulty, M.S., Curran, W.L., and McFerran, J.B. (1976b). The morphogenesis of a cytopathic bovine rotavirus in Madin-Derby bovine kidney cells. J. Gen. Virol. 33: 503-508.

McNulty, M.S., Pearson, G.R., McFerran, J.B., Collins, D.S., and Allan, G.M. (1976c). A reovirus-like agent (rotavirus) associated with diarrhoea in neonatal pigs. Vet. Microbiol. 1: 55-63

Mebus, C.A., Kono, M., Underdahl, N.R., and Twiehaus, M.J. (1971). Cell culture propagation of a neonatal calf diarrhoea (scours) virus. Canad. Vet. J. 12: 69-72.

Mebus, C.A., Underdahl, N.R., Rhodes, M.B., and Twiehaus, H.J. (1969). Calf diarrhoea virus (scours) reproduced with a virus from a field outbreak. Bull. Neb. Agric. Exp. Statn. 233: 1-16.

Mebus, C.A., Wyatt, R.G., Sharpe, R.L., Sereno, M.M., Kalica, A.R., Kapikian, A.Z., and Twiehaus, M.J. (1976). Diarrhoea in gnobiotic calves caused by the reovirus-like agent of human infantile gastroenteritis. Infect. Immun. 14: 471-474.

Middleton, P.J., Petric, M., Hewitt, C.M., Symanski, M.T., and Tam, J.S. (1976). Counter immunoelectroosmophoresis for the detection of infantile gastroenteritis virus (orbivirus group) antigen and antibody. J. Clin. Pathol. 29: 191-197.

Middleton, P.J., Szymanski, M.T., Abbott, G.D., Bartolussi, R., and Hamilton, J.R. (1974). Orbivirus acute gastroenteritis of infancy. Lancet i: 1241-1244.

Millward, S., and Graham, A.F. (1970). Structural studies on reovirus: Discontinuities in the genome. Proc. Natl. Acad. Sci. USA. 65: 422-429.

Much, D.H., and Zajac, I. (1972). Purification and characterization of epizootic diarrhoea of infant mice virus. Infect. Immun. 6: 1019-1024.

Newman, J.F.E., Brown, F., Bridger, J.C., and Woode, G.N. (1975). Characterisation of a rotavirus. Nature 258: 631-633.

Obijeski, J.F., Palmer, E.L., and Martin, M.L. (1977). Biochemical characterization of infantile gastroenteritis virus (IGV). J. Gen. Virol. 34: 485-497.

Palmer, E.L., Martin, M.L., and Murphy, F.A. (1977). Morphology and stability of infantile gastroenteritis virus: comparison with reovirus and bluetongue virus. J. Gen. Virol. 35: 403-414.

Pappenheimer, A.M., and Cheever, F.S. (1948). Epidemic diarrhoeal disease of suckling mice. IV. Cytoplasmic inclusion bodies in intestinal epithelium in relation to the diesease. J. Exp. Med. 88: 317-324.

Paver, W.K., Caul, E.O., Ashley, C.R., and Clarke, S.K.R. (1973). A small virus in human faeces. Lancet i: 237-240.

Petric, M., Tam, J.S., and Middleton, P.J. (1976). Preliminary characterization of the nucleic acid of infantile gastroenteritis virus (Orbivirus group). Intervirol. 7: 176-180.

.amia, S., and Sattar, S.A. (1979). Simian rotavirus SA-11 plaque formation in the presence of trypsin. J. Clin. Microbiol. 10: 609-614.

Ramig, R.F., Cross, R.K., and Fields, B.N. (1977). Genome RNAs and polypeptides of reovirus serotypes 1, 2, and 3. J. Virol. 22: 726-733.

Reed, D.E., Daley, C.A., and Shane, H.J. (1976). Reovirus-like agent associated with neonatal diarrhea in pronghorn antelope. J. Wild. Dis. 12: 488-491.

Rodger, S.M., Bishop, R.F., Birch, C., McLean, B., and Holmes, I.H. (1981). Molecular epidemiology of human rotaviruses in Melbourne, Australia from 1973 to 1979 as determined by electrophoresis of genome RNA. J. Clin. Microbiol. 13: 272-278.

Rodger, S.M., Schnagl, R.D., and Holmes, I.H. (1975). Biochemical and biophysical characteristics of diarrhea viruses of human and calf origin. J. Virol. 16: 1229-1235.

Rodger, S.M., Schnagl, R.D., and Holmes, I.H. (1977). Further biochemical characterization, including the detection of surface glycoproteins, of human, calf, and simian rotaviruses. J. Virol. 24: 91-98.

Rodger, S.M., and Holmes, I.H. (1979). Comparison of the genomes of simian, bovine and human rotaviruses by gel electrophoresis and detection of genomic variation among bovine isolates. J. Virol. 30: 839-846.

Rubenstein, D., Milne, R.G., Buckland, R., and Tyrrell, D.A.J. (1971). The growth of the virus of epidemic diarrhoea of infant mice (EDIM) in organ cultures of intestinal epithelium. Brit. J. Exp. Pathol. 52: 442-445.

Schnagl, R.D., Rodger, S.M., and Holmes, I.H. (1981). Variation in human rotavirus electropherotypes occurring between rotavirus gastroenteritis epidemics in Central Australia. Infect. Immun. 33: 17-21.

Schnagl, R.D., and Holmes, I.H. (1976). Characteristics of the genome of human infantile enteritis virus (rotavirus). J. Virol. 19: 267-270.

Schoub, B.D. (1979). Viral gastroenterits - A review. South Afr. J. Hosp. Med. 5: 248-255.

Shatkin, A.J., Sipe, J.D., and Loh, P. (1968). Separation of ten reovirus genome segments by polyacrylamide gel electrophoresis. J. Virol. 2: 986-991.

Smith, E.M., Estes, M.K., Graham, D.Y., and Gerba, C.P. (1979). A plaque assay for the simian rotavirus SA11. J. Gen. Virol. 43: 513-519.

Smith, M., and Tzipori, S. (1979). Gel electrophoresis of rotavirus RNA derived from six different animal species. Austral. J. Exp. Biol. Med. Sci. 57: 583-585.

Smith, M.L., Lazdins, I., and Holmes, I.H. (1980). Coding assignments of double-stranded RNA segments of SA11 rotavirus established by in vitro translation. J. Virol. 33: 976-982.

Snodgrass, D.R., Smith, W., Gray, E.W., and Herring, J.A. (1976). A rotavirus in lambs with diarrhoea. Res. Vet. Sci. 20: 113-114.

Spendlove, R.S., and Schaffer, F.L. (1965). Enzymatic enhancement of infectivity of reovirus. J. Bact. 89: 597-602.

Stannard, L.M., and Schoub, B.D. (1977). Observations on the morphology of two rotaviruses. J. Gen. Virol. 37: 435-439.

Storz, J., and Bates, R.C. (1973). Parvovirus infections in calves. J. Amer. Vet. Med. Assoc. 163: 884-886.

Syverton, J.T., and Olitsky, P.K. (1934). Bacteriological studies on an epizootic of intestinal disease in suckling and newly weaned mice. J. Exp. Med. 60: 385-394.

Tam, J.S., Szymanski, M.T., Middleton, P.J., and Petric, M. (1976). Studies on the particles of infantile gastroenteritis virus (Orbivirus group). Intervirol. 7: 181-191.

Theil, K.W., Bohl, E.H., and Agnes, A.G. (1977). Cell culture propagation of porcine rotavirus (reovirus-like agent). Amer. J. Vet. Res. 38: 1765-1768.

Thouless, M.E., Bryden, A.S., Flewett, T.H., Woode, G.N., Bridger, J.C., Snodgrass, D.R., and Herring, J.A. (1977). Serological relationships between rotaviruses from different species as studied by complement fixation and neutralization. Arch. Virol. 53: 287-294.

Todd, D., McNulty, M.S., and Allan, G.M. (1980). Polyacrylamide gel electrophoresis of avian rotavirus RNA. Arch. Virol. 63: 87-97.

Todd, D., and McNulty, M.S. (1976). Characterization of pig rotavirus RNA. J. Gen. Virol. 33: 147-150.

Todd, D., and McNulty, M.S. (1977). Biochemical studies on a reovirus-like agent (rotavirus) from lambs. J. Virol. 21:

1215-1218.

Tufvesson, B., and Johnsson, T. (1976). Immunoelectro-osmophoresis for detection of reo-like virus: methodology and comparison with electronmicroscopy. Acta Path. Microbiol. Scand. B84: 225-228.

Tzipori, S., Caple, I.W., and Butler, K. (1976). Isolation of a rotavirus from deer. Vet. Rec. 99: 398.

Verly, E., and Cohen, J. (1977). Demonstration of size variation of RNA segments between different isolates of calf rotavirus. J. Gen. Virol. 35: 583-586.

Von Bonsdorff, C.-H., Hovi, T., Makela, P., and Morttinen, A. (1978). Rotavirus infections in adults in association with acute gastroenteritis. J. Med. Virol. 2: 21-28.

Wood, H.A. (1973). Viruses with double-stranded RNA genomes. J. Gen Virol. 20: 61-85.

Woode, G.N., Bridger, J., Hall, G.A., Jones, J.M., and Jackson, G. (1976a). The isolation of reovirus-like agents (rotaviruses) from acute gastroenteritis of piglets. J. Med. Microbiol. 9: 203-209.

Woode, G.N., Bridger, J.C., Hall, G.A., and Dennis, M.J. (1974). The isolation of a rotavirus-like agent associated with diarrhoea in colostrum deprived calves in Great Britain. Res. Vet. Sci. 16: 102-105.

Woode, G.N., Bridger, J.C., Jones, J.M., Flewett, T.H., Bryden, A.S., Davies, H.A., and White, G.B.B. (1976b). Morphological and antigenic relationships between viruses (rotaviruses) from acute gastroenteritis of children, calves, piglets, mice, and foals. Infect. Immun. 14: 804-810.

Woode, G.N., and Bridger, J.C. (1975). Viral enteritis of calves. Vet. Rec. 96: 85-88.

Wyatt, R.G., Gill, V.W., Sereno, M.M., Kalica, A.R., Van Kirk, D.H., Chanock, R.M., and Kapikian, A.Z. (1976). Probable in vitro cultivation of human reovirus-like agent of infantile diarrhea. Lancet i: 98-99.

Wyatt, R.G., Greenberg, H.B., Dalgard, D.W., Allen, W.P., Sly, D.L., Thornhill, T.S., Chanock, R.M., and Kapikian, A.Z. (1978). Experimental infection of chimpanzees with the norwalk agent of epidemic viral gastroenteritis. J. Med. Virol. 2: 89-96.

Wyatt, R.G., James, W.D., Bohl, E.H., Theil, K.W., Saif, L.J., Kalica, A.R., Greenberg, H.B., Kapikian, A.Z., and Chanock, R.M. (1980). Human rotavirus type 2: Cultivation _in vitro_. Science 207: 189-191.

15. BUFFERS AND SOLUTIONS

Table 15-1. INGREDIENTS FOR POLYACRYLAMIDE GELS

Volumes in millilitres for a total final volume of 40ml.

GEL. CONC.	12% ACRYLAMIDE STOCK	10% TEMED	10% PER-SULPHATE	WATER
%	ml	ml	ml	ml
2.0	6.67	0.26	0.26	28.81
2.1	7.00	0.28	0.28	28.44
2.2	7.33	0.29	0.29	28.09
2.3	7.67	0.30	0.30	27.73
2.4	8.00	0.32	0.32	27.36
2.5	8.33	0.33	0.33	27.01
2.6	8.67	0.34	0.34	26.65
2.7	9.00	0.36	0.36	26.28
2.8	9.33	0.37	0.37	25.93
2.9	9.67	0.38	0.38	25.57
3.0	10.00	0.40	0.40	25.20
3.1	10.33	0.41	0.41	24.85
3.2	10.67	0.42	0.42	24.49
3.3	11.00	0.44	0.44	24.12
3.4	11.33	0.45	0.45	23.77
3.5	11.67	0.46	0.46	23.41
3.6	12.00	0.48	0.48	23.04
3.7	12.33	0.49	0.49	22.69
3.8	12.67	0.50	0.50	22.33
3.9	13.00	0.51	0.51	21.98
4.0	13.33	0.53	0.53	21.61

Gels up to 3.5% use acrylamide with 5% bis crosslinker.
Gels above 3.5% use acrylamide with 2.5% bis crosslinker.

ACRYLAMIDE STOCK SOLUTION

Acrylamide was recrystallized as follows:

70g acrylamide was dissolved in 1l chloroform at 50C and filtered hot without suction. The acrylamide was allowed to crystallize at -15C, and recovered by filtration. The crystals were washed briefly with cold chloroform, and dried at room temperature. The solid was stored cold and kept dark.

Bis (N,N'-methylenebisacrylamide) was recrystallized by dissolving in acetone (10g/l) at 40-50C, filtering hot, and cooling slowly to -15C. The crystals were collected by filtration, washed with cold acetone, and dried. Storage was as for acrylamide.

5% Crosslinked Stock	2.5% Crosslinked Stock
11.4% w/v acrylamide	11.7% w/v acrylamide
0.6% w/v bis	0.3% w/v bis

Made up in water and kept at 4C.

ELECTROPHORESIS BUFFERS

A. For Agarose Gels

(1) Tris-Borate Buffer

10.8g	Tris base
0.93g	Disodium EDTA
55.0g	Boric acid

per litre

(2) Tris-Acetate Buffer (Dugaiczyk et al., 1975)

Final Concentration		10x Stock
50mM	Tris base	60.6 g/l
20mM	Na Acetate	27.2 g/l
18mM	EDTA (free acid)	10.5 g/l

pH of 10x stock adjusted to 8.2 with glacial acetic acid.

(3) Phosphate Buffer (PB)

	20x Stock
Tris base	87.2 g/l
$NaH_2PO_4.2H_2O$	93.6 g/l
EDTA (disodium salt)	7.4 g/l
H_3PO_4	12.5 ml

B. For Acrylamide Gels

Final Concentration		10x Stock
30mM	Tris-HCl	36.3 g/l
30mM	Sodium dihydrogen phosphate	41.4 g/l
10mM	EDTA (disodium salt)	3.72 g/l

ETHIDIUM BROMIDE

A stock solution of 1mg/ml was kept in the dark at 4C. Contact with the skin was avoided.

RESTRICTION ENZYME BUFFERS

These were all made up according to the manufacturer's recommendations.

DNase BUFFER

20mM	Tris-HCl	(pH 7.4)
10mM	Magnesium chloride	

TE BUFFER

10mM	Tris-HCl
1mM	EDTA

pH 8.3 unless otherwise stated.

TES BUFFER

50mM	Tris-HCl	(pH 8.0)
50mM	NaCl	
5mM	EDTA	

SALINE SODIUM CITRATE (SSC) 1x

0.15M	NaCl
0.015M	Trisodium citrate
	pH 7.0

Kept as a 10x or 20x stock.

DNA EXTRACTION SOLUTIONS

A number of different detergent mixtures were employed in different applications. These are described under individual method protocols.

"Buffer A" and "Buffer B" were originally developed for the extraction of ssRNA from cell culture, but were used in several dsRNA extraction procedures for rotaviruses.

Buffer A

50mM	NaCl
0.1mM	EDTA
10mM	Na acetate
	pH 5.2

Buffer B

Same as Buffer A with the addition of 1% w/v SDS.

PHENOL REAGENT

Phenol was redistilled to remove oxidized tar-like impurities. The phenol was boiled in a round-bottomed Quickfit flask heated by a heating mantle, and the vapours passed down an air condenser and collected. Distillation was performed either under reduced pressure, or in an atmosphere of nitrogen, in order to inhibit oxidation. Reduced pressure also lowered the boiling point from ±174C (the boiling point at atmospheric pressure). Great care was taken to avoid spillage or contact with the phenol at any stage, and the apparatus was set up in a fume cupboard.

The molten phenol was poured into airtight bottles, allowed to solidify, and stored at 4C. When required, the phenol was melted at 60C, and saturated with water. In some applications the phenol was saturated with TE buffer. 1% 8-hydroxyquinoline was added as a colouring agent to help distinguish phases after an extraction. The phenol reagent was discarded after about one week.

CHLOROFORM REAGENT

Analar grade chloroform was saturated with water, and 1% v/v isoamyl alcohol added as an antifoaming agent. Storage was at room temperature.

TRICHLORACETIC ACID (TCA)

10% w/v in water

SCINTILLATION FLUID

Dissolve 40g TLA ("Fluoralloy", Beckman Products Inc.) in 1.5l scintillation grade toluene. Add 400ml Biosolve (Beckman) and mix. Make up to 5l with toluene.

CELL CULTURE MEDIA

Minimal Essential Medium

40ml	10x MEM with Earle's salts (Gibco Biocult).
340ml	Sterile water.
20ml	Sterile donor (or foetal) calf serum (Gibco).
4ml	20mM L-glutamine (filter-sterilized).
2ml	PSN (see below).
15ml	5% sodium bicarbonate (filter-sterilized).

Newman Tytell Medium

Purchased as complete 1x medium (Flow Laboratories).

Antibiotic Mix (PSN)

1.2g	Sodium benzylpenicillin (Crystapen)
1g	Streptomycin sulphate
1g	Neomycin sulphate

Dissolved in 100ml sterile saline and stored frozen.

Versene-Trypsin (VT) 10x Stock

4g	Sodium versenate (EDTA)
160g	NaCl
4g	KCl
23g	Disodium hydrogen phosphate
4g	Potassium dihydrogen phosphate
2g	Glucose

Filter sterilize and store frozen. Dilute with 90ml sterile water for use, and keep at 4C.

BACTERIAL CULTURE MEDIA

Luria Broth (LB)

10g	Bacto Tryptone
5g	Yeast extract
5g	NaCl

Dissolve in 1000ml water and adjust pH to 7.2. Sterilize by autoclaving. 10ml of filter-sterilized 10% glucose solution may be added prior to use.

Luria Agar (LA)

Add 15g/l "Oxoid" Agar No. 3 or equivalent to LB. Dissolve agar in a steam bath and sterilize by autoclaving. Glucose and/or 5ml of 0.5M calcium chloride solution per litre may be added if desired before pouring plates.

ANTIBIOTIC STOCK SOLUTIONS

Chloramphenicol - 200mg/ml in methanol. Stored at -20C.

Ampicillin - 25mg/ml prepared fresh.

Tetracycline - 10mg/ml stock in water, filter sterilized. Stored at -20C and kept in the dark.

Antibiotics may be added to media when they have cooled to below 50C after autoclaving.

16. ABBREVIATIONS

3H	- Tritium
32P	- Gamma-emitting phosphorus isotope
37C, etc.	- 37 degrees Centigrade
amp	- Ampicillin resistance gene
Ap(R)/(S)	- Ampicillin resistant/sensitive phenotype
BK(WT)	- Prototype BK human polyomavirus (papovavirus)
BK(MM)	- BK human polyomavirus strain MM
bp	- base pairs
BRL	- Bethesda Research Laboratories
BSA	- Bovine serum albumin, fraction V
Bis	- N,N'-methylenebisacrylamide
CCC	- Covalently closed circular
Chr	- Chromosomal DNA
CPE	- Cytopathogenic effect
cpm	- Counts per minute
CV1	- Established line African green monkey kidney cells
DNA	- Deoxyribonucleic acid
DNase	- Deoxyribonuclease
dsRNA, ssRNA	- Double-stranded RNA, single-stranded RNA
DTT	- Dithiothreitol
EDIM	- Epizootic diarrhoea of infant mice
EDTA	- Ethylenediaminetetra-actetate
EM	- Electronmicroscopy
EtdBr	- Ethidium bromide
HindIII, PstI, EcoRI, TaqI, etc.	- Restriction endonucleases
IGV, HGV	- Infantile (human) gastroenteritis virus
MEM	- Minimum essential medium
mu	- Map units
MW	- Molecular weight
NOC	- Nicked open circular
NTM	- Newman Tytell Medium
OA	- Offal agent
OD600	- Optical density at 600nm
ON	- Overnight
PAGE	- Polyacrylamide gel electrophoresis
pBR322	- *E. coli* plasmid cloning vector (*amp*, *tet*)
pMM-L1	- Recombinant clone of large PstI/EcoRI BK(MM) fragment and pBR322
pMM-S1	- Recombinant clone of small PstI/EcoRI BK(MM) fragment and pBR322
PFU	- Plaque forming units
PR, GR, WW, SN*	- South African isolates of human polyomavirus
PSN	- Penicillin/streptomycin/neomycin cocktail
RI	- Refractive index
RNA	- Ribonucleic acid
RNase	- Ribonuclease I
SA11	- Simian agent 11
SDS	- Sodium dodecyl sulphate (= lauryl sulphate)
SV40	- Simian virus 40
TCA	- Trichloacetic acid
TCID50	- 50% tissue culture infective dose
Tc(R)/(S)	- Tetracycline resistant/sensitive phenotype

* These correspond to patients P.R., G.R., etc., from whom the viruses were isolated.

22 JUL 1982

TEMED	- N,N,N',N'-tetramethylethylenediamine
tet	- Tetracycline resistance gene
Tris	- Tris-(hydroxymethyl)methylamine
UV	- Ultra-violet light
VT	- Versene-trypsin solution
WAS	- Wiskott-Aldridge Syndrome
MOI	- Multiplicity of infection
Md	- Megadaltons
h	- Hours
min	- Minutes
DGC	- Density gradient centrifugation
E.H.H.	- Professor E.H. Harley