Cloning of a putative human oncogenic virus, BK.

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INDEX

Acknowledgements	i
Fig. 1	ii
Fig. 2	iii
Chapter 1. - Literature review	1
Chapter 2. - Introduction	16.
Chapter 3. - Results and Discussion	23.
3.1. Preparation of DNA samples	23.
3.2. Extraction of viral DNA	32.
3.3. Ligation reactions	36.
3.4. Transformation of E.Coli.	53.
3.5. Transformations of E.Coli with BK (MM) and BK(WW) DNA	58.
3.6. Preparation of radioactive DNA probes	61.
3.7. Analysis of DNA extracted from putative BK(MM) clones.	70.
3.8. Investigation of human urines for viral DNA	80.
Chapter 4. - Summary	84.
Chapter 5. - Materials and Methods	86.
Abbreviations -	106.
References -	107.

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Fig. 1. Restriction endonuclease map of the prototype BK DNA.

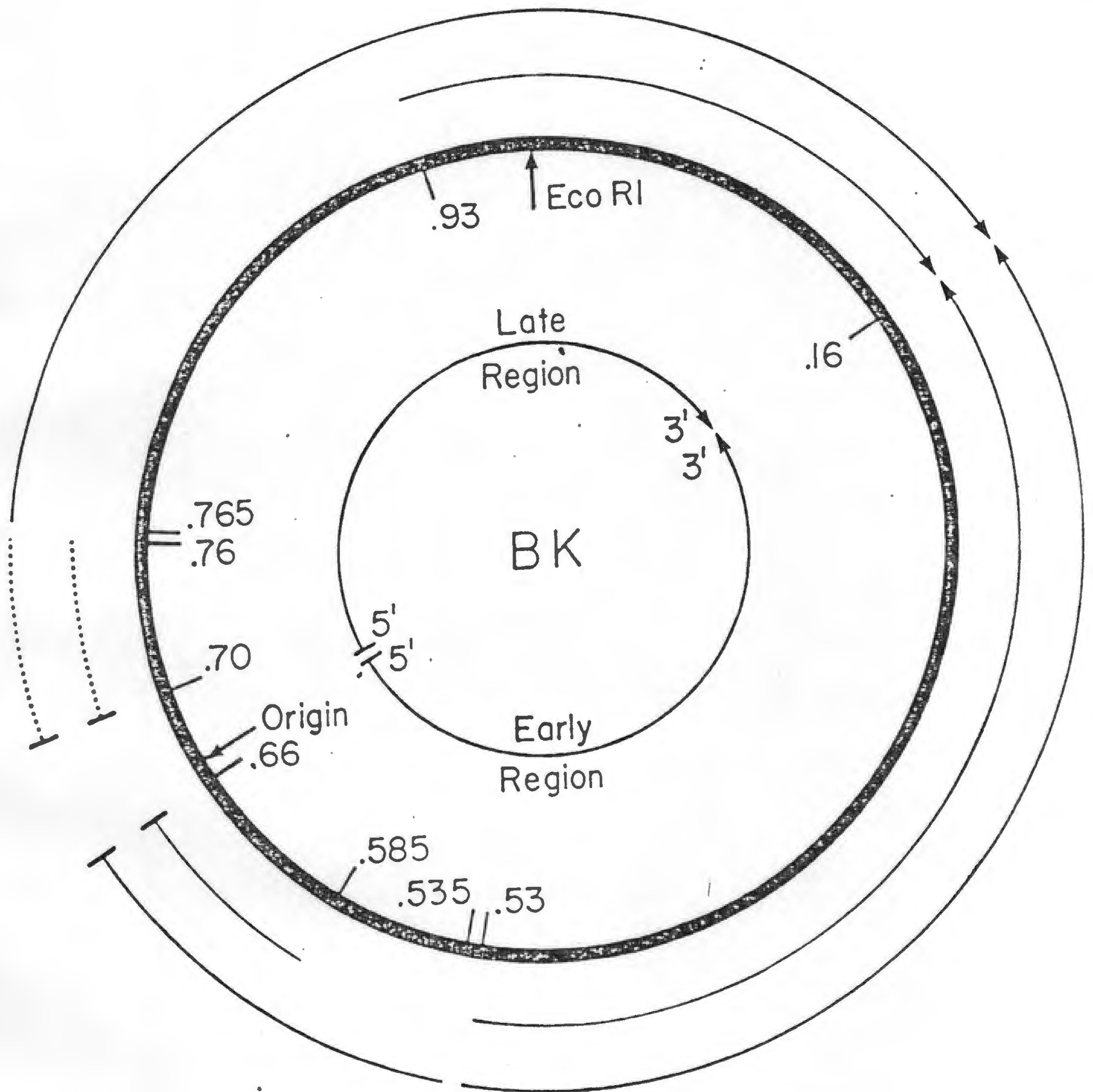


Fig.2. Organisational map of the prototype BK genome. Inner arcs indicate the early and late regions and the directions in which the stable mRNAs are transcribed.

Chapter I.

Literature Review

1. Literature Review

1.1. Papovaviruses are a group of non-enveloped icosahedral viruses which contain a double-stranded circular DNA genome in the supercoiled configuration. There are two subgroups, i.e. the papilloma and the polyoma viruses. The papilloma viruses are generally larger than the polyoma-viruses, having a genome of approximately 5×10^6 daltons compared with $3,3 \times 10^6$ daltons, and virions of approximately 55nm diameter as opposed to 41nm. The papilloma viruses generally produce benign epithelial proliferations in the host e.g. the human wart, and attempts to propagate these viruses in cells in culture have been unsuccessful. On the other hand, polyoma viruses can usually be propagated in tissue culture and do not appear to be associated with any widespread pathology in their natural hosts. Although there is no convincing evidence of polyoma viruses causing malignancies in their natural host, nonpermissive cells of other species may be transformed and these viruses therefore have oncogenic potential in particular laboratory animals.

Polyoma viruses infect eukaryotic cells, and investigation thereof should allow further elucidation of eukaryotic gene expression and regulation. Members of the polyoma group which have been extensively studied include polyoma virus itself, which infects mice, simian virus 40, (SV40),which infects rhesus monkey cells, and RKV which infects rabbits.

Interest in this polyoma group of viruses has increased ever since 1965 when a new papovavirus strain, JC, was isolated from brain glial cells of a patient with progressive multifocal leukoencephalopathy (PML), and was thus the first polyomavirus infection of humans to be discovered. (ZuRhein and Chou, 1965). In 1971, an immunologically distinct polyomavirus, BK, was isolated from the urine of an immunocompromised recipient of a renal allograft (Gardner et al., 1971). Interest in these two viruses in particular has been compounded by their potential oncogenicity in humans, (see section 1.8).

1.2. JC virus has been isolated from the urine of patients showing no signs of PML (Gardner, 1977) as well as from the glial cells of patients with PML (Weiner et al., 1972; Padgett et al., 1971). The only other known sites of polyomavirus infection are the endothelia of the ureter and the kidney.

BK virus strains have been isolated from both sources (Jung et al., 1975), as well as from the urine and the reticulum sarcoma of a patient with Wiskott-Aldrich Syndrome, a disease affecting both cellular and humoral immunity (Takemoto et al., 1974). BK virus may frequently be isolated from the urine of renal transplant recipients for two years following the transplant, although excretion of the virus has been known to persist for 8 years, after which period of time the raised antibody levels (lgM specific for BK Antigens) presumably diminish the virus levels (Coleman et al., 1973). Whereas transplant patients appear to excrete BK virions intermittently, Wiskott-Aldrich patients whose urines have been tested, continuously shed virus (Takemoto and Mullarkey - unpublished results in Gardner, 1977).

1.3. BK Virus.

Active infection of BK virus has so far only been associated with patients who are immunocompromised in some way. However, more than 70% of the general adult population in the U.K., (Gardner, 1973), U.S.A., (Shah et al., 1973), and Italy, (Portolani et al., 1974), and 37% of 5 year old children have antibodies to BK virus. (In contrast less than 3% of people have SV 40-neutralising antibodies.)(Lai et al., 1979). It is therefore suspected that infection with BK virus is a common occurrence, exposure occurring early in life, with immunocompromisation resulting in reactivation and possible excretion of the once-latent virus. Interestingly, a high incidence of productive infection is indicated in South Africa where 8 out of 18 renal transplant patients screened were found to have papovavirus particles in their urine. (Lecatsas et al., 1973). Switzerland seems to have a comparably high incidence of productive infection, i.e. where a third of renal transplant patients showed a significant increase in antibody titre to BK virus after transplant operations. (Coleman et al., 1973).

BK virions measure between 40.5 and 44,0 nm in diameter and the infectious particles have a density of 1,34 g/ml. (Gardner, 1971; Dougherty & di Stefano, 1974). The circular genome of the prototype BK has a molecular weight of $3,4 \times 10^6$ daltons and was shown to be in the supercoiled configuration by caesium chloride - ethidium bromide density gradient centrifugation, and by gel analysis.

1.4. BK genome

The genome of BK virus has been isolated from various sources and has been

found to be heterogenous after passage through Vero (monkey kidney) cells. In contrast, when propagated in human embryonic cells, the genomes were found to be homogenous (Howley et al., 1975a). Since human cells are more susceptible to BK infection than Vero cells are, the heterogeneity discovered was proposed to be due to passage through Vero cells. Serial passage of papovaviruses at a high multiplicity of infection has been shown to cause significant rearrangements of viral DNA, particularly when non-plaque-purified virus is used; genomes were often heterogenous in size and had defective abilities to replicate autonomously. (Seif et al., 1979b; Howley et al., 1975a; Yang & Wu, 1979a).

The mapping of BK genomes with restriction endonucleases, after amplification in tissue culture, resulted in the discovery of a number of variants of the virus. Although immunological differences have been detected amongst a few variants, most differences are primarily due to deletions or alterations of DNA sequences, particularly in the tandem repeats just clockwise to the origin of DNA replication. These differ in size and pattern in the various strains of BK virus. The genomes of the prototype virus and the MM strain have been extensively mapped using the EcoR1 site as an arbitary zero map position. Enzymes employed include Hind III, HpaII, HhaI, Kpn I, Bam HI, Hph I, Bgl II, AvaII, Hinf I, Hae III, Xba I, and Pvu II (Yang & Wu, 1978b&c; Freund et al., 1979; and Howley et al., 1975b). Sites on the genome do not exist for Hind II, Hpa I, Hae II, Bgl I or Taq I. (Figure 1.)

The prototype virus,(MM)and Dunlop (Dun) strains more recently have been completely sequenced. (Howley, 1980b; Yang and Wu, 1979a; Seif et al., 1979c). The BK (Dun) strain has a 42 base pair deletion at map position 0.70 in the non-coding region, clockwise to the region of tandem repeats near the origin of DNA replication. The BK (JL) variant has an extra Eco RI site and four Hind III fragments, different in size from those of the prototype genome. Various isolates of BK (MM) show a high degree of conservation of coding sequences, particularly in the early coding region, whereas genomic heterogeneity is located mainly in the non-coding regions and accounts for less than 10% of the genome. The BK (MM) strain has a 262 base pair deletion at the region coding for the carboxy terminal of the small t Ag. There are three Hind III sites as opposed to the four of the prototype and BK (Dun) strains. Our own isolate,BK(WW),differs in the number and size of Hind III fragments from both the BK (MM) and prototype genomes. BK (WW) has three Hind III sites whereas the prototype has four, and (MM) has three. The

size of the fragments of (MM) differ however, from those of (WW) (See section 2.1).

The BK (MM) nucleotide sequence shows various characteristics similar to those of the SV 40 genome. Estimates for the extent of sequence homology between the genomes had been based on various hybridisation methods and ranged between 11% and 92% depending on the stringency of the technique. (Osborn et al., 1976; Howley et al., 1979; Khoury et al., 1975; Takemoto and Mullarkey, 1973; Seehafer et al., 1975). Since this technique gave inaccurate and varying results, it was only when nucleotide sequencing was available that reliable homologies could be ascertained. Complete sequence analysis (Yang and Wu, 1979b) showed an overall degree of sequence homology with SV 40 of 69%, which correlated well with values predicted by heteroduplex analysis, but only after certain allowances had been made. These extensive sequence homologies and the antigenic cross-reactivity between SV 40 and BK (MM) allowed extrapolations for the localisation of coding regions and of regulatory functions for lytic growth and transformation by BK virus, after comparison with analogous regions of the SV 40 genome, which has been extensively studied and completely sequenced. (Fiers et al., 1978; Kelly and Nathans, 1977; Dhar et al., 1978).

1.5. Transcription

The BK genome is transcribed bidirectionally from close to the origin of DNA replication. Distinct mRNAs for the "early" proteins are transcribed anticlockwise off the early genes. The two early proteins, which are coded for by overlapping genes, are the small t and the large T antigens. (Yang et al., 1980; Dhar et al., 1979). The first 82 amino acids of t antigen are identical to those of T antigen, and this sequence appears to be highly conserved within BK variants (Simmons and Martin, 1978). The remainder of the T antigen is coded for by a noncontiguous sequence of DNA distal to the t antigen (Yang et al., 1980). An intervening sequence, which includes a number of termination codons including that for the small t antigen, is removed by splicing. Splice sequences (AGGT) are identical to those in SV 40 implicating that similar enzyme(s) splice SV 40 and BK encoded mRNAs (Seif et al., 1979a). The late genes are transcribed clockwise off the complementary strand of DNA, with the coding region stretching from map position 0.707 to 0.163. Three capsid proteins are coded for; VP1, VP2 and VP3. The latter has its initiation codon within the sequence coding for VP2 and is

transcribed off the same reading frame. In contrast, the VP1 gene is read in a different frame although there is a 113 base pair overlap with the VP2 gene sequence. The use of overlapping genes interestingly however, does not seem to result in an increased conservation of sequence or in a reduction in the accumulation of mutations (Ghosh et al., 1978).

Immediately following the potential late proteins' initiation codon at nucleotides 3510-3512, is a sequence coding for an RNA species capbable of being translated into a 66 amino acid protein (62 amino acids in SV 40), (Yang and Wu, 1979d). SV 40 has abundant RNA species complementary to this DNA sequence and the "agnoprotein" has only very recently been identified (Jay et al., 1981) and is a DNA binding protein (see section 1.6.2). This "agnogene" region is highly conserved between SV 40 and BK and also between BK variants; 40 amino acids of the putative BK (MM) protein would be identical to those in SV 40. Differences in this region amongst the genomes of various strains, arise mainly at the carboxy-terminal end of the protein. This region seems to be dispensable for lytic growth and transformation in tissue culture (Bouck et al., 1978).

A second open reading frame, about 300 base pairs long, exists at the 3' ends of BK virus and SV 40 early regions, where the two genomes share very little sequence homology. (Osborn et al., 1976; Khoury et al., 1975). This DNA sequence would code for a highly hydrophobic protein which could associate with membranes. However, such peptides have not yet been demonstrated. (Yang and Wu, 1979a)

The use of codons is essentially similar in the early coding regions of BK (MM) and prototype BK genomes (Dhar et al., 1979). The deficiency of the dinucleotide CG in sense codons is found in BK genomes as well as in SV 40 and the β-globin mRNA of rabbits and humans. This codon usage is presumably dependent on factors such as tRNA distribution and mRNA structures, as well as on the interaction energy between mRNA and tRNA. It may also be related to methylation of DNA. A strong preference for AAA over AAG codons for lysine and for UUU are UUC codons for phenylalanine was found. In general, all NNPy codons showed selection for U over C, e.g. NUU occurs 82 times whereas NUC occurs 8 times (Yang and Wu, 1980). This is also observed in SV 40 DNA. (Fiers et al., 1978; and Reddy et al., 1978).

The origin of replication contains GC-rich inverted repeats of GCCTC, which may form secondary structures of hairpin loops. It is possible that the T antigen regulates the initiation of early and/or late transcription of DNA synthesis by binding to these sequences in BK as well as SV 40. (Dhar et al., 1978).

The palindromes, symmetrical segments and AT-rich stretches surrounding the origin of replication, have possible functional significance with respect to transcriptional regulation, being possible promoter or processing signals for early viral RNA transcription. For comparison, bacterial promoters are located near AT rich sequences closely preceding the 5' end of the message. Evidence also exists suggesting that the short stretches of A residues in the 3' early region may serve as terminators for transcription, or as processing signals for the 3' terminal ends of messages. These short A-residue stretches occur on the early strand of BK and SV 40 DNA, and may alternatively signal the termination of a small (40-50 nucleotide) RNA species capable of functioning in the control of transcription, or in the initiation of viral DNA replication (Dhar et al., 1979).

The noncoding regions account for 12% of the genome and include the origin of replication as well as the surrounding palindromes and tandem repetitive sequences. The origin of replication itself contains a palindrome of 17 nucleotides, followed by two symmetrical segments with intrastrand homology. The sequence of the first segment diverges greatly between BK and SV 40 DNA whereas there is extensive sequence homology in the second segment, with a high GC content. The non-coding tandem repeats are not highly conserved and differ in repeat sequence and pattern between BK strains, and between BK and SV 40 genomes. Deletions in this area do not all appear to have deleterious effects on viral growth. (Dhar et al., 1978).

The high degree of sequence homology between SV 40 and BK virus genomes is either representative of a close evolutionary relationship, or of a functional requirement for these specific DNA sequences for bidirectional replication. The latter is probably more likely since the overall topology is conserved despite small changes in sequence. (Dhar et al., 1978).

1.6 BK Virus Proteins

1.6.1 The BK genome codes for two early proteins, the small t antigen and the large T antigen. The gene localisation of these proteins was made possible by comparison firstly of the primary nucleotide sequences of BK and SV 40, and secondly, of the amino acid homologies of the respective proteins.

The open reading frame in the prototype BK from map position 0,64 to approximately 0,535, codes for a protein of 172 amino acids, with a molecular weight of 17 000 daltons; this is the small t antigen. These 172 amino acids in prototype BK (or the equivalent 100 in the MM strain) show a high degree of similarity with the 174 amino acids of the SV 40 t antigen. Approximately 30% of the methionine containing tryptic peptides are identical in the t and T antigens and four of these peptides common to both antigens are identical in BK and SV 40. The t antigen of prototype BK shows 87% amino acid sequence homology and 79% nucleotide sequence homology in the coding region for the amino terminal. (Dhar et al., 1979). The carboxy terminal diverges more, with 66% of the nucleotide and 58% of the amino acid sequences identical, with an abundance of cysteine in the t antigen of both BK and SV 40. (Lai et al., 1979, Wright et al, 1976).

The region between map positions 0,64 and 0,595 is represented in the mRNAs coding for both t and T antigens and encodes amino acids common to both proteins. The region between map positions 0,595 and 0,535 shares 192 out of 291 nucleotides with SV 40 (58% of the amino acids) and corresponds to the region spliced out of the t antigen mRNA, with splice sequences identical to those in the SV 40 genome (Seif et al., 1979a; Lai et al., 1979).

The large T antigen has a molecular weight of 94 000 daltons, comprising 708 amino acids, with the coding region terminating at map position 0,172. The region from map position 0,54 to 0,21 is essential for the expression of T antigen in SV 40, with no known viable deletions occurring in the amino terminal region. However deletions between positions 0,21 and 0,18 give viable mutants in SV 40 (Benoist and Chambon, 1980); in this region the amino acid sequence homology between the SV 40 and BK T antigens is decreased with respect to the highly conserved amino terminal region. In addition to the similarities in size (94 000 and 100 000 daltons) and in amino acid

composition, the T antigen of BK virus is immunoprecipitated with SV 40 T-antisera (Simmons and Martin, 1978).

Transcription and/or translation of the T antigen mRNA may be regulated in part by a potential secondary structure of an 8 base pair double stranded stem adjacent to a stable hairpin loop, immediately adjacent to an AUG initiation codon.

1.6.1.2. Functions of early proteins

The large T antigen is localised primarily in the nucleus of infected cells and is in the phosphorylated state. (Takemoto et al., 1973; Farrell et al., 1978). The BK T antigen may play a role similar to that of SV40 T antigen in binding to the genome at the origin of replication to regulate DNA synthesis, and in the region of initiation and transcription of the early and late mRNAs. This binding may be via a stretch of aromatic amino acid residues in the amino-terminal region. The T antigen is essential for the initiation of viral DNA replication, for the induction of cellular DNA synthesis, and the initiation and maintenance of transformation of infected cells. (Seif and Martin, 1979b and d). SV 40 T antigen is thought to be the primary regulator of the ratio of early versus late transcriptional initiation events in late lytic infection;BK T antigen restores the normal SV 40 transcriptional pattern when SV 40 T antigen mutants are involved. Both T antigens are thus thought to be comparable in function. In conjunction with the small t antigen, T antigen is involved in the regulation of the switch to late lytic BK genome transcription. (Lai et al., 1979; Sleigh et al., 1978; Seif et al., 1979b; Bouck et al., 1978; Kelly et al., 1977). In addition, BK T antigen carboxy-terminal appears to have a helper function for the growth of human adenovirus type 2 in monkey cells, as does that of SV 40 (Miyamura and Takemoto, 1979). Also, preinfection of monkey cells by BK virus restores a number of biological properties, (including initiation of DNA replication), of the early SV 40 tsA mutant grown at nonpermissive termperatures. (Sleigh et al., 1978; Mason and Takemoto, 1976).

Early viable deletion mutants of t antigen in SV 40 have shown that the t antigen is dispensable with respect to growth in tissue culture and the induction of tumors in newborn hamsters. (Sleigh et al., 1978). These

mutants however, do not affect T antigen production (Benoist and Chambon, 1980). BK (MM) does not encode a normal sized t antigen of 17 000 daltons, but a 10 000 dalton t antigen. It can however replicate in tissue culture, lytically infect human cells, stably and fully transform growing cells _in vitro_, and induce tumors _in vivo_ in rodents. (Howley, 1980a). It is therefore probable that the small t antigen is dispensable for these functions of BK virus. The high cysteine content in the middle region of the t Ag in the prototype BK, BK(Dun) and (MM) strains appears in a pattern similar to that in a soyabean protease inhibitor,suggesting the corollary that the t antigen may likewise have two sites for protein - protein interactions; the protease inhibitor contains a functional duplication with each of the paired cysteine units having a reactive site for a different protease (Seif et al., 1979c). The t antigen does not bind DNA and is not localised in the nucleus of infected cells. It is possible however, that BK and SV 40 t antigens have transient roles in the transformation of stationary cells, (Seif et al., 1979b and c).

1.6.2. The late genes of BK code for three capsid proteins' mRNAs. The proteins are detected in BK infected cells after the onset of viral DNA synthesis. These proteins are immunologically distinct from those of SV 40, and tryptic peptides of VP1 and VP3 of a number of BK strains (e.g. prototype RF, GS, DW, MG) revealed minor variations only. (Wright et al., 1976). The genetic organisation of the late regions of SV 40 and BK genomes appears, however to have been conserved. VP1 comprises 69-84% of the protein mass of the virion, and is an intranuclear antigen of molecular weight of 39 000 - 44 000 daltons, observed late in the lytic cycle (Wright et al., 1976). It is the most highly conserved protein between the SV 40 and BK viruses, showing an 83% amino acid homology with SV 40 VP1 (Ghosh et al., 1978). It has a very hydrophobic amino-terminal and a very basic carboxy-terminal.

VP2 (35 000 daltons) shows 77% amino acid homology with SV 40 proteins,and VP2 with VP3 constitute the minor capsid proteins,(Wright et al., 1976). One of the two basic amino acid stretches occurring in VP2 and VP3 may be involved in DNA interaction, and is conserved in BK, SV 40, and polyoma virus. (Dhar et al., 1979; Ghosh et al., 1978).

The VP1, VP2, and VP3 capsid proteins are arranged in a T=7 lattice enclosing the circular DNA genome. The "triangular number," T, describes the arrangement of protein units into capsomeres. The genome is associated in a nucleosome-

like structure (similar to that described for SV 40), with the host histone proteins of 14 500, 16 000 and 18 000 daltons i.e. H2a, H2b, H3. (Seehafer et al., 1975).

The recently identified agnoprotein of SV 40 appears and accumulates late in the lytic cycle of infected African green monkey cells. It has a MW of 79 000 daltons, is highly basic and has a strong affinity for both single and double stranded DNA. In addition, its short half-life of two hours suggests that the agnoprotein may have a transient regulatory role in nucleic acid-protein interactions, possibly in the association of the nucleic acid viral core. Mutants with deletions within the agnogene region are viable. The high conservation in DNA sequence in SV 40 and BK virus suggests this protein has a significant function, possibly also involving transcription or processing of late mRNAs (Jay et al., 1981).

1.6.3. Antigenic cross-reactivity occurs between the T antigens of SV 40, BK and JC viruses, as determined by indirect immunofluorescence (Dhar et al., 1979). The capsid proteins show less cross-reactivity as measured also by immune electron microscopy, although there is a certain degree of sequence homology between the late regions of the BK and SV 40 genomes (Simmons and Martin, 1978). Evidence exists showing that BK transformed mice and hamster cells induce a particular tumor-specific transplantation antigen which is immunologically related to that induced by SV 40 in mice, but not in hamster cells (Kato et al., 1979). BK virus agglutinates human type O and guinea pig erythrocytes, whereas SV 40 does not (Gardner et al., 1971).

1.7. Lytic Infection of Cells

Human cells in culture are permissive for BK virus, e.g. human fibroblasts, primary embryonic kidney, brain and endothelial cells are lytically infected, but usually no transformation occurs. (Wright et al, 1976; Yang and Wu,1979a; Takemoto et al., 1979). BK replicates through passage level 9 im embryonic kidney cells, and 12 in both muscle and lung cells. Plaques are visible within 20 days in embryonic kidney cells, whereas Vero and BSC-1 monkey kidney cells yield plaques only after 28 days, with those of the Vero cells being poorly defined and producing a titre of 1 x $\log_{10}$ lower than embryonic kidney or

BSC-1 cells. BK virus however is a slow developing virus, so host cells for plaque assays must survive 3 to 4 weeks under nutrient agar to allow plaque development. Plaque assays are therefore usually performed with embryonic kidney later passage cells, since strong association of the virus occurs with the cell membranes of embryonic muscle or lung cells, such that infected cells release low yields of progeny virus. (Seehafer et al., 1978).

Virus particles are adsorbed and the DNA transported to the nucleus, where early mRNA transcripts are made and then transported out of the nucleus. Viral DNA synthesis begins 18 hours after infection and continues until 48-60 hours after infection. Following the onset of viral DNA replication, late mRNAs are detected in the cytoplasm. Progeny virions are assembled in the nucleus and cellular degeneration and lysis follow. Cytoplasmic vacuolisation and the appearance of intranuclear basophilic bodies occur within approximately seven days of infection of secondary embryonic kidney cells. (Mason et al., 1976). Studies on the BK growth cycle are limited, but the infectious cycle appears to be somewhat longer than that of SV 40, with variable dependency on the input multiplicity, viral strain, and the presence of defective particles in the virus stock (Hogan et al., 1980).

1.8 Persistent infection of human cells

Thirty days post infection of secondary embryonic kidney cells, multilayered colonies of semi-transformed cells were observed. They produced infectious virus as well as being positive for both the t and T antigens, but contained, on average, 20 copies / cell of an episomal superhelical viral genome. No colonies grew on soft agar and the cells were not tumorigenic in nude mice. In addition, the cells reached a saturation density of $1{,}3 \times 10^{8}$ cells/cm^{2}. Late mRNAs were not efficiently translated and only 1% of cells were V-Ag positive. Serial dilution of these cells allowed detection of T-antigen positive cells containing integrated BK sequences corresponding to the early region, and from which stable early mRNA transcripts were made (Purchio and Fareed, 1979). Embryonic brain cells by contrast contained episomal sequences and were deficient in T antigen expression; they grew indefinitely in culture and were tumorigenic in nude mice. At the same time, the cells were persistently infected, and this represents a unique association of papovavirus DNA with the host cell. This state is known as persistent infection, or semitransformation since the cells release virus after removal of antiserum

from the medium. (Takemoto et al., 1979). After several years in culture, homogenous free BK genomes persisted, in contrast to the heterogenous population of free and integrated sequences in SV 40 - transformed rodent cells and in polyoma-transformed rat cells. (Botchan et al., 1976).

1.9 Transformation and Oncogenicity

BK virus is capable of stable transformation of nonpermissive cells in culture e.g. monkey kidney cells, rat and rabbit kidney cells, and hamster, Swiss and BALB/C mouse cells. (Major and di Mayorca, 1973). These cells are generally V antigen negative, but t and T antigen positive. They are tumorigenic in nude mice and in hamsters, *in vivo*. The presence however of t and T antigens *per se* is not necessarily associated with malignancy since such cells have been non-malignant in nude mice and also unable to grow in soft agar. (Seif et al., 1979 b and d; Yang and Wu 1979a; Bouck et al., 1978).

A linear relationship exists between the multiplicity of infection, (between 1 and 100 pfu/cell) and the transformation frequency. Above this level of 100 pfu/cell the transformation frequency plateaus off (Wright et al., 1976). BK-transformed cells induce a high incidence of tumors when innoculated intracerebrally or intravenously into hamsters or mice. Ependymonas were found to be the most frequent (72%) tumors induced and all showed tandem integration of full size linear BK DNA. Osteosarcomas (10%) and pancreatic insulinomas (12%) showed patterns suggesting individual integrations, or short tandems of defective viral molecules. The polyoncogenic capacity of BK virus is accounted for by the presence of BK mutants; e.g. a BK mutant having a deletion and insertion at map position 0,72 (lying in the leader region of the late mRNA in SV 40) produces brain tumors and insulinomas in hamsters (Watanabe et al., 1979). In comparison,cloned prototype BK induced brain tumors and osteosarcomas (Howley and Martin, 1977; Chenciner et al., 1980). Cultured cells probably give only an incomplete account of the state of viral DNA in the virus-induced tumors, since losses and re-arrangements of viral DNA sequences have been shown to occur in cell culture (Seif et al., 1979c; Howley et al., 1975a). However, analysis of the mode of integration of viral DNA in cells of virus-induced tumors in principle, should offer a valuable means to investigate the problem of the clonal origins of tumor cells. BK transformed hamster cells and tumor tissues generally do not contain detectable quantities of the circular forms of

viral DNA. Instead, they invariably carry viral DNA sequences integrated into their chromosomes - usually the amount of these sequences in the genomes of transformed cells is both constant and small (i.e. less than ten viral genome equivalents per diploid cellular DNA) (Botchan et al., 1976; Purchio and Fareed, 1979). Integration patterns differ suggesting that many integration sites exist on the cellular and/or viral DNA. DNA transformed cells represent uniform amounts of viral genome in stable association with the cellular DNA. Although undetermined as yet, selective amplification of certain sequences may occur after continued passage in culture. However, integration of the entire viral genome is not necessary for transformation. Expression of the early gene of SV 40 is necessary for establishment and maintenance of the transformed state (Bouck et al., 1978; Seif and Martin 1979b). The significance of integration of the genome for early gene expression is unknown. (Botchan et al., 1976).

BK DNA linearised by cleavage with EcoRI, Bam HI , Kpn I or Hha I, has unchanged transforming capacity, whereas cleavage with Pst I significantly reduces this capacity. T antigen induction by linearised DNA is less efficient than with supercoiled DNA. T-antigen induction by Pst I linearised DNA, however, is particularly low, implicating that this region (0.31 map units) is indispensable for transformation. This is supported by the fact that mutants of BK lacking the segment from map position 0.28 to 0.53 (and thus the Pst I site) cannot induce T antigen production, or transformation, yet can only interfere with the replication of prototype BK DNA (van der Noordaa et al., 1979).

The efficiency of transformation of cells is greater and more rapid when DNA, rather than infectious virus, is used. T-antigen is synthesised, the cells contain rescuable virus and are tumorigenic in mice. A viral specific anti-nuclear antibody is produced in infected animals, and it reacts against untransformed cells but is not found in hamsters with SV 40, JC, polyoma or adenovirus-induced tumors (Takemoto and Martin, 1976). It is possible that DNA infection is more effective with rodent cells, than infection with virus particles is,due to insufficient virion uncoating or penetration of cells. The DEAE dextran method gave preferable infection results to the calcium phosphate co-precipitation method, although the latter yielded better transformation results (Van der Noordaa, 1976).

The establishment of BK virus as an oncogenic virus in nonpermissive hosts, and as an inducer of the semi-transformed state in human cells, raises the question as to the possible etiological role of BK virus in human neoplasia.

1.10 Association of BK virus with human diseases

BK virus has been detected in human urine by viral culture, immune electron microscopy, immunofluorescence microscopy, and viral serology (Gardner 1971; Hogan et al., 1980; Jung et al., 1975; Coleman et al., 1973; Lecatsas, 1973). BK cytopathic effects are assayed by tube titration, plaque formation and fluorescent focus formation (Howley, 1980a). Fiori and di Mayorca (1976) found DNA sequences homologous to BK DNA in DNA preparations from 5 out of 12 human tumor tissues, and 3 out of 4 human tumor cells lines. Normal human tissues showed no BK DNA sequences, neither did the heterodiploid Hela cell lines. 7 out of 8 BK DNA positive tissues contained 0,4 to 1,6 BK genome equivalents per diploid cell, and one tissue contained 11,0 genome equivalents. However, Israel et al., 1978, were unable to detect any BK sequences in any of the human tumors or cell lines examined. None were T-antigen positive and no antibodies specific for the BK intranuclear T antigen were found in any patients with known malignancies, whereas animals with BK - induced tumors express an anti-nuclear T antibody. (Costa et al., 1977). Tissues examined included urologic tumors, renal tumor cell lines, kidney tumor lines and human brain tumors. (Corallini et al., 1976; Sleigh et al., 1978). The only explanation for this discrepancy was that in the latter cases, cell lines at earlier passage levels were used, and a more stringent assay for BK DNA was used. It is therefore unlikely that BK is commonly associated with human neoplasia.

Epidemiological studies have indicated that immunosuppressed renal transplant and Wiskott-Aldrich syndrome patients develop cancer at 19 times the incidence of the normal population, with very rare types of cancer often occurring, e.g. reticulum cell sarcomas occur at 150 times the normal incidence. To exclude BK virus as an agent of these types of cancer, additional tumors must be analysed. (Wold et al., 1978; Yang and Wu, 1979b). BK-like viruses have been detected in urines of two systemic lupus erythematosus patients (Taguchi et al., 1979), by haemagglutination. Results indicate that urinary sediments contained large, abnormal cells with intranuclear inclusion bodies (Taguchi et al., 1980).

1.11 In addition to the potential association of BK virus with human diseases and particularly its putative oncogenicity, this papovavirus warrants interest due to other aspects. The regulation and expression of eukaryotic genes may be investigated using this eukaryote-infecting virus. The early genes of SV 40 have been inserted into the plasmid vector pBR 322, and hereby introduced into eukaryotic cells where the early genes were expressed (Benoist and Chambon, 1980). SV 40 and BK viral genomes provide good models for the analysis of the structure of eukaryotic promoter sites, due to the fact that the entire sequences of these small genomes have been determined. In addition, the viral genomes themselves, after in vitro modification, or in vivo mutation, may be used as vectors for the introduction into, and expression of eukaryotic genes in eukaryotic host cells. Such hybrid genomes have been encapsidated into progeny virions (Ganem et al., 1976).

After deletion of the t and T antigen transforming genes, the origin of replication (an 880 base pair segment in SV 40) may be ligated, by in vitro recombination catalysed by bacteriophage T4 polynucleotide ligase, to a segment of foreign DNA of appropriate size (e.g. a 520 base pair Hind III fragment of bacteriophage lambda DNA) by joining of the cohesive termini generated by specific restriction endonucleases or by poly d (A) d(T) tailing.

Prototype SV 40 DNA has been used to supply the helper gene function in conjunction with the recombinant SV 40 molecule, to infect cultured monkey cells, following which the inserted sequences were seen to replicate. Hybrid genomes were encapsidated into progeny virions with resultant amplification of the foreign inserted DNA sequences. Defective genomes of SV 40 exist, which contain the origin of replication in tandem reiterations, but which yield molecules of approximately prototype genome size. The origin of replication of SV 40 DNA has been shown to be the only function required in cis for the replication of these reiteration mutants (Khoury et al., 1974). Similar mutants of BK virus may be constructed and used as vectors for infection of human cells in culture and the subsequent study of eukaryotic gene expression and regulation in human cells.

Chapter II

Introduction

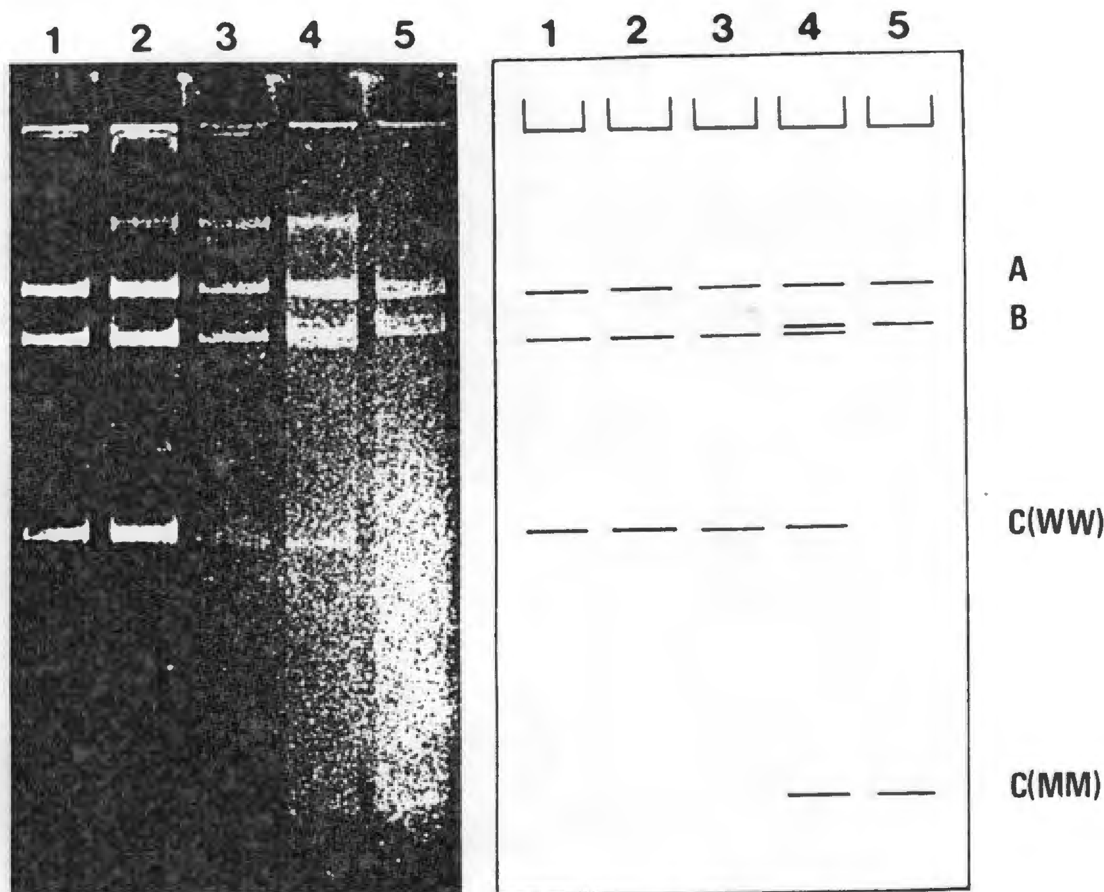
2. Introduction

2.1 Viral material extracted from human urine was being investigated as a screening procedure of the urine of (amongst others) renal transplant patients by G.Lecatsas and E. Harley. Electron microscopy of the sediments obtained by high speed centrifugation of urines of many renal transplant patients allowed morphological identification of papovavirus-like virions e.g. large numbers of particles typical of papovavirus virions were seen in the isolates of two immunocompromised renal transplant patients. (Lecatsas and Van Wyk, 1978). Sufficient DNA was extracted from these two isolates for limited restriction endonuclease mapping. Direct preliminary mapping was performed by Ron Mew and showed that the same variant of BK virus had been extracted from these two patients (WW and SN; the isolates are jointly hereafter referred to as BK(WW)).

Attempts to infect primary human foetal fibroblasts in culture with the virions or DNA of these isolates were unsuccessful, i.e. no cytoplasmic effects were observed, no nuclear inclusion bodies were visualised by the staining of flying coverslips, and frozen and thawed subcultures showed negative haemagglutination assay results (Gardner et al., 1971). However, available BK (MM) successfully and lytically infected these as well as human embryonic brain glial cells, in culture. The infectivity of the DNA may have been reduced by nonspecific inhibition, or by antibodies in the urines. The infectivity of further direct isolates will need to be quantitated for comparison.

Mapping of BK (WW) divulged information indicating that certain rearrangements of the genome had occurred to distinguish this from the MM and prototype BK strains.

Fig.3. Comparison of Hind III digests of BK (MM); BK(WW); and BK(SN) by 2.7% polyacrylamide gel electrophoresis.

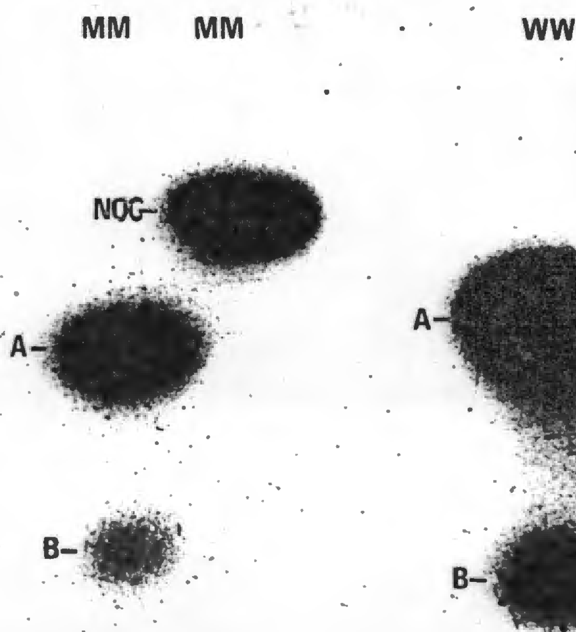


Lanes 1 - 5 Hind III digests; 1. BK(SN)
2. BK(SN) & BK(WW)
3. BK(WW)
4. BK(WW) & BK(MM)
5. BK(MM)

The restriction patterns of the two isolates are similar to those of the BK virus group; they are identical to each other but significantly different from those of the BK(MM) and prototype cell-culture-adapted strains. Their three Hind III fragments initially suggested a closer resemblance to the MM than to the prototype strain (which has four Hind III fragments). However, coelectrophoresis of the fragments of BK (MM) and BK(WW) allowed detection of a marked difference in electrophoretic mobility, and therefore in size, between the Hind III C fragments, and a lesser difference in mobility of their Hind III B fragments. That of the WW strain appears to be intermediate

in size between those of the MM and prototype strains. Verification of this will however require coelectrophoresis with the prototype DNA Hind III fragments. The size of the Hind III A fragment is identical to those of both MM and prototype strains. The C fragment is larger than that of the MM strain, but similar in size to that of the sum of prototype C and D fragments. It is proposed therefore that the third Hind III site clockwise to the EcoRl site of prototype BK DNA, is deleted in WW DNA.

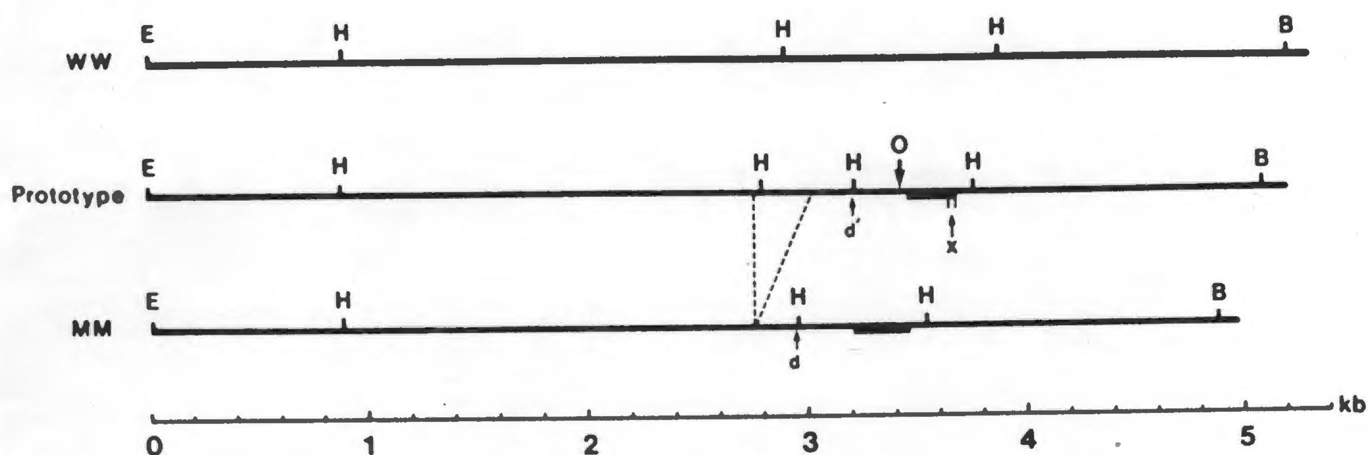
Fig. 4. Comparison of the Pst I EcoRI digest fragments of BK(MM) and BK(WW) by agarose gel electrophoresis followed by autoradiography of a Southern blot of the gel hybridised to a BK(MM) ^{32}P probe



The Pst I EcoR I A fragment of the WW strain appears larger than that of the MM strain whereas the B fragment appears significantly smaller than that of the MM strain.

Study of the nucleotide sequences of BK(MM) and the prototype DNA suggested that the WW strain contained an insertion near the origin of replication.

Fig.5 Comparison of the restriction endonuclease maps of BK prototype, BK(MM) and BK(WW) strains.



E-Eco RI B-Bam HI H-Hind III kb-kilobase pairs
O-Origin of replication
d-deletion in (MM) d'-corresponding area in prototype
blocks-tandem repeats

The two BK(WW) isolates represent the only two out of many from which sufficient DNA was extracted for restriction endonuclease mapping. The fact that both have identical restriction patterns suggests that this strain is not rare. Possible reasons for its not having been previously described are that 1) WW represents a strain peculiar to the area from which both the patients and donors came, i.e. Southern Africa; this explanation will require further analysis of BK DNA extracted directly from urine, in various areas of the world;

2) WW represents a non-tissue culture adapted strain, and that others, including the prototype, are selected for during passage in culture. (Previously all strains have been amplified in cell culture prior to biochemical characterisation, and this might preclude detection of the WW strain.)

3) The third possible explanation is that WW represents the original strain from which others evolved due to the selective pressures of growth to high titre in cell culture. However there are no simple relationships between the genomes suggesting evolution of the prototype (or other strains) from WW. Detailed investigation and characterisation of the genome

(including nucleotide sequencing) will be required to establish the relationship between the virus strains. Since the two patients are no longer excreting the virus, available DNA must be amplified. The particular significance of this strain is inherent in its origin i.e. it has been directly extracted from human urine, and therefore amplification may not be through cell culture. Amplification may however be achieved by use of recombinant DNA techniques to insert the DNA directly into a host cell as part of a hybrid viral-vector molecule. Passage through cells in culture is obviated, and the cloned homogenous BK DNA genome will be unique papovavirus material, unadapted to cell culture.

Additional reasons for obtaining a cloned BK genome include the following:

1. The unique cloned genome would serve as a radioactive probe for rapid and specific detection of BK viral genomic material in crude extracts of human urine, and possibly in available tissue extracts, particularly in tumor tissues. DNA extraction, purification and gel electrophoretic analysis of restriction digests would be unnecessary and thus screening would be more rapid.

2. Subgenomic BK clones could be manipulated to generate cloning vector material for use in (a) the introduction of foreign DNA segments into eukaryotic (possibly human) cells as discussed in the previous section, or

   (b) simply the study of the expression of the uniform cloned genes.

   Study of the BK(WW) genome may lead to correlation of its lack of infectivity with an altered region of the genome; the expression of particular genes (and simultaneously the function of their products) involved in cell transformation and infection may then be elucidated, with concomitant increased understanding of the processes of viral infection and transformation of cells.

Primarily cloning techniques were developed and established using the BK(MM) strain, of which a substantial supply of DNA was available via tissue culture. Subsequently, a stock of homogenous BK(WW) DNA could be generated through cloning amplification.

2.2. Cloning strategy.

E.Coli K.12 strains C600 and HB101 were available as EK1 hosts, and the disabled strain 1776 as an EK2 host (Curtiss et al., 1977). The latter was worked with as a recipient of foreign DNA until it was published by van der Noordaa et al., 1979, that Pst I cleaves the transforming gene of BK viral DNA. Initially, the approaches to cloning BK DNA was either a full genomic one, using a single restriction endonuclease, e.g. Hind III, or Bam HI (in the tetracycline gene), or a subgenomic one using Bam HI and EcoR I simultaneously, followed by separation of the 2 viral fragments and separate cloning thereof. Both approaches necessitated use of an EK2 host due to the oncogenic potential of the virus and in accordance with the regulations of SA GENE. However the extreme sensitivity of χ1776, particularly with respect to ionic detergents and vortexing, and the reduced transformation frequency obtainable with this strain, rendered it unfavourable as a recipient when dealing with limited amounts of foreign DNA to be cloned. In contrast, the transformation frequencies obtainable with both EK1 hosts, C600 and HB101, were considerably higher, as well as the hosts being more robust. It was therefore greatly advantageous to drop containment levels to EK1. A subgenomic cloning approach, of BK DNA cleaved within the transforming gene by Pst I, would allow this reduction. The Pst I EcoR I double digest subgenomic approach was suitable in a number of ways. Firstly, cleavage at the Pst I site of BK DNA drastically reduced T-antigen induction and transformation ability, allowing use of EK1 containment levels. Secondly, circularisation of the large fragment of Pst I EcoR I digested pBR322 and transformation thereby to yield Amp^S Tet^R colonies, could be reduced by treatment with bacterial alkaline phosphatase. This would significantly reduce the background of colonies not representing BK insert recombinants, and would simultaneously improve the probability of BK DNA recombining with pBR322. In addition, alkaline phosphatase treatment (or removal of the small fragment of pBR322) should remove the possibility of losses of pBR322 (with respect to ligation with BK DNA) by its ligation to form full length pBR322. Moreover, the particular section of BK(WW) DNA which appears to differ from those of other strains could be prepared as a single entity separate from the larger BK fragment. This smaller fragment would be more amenable to less complex purification and subsequent characterisation, particularly sequencing. It is also known that it is less difficult to clone smaller inserts of DNA and in this subgenomic cloning approach BK molecules of 1,09 and 2,17 x 10^6 daltons

respectively would be involved as opposed to the full length 3,26 x 10^6 dalton genome. The full length linear viral genome could be reconstructed after identification of clones containing the small or large inserts. DNA could be extracted and BK inserts separated from pBR322 by Pst I EcoR I digestion and separation of these fragments from pBR322, e.g. by electroelution from gels, followed by ligation of the viral fragments at the appropriate j/i ratio to obtain CCC monomers in the correct orientation, thus providing the original DNA genome.

Chapter III

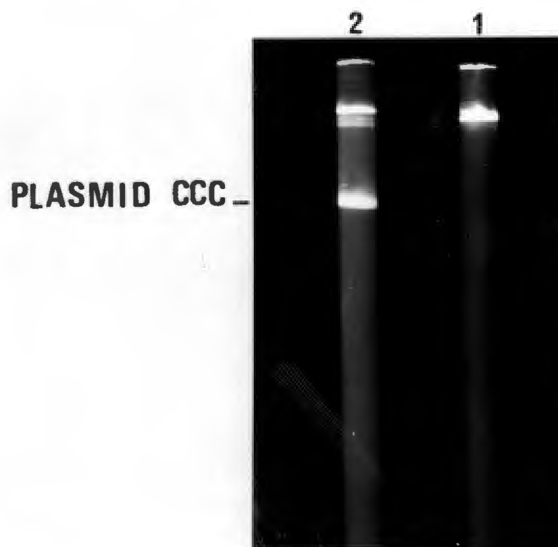
Results and Discussion

3.1. Preparation of DNA samples

3.1.1 Extraction of plasmids from Escherichia Coli

Extraction of plasmid pBR322 for use as a vector in the transformation of bacteria presented various problems which required resolving. The extent of cellular lysis was found to depend partially on use of a fresh lysozyme solution which cleared the bacterial suspension within an hour, whereas frozen and thawed solutions of lysozyme at the same final concentration cleared the suspension in only three hours. Lysis was aided by washing the initial pellet of cells in a larger volume (250 - 500 ml) of buffer as opposed to 20 ml as described by Clewell and Helinski, 1969. This wash probably removed extraneous material, cell debris and medium from the bacteria, thus rendering the bacterial walls more susceptible to lysozyme treatment. The relative yield of circular plasmid DNA with respect to linear chromosomal DNA was found to be critically dependent on the duration of incubation in detergent. The principle of the detergent treatment is disruption of the cell membrane, to which the chromosomal DNA is attached and is therefore separated from plasmid DNA by centrifugation. Plasmid DNA appears to attach to the cell membrane only while replicating. Over-exposure to detergent releases chromosomal DNA from the membrane; this DNA then does not precipitate with the cell membrane but contaminates the plasmid DNA in the supernatant. For the same reason, and also to prevent introduction of single or double-stranded nicks into circular DNA, extraction should be gentle without vortexing. The following figure demonstrates a comparison of relative yields of circular plasmid versus chromosomal DNA after 10 minutes in detergent solution, with or without vortexing.

Fig.**6** DNA analysed by electrophoresis in 1% cylindrical agarose gels after 1) 10 minutes in detergent with vortexing
2) 10 minutes in detergent without vortexing.



The use of the acetate salt of chloramphenicol was found not to induce plasmid amplification since it cannot enter the cell whereas amplification with chloramphenicol increased plasmid yield 20 fold i.e. from 1 to 20ug from 500 ml starting material of bacterial culture (Lewin, 1977; Clewell and Helinkski, 1972).

Aliquots of plasmid DNA extractions were quantitated by comparison of the ethidium bromide fluorescence under UV illumination, compared with that of known amounts of commercially prepared DNA, after electrophoresis on 1% agarose gels, and staining with ethidium bromide.

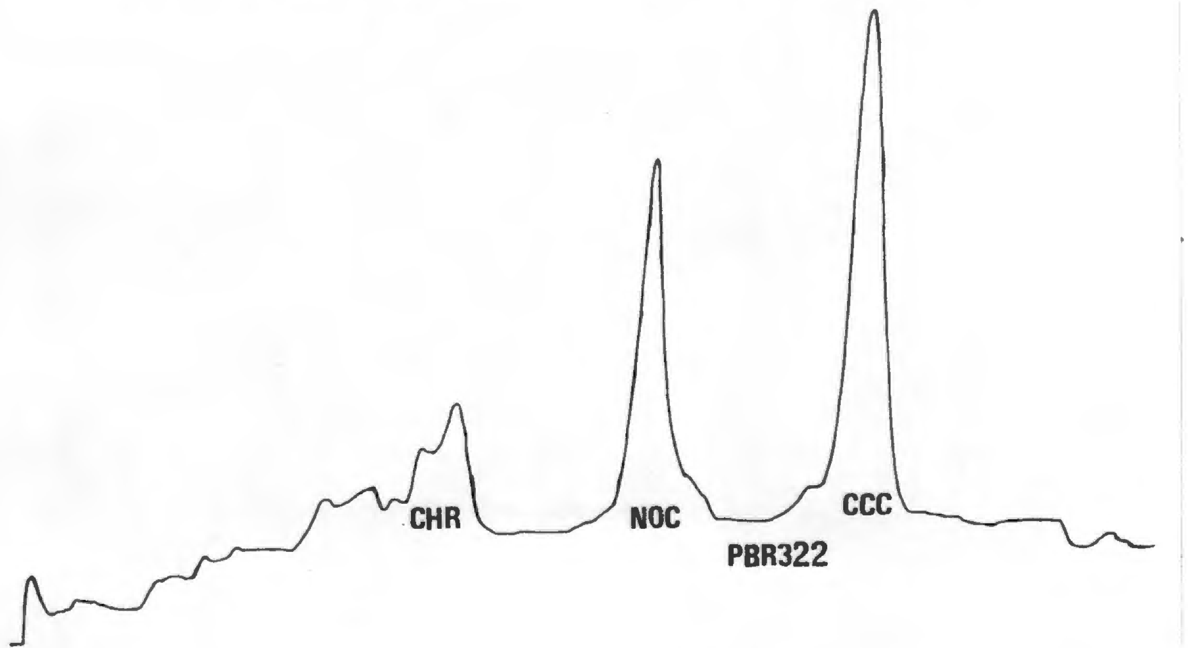


Fig. 7 Densitometric scan of the ethidium bromide fluorescence of bands on a cylindrical agarose gel, containing pBR322 and showing chromosomal, NOC and CCC plasmid DNA in approximate relative proportions of 1:3,3; 4,7 respectively.

The homogeneity of extracted plasmid DNA was checked by gel electrophoretic analysis of restriction endonuclease digests of the preparations. An enzyme with a single site on the DNA was used in order to linearise the DNA, giving a unique band on analysis.

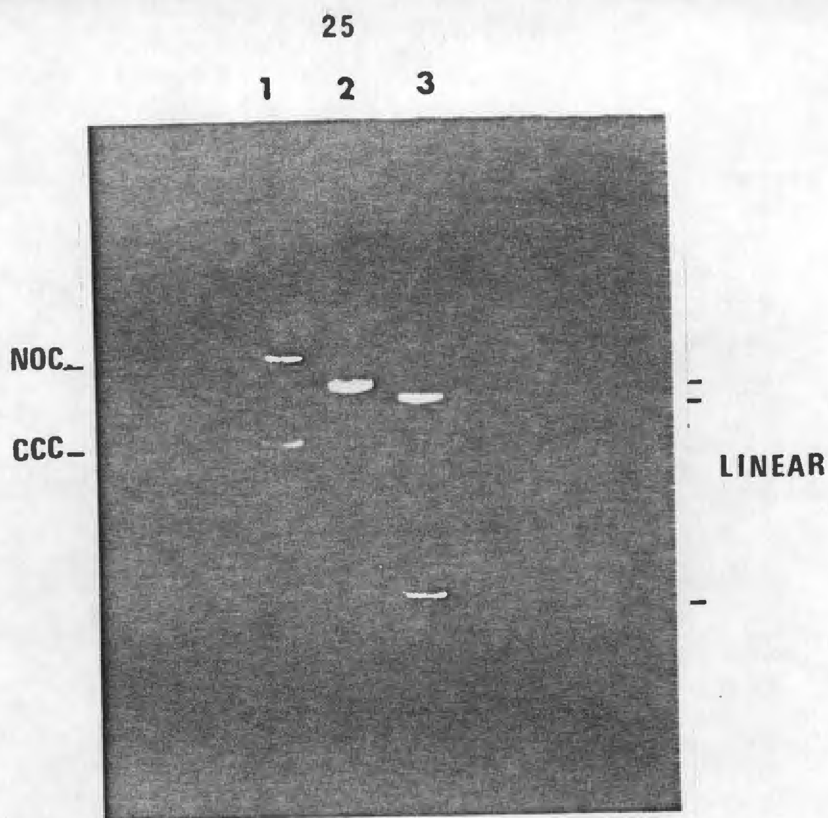


Fig.8 1% cylindrical agarose gel electrophoresis of
1) pBR322, showing NOC and CCC forms of the dimer molecule as well as the monomer.
2) Pst I digested pBR322, showing linear pBR322 only
3) Pst I EcoRI digested pBR322, showing two linear pBR322 fragments, as expected.

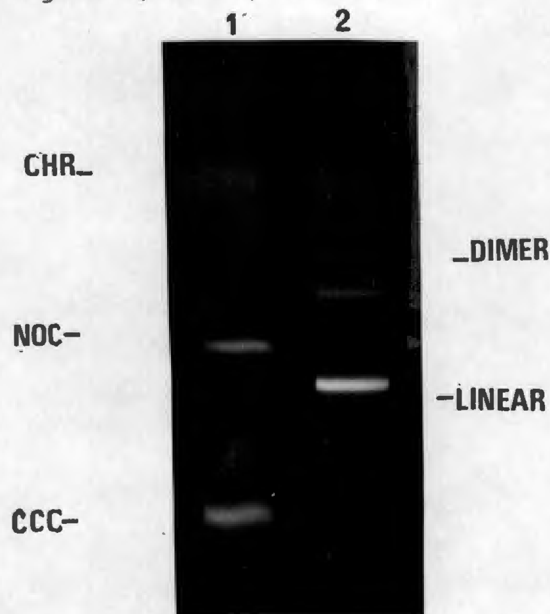


Fig.9 1% cylindrical agarose gel electrophoresis of
1) pBR322, showing chromosomal, NOC and CCC molecules
2) Bam HI digested pBR322 showing a smear of digested chromosomal DNA, and incompletely linearised dimer pBR322 molecules.

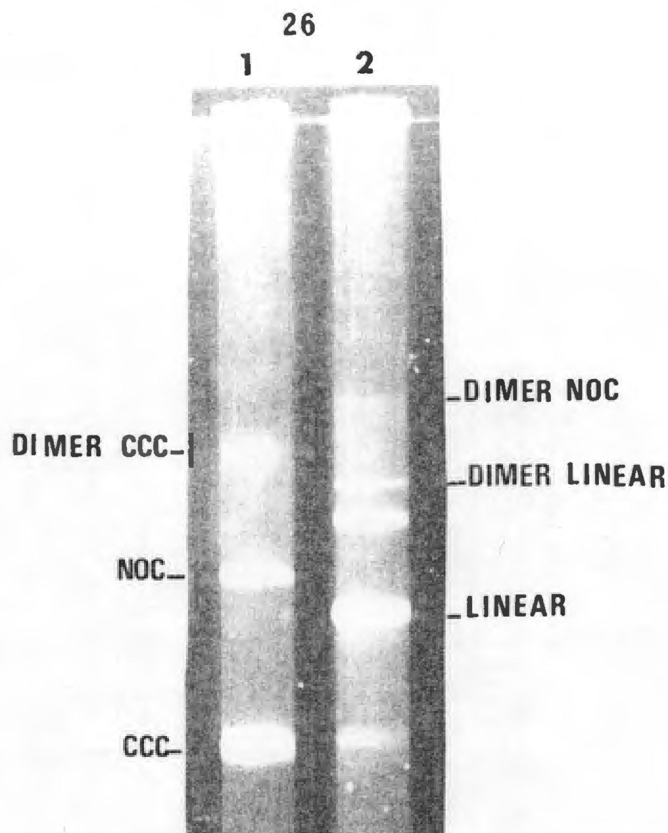


Fig.10 1% cylindrical agarose gel electrophoresis of
1) pBR322, showing CCC dimer, and NOC and CCC monomer bands.
2) Bam HI digested pBR322 (incomplete digest) showing linear monomer and dimer bands, as well as a residual NOC dimer band.

3.1.2. DNA purification procedures

3.1.2.1 Initially, CsCl-EtBr density gradient centrifugation was the method of choice for separation of CCC plasmid DNA from residual linear DNA : Clean preparations of CCC plasmid DNA were obtained with this method, however the high yield of purified plasmid DNA was not consistently reproducible in spite of identical gradient and centrifugation conditions. In certain cases microgram quantities of extracted or commercially prepared DNA were not visible, possibly due to the inadequate ultraviolet illumination available at the time. Compounded by the expense and lengthy duration of the technique, alternative procedures were investigated.

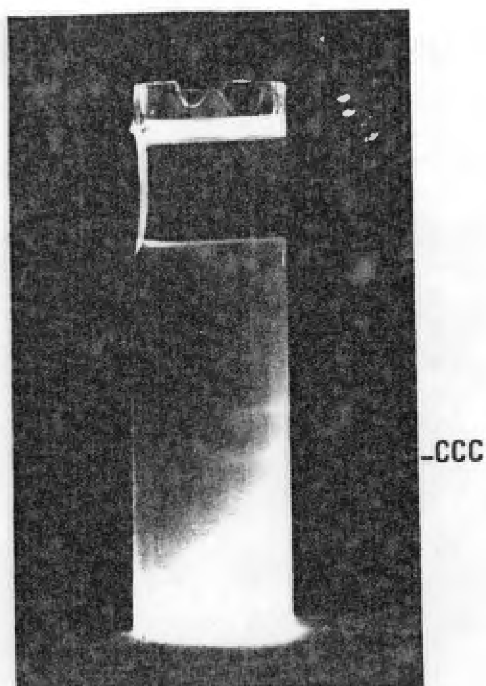


Fig.11 CCC and linear bands separated according to density. Displacement of caesium ions by ethidium lightens the DNA; in the case of linear DNA this process is less limited than in the case of CCC DNA where conformational restraints limit the number of caesium ions displaced, and the subsequent unwinding of DNA. CCC DNA therefore remains denser.

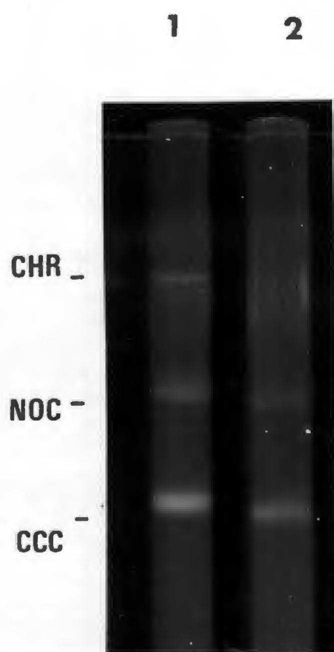


Fig.12 1% cylindrical agarose gel electrophoresis of pBR322

1) after one CsCl-EtBr density gradient centrifugation
2) after two centrifugations, showing removal of all linear DNA (plasmid and chromosomal) from the preparation.

3.1.2.2. Purification of DNA preparations generally included phenol and chloroform extractions to remove protein, followed by precipitation of DNA from the aqueous phase. The presence of phenol was detected in bulk DNA preparations by its absorbance at 270nm; and extensive dialysis was occasionally necessary to remove all phenol. Significant (50%) losses of DNA were incurred at the ether-chloroform interface; this ether extraction was subsequently omitted with no deleterious effects on enzyme reactions carried out on the DNA. Chromosomal DNA was removed efficiently by acid phenol extractions. The advantages of this method include its rapidity and the fact that DNA losses are minimal.

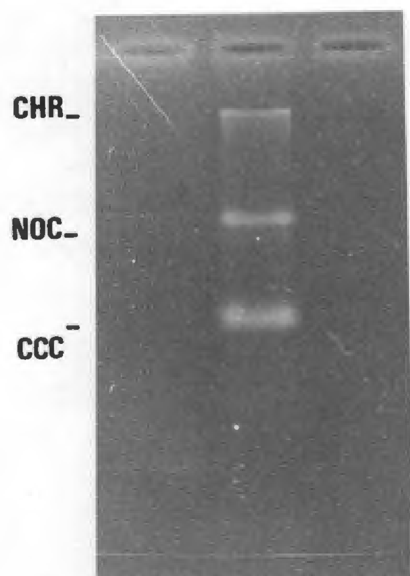


Fig.13 Agarose gel electrophoresis of acid phenol treated BK(MM) DNA, showing residual chromosomal DNA (which is however significantly reduced in amount), NOC and CCC BK(MM) DNA.

3.1.2.3. Preparation of specific DNA sequences e.g. restriction endonuclease fragments, or DNA in a particular molecular conformation (e.g. CCC versus linear or NOC) was carried out by electroelution of DNA-containing bands from agarose or acrylamide gels, the latter distinction

being made according to the purpose for which the DNA species was intended. Electroelution (with yields of 30 - 85%) proved preferable to treatment with isoamylalcohol (with losses of more than 80%) for removal of agarose. However, the yields obtained by electroelution were variable, and considering the following factors, electroelution was not used as a method of choice in most cases where enzymatic reactions were to follow, or where limited amounts of DNA were available.

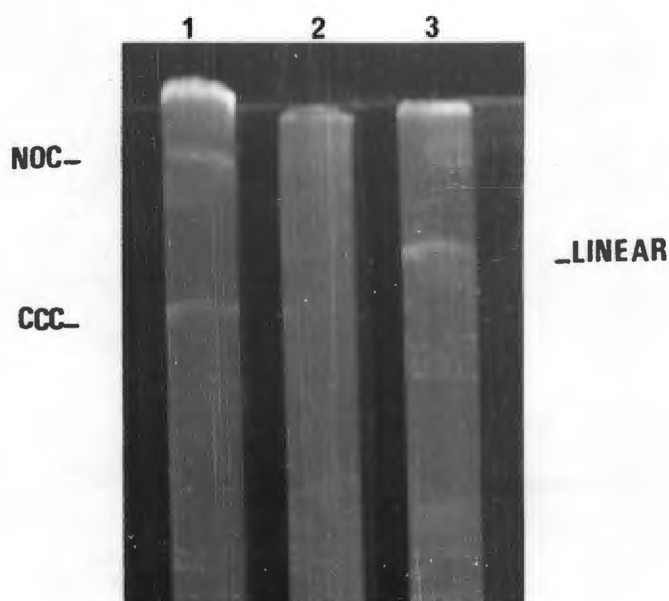


Fig.**14** 1% cylindrical agarose gel electrophoresis of

1) CCC and NOC pBR322
2) Electroeluted Bam HI linearised pBR322 and this representing the washes of the dialysis membrane i.e. no detectable DNA
3) Bam HI linearised pBR322 electroeluted; this representing the eluate and showing linear pBR322 only.

Agarose contaminating the eluate was removed by phenol and chloroform extractions and alcohol precipitations. Purification was monitored by the efficiency of restriction digests on the purified DNA. However, T4 DNA ligase was found to be highly sensitive to agarose contamination, and further washes with alcohol and 70% alcohol - 30% TES were essential for successful ligation of the DNA sample. Wherever possible, electroelution was avoided with DNA preparations intended for ligation. When

necessary however, DNA was eluted from agarose gels when prepared for ligation or restriction since acrylamide gels contain constituents which appear to introduce single-stranded nicks into DNA. This property is however useful for nick translation substrate DNA;the requirement for DNAse I is obviated whilst reaction and labelling efficiency appear to improve (see section 3.6.3). Exposure of DNA to be electroeluted to ultraviolet light was always minimised since it is known to damage DNA.

3.1.2.4 The frequency of tbansformation of E.Coli was significantly decreased by DNA samples containing residual agarose. This was avoided since transformation frequencies had to be reliably maximised, particularly when using DNA in limited supply. For this reason also, ligation of foreign DNA to vector DNA had to be optimised. Religation of the two constituent pBR322 fragments needed to be avoided to increase the chances of pBR322 ligating to BK DNA (see sections 3.5.2. and 2.2.).

3.1.2.4.1 Religation of Pst I EcoR I digested pBR322 fragments was minimised by separation of the two linear fragments by glycerol density gradient centrifugation or by electroelution, for use of the larger one only, for ligations. Disadvantages of the latter method have been discussed (3.1.2.3). 30ul aliquots of twenty 0,6 ml fractions of density gradients were analysed for DNA fragments by gel electrophoresis. The $2,14 \times 10^6$ dalton fragment of pBR322 was completely recovered in three fractions, whilst the less dense fractions were pooled and concentrated to detect the $0,46 \times 10^6$ dalton fragment. Contamination of the larger fragment with small amounts of the other fragment was significant, and resolution of the separation was not optimal. The duration of this method and this unsatisfactory result led to investigation of the following technique.

3.1.2.4.2. The alternative procedure of bacterial alkaline phosphatase treatment of Pst I EcoR I digested pBR322 was performed in order to minimise religation of pBR322. Gel analysis of untreated Pst I EcoR I digested pBR322 ligated to alkaline phosphatased - pBR322 at a j/i of greater than one, (and thus favouring formation of circular molecules of MW = $2 \times (2,6 \times 10^6)$ daltons - see section 3.3.1) showed the formation of monomer-

sized circles. If bacterial alkaline phosphatase activity was low, and the removal of 5' phosphate groups was incomplete, there should be an excess of pBR322 capable of forming monomeric circles instead of necessarily forming linear dimers first. This would result in preferential formation of monomeric circles, as occurred in this case.

It was concluded that the reaction was incomplete so a more highly purified and DNAse-free preparation of the enzyme was used in subsequent experiments. Use of this enzyme did not inhibit ligation reactions, and transformation frequencies actually increased (see section 3.3. and 3.5.2.)

3.2.1. Infection of human embryonic glial cells with BK(MM) virus.

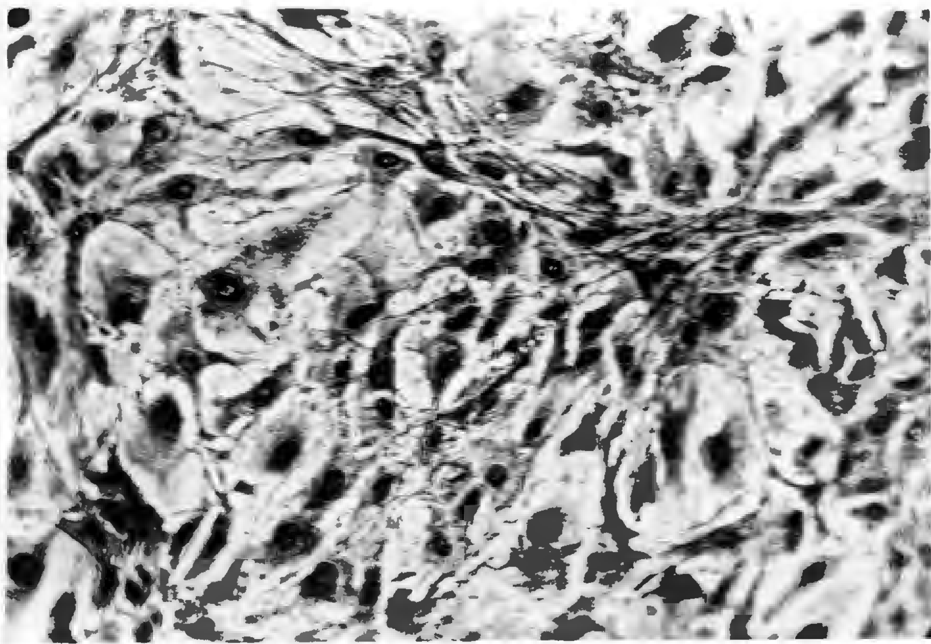


Fig.15 Control human embryonic glial cells stained with haemotoxin and eosin.

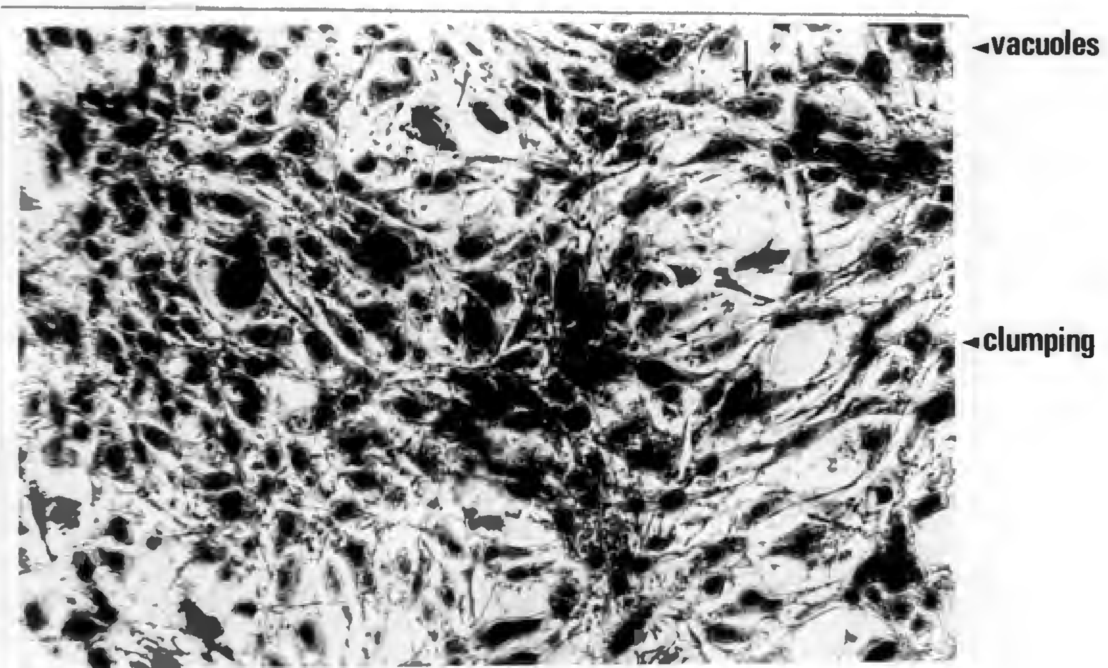


Fig.16 Human embryonic glial cells infected with BK(MM) virus and stained with haemotoxin and eosin to show cytopathic effects.

3.2.2 Extraction of BK (MM) viral DNA from human brain glial cells in culture

Maximum yield of viral DNA was obtained from infected cells, whilst an insignificant amount of DNA was extracted from the medium. Small amounts of viral DNA were lost into the Hirt's pellet (as detected by gel electrophoretic analysis), but the majority of cellular DNA was precipitated into the pellet and thus separated from viral DNA in the supernatant. Viral DNA was retrieved mostly in the CCC form with less intense bands in the NOC position on gel analysis of extracts.

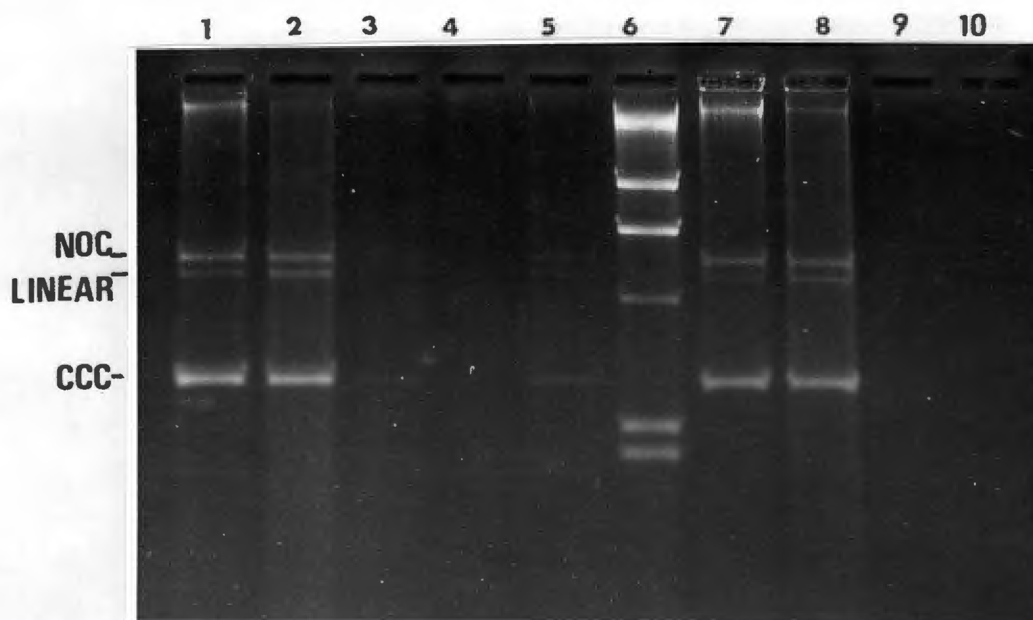


Fig.17 Agarose gel electrophoresis of Hirt's lysates of BK (MM) infected glial cells.

1. Extraction from cells infected at 10^{-2}
2. " " " " " 10^{-3}
3. Extraction from medium of cells infected at 10^{-2}
4. " " " " " " " 10^{-3}
5. " " " " " " " 10^{-3}
6. Hind III digested lambda DNA as markers.

7 - 10. Hirt's pellets of the samples in 1 - 4 respectively.

4ug of viral DNA were extracted from three 75ml Falcon flasks infected with BK (MM) DNA at a dilution of 10^{-2}. This yield was sufficient for several restriction endonuclease digestions, nick translations for probe preparation, ligations, and several transformation experiments.

Simultaneous parallel extractions of DNA from control, uninfected glial cells in culture showed only chromosomal DNA on gel analysis. Restriction analysis showing patterns expected of BK (MM) DNA confirmed that DNA extracted from infected cells was BK (MM) DNA.

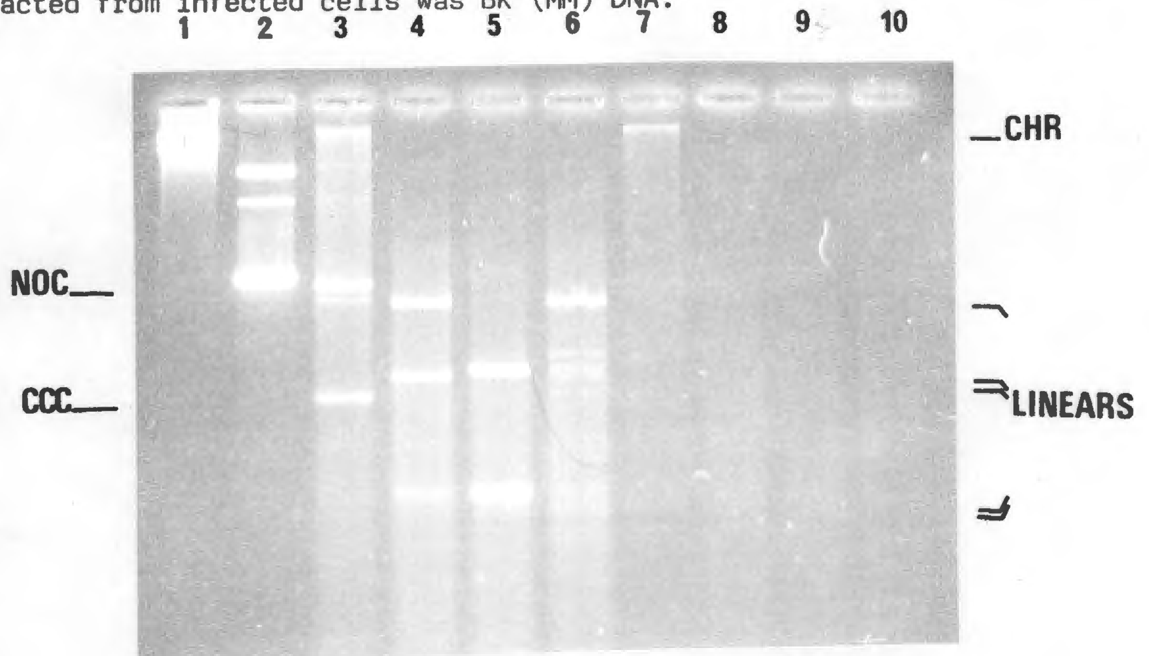


Fig.18 1% agarose gel electrophoresis of restriction digests of Hirt's extracts of BK (MM) infected glial cells.

1. Lambda DNA
2. pM2 DNA
3. DNA extracted from Hirt's lysate of infected cells
4. Hind III digest of 3.
5. Pst I " " "
6. Eco RI " " "
7. DNA extracted from uninfected cells.
8. Hind III digest of 7.
9. Pst I " " "
10. Eco RI " " "

3.2.3. Extraction of BK (WW) viral DNA from human foetal brain cell cultures infected with BK virus.

An attempt was made to infect human foetal brain cells with BK (WW) DNA in the hope of amplifying supplies. However the yield of non-chromosomal DNA was poor but initial gel analysis showed a very faint band having greater mobility than chromosomal DNA and therefore possibly the same as viral CCC DNA. It was not possible however to extract sufficient of this DNA to prepare restriction digests for further analysis or evidence that the DNA was of BK (WW) viral origin, and we decided not to use more (WW) DNA to attempt further infections.

3.2.4. Extraction of human polyoma virus DNA from two urine pellets.

0,8% horizontal agarose gel electrophoresis of the extract (derived from two litres of urine of an immunocompromised patient) showed a small quantity of CCC DNA in addition to chromosomal DNA. However after further purification by CsCl density gradient centrifugation, only a very faint band of CCC DNA was seen, with respect to the intensity of the chromosomal DNA band. Analysis of the restriction pattern for comparison with those of BK (MM) and (WW) DNA was therefore not performed.

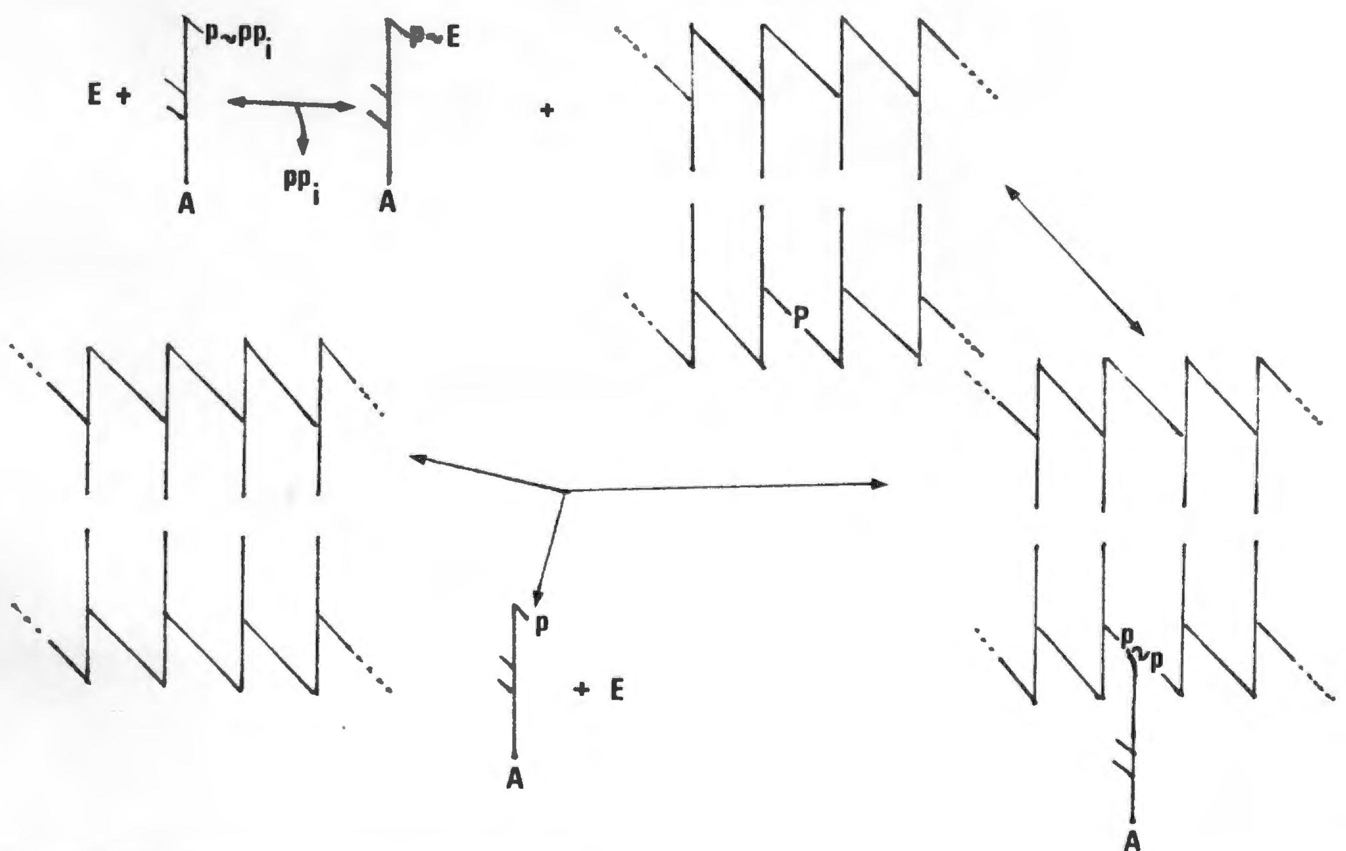
3.3. Ligation Reactions

3.3.1 T4 DNA ligase is a single polypeptide chain of 68000 daltons purified from E.Coli cells lysogenic for lambda phage chimera T4 45. T4 DNA ligase catalyses the repair of single-stranded nicks with adjacent 3' hydroxyl and 5' phosphoryl termini, as well as the end to end joining of DNA fragments with cohesive or with blunt termini . Phosphodiester bond cleavage is coupled to the cleavage of a pyrophosphate bond in ATP. The reaction has three steps;

1) The adenylation of the free enzyme with release of pyrophosphate, due to the epsilon amino group of a lysine forming a phospho-amide link with ATP. The high energy bond necessary to drive phosphodiester bond synthesis is actually stored in the enzyme before it encounters a polynucleotide chain ;
2) The transfer of the AMP moiety from the enzyme to the 5' phosphoryl group of the DNA substrate thereby recreating a pyrophosphate linkage and preserving the high energy bond; and
3) The nucleophilic attack of the adjacent 3' hydroxyl to form a phosphodiester bond and eliminating AMP.

T4 DNA ligase differs from the E Coli ligase, which uses NAD instead of ATP as cofactor.

Fig.19 Diagrammatic representation of the reaction catalyzed by T4 DNA ligase (E)



Reactions catalysed by T4 DNA ligase are more permissive than those catalysed by E.Coli DNA ligase and include the following substrates :

1) oligo(dT) - poly(dA) or poly(A)
2) oligo(dA) - poly(dT)
3) blunt ended duplexes (the nucleotide sequence of the substrate is altered here)
4) cohesive ended duplexes e.g. as generated by type II restriction endonucleases
5) RNA-DNA linkages are catalysed
6) RNA-RNA linkages at a rate of at least 2 orders of magnitude lower than with DNA substrates.

The turnover rate for T4 DNA ligase is 1 mol/min per mole of ligase with cohesive termini. The apparent Km for blunt ends, in terms of 5' termini is 50uM (with RNA ligase present), which is two orders of magnitude higher than for substrates with cohesive ends (Sugino et al., 1977; Higgins et al, 1979). Ligations of cohesive ends is not affected by T4 RNA ligase but at low concentrations of DNA ligase, the ligation of blunt ends is stimulated by T4 RNA ligase.

Joining of cohesive ends is highly efficient with all sizes of molecules participating, but the degree of inter- or intra-molecular ligation depends on the DNA concentrations. In general it can be said that circularisation and intramolecular ligation is favoured by low substrate concentration whereas linearisation or intermolecular ligation is favoured by high substrate concentration. The distribution of molecular species formed during ligation can be calculated by a method described by Dugaiczyk et al, 1975. Knowledge is required of the DNA concentrations and the molecular weights of the fragments involved since the size of the molecules imparts physical constraints on them, and affects their ability to form intramolecular complexes by ligation. Two parameters must be considered :

1) j, the effective concentration of one end in the neighbourhood or volume of the other end of the same molecule, and $j = \left(\frac{3}{2\pi lb}\right)^{3/2}$ mols/ml

   where l = contour length and b = random coil segment length of a DNA molecule determined by sedimentation coefficients of the DNA. b is a parameter which is dependent on ionic strength which is standardised in the presence of the enzyme.

2) i, the total concentration of single-stranded self-complementary ends of a duplex linear DNA molecule/ml.
$i = 2\ N_0 M \times 10^{-3}$ ends/ml where N_0 = Avogadro's number and
M = molar concentration of the DNA molecules.

The above constants are known for lambda DNA; consequently the j/i ratios for any DNA containing solution can be calculated :

$$j/i = \frac{j_\lambda \left(\frac{MW_\lambda}{MW}\right)^{3/2}}{2\ N_0 M \times 10^{-3}} = \frac{51,1}{[DNA]\sqrt{MW}}$$

Ligation of DNA fragments when j is calculated to equal i, was found to lead largely to concatemerisation of linear molecules, which leads to the production of multimers up to decamers, representing all the vectorial combinations possible with the vectorial ends involved (i.e. $\longrightarrow$ $\longrightarrow$; $\longleftarrow$ $\longleftarrow$ joining of ends). In theory, when i is smaller than j, circularisation should occur, whilst when i is greater than j, linearisation or intermolecular ligation should be favoured. In practice it was found that i should be considerably less than j before circularisation predominates over linearisation, i.e. j/i is greater than or equal to 2. This may be due to the small size of the original linear molecules e.g. pBR322 and BK DNA have fewer than twenty segment lengths, with the result that j increases and therefore the j/i value necessary for circularisation increases. The interaction of ligase with DNA may increase the value of b with the result that j decreases and j/i decreases; with consequent reduction of circularisation yet unaffected linear polymerisation of molecules. In addition, the production of circular molecules increases with time since linearisation results in decreased i values and therefore an increased j/i ratio of the solution. As a result, the majority of linear molecules eventually circularise and the relative amount of circular products increases. The circular products are not substrates for ligase.

Considering cross-ligation of DNA species with self-complementary ends, j remains unaltered by the presence of a second DNA species, whilst i is taken as the sum of the i values of each DNA species. Molar ratios of the two DNA fragments equals the ratio of their i values.

3.3.2. Ligase reactions were initially studied on Hind III digests of commercially prepared lambda DNA. A change in banding pattern was taken to indicate that ligation had occurred e.g. Fig. **20** shows a gel analysis of a five hour ligation at 23°, of 800ng Hind III lambda DNA ligated at a j/i ratio of 1,5 and using two Weiss units of T4 DNA ligase.

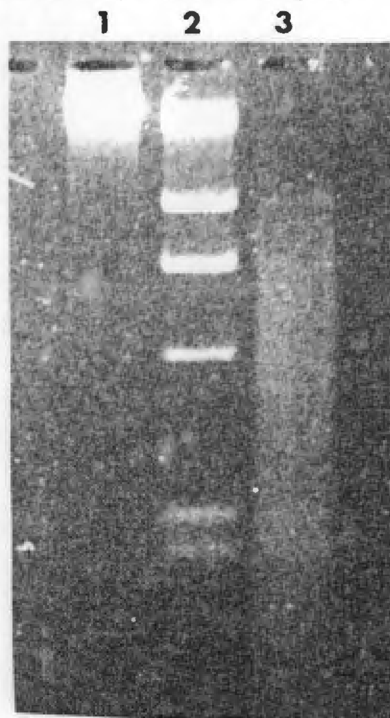


Fig.**20** 1% horizontal agarose gel electrophoresis of

1. Lambda DNA
2. Lambda DNA digested with Hind III
3. Lambda DNA digested with Hind III and ligated at j/i of 1,5.

It can be concluded that ligation was incomplete due to the residual two smallest MW fragments running in unaltered positions. However evidence of ligation included disappearance of the other bands, and the smear of fluorescence represents a range of molecules of varying molecular size formed by ligation of fragments to form linear molecules. In this particular case the probability of DNAse contamination arose, due to the smear of fluorescence below the smallest Hind III digest fragment, indicating a certain amount of DNA degradation.

3.3.3. Ultimately, the desired ligation products for transformation of cells would be circular dimers formed by intermolecular ligation. To distinguish between inter- and intra-molecular ligation and between cir-

cularisation and linearisation, it was necessary to study ligation product profiles of circular molecules which had been linearised with restriction endonucleases having a unique site on the molecules. These results were expected to be less complex to interpret than those obtained with multiple linear substrates of various sizes. Substrates included pBR322, Col EI DNA, and BK(MM) DNA. An EcoRI digest of Col EI DNA was ligated at j/i = 1,2 and was analysed after 2 and 6 hours at 12° with either 0,1 or 0,2 Weiss units of ligase.

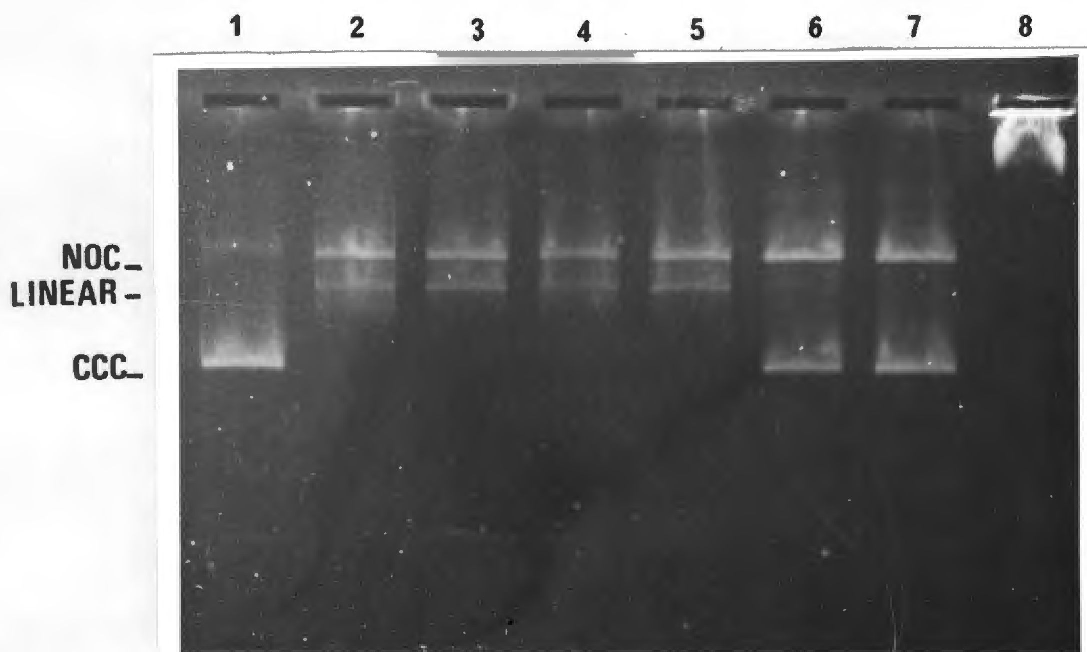


Fig.21 1% horizontal agarose electrophoretic analysis of
1. Col EI DNA
2. & 3. Eco RI digested Col EI DNA
4. & 5. " " " " " ligated for 2 hours
6. & 7. " " " " " ligated for 6 hours
8. Lambda DNA

Fig.21 shows no change in banding pattern after two hours, but after six the linear band disappeared concomitant with the appearance of a monomer CCC band. The band running in the NOC position is as intense as the CCC band, although this was not the case originally, when CCC was predominant. (Band intensity represents to some extent the relative abundance of DNA molecules). This band may represent a dimer CCC molecule although no dimer linear molecules are seen running slower than the NOC position,

where they would be expected to run. It is therefore probable that the band represents NOC monomeric molecules. Two hours at 12° appears to be insufficient for complete ligation, yet there is no difference between the ligation products obtained with 0,1 and 0,2 Weiss units of ligase. Digestion of Col EI DNA with Bam HI yields two linear fragments and ligation of these at j/i = 2,4 at 12° allowed complete ligation of the linear molecules to form a monomeric CCC molecule with the majority in the non-supercoiled form and therefore moving in the NOC position.

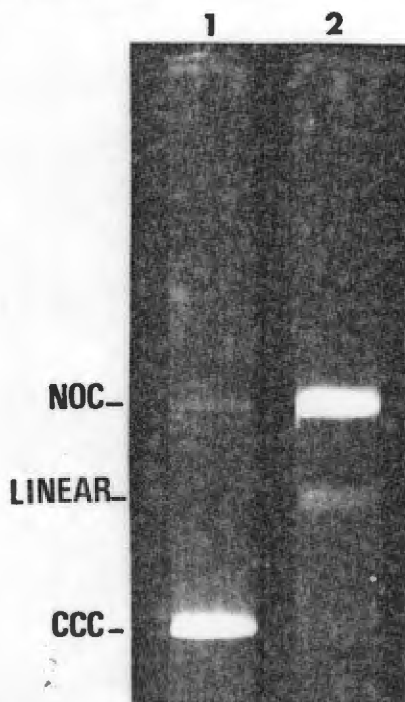


Fig.22 1% agarose gel electrophoretic analysis of
1. Col EI DNA
2. Col EI DNA digested with Bam HI and ligated.

A Hind III digest of pM2 DNA ligated at j/i = 2,0 at 20° for five hours showed multiple bands representing various ligated combinations of the linear molecules, whilst no original digest bands remained, indicating successful ligation. Bands are evident coinciding with the full length monomer and dimer pM2 CCC molecules, which would be expected to increase in intensity with time as linear molecules circularise.

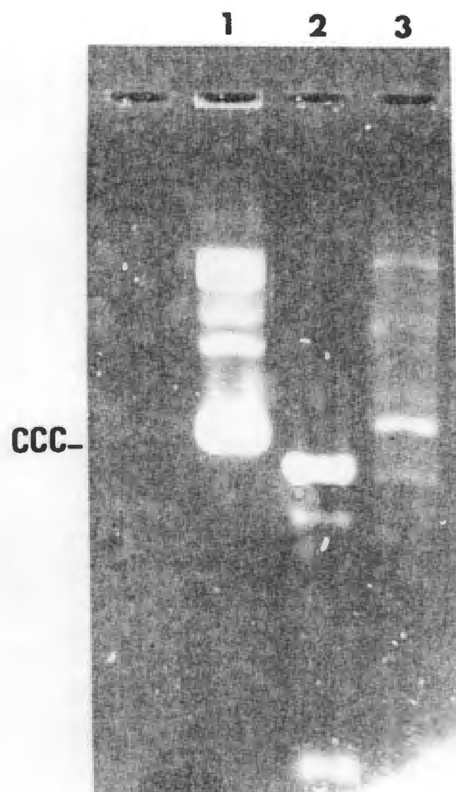


Fig.**23** 1% horizontal agarose electrophoretic analysis of

1. pM2 DNA
2. Hind III digested pM2 DNA
3. Hind III digested pM2 DNA ligated at j/i = 2,0

The degree of supercoiling, or the winding number, of CCC molecules after ligation would not be expected to be the same as that obtained <u>in vivo</u> , since various specific enzymes, e.g. - DNA gyrase, introduce a specific number of turns into the DNA molecule as it circularises. These enzymes are, however, not present <u>in vitro</u>, and it would therefore be expected that the NOC molecule would be ligated in a relaxed conformation and have electrophoretic mobility of the NOC molecule. This occasionally happens, but not consistently as seen by comparing fig. **24** and figs.**21 & 23**, where CCC molecules migrate as though they have the same number of turns as undigested molecules have.

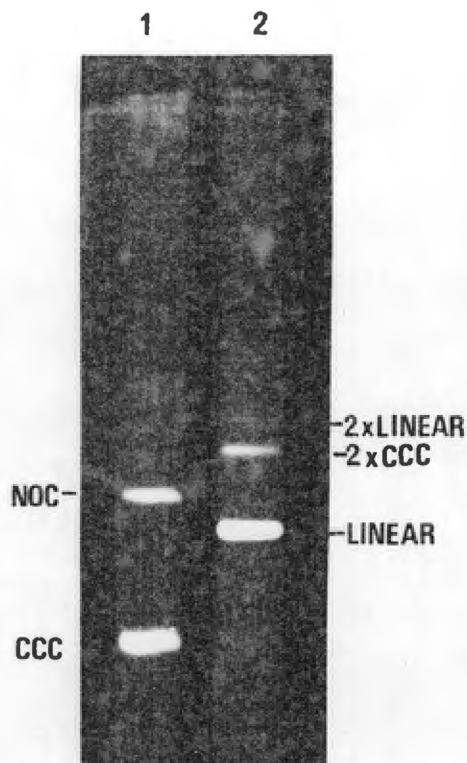


Fig.**24** 1% cylindrical agarose electrophoretic analysis of
1. pBR322
2. Bam HI digested pBR322, ligated at j/i = 5,8 favouring formation of CCC monomers, dimers.

It may be that certain constraints exist on the circular molecules in the reaction mixture such that the number of turns varies continuously with the result that a random number of turns exist at the ligation event. Alternatively, the local environment's ionic strength prevailing at the time of ligation, or the binding of ligase itself may induce a different and specific degree of supercoiling with the result that ligated monomeric circles migrate slower than undigested circular monomers although still faster than NOC molecules.

3.3.4. The effect of varying j/i ratios on the relative abundance of ligation products was investigated using Bam HI linearised pBR322.

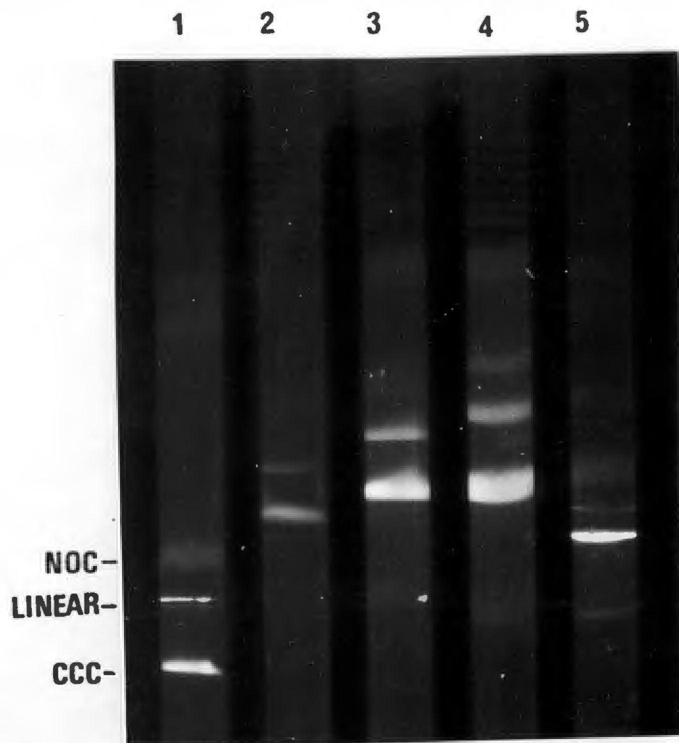


Fig.**25** 1% cylindrical agarose gel electrophoresis of
1) pBR322,
and Bam HI digested pBR322 ligated at a j/i = 2) 5
3) 1
4) 0,8
5) 0,5

A j/i ratio of 5 (favouring circularisation of monomers) yielded no linear monomers but two species probably representing dimer and trimer CCC molecules. Linear monomers are seen at j/i of 1; 0,8; and 0,5, in addition to higher MW molecules of linear dimers and trimers (which move more slowly than their CCC counterparts) with less abundant higher multimers. As the j/i ratio decreases, larger linear molecules appear. A j/i ratio of 0,5 gave an interesting picture of linear monomers, dimers, trimers and tetramers, but the most intense band ran in the position of a NOC monomer. This is unexpected and it is quite possible that this extremely distinct band is artefactual.

3.3.5. After gel analysis to check completion of digests, a Pst I and a Pst I Eco RI double digest of pBR322 were separately subjected to ligation whilst varying 1) j/i ratios, i.e. j/i of 1,44 or 3,5

2) time i.e. 2 or 16 hours

3) temperature, i.e. 4°, 12° or 20°,

using 0,5 Weiss units of ligase per reaction volume of 20 or 50ul. The following results were obtained after analysis by agarose gel electrophoresis.

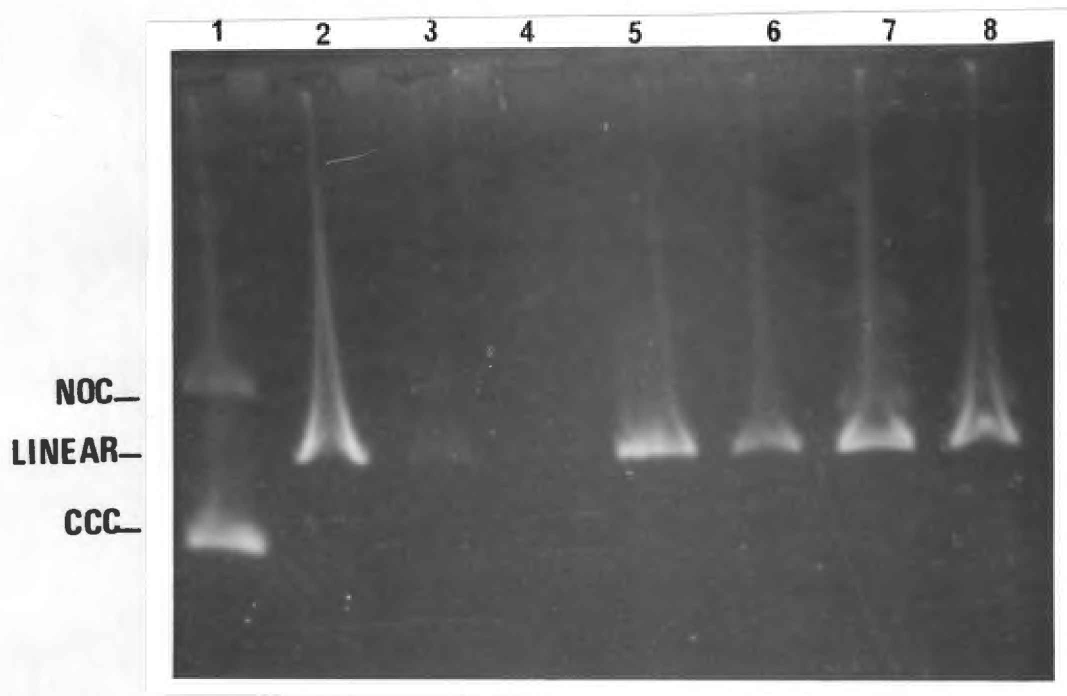


Fig.**26** 1% horizontal agarose gel electrophoresis of

1) pBR322
2) Pst I digested pBR322
3) " " " ligated at 4° for 2 hours)
4) " " " " " " " 16 hours)
5) " " " " " 12° " 2 hours)
6) " " " " " " " 16 hours) at j/i of 1,44
7) " " " " " 20° " 2 hours)
8) " " " " " " " 16 hours)

At 4° and 20° predominantly full length linear molecules were obtained. Dimer CCC molecules appeared after two hours at 12° and increased in intensity after 16 hours. In addition, at 20° a slower moving band

appeared but decreased in intensity after 16 hours and therefore possibly represented a dimer linear molecule. It appears therefore that a compromise (of 12°) between the enzyme's optimal temperature of 37° and the melting temperature of the noncovalently joined cohesive ends i.e. 5-6°, yields monomeric circles in preference to dimeric circles as found at 4° where temporary binding of separate molecules is less readily broken than at 12°. It appears also that intermolecular noncovalent binding is too transitory at 20° to allow ligations to occur.

The following results were obtained after ligating Pst I Eco RI digested pBR322 at j/i ratios of 1) 3,5 with respect to a molecule of 2.6×10^6 daltons; of
2) 8 with respect to one of $0,46 \times 10^6$ daltons; of
3) 3,9 with respect to one of $2,14 \times 10^6$ daltons; and of
4) 2,5 with respect to a dimer of $5,2 \times 10^6$ daltons.

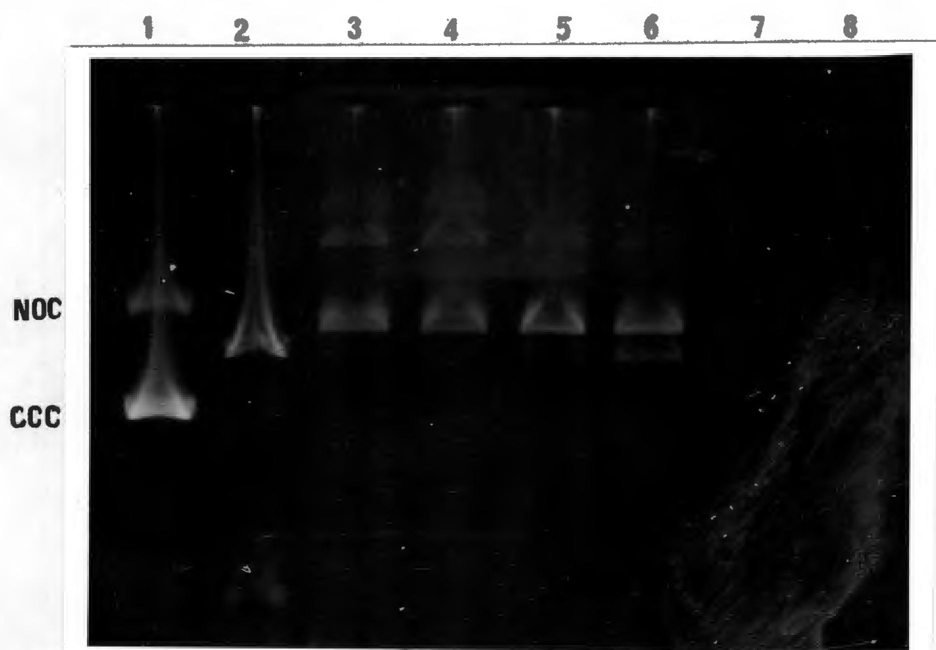


Fig.**27** 1% horizontal agarose gel electrophoresis of
1) pBR322
2) Pst I Eco RI digested pBR322
3) Pst I Eco RI digested pBR322 ligated at 4° for 2 hours
4) " " " " " " 16 "
5) " " " " " 12° 2 "
6) " " " " " " 16 "
7) " " " " " 20° 2 "
8) " " " " " " 16 "

Predominantly monomer CCC molecules were produced with a small amount of dimer CCC and larger linear molecules found, particularly at 4° and 20°. Again, 12° incubation gave an optimum of monomer circles, whilst more multiple linear molecules are formed at 4°. No great difference in the relative amounts of products is observed between 2 and 16 hour incubations.

In conclusion, the relative amount of CCC ligation products increases from 4° to 12° but a further increase in temperature to 20° makes no significant difference, or may even yield fewer CCC molecules with respect to linear molecules. This confirms observations made by Dugaiczyk et al, 1975.

3.3.6. An incomplete Pst I digest of pBR322 with linear and NOC species present in approximately equimolar amounts, was ligated at j/i = 1,0 or 2,0 with either 1,0 or 2,0 units of ligase.

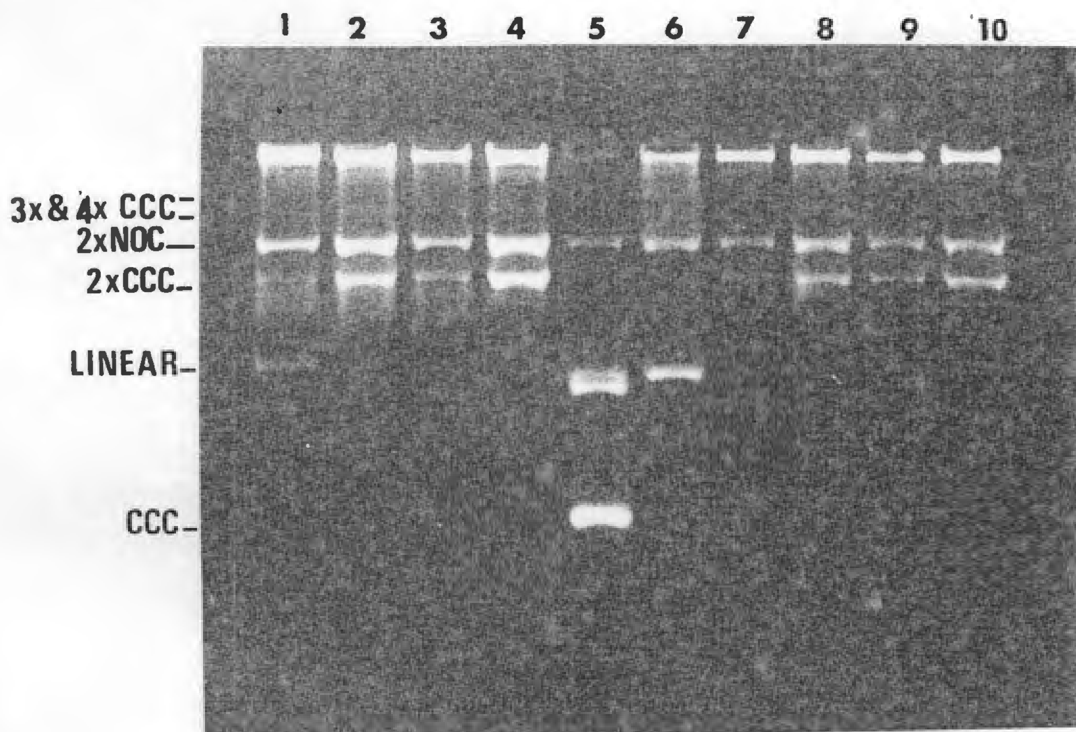


Fig.28 1% horizontal agarose gel electrophoresis of

1) Pst I digested pBR322 ligated at j/i = 1 with 1,0 unit ligase for 2 hours
2) Pst I digested pBR322 ligated at j/i = 1 with 1,0 unit ligase for 6 hours
3) Pst I digested pBR322 ligated at j/i = 1 with 2,0 unit ligase for 2 hours
4) Pst I digested pBR322 ligated at j/i = 1 with 2,0 unit ligase for 6 hours
5) pBR322

6) Pst I digested pBR322
7) Pst I digested pBR322 ligated at j/i = 2 with 1,0 unit ligase for 2 hours
8) Pst I digested pBR322 ligated at j/i = 2 with 1,0 unit ligase for 6 hours
9) Pst I digested pBR322 ligated at j/i = 2 with 2,0 unit ligase for 2 hours
10) Pst I digested pBR322 ligated at j/i = 2 with 2,0 unit ligase for 6 hours.

The same product profile was achieved using 1 or 2 units of ligase, however use of more ligase speeded up the reaction. A j/i ratio of 1,0 yielded more high MW linear molecules than a j/i of 2,0 did, showing a tendency to form linear molecules before circularisation. The smear of bands seen in lane 1) and representing dimer and trimer linear molecules disappeared after 6 hours to give two bands taken as being dimer NOC and CCC molecules. At a j/i of 2,0 the NOC band was more intense than the CCC band was at 2 hours, but reached equal intensity after 6 hours incubation. Here an example is shown of linearisation occurring until i is reduced to an extent which increases the j/i ratio to favour circularisation of dimers and trimers.

3.3.7. Trial ligations were performed with Pst I Eco RI digested pBR322 and lambda or BK(MM) DNA, whilst varying vector : insert ratios.

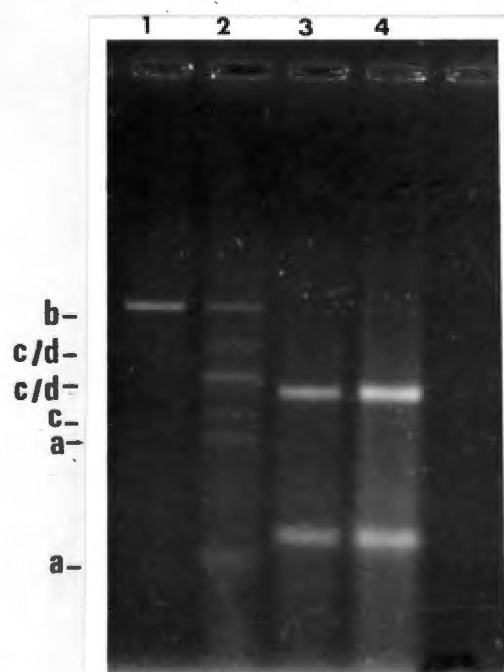


Fig.29 1% horizontal agarose electrophoresis of
1) Pst I digested BK (MM) DNA
2) Pst I Eco RI digested BK(MM) DNA ligated with six-fold

excess Pst I Eco RI digested pBR322

3) Pst I Eco RI digested BK(MM) DNA
4) Pst I Eco RI digested pBR322 ligated with 1,6 fold excess Pst I Eco RI digested BK(MM)

With a 1,6 times excess of BK(MM) over pBR322 DNA, at a j/i of 4,5 with respect to pBR322, no ligation was evident in that only the two fragments of BK (MM) DNA were seen after 18 hours. However, a six-fold excess of pBR322 at j/i of 1,4 showed (Fig.**29**),

a) residual unligated pBR322;
b) full length linear BK(MM) or possibly CCC pBR322 not supercoiled and running in the NOC position;
c) a recombinant of either the large fragment of BK(MM) and the small fragment of pBR322, or the large fragment of pBR322 and the small fragment of BK(MM); and
d) full length linear pBR322.

In addition bands seen could represent full length pBR322 plus the BK(MM) small fragment, or the large fragment of BK(MM) ligated to two small fragments of pBR322 and two small fragments of BK(MM), or a recombinant of the small BK(MM) fragment and the small pBR322 fragment. In conclusion, a j/i ratio of 1,45 favours linearisation but not circularisation, which infers that recombination for transformation requires a j/i ratio greater than 1,45.

3.3.8. The large Pst I Eco RI fragment of pBR322 was extracted by glycerol density gradient centrifugation and ligated at j/i ratio of 3,5 with Pst I Eco RI digested lambda DNA, using 2,5 Weiss units of ligase in a total volume of 25ul. Previous experiments had shown the ligase to be active, however the reaction in this case was unsuccessful; it was suspected that high glycerol concentrations (in which ligase is stored) could inhibit the enzyme. Successful use of more dilute enzyme (0,5 Weiss units in a 50ul reaction volume) showed this to be correct. e.g. Hind III digested pM2 DNA ligated for five hours gave a myriad of bands representing linear DNA, circular monomers and dimers. Subsequently therefore T4 DNA ligase was diluted with ligation buffer prior to use (even if this resulted in larger reaction volumes) to avoid inhibition of the enzyme by high glycerol concentrations.

3.3.9 Instead of, or in addition to monitoring ligation reactions by biochemical characterisation of the products by gel electrophoresis, the success of ligation was determined by transformation efficiencies. NOC and CCC molecules only will transform competent cells whilst linear DNA actually inhibits the uptake of NOC or CCC DNA possibly by coating the cell walls, (see section 3.4.3). Obtaining colonies exhibiting correct antibiotic sensitivities is indicative therefore of circularisation having occurred during ligation, although proof of actual recombination events requires further screening of colonies.

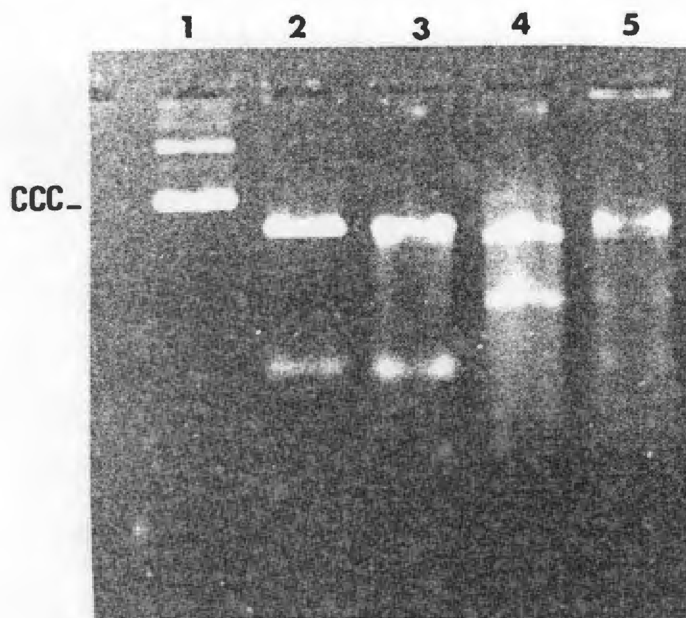


Fig.30 1% horizontal agarose electrophoresis of

1) pBR322
2) Pst I Eco RI digested pBR322
3) Pst I Eco RI digested pBR322 ligated with Pst I Eco RI digested pBR322
4) Pst I Eco RI digested BK(MM) DNA
5) Pst I Eco RI digested BK(MM) DNA ligated with Pst I Eco RI digested pBR322

Pst I Eco RI digested pBR322 was ligated with itself at a j/i ratio of 2,0 and with similarly digested BK(MM) or (WW) at a j/i ratio of 3.1.

Transformation frequency was very low therefore biochemical characterisation of the ligation products was necessary. This revealed that poor ligation had occurred in that the majority of original linear bands remained whilst very faint CCC bands were evident with BK(MM) DNA (ligated with pBR322) only. The decreased transformation frequency obtained was attributed to the addition of minimal amounts of CCC DNA to the cells since the (WW) viral DNA was in short supply.

A transformation frequency of 10^2 colonies/ug DNA was obtained with χ1776 exposed to Pst I Eco RI digested pBR322 and lambda DNA which had been ligated at a j/i ratio of 4,6 with respect to the large fragment of pBR322. All colonies however were pBR322 transformants as opposed to recombinants since all were A^R T^R i.e. no insertional inactivation of the ampicillin gene had occurred. Digests were known to be complete as analysed by gel electrophoresis. It was concluded therefore that ligation to yield CCC molecules had occurred to an extent where residual linear DNA did not inhibit uptake of DNA.

3.3.10. Ligation results were more reproducible when a purer and more active commercial preparation of T4 DNA ligase was obtained. 0,25 Weiss units of this enzyme were used to ligate Pst I Eco RI digested pBR322 with itself at a j/i ratio of 4,6, or with similarly digested BK(MM) DNA at a j/i ratio of 2,4. Fresh ligation buffer and ATP were used and no EDTA was used to stop the reaction. After two hours incubation at 12,5°, products were electrophoresed for analysis, see figure **31**.

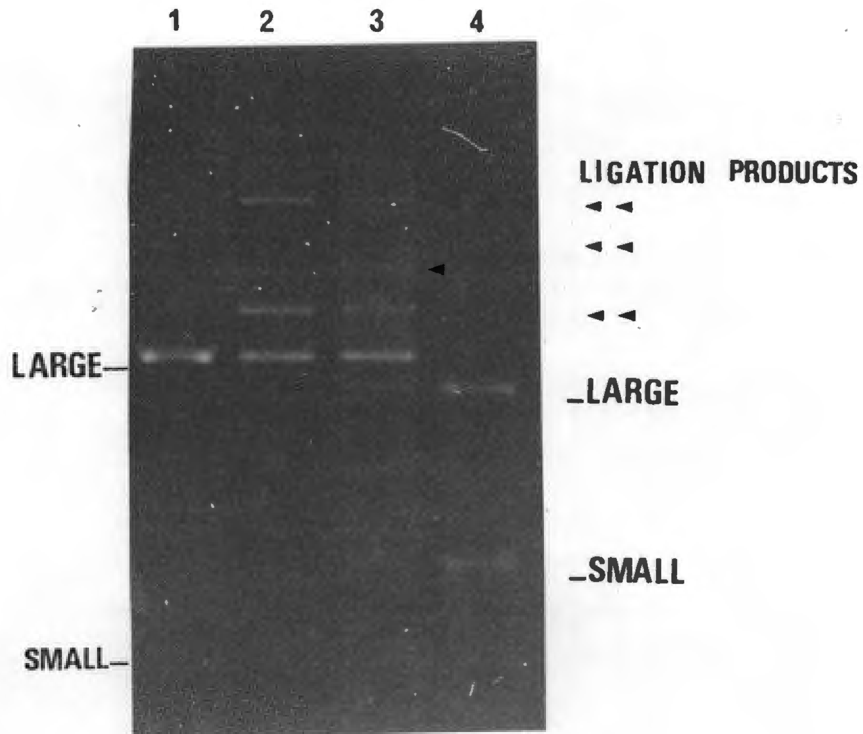


Fig.**31** 1% horizontal agarose electrophoresis of

1) Pst I Eco RI digested pBR322
2) " " " " " ligated with Pst I Eco RI digested pBR322
3) Pst I Eco RI digested pBR322 ligated with Pst I Eco RI digested BK(MM) DNA
4) Pst I Eco RI digested BK(MM) DNA.

Since this ligation was successful the same conditions were subsequently used for ligations of limited quantities of DNA to be used for transformation experiments. Optimal conditions for production of circular molecules of 4,84 x 10^6 daltons and of 3,04 x 10^6 daltons were considered to be the following :

1) j/i ratios between 2,5 and 5,8 with respect to a 4,84 x 10^6 dalton molecule.
2) " " " 5,9 and 7,0 " " " " 3,04 x 10^6 " "
3) equimolar amounts of the two DNA species involved
4) 0,5 Weiss units of T4 DNA ligase in a 20ul reaction volume, or per a maximum of 200ng DNA. These considerations were confirmed by successful transformation of C600 and HB101 with recombinant pBR322 - BK(MM) DNA molecules (see section 3.7).

3.4 Transformation of Escherichia Coli.

3.4.1 Transformations had not previously been performed in this laboratory, so methods were investigated and developed from numerous protocols in the literature in order to obtain the optimum methods. Transformation frequencies had to be maximised simply due to the small amount of BK(WW) DNA available, limiting the possible number of transformation attempts to at most, two. Prior to van der Noordaa's publication in 1977 and the subsequent decision to adapt a subgenomic cloning approach at the EK1 level of containment, χ1776 was used as the recipient strain. (See section 2.2.) Attempts to increase the transformation frequency made use of methods described by Curtiss et al., 1977, and Norgard et al., 1978, who had investigated many parameters of the procedure used to render χ1776 competent. A frequency of 10^7 transformants /ug DNA was claimed, however this was not equalled in my attempts with χ1776.

3.4.2 DNA used in the preliminary experiments was undigested pBR322 which was either commercially prepared or extracted from transformed C600 and HB101; this eliminated all variables attributable to restriction and ligation of DNA. In many cases the cell viability was high but no uptake of DNA occurred, shown by the fact that no cells grew on selective antibiotic-containing plates. Successes with the transformation of χ1776 were always at a low frequency, which was moreover, not reliably reproducible. This was unsatisfactory for use with our limited supply of viral DNA. Preliminary optimisations of the method were performed with comparably small amounts of pBR322 for direct comparison with the conditions to prevail when BK(WW) DNA was to be used.

A comparison was made therefore between the transformation frequencies obtained using 5ug amounts of 1) unrestricted circular pBR322,
2) Bam HI linearised pBR322, and
3) Bam HI digested pBR322 ligated at a j/i ratio of 5 to induce circularisation of monomer and/or dimer molecules.

Although cell viability was not optimal (growth on nonselective plates was not quite confluent), the frequency of transformation was 4×10^3 colonies/ug pBR322, slightly lower with ligated Bam HI digested pBR322, and 2×10^2 colonies/ug linearised pBR322. As expected, the latter is lower than with circular DNA since linear DNA is inefficient at cell transformation. (see section 3.4.3).

A transformation frequency of 2,8 x 10^3 colonies/ug DNA was obtained using Norgard's procedure with 10ng CsCl-EtBr purified pBR322 DNA resuspended in $CaCl_2$ buffer. Extraction of the DNA of an Amp^R Tet^R clone showed that the dimer molecule was taken up, although the sample DNA was circular undigested pBR322. Concatemers of pBR322 are known to exist in E.Coli hosts and the DNA is often extracted as multimer CCC forms of the monomer. Commercial preparations of pBR322 state that up to 10% of preparations consist of dimer molecules. The general capacity for recombination in the E.Coli cell appears to be constituted in the rec system(s) which comprise(s) of two pathways governed by three genetic loci; A,B and C. Both pathways rely on an active recA gene product, and in addition this product is implicated in single strand DNA integration, repair, and sex factor insertion into the bacterial chromosome. Two possible roles have subsequently been proposed for the recA gene product; it may be an enzyme catalysing a step common to all these pathways, or it may be a control factor regulating necessary activities of the pathways. $RecA^-$ E.Coli therefore cannot integrate DNA into the recipient genome and lack virtually all ability to form recombinants when used as recipients for transformation or conjugation. Such $recA^-$ bacteria, e.g. C600, are used as hosts for cloning experiments since the foreign DNA will not be integrated into the host chromosome, but will be maintained as episomal DNA. The DNA should theoretically not be altered whilst in the host cell; however the occurrence of concatemers of pBR322 in C600 and HB101 raises the possibility of these hosts utilizing alternative recombination pathway(s) not yet characterised.

3.4.3. Transformation frequencies of X1776 and of C600, obtained using Norgard's method, were compared using various DNA samples:

1. commercially prepared CCC pBR322
2. Pst I digested and ligated pBR322, and
3. the two fragments of pBR322 digested with PstI and EcoR I ligated with the 18 fragments of Pst I and EcoR I digested lambda DNA.

The uptake of DNA in the third case would be expected to be the least, since residual linear DNA (as opposed to CCC DNA), inhibits the process of DNA uptake, possibly by competing with circular molecules for recognition sites on the cell surface, (Bolivar and Backman, 1979). e.g. 1,25ng linear DNA/ng plasmid DNA has been shown to reduce transformation efficiencies to about 75% of maximum values (Norgard et al., 1978). Competent cells were found to have

high viability with minimum contamination (tested by growth on Blood Agar and McKonckey's Agar plates, on which χ1776 will not grow.) $1{,}3 \times 10^4$ transformants/ug commercially prepared pBR322 were obtained using χ1776 whilst $5{,}6 \times 10^7$ were obtained with C600. The number of transformants was reduced by up to 10^3 using Pst I digested and ligated pBR322, i.e. $5{,}3 \times 10^3$/ug with χ1776 and $3{,}3 \times 10^4$/ug with C600. (Cell viability however was simultaneously reduced, possibly by the ligation buffer and/or EDTA present; see section 3.4.4). Pst I EcoRI digested and ligated pBR322 yielded 4×10^2/ug DNA with C600 but no colonies were obtained with χ1776. It was concluded that use of ligated DNA after a single digestion reduced transformation frequency by an order of magnitude of from one to three, whilst doubly digested and ligated material reduced the number of transformants by at least 10^4. These results correlate with those of Norgard et al., 1978. Transformation with pBR322 prepared in another laboratory yielded a similar number of transformants ($0{,}28 - 0{,}36 \times 10^3$/ug Pst I EcoR I or Bam HI EcoR I lambda DNA ligated with similarly restricted pBR322). C600 was found therefore to have a transformation frequency 10^4 times greater than χ1776, emphasising the preference for use of this EK1 host where possible.

Comparison of Transformation Frequencies

DNA sample	E.Coli recipient strain χ1776	C600
1. Commercially prepared pBR322	4×10^3/ug	-
	$1{,}3 \times 10^4$/ug	$5{,}6 \times 10^7$/ug
2. pBR322, extracted and purified	$2{,}8 \times 10^3$/ug	6×10^7/ug
3. Pst I digested;ligated pBR322	2×10^2/ug	-
	5×10^3/ug	$3{,}3 \times 10^4$/ug
4. Pst I EcoR I digested commercial pBR322 ligated with Pst I EcoR I digested lambda DNA	0/ug	4×10^2/ug
5. As in (4) but with extracted and purified pBR322	$0{,}2 \times 10^2$/ug	$0{,}3 \times 10^3$/ug

Transformation frequencies expressed as Tet^R colonies/ug DNA.

3.4.4 Various preparations of DNA in different buffers were investigated with respect to their effect on cell viability and their susceptibility to uptake by competent C600 cells. The modified $CaCl_2$ method was used (see section 5.2.13.2) which claims a transformation frequency of 10^6/ug pBR322. 12ng of commercially prepared CCC pBR322 yielded 6 x 10^7 Tet^R colonies/ug DNA, which was in the same order of magnitude as the yield obtained using pBR322 which had been extracted from bacteria and purified. Addition of ligation buffer and $CaCl_2$ to competent cells very much reduced their viability compared with cells exposed to $CaCl_2$ buffer only. Uptake of 12ng of commercially prepared CCC pBR322 was reduced to yield 10^4 transformants/ug DNA by the addition of a small volume (3ul in a total volume of 30ul) of ligation buffer to the $CaCl_2$ buffer. Removal of all ligation buffer was achieved by 70% ethanol 30% $CaCl_2$ washes, followed by thorough removal of ethanol by vacuum drying the DNA.

192ng of Pst I EcoR I digested pBR322 ligated with similarly restricted lambda DNA treated in the latter manner to remove ligation buffer constituents yielded 2 x 10^5 transformants/ug DNA, although only 2 x 10^2 of these were transformants containing recombinant molecules (as opposed to containing pBR322 only). This increased frequency of transformation eventually achieved was contributed to by a number of factors;

(a) a method more suited to C600 than Norgard's method was,
(b) the overnight incubation of the cells in $CaCl_2$ increased their competency (Dagert and Erhlich, 1979),
(c) 10ul aliquots of a 1:10 dilution of the stock DNA-cells in Luria broth, were plated out in contrast to plating out undiluted cells;
(d) ligation reactions were not terminated with EDTA, which quite probably reduces the transformation frequency by chelating Ca^{2+} ions, (Bolivar et al., 1977), and
(e) minimal volumes of ligation buffer (in the DNA solutions) were added to the cells.

Methods described by Molholt and Doskočil,1978, claimed a six-fold increase in the transformation frequency by growing cells in 0.5M sucrose to weaken the bacterial murein layer, and then adding 1 ug/ml lysozyme to the DNA solution. A three-fold increase was found in the transformation frequency of HB101 but we obtained no increase with C600.

Storage of cell cultures in broth at 4° for 24 hours had no noticeable effect on the viabilities of C600, C600 with $CaCl_2$ buffer, or C600 with $CaCl_2$ and pBR322. However, few cells survived after storage in ligation buffer and $CaCl_2$, or in the presence of ligation buffer and $CaCl_2$ and DNA. The viability of cells in the presence of commercially prepared pBR322 and in $CaCl_2$, was slightly reduced, whilst that of cells in the presence of Pst I EcoR I digested-pBR322 and - lambda DNA with very little ligation buffer (at most 5ul) was greatly reduced. It appears that there is a toxic or an inhibitory component in the ligation buffer, storage of cells in which or exposure to which, greatly reduces cell viability and ability to take up foreign DNA.

Whilst testing various methods for achievement of an increased transformation frequency, results were not accurately quantitated. Nevertheless the following additional points were found to be significant;

1. In combination with a 1:10 dilution of L-broth cell cultures prior to plating out, a 2 minute 45° heat shock increased transformation frequency, although the presence of EDTA in the DNA solution can counter this treatment e.g. C600 showed up to 10 fold increases whereas HB101 exhibited smaller increases, if any at all.

2. Cells grown to an OD_{600} of 0,2 to 0,3 (early late log phase) transformed at a higher frequency than those grown to an OD_{600} of 0,6 or higher. This correlates with the results of Dagert and Erhlich, 1979, where two times lower competence was found with cultures grown to an OD_{650} of 0,5 instead of 0,2.

3.4.5. Selective plates were used fresh, but if necessary were stored in the cool and the dark to prevent degradation or inactivation of the antibiotics, particularly tetracycline. The endogenous resistance of E.Coli strains to tetracycline (both oxy - and chlor - tetracycline) was found to differ i.e. HB101 was sensitive to as little as 12,5ug/ml whereas C600 was resistant to as much as 20ug/ml. Incubation of selective plates at 37° was not carried out for more than two days, since by this stage, the bacteriostatic effect of tetracycline (Atherly, 1974) was reduced somewhat such that background colonies were able to grow and imply falsely high frequencies of transformation.

3.5 Results of transformation of C600 and HB101 with pBR322, digested with Pst I and EcoR I and ligated to Pst I EcoR I digested BK(MM) or BK(WW) DNA

3.5.1 C600 was found to transform more proficiently than HB101 with undigested pBR322 (i.e. 1000 versus 400 Tet^R colonies/100ng DNA), however with all other DNA samples a greater transformation frequency was obtained using HB101. The difference varied between 5 and 8 times as many Tet^R colonies with HB101 as with C600. There is no obvious reason for this discrepancy which reflects the ability of HB101 to become more susceptible than C600 to the state of competence with this method of transformation. Bacterial strains are however known to differ in their susceptibility to the uptake of foreign DNA; (a relevant example is that of χ1776 compared with C600, see section 3.4.3).

An overall frequency of transformation of approximately 2×10^3 Amp^S Tet^R colonies/ug BK(MM) DNA, or of $0,5 \times 10^3$ Amp^S Tet^R colonies/ug total DNA added, was achieved. This is an acceptable level considering the small quantities of DNA used as well as the number of manipulations preceeding the transformation of bacteria. A second transformation experiment yielded a frequency of 10^3 Tet^R colonies/ug DNA. These were not all screened for ampicillin sensitivity so many may represent Amp^R transformants containing religated pBR322. Ultimately the significance of these transformation results lay not in the frequency obtained, but in the characteristics of the BK(MM) recombinants obtained. In correlation with this interest the frequency of transformation was not accurately quantitated. In contrast it was decided to screen the putative recombinant colonies immediately for ampicillin sensitivity and for viral inserts thereafter.

3.5.2. Toothpicking Tet^R colonies onto ampicillin-containing plates revealed that many (almost 50%) of the colonies were actually Amp^R and therefore represented religated pBR322 vector molecules. It was noted however that, although the frequency of Tet^R colonies was four times greater with viral DNA ligated with alkaline-phosphatased pBR322 as opposed to untreated pBR322, the majority of the former were Amp^R whereas the majority of the latter were Amp^S. This result is in opposition to that expected in theory since bacterial alkaline phosphatase is employed to prevent religation of pBR322.

Foreign DNA to be inserted into vector molecules is often in short supply (e.g.cDNA), and ligation of these to vector molecules must therefore be preferential to vector religation. The latter occurs to a greater extent

when excess vector is added with a view to increasing chances of recombination with foreign DNA. To avoid this and the extensive screening required thereafter to detect insert-recombinants, alkaline phosphatase-mediated removal of 5' phosphate groups of the vector necessitates vector ligation to foreign DNA which provides the necessary 5' phosphate substrate groups for ligase action. The NOC molecules subsequently formed transform cells more efficiently than the unligated, linear DNA does, and is most probably covalently closed within the cell by repair mechanisms. Transformation by recombinant vector-insert molecules therefore overrides the lesser amount of transformation by religated vector molecules. In this way the chances of foreign DNA being inserted into vector DNA and of its subsequently transforming recipient cells, are optimised. (Bolivar and Backman, 1979).

Possible reasons for this unexpected result include the following:

1. the alkaline phosphatase treated pBR322 preparation may contain unrestricted CCC pBR322 molecules which transform with higher frequency than any digested and ligated molecules do. This appears possible after consideration of the electrophoretic analysis of the pBR322 used. (This digest was a different preparation from the untreated digested pBR322, which appears to be completely restricted).

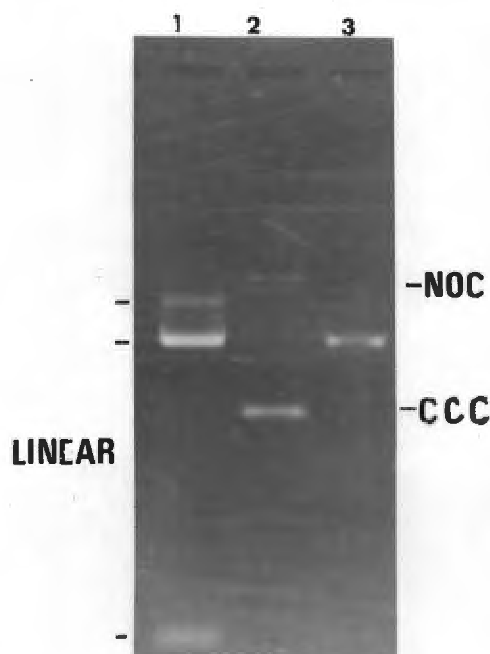


Fig.**32** 1% agarose gel electrophoresis of
lane 1. alkaline phosphatased, Pst I EcoR I digested pBR322 showing residual full length linear pBR322 (incomplete digest).
2. commercial pBR322 preparation with NOC and CCC molecules.
3. Pst I EcoR I digested pBR322.

2. The purification of alkaline-phosphatased pBR322 was more extensive than the untreated preparation (see section 5.2.12.3). It is quite likely that the more highly purified DNA ligated more efficiently and therefore transformed more efficiently. If the removal of the vector's 5' phosphate groups was incomplete, these highly purified pBR322 molecules would ligate to each other very efficiently, and thus increase the number of Amp^R Tet^R colonies obtained. Simultaneously however the ligation with viral DNA would be more efficient with pure pBR322 and was moreover optimised by j/i ratios (see section 3.3). In support of this, it was noted after DNA analysis, that the majority of BK(MM) clones originated from ligation with alkaline-phosphatased pBR322 molecules.

3.5.3. BK DNA inserts in pBR322 were screened for by colony hybridisation. Colonies containing back-to-back recombinants of the two large fragments of pBR322 would be Amp^S Tet^R; the DNA of these clones should not however hybridise to a BK(MM) DNA specific probe. In this way it was intended to distinguish pBR322-BK DNA recombinants from colonies containing only ligated pBR322 molecules. Colonies screened included Amp^S Tet^R colonies expected to harbour BK DNA, as well as a few Amp^R Tet^R colonies not expected to contain viral DNA, as control material.

Results obtained from autoradiography of the filters after hybridisation showed that many colonies had hybridised to some extent but to varying degrees with the probe, shown by different intensities of the spots.

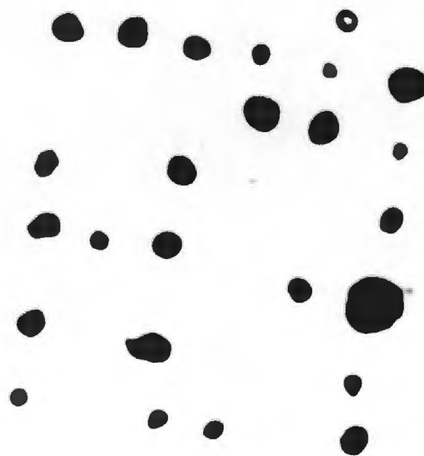


Fig.33 Autoradiograph of the colony hybridisation filters.

Those colonies containing inserts of the larger Pst I EcoR I fragment of BK DNA would be expected to show up more intensely than those with the smaller fragment, simply due to the fact that the former contain almost double the amount of complementary DNA sequences. Inserts of BK(WW) DNA fragments would be expected to hybridise to the same extent as BK(MM) inserts do, since no significant difference was found between the Southern blot hybridisations of (MM) and (WW) DNA to the same BK(MM) DNA probe used here. In addition, putative (WW) containing colonies were originally smaller than the (MM) ones and one would therefore expect smaller, less intense spots. (The colony size contributes largely to the degree of hybridisation to available DNA,and therefore to the intensity of the spot.) Differing intensities were accentuated by decreasing the exposure time of the autoradiographs, and also by increasing the stringency of the post-hybridisation washes from 0,66 to 0,1 x SET. The latter step cleared the background but did not ultimately change any information obtained or any interpretations of the radioactive spots. Bigger positive colonies were largely derived from bacteria exposed to alkaline phosphatased pBR322 ligated to viral DNA, which indicates, despite the above observations, that this treatment assists the ligation of vector-insert DNA molecules.

Colony hybridisation results are generally known not to be definitive but can be anomalous and therefore are used as more of a guideline for screening hundreds (or more) of colonies before DNA is extracted from the colonies for gel electrophoretic analysis.

Colonies chosen for DNA extraction therefore included some giving strong positive results (intense spots) which could contain large Pst I EcoR I fragments of BK(DNA), as well as those giving weaker responses and which could therefore contain small fragments of Pst I EcoR I digested BK DNA. Untested colonies were also investigated.

3.6. Preparation of radioactive DNA probes

3.6.1. A radioactive probe labelled to high specific activity was necessary for hybridisation to Southern blots and colony transfers to detect specific sequences of DNA present in small amounts. Initially, 5' end labelling of cohesive ends generated by Class II restriction endonucleases was attempted,

using a single tritiated nucleotide triphosphate and E.Coli DNA polymerase I. The substrate was Sal I digested lambda DNA (which has two sites for the enzyme), with cohesive ends generated by cleavage at the following sequence: G↓TCGAC. The negative control was EcoR I digested lambda DNA; the labelled nucleotide triphosphate, (methyl 1',2' - ^{3}H) - thymidine triphosphate will not be incorporated into the EcoR I 5' sequence G↓AATTC when adenine is absent from the reaction mixture. Scanning for tritium in the DNA products after agarose gel electrophoresis, showed that the specific activity of the Sal I product was 2,6 x 10^4 dpm/ug, with two sites labelled (or 1,3 x 10^4 dpm/ug with one site labelled). Since 22 x 10^6dpm (1,6 x 10^4 umoles) ^{3}H-dTTP were present, and a maximum of 1,58 x 10^4 dpm (1,2 x 10^{-7} umoles) can be incorporated into two micrograms lambda DNA, a 60% incorporation of ^{3}H-dTTP had been achieved.

The technique of end-labelling is optimised by use of the Klenow fragment, which lacks the 5'→3' exonuclease activity of E.Coli DNA polymerase I, and thus does not degrade the extended DNA or the DNA template, allowing a greater specific activity of labelled DNA to be obtained. Pst I was chosen as one of the restriction endonucleases to be used (see section 2. 2.), and a 5' hydroxyl is generated by this enzyme, which does not serve as a template for the polymerase (which requires a 3' hydroxyl for polymerisation). In addition, since the Klenow fragment was not available at the time, end labelling was superceded by nick translation as the method of choice for labelling DNA to high specific activity.

3.6.2. 100ng of Pst I EcoR I digested, commercially prepared pBR322 was nick translated using 5,5U E.Coli DNA Polymerase I, 200pg DNAse I, and a 2-fold excess of unlabelled nucleotides (16uM) with respect to ^{3}H-dTTP (8uM). Fractions of the sample after glycerol density gradient centrifugation were analysed for TCA precipitable counts.

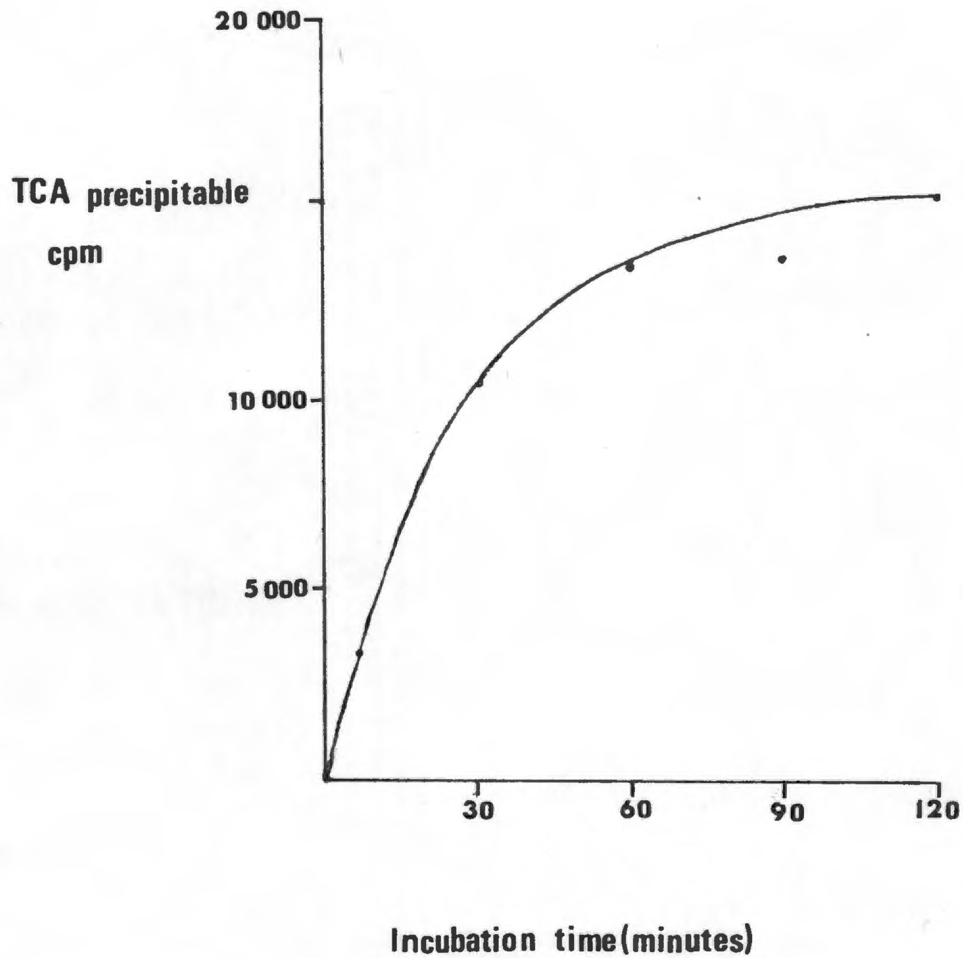


Fig.**34** TCA precipitable counts versus duration of reaction. One expects the reaction to be visually linear for 2 to 4 hours, after which the incorporation of counts should plateau off.

Here, the incorporation of counts appears too rapid, indicating extensive nicking of the DNA to allow such a high rate of labelling.

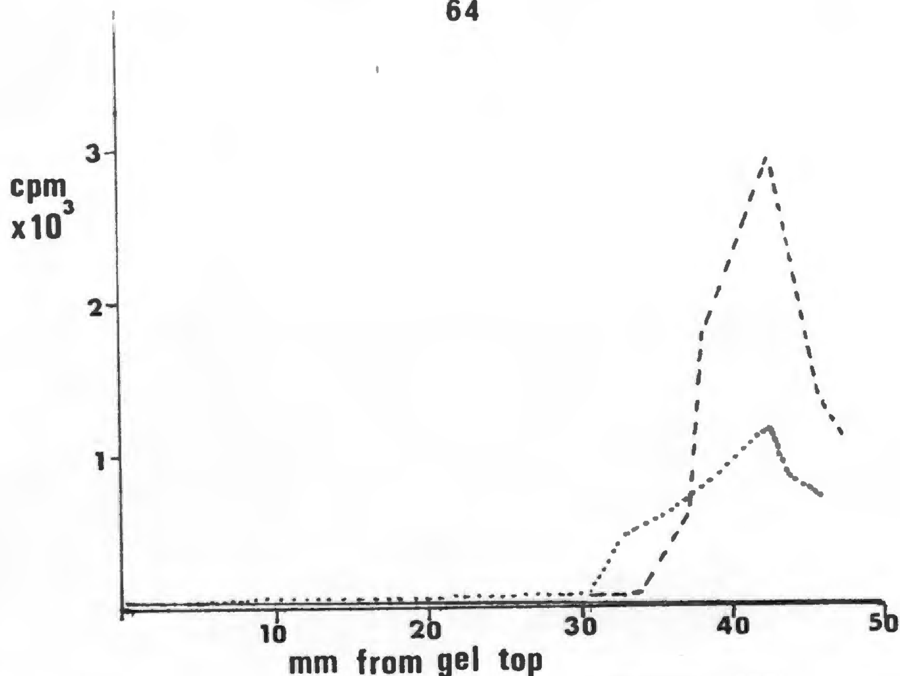


Fig.35 Radioactive profiles of sliced cylindrical agarose gels containing 1. unfractionated nick translation sample......
2. fractions 11-13 ———)
) of the glycerol density gradient
3. fractions 17-20 - - - -) centrifugation

The appearance of all radioactivity near the bottom of the gels indicates that smaller fragments of DNA had been labelled. The results support the hypothesis that DNA nicking and fragmentation was extensive, allowing rapid and efficient incorporation of ^{3}H-dTTP into the DNA.

Very small DNA fragments are not suitable for use as probes since specificity is reduced and hybridisation is not optimal. The effect of varying the DNAse concentration was therefore investigated in order to reduce DNA fragmentation. 1% agarose gel electrophoresis of the nick translated DNA showed that high MW bands of lambda DNA were labelled when using 0 or 1pg DNAse per 100ng DNA, whereas various different sizes of products, smaller than full length lambda DNA, were labelled when 10 or 100pg DNAse were used per 100ng lambda DNA. Incorporation of label ranged from approximately 1.5 to 3.0 times that obtained with lower DNAse concentrations. The lower incorporation of label into DNA was sufficient however for its use as a probe, and since full length or near full length probe DNA was required for increased specificity, it was decided to avoid fragmentation by omitting DNAse from the nick translation procedure.

3.6.3. Methods of preparation of DNA for nick translation were investigated using commercially prepared lambda DNA, which was either

1. electroeluted from (a) 3% polyacrylamide gels or
   (b) 1% agarose gels,and then precipitated with alcohol, or
2. simply alcohol precipitated and resuspended in the appropriate buffer.

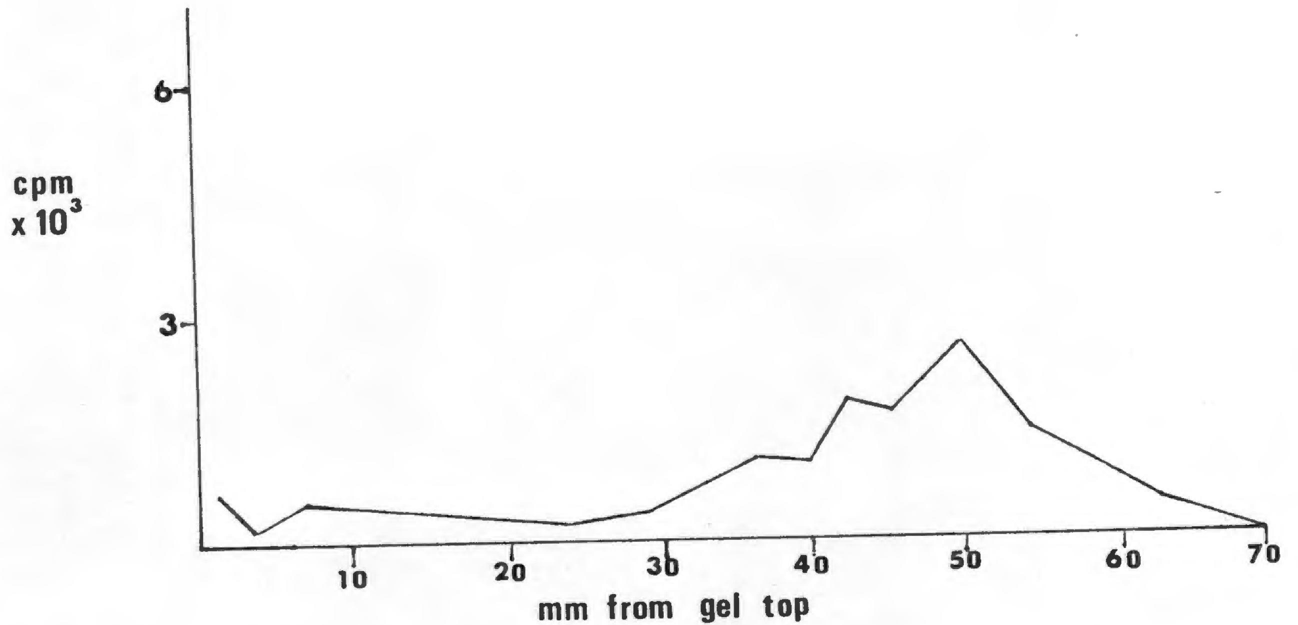


Fig.36 Lambda DNA electroeluted from 1% agarose gels prior to nick translation. The radioactive profile of the gel shows a great degree of inhibition of incorporation of label. Residual agarose is quite probably preventing access of DNA polymerase I to the DNA molecules, or inhibiting the enzyme itself. Absolute removal of agarose is therefore essential before use of DNA for nick translation.

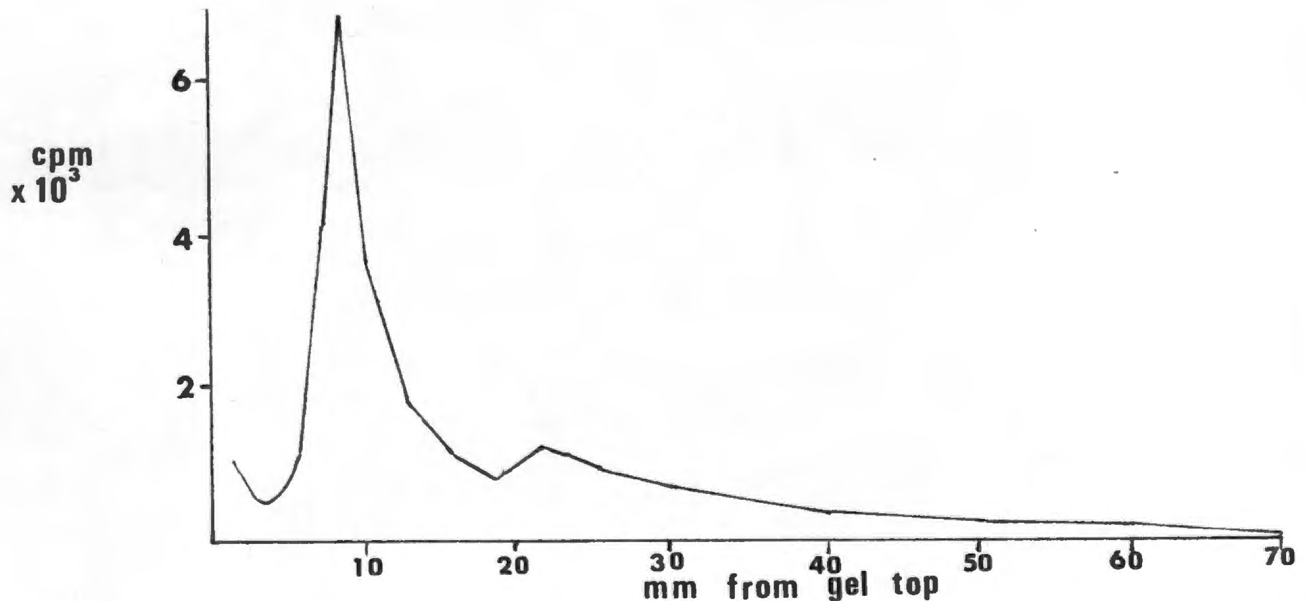


Fig.37 Lambda DNA electroeluted form 3% polyacrylamide gels prior to nick translation. The radioactive profile of the gel shows a high degree of incorporation of ^{3}H-dTTP, predominantly into full-length DNA with less degradation occurring than with non-electroeluted DNA. In this case, nicks do not appear to be

limiting; it does appear however that constituents of the polyacrylamide gel introduce single-strand nicks into the DNA but do not hinder access of the polymerase to the DNA, neither do they inhibit the enzyme.

Acid precipitable counts of the nick translated samples were vastly different:

1. non-electroeluted DNA gave a specific activity of 1,4 x 10^4 cpm/ug DNA (1,1% incorporation)
2. DNA electroeluted from agarose gave a specific activity of 0,3 x 10^4 cpm/ug DNA (0,2% incorporation)
3. DNA electroeluted from polyacrylamide gave a specific activity of 12 x 10^4 cpm/ug (9,2% incorporation).

Nick translation of Pst I EcoR I digested BK(MM) DNA electroeluted from 3% polyacrylamide gels, yielded a high specific activity of 4,8 x 10^6 cpm/ug DNA. Incorporation of acid precipitable counts with increasing time was once again unexpectedly rapid, although further investigation showed degradation not to be extensive.

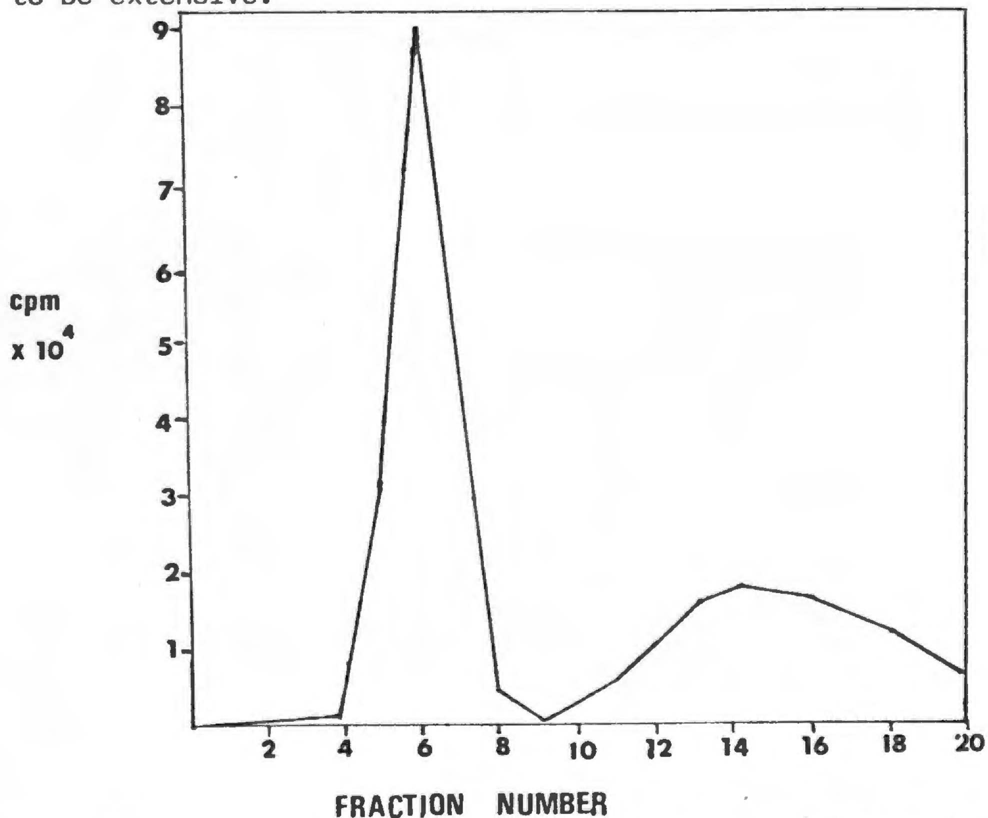


Fig.38 Acid-precipitable ^{3}H counts of fractions of a Sephadex G50 column.

Separation of tritiated nucleotides from labelled DNA using a Sephadex G50 column was highly efficient.

The majority of the label was incorporated into products which have mobilities (on a 1% agarose gel) expected of Pst I EcoR I DNA fragments of BK(MM), i.e. 2.17 and 1.09 x 10^6 daltons. There was however labelling of fragments of smaller sizes, indicating that a certain amount of fragmentation occurred. There were also some residual nucleotides in the sample; these however will wash off filters used for Southern blots and colony hybridisations. A greater degree of labelling could be expected in the larger, slower moving fragment of BK(MM) DNA, than in the smaller fragment. It is possible that the small fragment appears more highly labelled due to fragmentation of the larger one with the result that the two are not in equimolar amounts, but that the smaller are in excess of the larger. There is no reason to expect preferential labelling of a smaller DNA fragment.

3.6.4. 1200ng CCC BK(MM) DNA was electroeluted from 3% polyacrylamide gels, and nick translated for use as a full length probe, using 7,2 U DNA polymerase I, and 30pmols deoxynucleotide triphosphates in comparison with 16pmols ^{32}P- dCTP. ^{32}P was used to obtain greater sensitivity of the probe and to speed up screening of recombinant clones. Specific activity achieved was 2,5 x 10^7cpm/ug DNA. Labelled DNA was separated from nucleotides on a Sephadex G50 column as before, and the fractions were run on a horizontal agarose gel which was then autoradiographed to determine the sizes of the labelled products. These run in positions expected of full length CCC and NOC BK(MM) DNA.

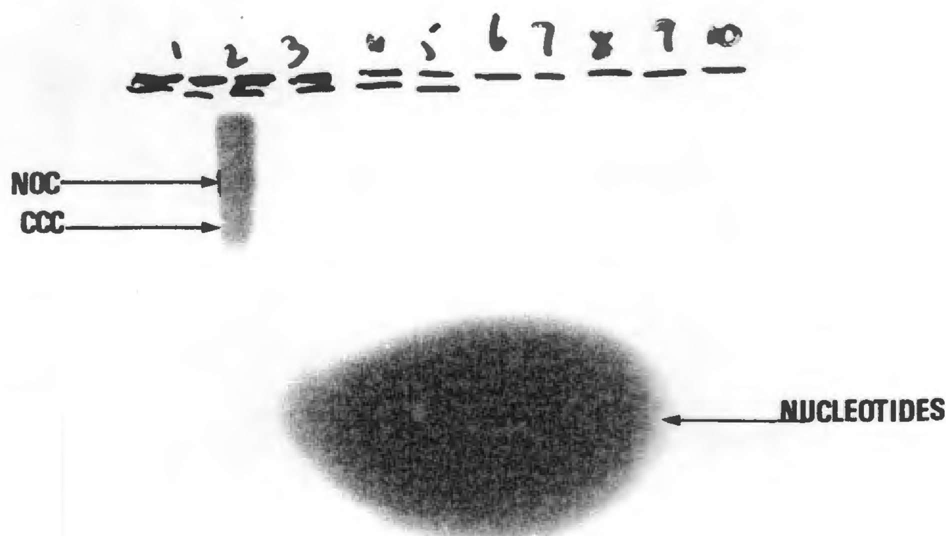


Fig.39 Autoradiograph of 1% horizontal agarose gel containing fractions of Sephadex G50 column.

Probe specificity was checked by hybridisation to a Southern gel blot of a slab gel containing BK(MM) and BK(WW) DNA extracted from human brain cells and urine respectively, and pBR322 DNA extracted from E.Coli C600. No cross-reactivity was apparent with either human or bacterial chromosomal DNA, nor with pBR322.

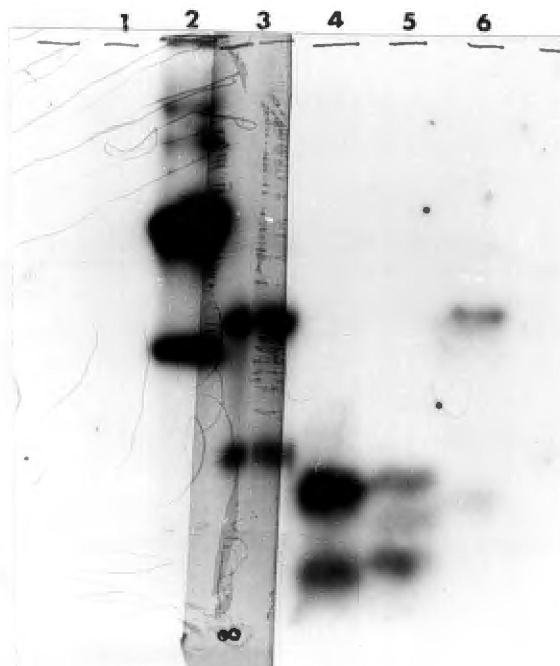


Fig.40 Autoradiograph of a Southern blot of an agarose gel, hybridised to the ^{32}P- BK(MM) probe.

Lane 1 pBR 322
2 BK(MM) DNA
3. Pst I Eco RI digested BK(MM) DNA
4. Pst I Eco RI Hind III digested BK(MM) DNA
5. " " " " " " BK(WW) DNA
6. " " " digested BK(WW) DNA.

The gel contained insufficient DNA to be detected by staining; however the sensitivity of the ^{32}P-probe allowed detection of particular DNA sequences.

A second probe of BK(MM) DNA was prepared after purification of the DNA by G200 Sephadex chromatography (to remove RNA), followed by acid phenol extraction to remove chromosomal linear DNA and alcohol precipitation. i.e. DNA was not electroeluted from acrylamide. 2-fold excess unlabelled nucleotides were used in comparison with ^{32}P- dCTP, and 7,5U DNA polymerase 1 were used with approximately 1200ng DNA. Specific activity achieved was 3,6 x 10^7 cpm/ug DNA, which is higher than that achieved with the previous probe electroeluted from acrylamide. 25uCi instead of 16uCi was added initially, although this represented 16 pmols dCTP in both cases, and the specific activity of both ^{32}P-dCTP and labelled DNA was 1,5 times greater with the latter probe. Incorporation was comparable therefore after electroelution of DNA from acrylamide and after the above purification steps. Once again the probe specificity was checked and no cross-reactivity with human or bacterial chromosomal DNA was found.

3.7. Analysis of DNA extracted from putative BK clones

Single colonies (obtained by toothpicking Tet^R Amp^S colonies onto tetracycline-containing plates) were amplified with chloramphenicol in L-broth before selective extraction of plasmid DNA by the rapid alkaline extraction method, or alternatively by the detergent lysis method.

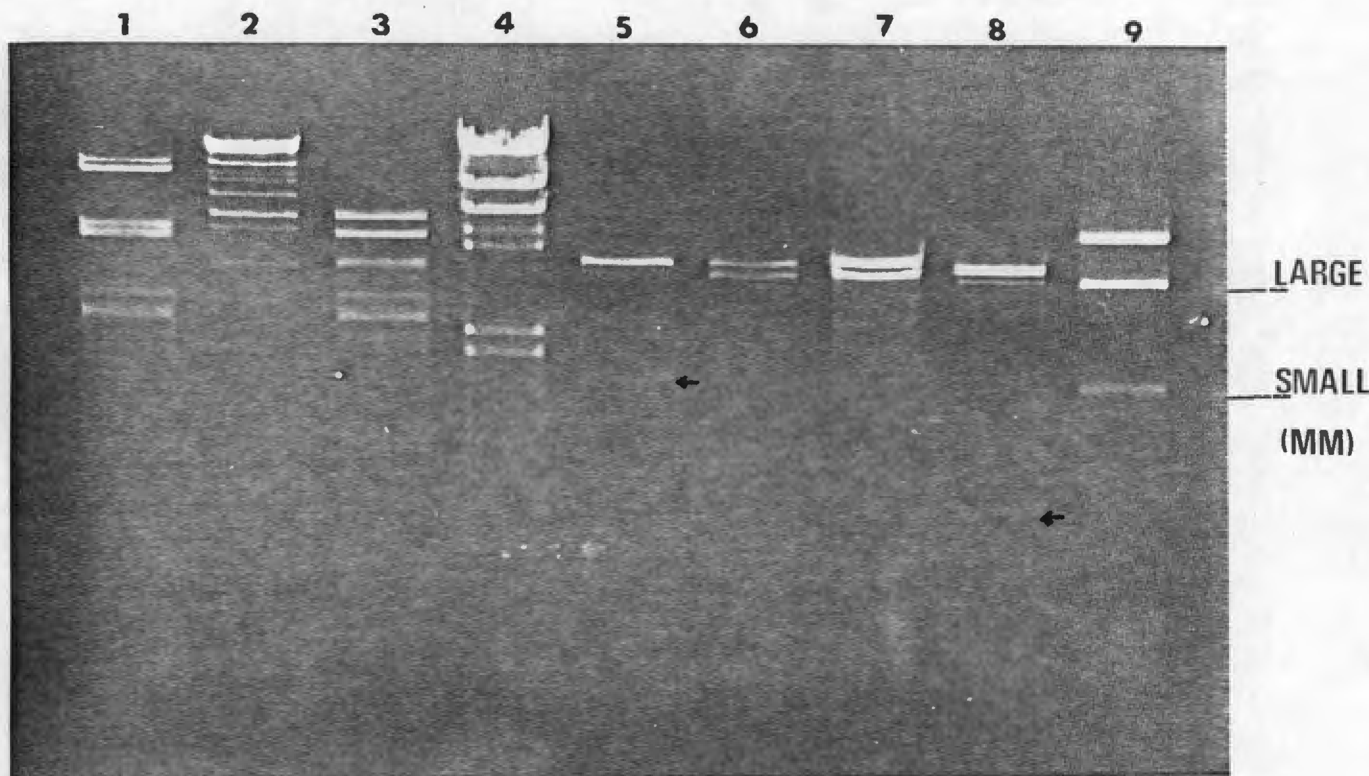


Fig.41 1% agarose gel electrophoresis of
1. Pst I lambda DNA
2. Eco RI " "
3. Pst I Eco RI Lambda DNA
4. Hind III Lambda DNA
5,6, 7. BK(MM) recombinant cloned DNA digested with Pst I Eco RI
8. Pst I Eco RI digested pBR322
9. Pst I Eco RI digested BK(MM) DNA isolated from infected cells.

Clones of inserts of both the small and the large Pst I Eco RI fragments of BK(MM) DNA in pBR322 were demonstrated here.

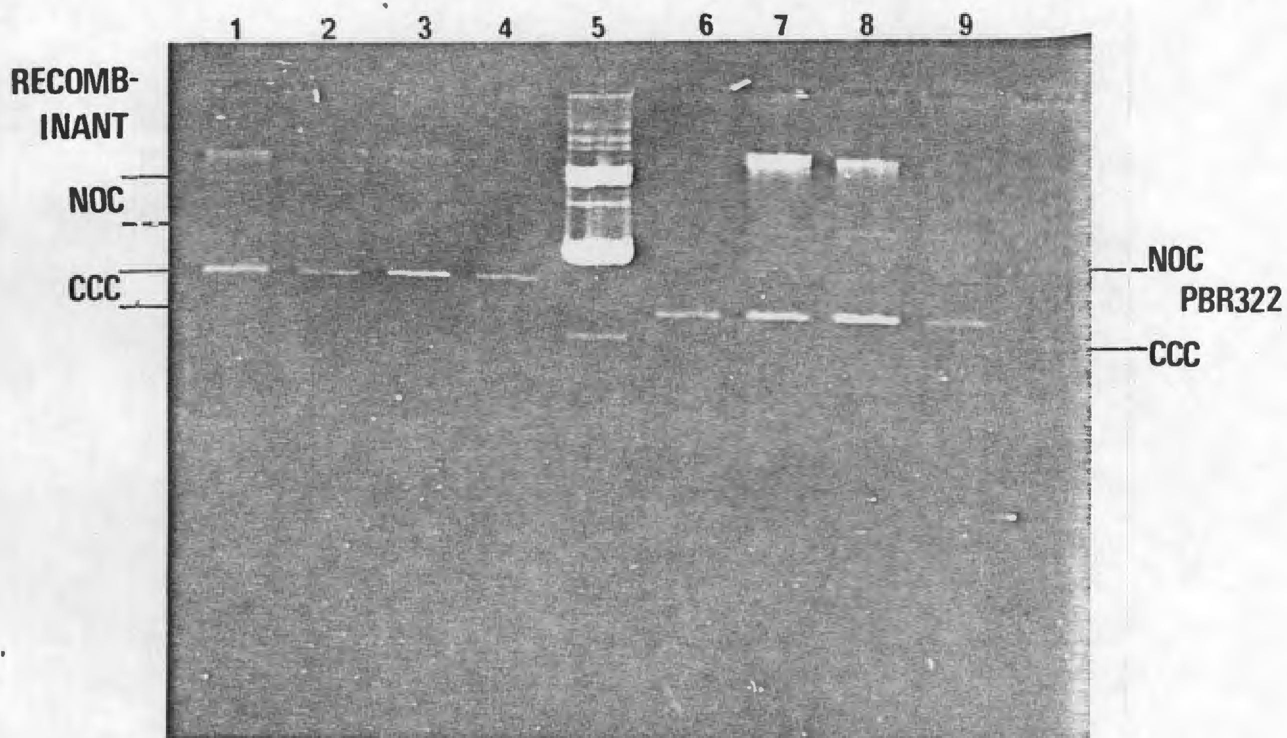


Fig.**42** 1% agarose gel electrophoresis of
1-4; 6-9; DNA extracted from Tet^R Amp^S putative BK(MM) clones
5. pBR 322

The last four lanes show plasmids moving slower than the pBR322 plasmid whilst the first four lanes show plasmids moving slower still. The bands therefore probably represent recombinant plasmids containing inserts of the small and large Pst I Eco RI fragments of BK(MM) DNA, respectively, in pBR322. Confirmation of this proposal required restriction to separate pBR322 and BK(MM) molecules.

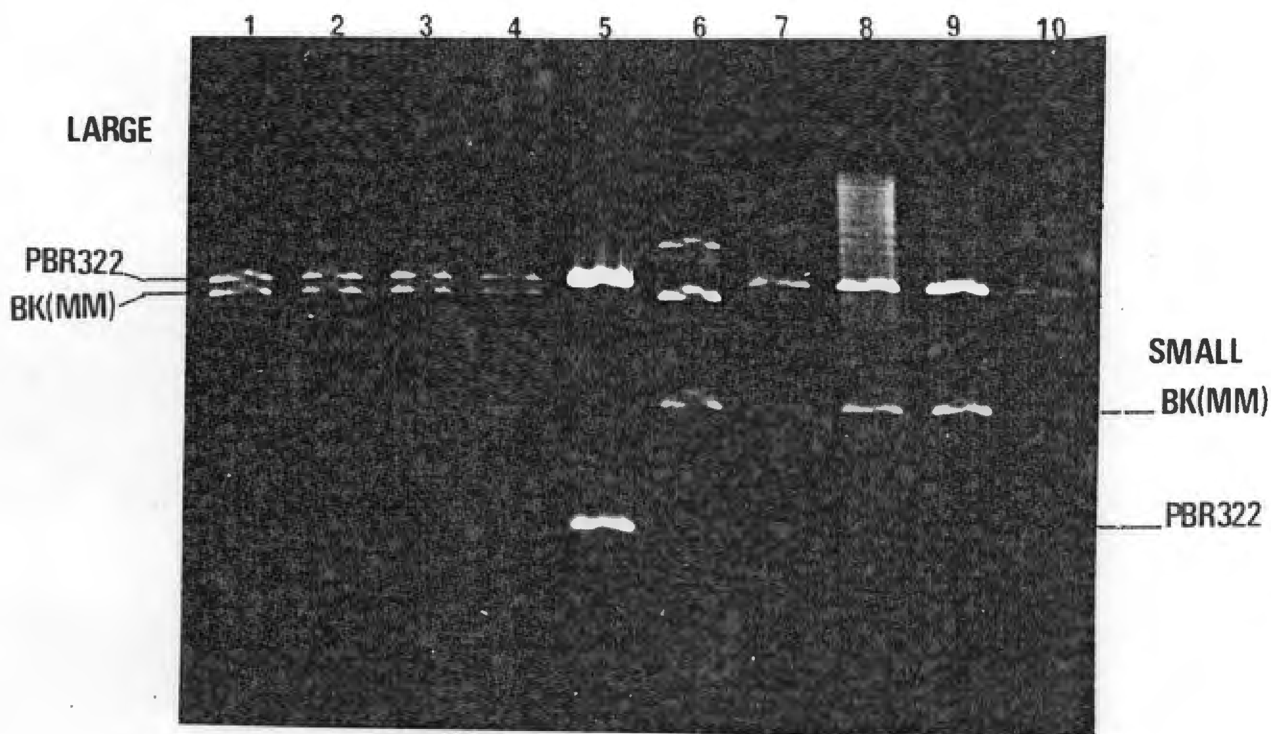


Fig.**43** 1,2% agarose gel electrophoresis of Pst I Eco RI digests of

1-4 DNA extracted from putative BK(MM) clones proposed to contain the large Pst I Eco RI fragment of BK(MM) DNA in pBR322.

5. pBR322

6. BK(MM) DNA

7-10. DNA extracted from putative BK(MM) clones proposed to contain the small Pst I Eco RI fragment of BK(MM) DNA in pBR322.

These results confirm the proposal that BK(MM) clones had been isolated, containing either the large or small fragment inserted into pBR322. Further confirmation was obtained with an autoradiograph of a Southern blot of this gel, hybridised to ^{32}P - BK(MM) DNA and showing the allocated bands to contain sequences complementary to BK(MM) DNA. Moreover, no hybridisation to pBR322 occurred.

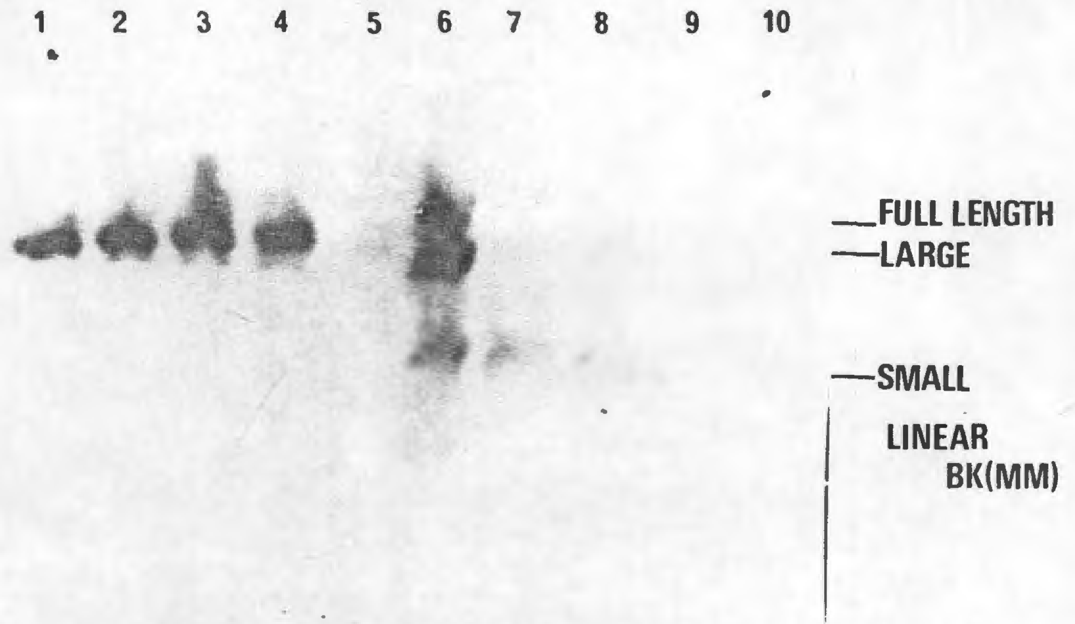


Fig.44 Autoradiograph of a Southern blot of the above gel, hybridised to ^{32}P- BK(MM) probe derived from BK DNA extracted from infected cells.

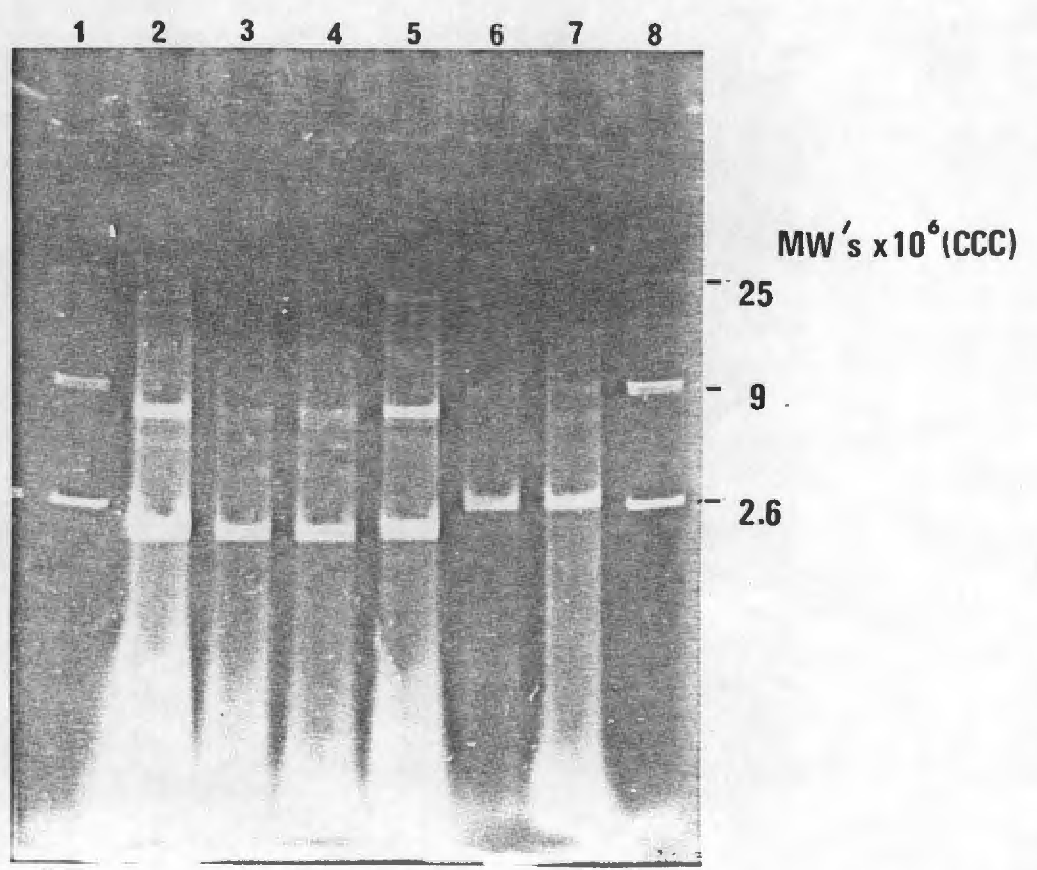


Fig.45 1% vertical agarose gel electrophoresis of DNA extracted from putative recombinant clones.

Lane 1. pBR322 DNA& markers.

2. pBR322 extracted from HB101

3. " " " C600

4,5,6,7. Amp^S Tet^R putative BK(MM) recombinant C600 clones' DNA
8. DNA molecular weight markers.

Lanes 6 and 7 show CCC and NOC plasmid bands of larger MW than pBR322. Comparison with corresponding gels, and of the linear band with the markers and with pBR322 ($2,6 \times 10^6$ daltons) indicates a MW of $3,7 \times 10^6$ daltons, which infers insertion of the small fragment of BK(MM) DNA into pBR322 in this particular colony. Lanes 4 and 5 however appear to contain pBR322 only, since their CCC and NOC plasmids have the same electrophoretic mobilities as those of pBR322 extracted from HB101 or C600.

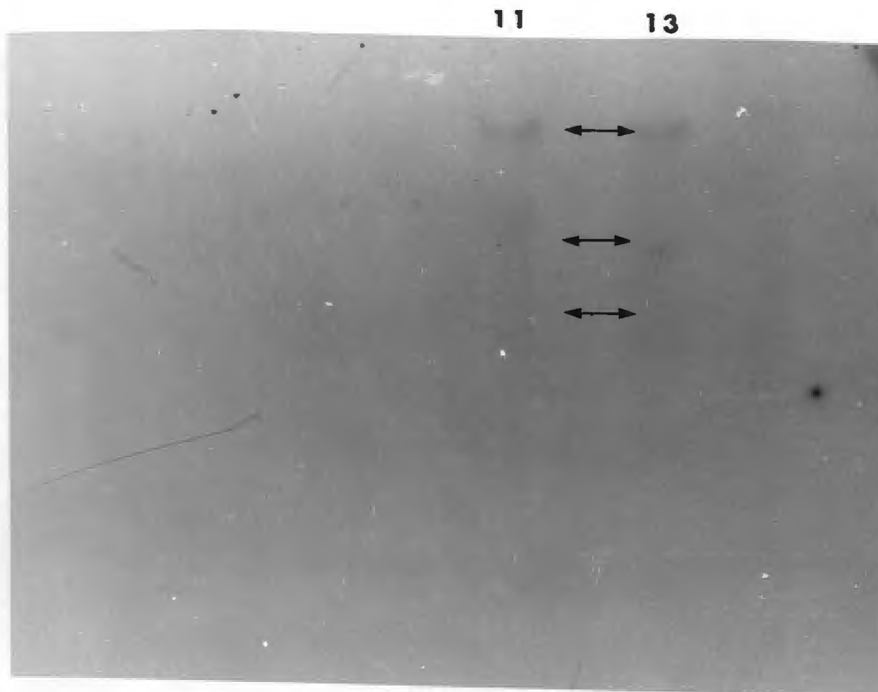


Fig.**46** Autoradiograph of hybridisation of a ^{32}P- BK(MM) DNA probe to a Southern blot of a 1% agarose gel containing DNA extracted from putative recombinant clones.

1. Hind III pBR322

2,3 & 7-12. Amp^S Tet^R, colony-hybridisation-positive putative BK(MM) recombinant clones' DNA

4,5,6. Amp^S Tet^R, colony-hybridisation-questionably-positive putative BK(WW) recombinant clones DNA

13,14. Amp^S Tet^R putative BK(WW) recombinant clones DNA

15. HB101 containing pBR322

16. Lambda DNA.

This result demonstrates the anomalous results obtained with colony hybridisation, since nine of the fourteen clones were expected to contain viral DNA inserts, based on colony hybridisation results. However, the extracted DNA did not hybridise in twelve cases to the specific viral DNA probe, indicating absence of complementary DNA sequences. However, lanes 11 and 13 show plasmids in the NOC and CCC forms whose sizes indicate insertions of the large BK(MM) fragment and the small BK(MM) fragment respectively. Also demonstrated is the absence of cross-reactivity of the BK(MM) probe to E.Coli bacterial DNA, pBR322, or lambda DNA.

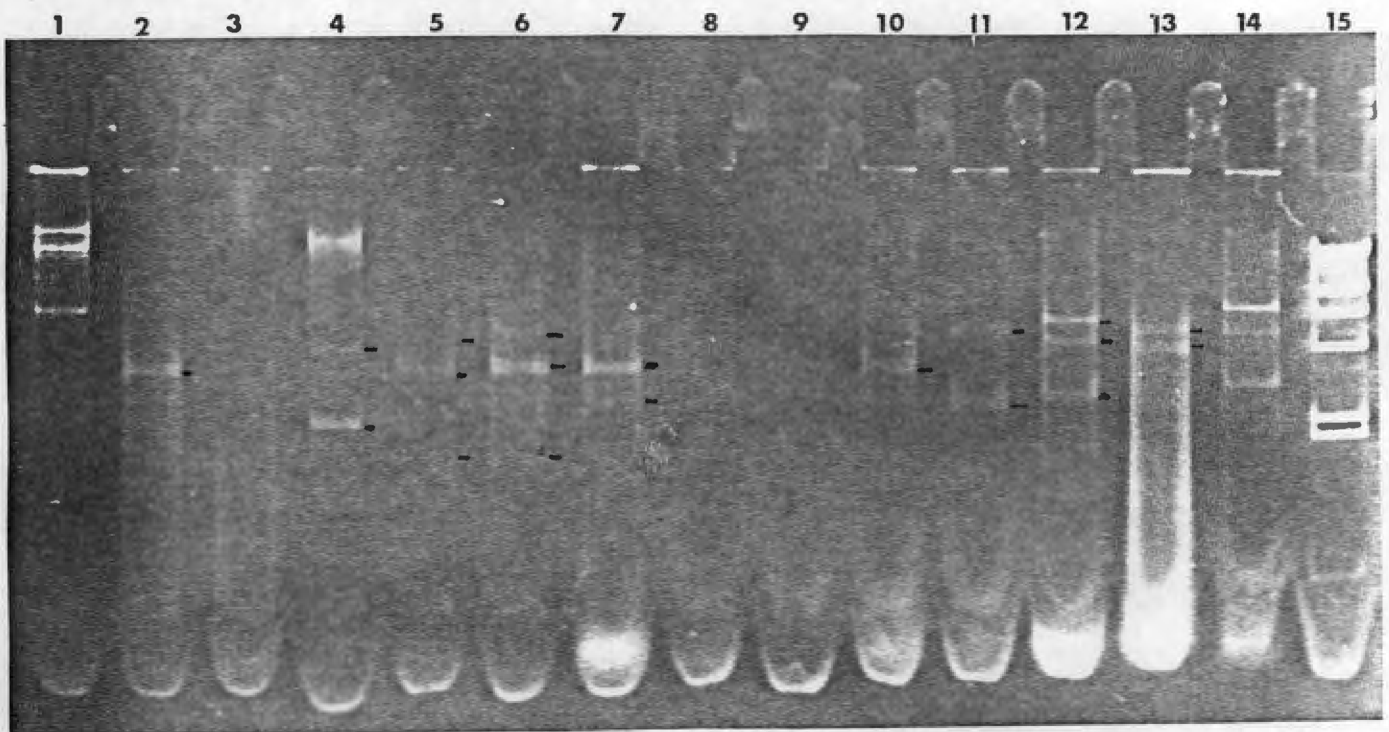


Fig.47 1% agarose gel electrophoresis of Pst I Eco RI digests of DNA extracted from Amp^S Tet^R putative recombinant clones.

Lanes 1-10. Pst I Eco RI digests of DNA
11-14. Undigested DNA

Lanes						
1,2.	Amp^S Tet^R putative	C600	recombinant	BK(MM)	clones.
3-6.	" " "	HB101	"	"	"
7.	" " "	"	"	BK(WW)	"
8,9.	" " "	C600	"	"	"
10.	HB101 containing pBR322				
11.	C600				
12.	Amp^S Tet^R putative	HB101	"	BK(MM)	clone
13.	" " "	"	"	BK(WW)	"
14.	" " "	"	"	BK(MM)	"
15.	Hind III digest of lambda DNA				

The large Pst I Eco RI fragment of BK(MM) DNA has a MW of 2,17 x 10^6 daltons whilst the small fragment has a MW of 1,09 x 10^6 daltons. Those of BK(WW) are expected to be respectively slightly bigger and small (see section 2.). The two Pst I Eco RI fragments of pBR322 have MW values of 2,14 and 0,46 x 10^6 daltons. By comparison of expected MW's of the bands seen, various predictions concerning the inserts can be made. Lane 1 shows apparent concatemers of plasmid DNA, known to occur in E.Coli (see section 3.4.2.), as well as a faster moving band smaller than the pBR322 digest. Since some of these digests are obviously incomplete (e.g. lanes 7 and 10 show residual CCC and lanes 5 and 6 show residual NOC bands), it is possible that the two fastest moving bands represent CCC and NOC molecules similar in size to those in lane 14, and thus quite possibly indicate a recombinant with the large BK(MM) insert. Lane 14 shows CCC and NOC bands considerably larger than pBR322, and also noticeably larger than those in lane 12. These most probably represent the large insert (lane 14) and the small insert (12) of BK(MM) DNA.

Lanes 5 and 6 show, in additon to the linearised large pBR322 fragment, a faster moving fragment, in a position indicative of a 1 x 10^6 MW fragment, which is consistent with the proposal that this represents the small fragment of BK(MM) DNA in both cases. The position of the slower band in lane 4 indicates full length pBR322 whilst the smallest fragment could represent inserts of incompletely digested and therefore linear (1,5 x 10^6 daltons) BK(MM) DNA (1,09 x 10^6 daltons) and pBR322 (0,46 x 10^6 daltons). However the relative intensities of the bands indicate that the fastest band may be CCC with linear and NOC moving more slowly. Since this CCC plasmid is smaller than pBR322, it may represent the recircularised large Pst I Eco RI fragment of pBR322, although this ligation between noncomplementary sequences should not occur. Lanes 2,3,7,8,9 show no additional bands and therefore most probably represent religated pBR322 recombinants.

In certain cases identification of bands was extremely complex. In the following case, an agarose gel of Hind III digests of DNA extracted from putative recombinant clones was blotted, using the Southern technique and hybridised to a ^{32}P-BK(MM) radioactive probe. Since small quantities of DNA were applied to the gel, visualisation of the bands was very difficult. Once again certain digests were incomplete.

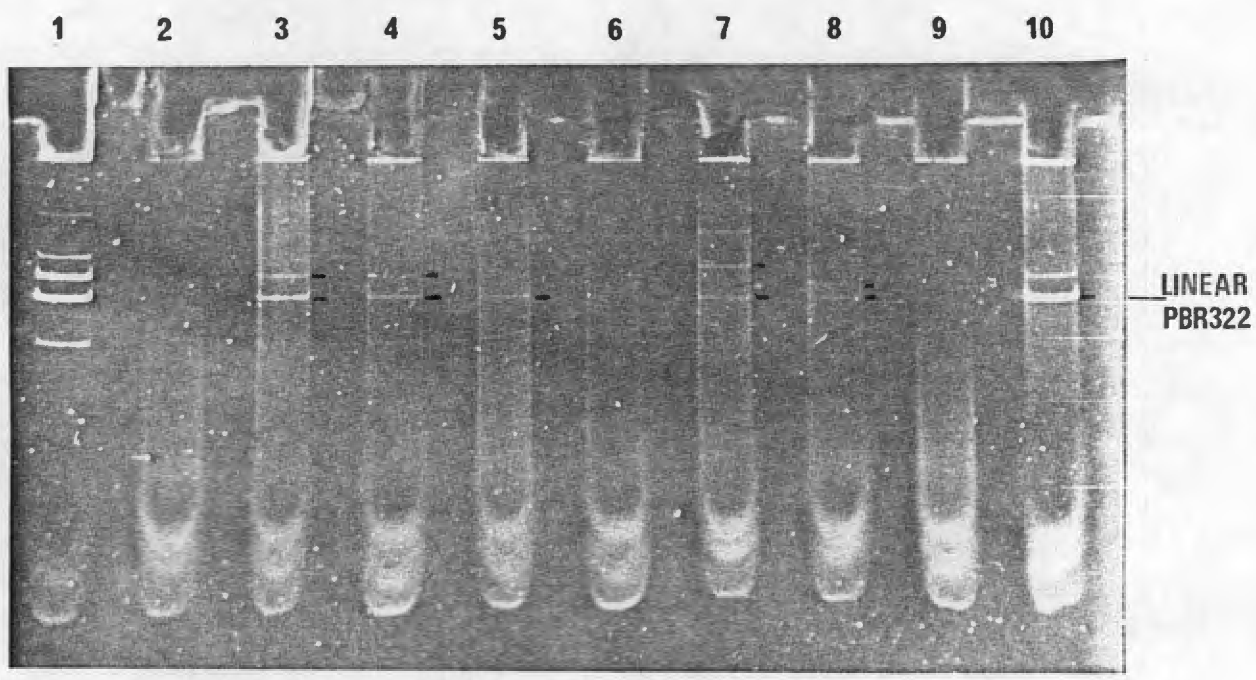


Fig.**48** 1% agarose gel electrophoresis of Hind III digested DNA extracted from putative recombinant BK(MM) clones.

Lane 1. Commercial preparation of pBR322 containing 10% dimers.

2. Hind III digested DNA extracted from an Amp^S Tet^R HB101 putative BK(MM) recombinant clone.

3-5. Hind III digested DNA extracted from a Tet^R HB101 putative BK(WW) recombinant clone

6-9. Hind III digested DNA extracted from an Amp^S Tet^R HB101 putative BK(MM) recombinant clone.

10. Hind III digested DNA extracted from HB101 containing pBR322

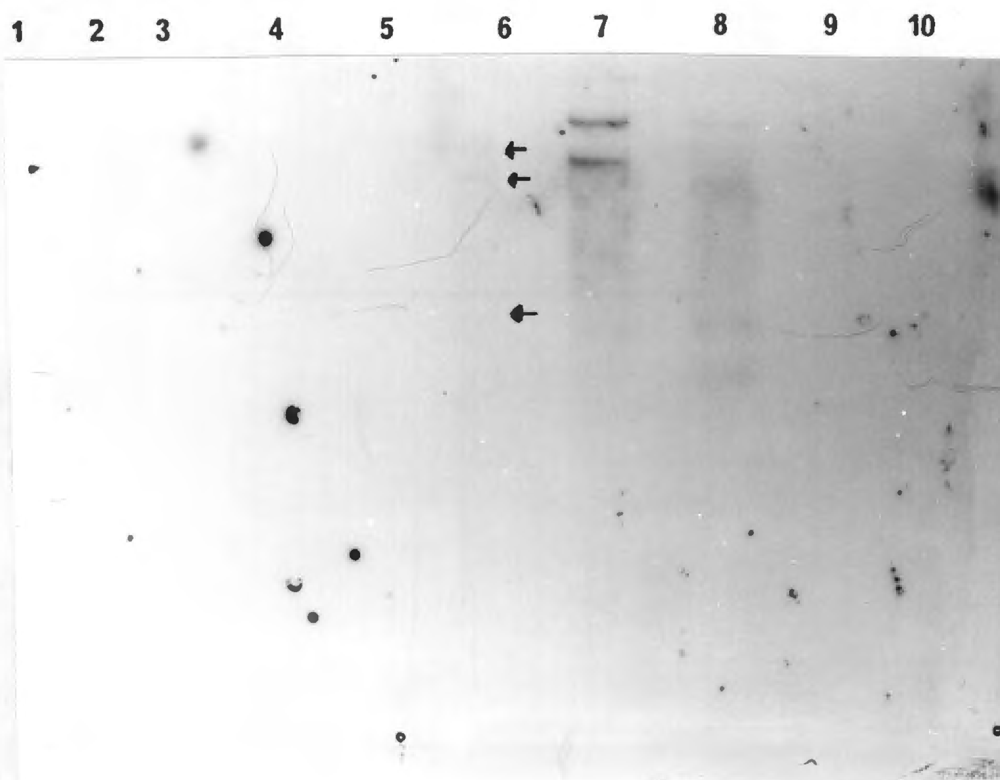


Fig.**49** Autoradiograph of Southern blot of above gel hybridised to ^{32}P-BK(MM) DNA.

Table 3.1

Expected MW's of fragments of various Hind III digested recombinant molecules

Antibiotic sensitivities	pBR322 fragment	BK(MM) fragment	MW's of digested recombinant
1. A^S T^R	2.14	-	4.24;0.04
	2.14		
2. A^S T^R	2.14	2.17	3.02;0.94;0.46
3. A^S T^R	2.14	1.09	2.61;0.62
4. A^S T^R	2.14	1.09	3.02;2.61;0.94
	2.14	2.17	0.62;0.46

From the expected MW's of the fragments, it is consistent to propose that lanes 7 and 8 represent large inserts of BK(MM) DNA. The radioactive bands could represent

(a) incompletely digested DNA; 3.02 fragment + 0.94 fragment (3.96×10^6)daltons
or 3.02 " + 0.46 " (3.48×10^6)

(b) 3.02×10^6 fragment

(c) 0.94×10^6 "

(d) 0.46×10^6 "

Lane 6 could in turn show bands arising from an incomplete digest of the small insert of BK(MM) DNA and pBR322 (large fragment), where the bands could represent (a) 3.23×10^6, made up of 2.61 and 0.62×10^6 fragments

(b) $2,61 \times 10^6$ molecule

(c) 0.62×10^6 fragment too faint to identify.

Alternatively;
Lanes 7 and 8 could both represent a large BK(MM) insert with an incomplete Hind III digest in lane 7 giving NOC ($4,31 \times 10^6$ daltons) and linear ($4,31 \times 10^6$ daltons) bands and an incomplete digest in lane 8 representing residual NOC and linear bands in addition to smaller bands of

(a) e.g. 3.96×10^6 (3.02 + 0.94)

(b) 0.94×10^6

(c) 0.46×10^6 daltons.

Lane 6 could equally well represent an incomplete digest of a small insert of BK(MM) into pBR322 i.e. (a) NOC - $3,23 \times 10^6$

(b) Linear - $3,23 \times 10^6$

Molar equivalents of smaller fragments (2.61 and 0.62×10^6 daltons) are, not surprisingly, present in amounts not large enough for detection. Markers on the same gel (CCC, linear, NOC pBR322 and CCC,NOC dimer pBR322) do not allow absolute distinction between the above two alternative interpretations of the autoradiograph, since this requires distinction between expected positions of a 4.31×10^6 and a $3.96/3.48 \times 10^6$ molecule, and also between a 2,61 and a $3,23 \times 10^6$ molecule. However from comparisons with previous gels and autoradiographs it appears more likely that the former alternative is correct, particularly with respect to the assignation of MW to the second largest hybridisation-positive band.

3.8. Unfortunately, no BK(WW) clones were identified by DNA analysis. It is probable that this DNA (extracted 18 months previously) is neither efficiently restricted nor ligated, possibly due to loss of 5' phosphate groups. Inhibitory chemicals may remain despite thorough purification by phenol and chloroform extractions and alcohol precipitations. The small quantity of available DNA limited the number of possible manipulations since even minimal losses are inevitable (and undesirable) in these procedures. The number of transformation experiments with BK(WW) DNA was limited to two. It is hoped that additional excretors may be found since both patients, WW and SN, have long since stopped excreting the virus. Identification of BK(WW) isolates in urine samples of immunocompromised patients may be made more rapid and more specific by hybridisation of Southern blots of gels containing crude DNA extracts, to a radioactive BK(MM) probe derived from the cloned material. This unique and uniform viral DNA will be useful as a diagnostic probe, firstly for detection of DNA sequences complementary to BK DNA in a short space of time, thus obviating primary requirements for DNA purification, and secondly, restriction of DNA from the urine extracts followed by hybridisation to the radioactive probe, will allow identification of the viral strain. Advantages of using cloned BK(MM) to prepare the probe (as opposed to BK(MM) DNA extracted from infected cells) include the fact that there will be no contamination with human DNA, and modifications of the viral genome, as they tend to occur in infected eukaryotic cells, are likely to occur. In addition, either the large or small Pst I Eco RI fragment of BK (MM) are already separately available to be used to detect particular sections of the extracted viral DNA. This will assist in determination of viral strains.

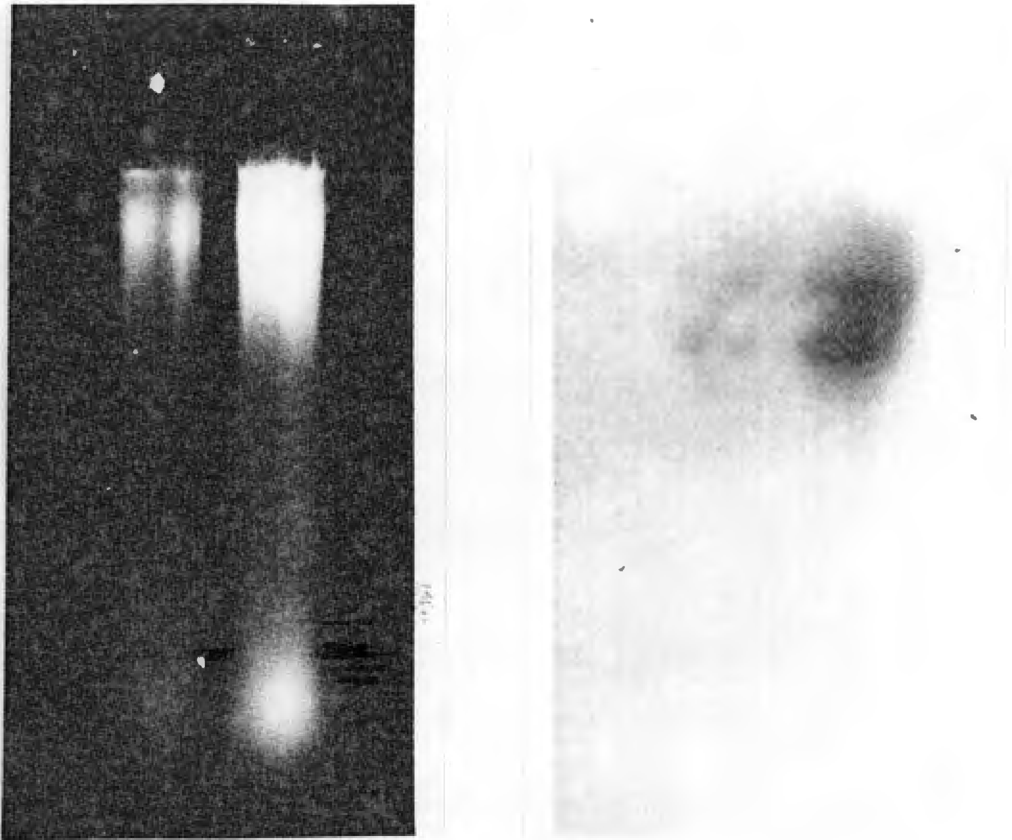


Fig.50 Agarose gel electrophoresis of a crude extract of total DNA from urine, showing a smear of concentrated DNA; and an autoradiograph of a Southern blot of this gel hybridised to a ^{32}P- BK(MM) DNA probe derived from both fragments of cloned Pst I Eco RI BK(MM).

This provides evidence for the presence of DNA sequences complementary to BK(MM) DNA. These bands have a mobility characteristic of linear and NOC BK DNA and it is moreover unlikely that such complementary sequences exist (in this position) in undigested chromosomal DNA. Confirmation of the presence of BK DNA requires restriction analysis which will assist, moreover, in assignation of the BK strain present.

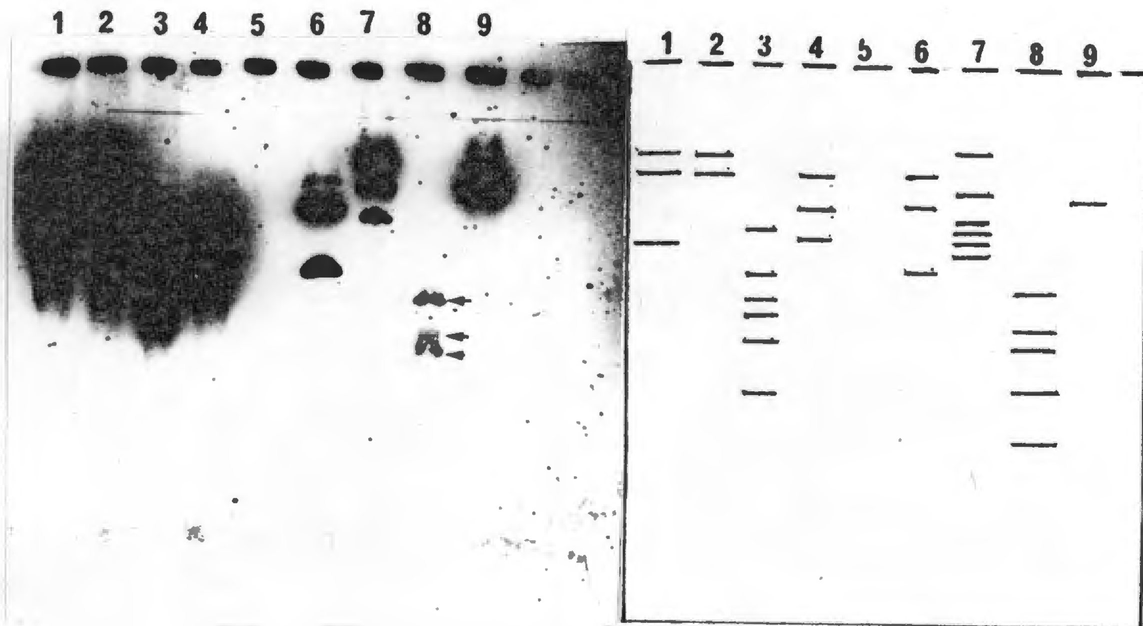


Fig.51 Autoradiograph of a Southern blot hybridised to ^{32}P -BK(MM) DNA derived from cloned material.

1. Pst I Eco RI digest of a crude extract of DNA from human urine
2. Hind III " " " " " " "
3. Ava II " " " " " " "
4. Bgl II " " " " " " "
5. Hind III lambda DNA, which does not hybridise to BK(MM) DNA
6. Pst I Eco RI digest of BK(MM) DNA
7. Hind III " " "
8. Ava II " " "
9. Bgl II " " "

Hybridisation of DNA of the urine extracts to BK(MM) DNA provides evidence for the presence of complementary DNA sequences. The high degree of

similarity between the respective restriction patterns allows identification of the extracted DNA as BK DNA. However the lack of correlation of the latter's restriction pattern with that of the MM strain, particularly with respect to the Ava II and Bgl II digests, allows elimination of the possibility of this BK DNA being of the (MM) strain. It is highly likely that the deletion of the Hind III site close to an Ava II site in BK(MM) actually includes the Ava II site (see Fig. I); digestion of BK(MM) DNA therefore yields six Ava II fragments whereas prototype and most probably BK(WW) DNA also, yield seven fragments on digestion with Ava II. By the same token, MM DNA is highly likely to lack the Bgl II site located near the deleted Hind III site, therefore yielding a single Bgl II fragment. (The above restriction pattern supports the deletion of the second Bgl II site in MM DNA). Prototype and WW DNA, in contrast, have two Bgl II sites, as seen in the autoradiograph. Final determination of the strain involved will require further restriction pattern analysis. Although one would expect the smaller Ava II fragment of extracted DNA to appear less intense than the larger fragment, this is not the case here and this may provide additional information about the DNA strain present. There may be a greater sequence divergence from that of MM in this smaller fragment, which consequently hybridises less intensely to thw MM probe. A specific area of the DNA is therefore implicated to differ from the MM strain, and it would be interesting to aim further investigation in this region. In this way it is highly probable that a new source of BK(WW) DNA will be obtained; further research into this strain of BK will consequently continue.

Chapter IV

Summary

4. The work described delineates the detailed development and establishment of a number of techniques basic to and indispensible for recombinant DNA research. These techniques were successfully applied to the cloning of both Pst I Eco RI fragments of BK(MM) DNA inserted into the vector pBR322 and amplifiable in the E.Coli host cells. The characterisation of cloned viral material by restriction analysis confirmed the insertion of both fragments separately from each other. Although cloning of BK(Dun) strain has been reported, there are no publications on the cloning of BK(MM); this work therefore represents the first cloning of this particular strain of BK virus.

The separated fragments of BK(MM) are now available in ample supply as uniform molecules. They do not pose a hazard since firstly, the material is stored in the subgenomic form, and secondly the restriction used to generate subgenomic fragments specifically inactivated the transforming gene of the virus. Nevertheless the full genome may be readily regenerated by ligation of the two fragments using appropriate containment precautions. The fact that the genome is fragmented may well assist research into the possible use of BK DNA as vector material for cloning in eukaryotic cells.

The transforming ability of the genome is considerably reduced; (it would be essential to verify annihilation of the malignant property prior to use of BK as a vector in order to obviate increased containment levels), and the origin of replication and surrounding regions may be manipulated in isolation from the rest of the genome. In parallel with the use of the SV 40 genome of tandem repeats of the origin of replication as a vector in eukaryotic cells, similar BK(MM) molecules may, in time, be constructed (see section 2.2.). Lines of human cells in culture are available as recipients of the vector, although preliminary transformations of bacteria with derivatives of the viral genome may be necessary. Manipulations with cloned material will not carry the risk of concomitant induced charges of human DNA since there is no contamination with the latter; BK DNA extracted from infected cells would however be suspected of containing human DNA sequences.

The latter point is advantageous also to the screening of DNA extracts from urine of immunocompromised patients. This use of BK(MM) cloned material has been shown to be powerful (section 3.8.) and portends to allow detection

of a further source of BK(WW), or possibly of variants of BK(WW) or other strains. At such a stage the established techniques could be applied to cloning of the non-cell-culture-adapted BK(WW) strain of BK virus. Thereafter the relevance of this strain's genomic deletions and alterations to its properties (e.g. its inability to infect cells in culture) can be thoroughly studied. Comparison of this strain with the cell culture adapted strains could yield information concerning essential genomic areas and their expression, particularly with regard to transformation of human cells in culture e.g. integration of specific DNA sequences into the host genome, roles of specific regions of the genome and the products made, etc.

Cloned BK(MM) DNA may be used in yet another field i.e. concerning the screening of human cellular DNA; in this way infection with this papovavirus may be correlated with the incidence of certain diseases, particularly malignancies. For this use, the absolute absence of human DNA sequences is essential to exclude the possibility of hybridisation of the viral probe to endogenous normal human DNA, and thus to maintain reliability of results. Such research could be extended to the study of integration of viral sequences into host genomes, and the resultant effects.

In general there are unlimited applications of the recombinant DNA methodology now efficiently set up in the laboratory.

Chapter V

Materials and Methods

5.1 MATERIALS

Restriction endonucleases PstI, Bam H I and Hind III, ColEI and pM2 DNA and tRNA (extracted from E.Coli MRE 600) were obtained from Boëhinger Mannheim. Sal I, Hin II, Bgl II, EcoR I, E.Coli B DNA polymerase I, DNAse I, Pronase, T4 DNA ligase, and agarose were purchased from Miles Laboratories. T4 DNA ligase, nuclease-free bovine serum albumin (fraction V) and bacterial alkaline phosphatase were supplied by Bethesda Research Laboratories. Rinonuclease A, polyvinylpyrrolidone, Ficoll and lysozyme were bought from Sigma, and TEMED and a Hinc II digest of ϕX174 were obtained from Biolabs. Sephadex G50 and G200 were obtained from Pharmacia (Uppsala, Sweden). Bacteriological growth medium was Nutrient Broth No.2., supplied by Oxoid, or Luria broth made up in the laboratory with Difco products (10g Bactotryptone, 5g yeast extract and 5g NaCl, per litre.) Chloramphenicol came from Parke-Davis,Rolitetracycline from Hoescht Pharmaceutica, Chlortetracycline from Sigma and Ampicillin from Beecham or Sigma, diaminopimelic acid from Sigma. All other chemicals were of Analar (or equivalent) grade.

Radioactive chemicals were obtained from the Radiochemical Centre, Amersham. The Packard scintillants, Dimilume and Instagel, were used. En³Hance was obtained from New England Nuclear. Ultracentrifugation was performed in a Beckman model L2.65B ultracentrifuge using S.W. 41 Ti or SW 50.1 rotors with cellulose nitrate tubes. Lower speed centrifugation was carried out either in a Beckman model J2.21 or in a Sorvall model RC-2B using the appropriate rotors.

5.2 METHODS

5.2.1 Bacterial cultures were stored at 4° on Nelson's egg slopes or at -80° in the appropriate broth containing 5% glycerol. Media for χ1776 was supplemented with 100μg/ml diaminopimetic acid, 5μg/ml thymidine, and 1% peptone. Stock solutions of antibiotics were filter-sterilised and stored in the dark for not more than 24 hours. Bacteriostatic levels of antibiotics were determined to be 50μg/ml ampicillin and 30 μg/ml tetracycline for C600, 50 μg/ml ampicillin and 15 μg/ml tetracycline for HB101, and 25μg/ml ampicillin 12,5 μg/ml tetracycline for χ1776. All glassware and media were sterilised by autoclaving. Sterile gloves were worn when handling DNA solutions and enzyme preparations, as well as when plating out bacterial cultures.

5.2.2 Detection of radioactive counts in DNA-containing solutions or gels.

Approximately 800ng of carrier lambda DNA was added to 0,5ml aliquots of radioactive samples which were then spotted onto $1cm^2$ squares of filter paper which, when dry, were washed three times for 2 - 3 minutes in cold 5% TCA, air-dried and neutralised by the addition of 50 μl of a 1:5 solution of $NH_3OH:H_2O$ to reduce quenching by TCA. 5ml scintillant was added to the filters in counting vials and counting was performed to 2% error in a Beckman model LS-233 liquid scintillation counter. Dpm were calculated using an efficiency value of 30% with tritium and the formula of : dpm x efficiency (%) = cpm.

TCA-precipitable counts were more reproducibly determined when 1μl aliquots of sample were added to 2ug lambda carrier DNA and 2ml cold 5% TCA, centrifuged at 5000 rpm at 4° for 15 minutes. The process was repeated on the precipitate, which was then vacuum dried, 50μl 1:5 NH_3OH : H_2O and 5ml scintillant added and then counted.

^{32}P-fractions off a Sephadex G50 column were collected in sterile Eppendorf tubes and counted directly by Cerenkov counting of ^{32}P Cylindrical gels were sliced using a Mickle gel slicer to obtain 1mm slices. Agarose gel slices were preferably melted by heating in 0,5 - 1,0ml distilled water in vials in a pressure cooker for 20 minutes before adding scintillant and counting. Alternatively, slices were disintegrated by the addition of 0,2ml 1N HCl at 51° overnight followed by 0,2ml 1,5N NH_3OH to neutralise the solution before counting. Polyacrylamide gel slices were always treated in the latter manner.

Dried slab gels containing radioactive samples, and nitrocellulose filters hybridised to a radioactive probe, were scanned for radioactivity using the Berthold LB 2723 Dünnshicht-Scanner II powered by the Berthold Power supply BF 1015-2 with a tritium filter of 1mm width, ΔE 1000, and E 169.

Non-radioactive DNA was quantitated either by measuring OD_{260} on the Pye Unicam SP 1700 spectrophotometer or on the Varian Techtron model 635. DNA concentrations, in μg/ml, were calculated using an extinction coefficient of 58. If very small amounts of DNA were available, ethidium bromide stained gels were scanned densitometrically using the Vitatron TLD 100 with filters B and U4, and these scans were then compared with those of gels loaded with known amounts of commercially prepared DNA.

5.2.3 Autoradiography

Tritium-labelled DNA radioactivity was enhanced by exchanging the water of agarose slab gels for a) 100% methanol for 3 hours, then

b) for a methanol-fluor solution for 8 hours (made up of equal volumes of methanol and the supernatant of a saturated solution of methanol and Beckman Fluoralloy Dry Mix A), then

c) for methanol and then

d) for distilled water overnight, before the gel was dried down under a flow of warm air whilst laid flat on a glass plate. (Randerath, 1970 and Laskey and Mills, 1975).

Alternatively, En^3Hance was used to enhance tritium signals in gels. Agarose slab gels were soaked with gentle stirring in 30ml En^3Hance for $2^1/2$ hours at room temperature, rinsed with distilled water for $2^1/2$ hours and dried flat on aluminium foil on a glass plate using a hairdryer. LKB tritium-sensitive film was used for tritium autoradiography whilst Kodak X-Omat MA was used for ^{32}P signals. Kodak Intensifying screens were used in autoradiography cassettes and exposure was at -20° for 3 - 62 hours depending on the ^{32}P signal intensity, or 3-10 days depending on the ^3H signal intensity. Kodak liquid X-ray developer was used with Kodak liquid X-ray fixer FX40.

5.2.4 Photography

Ethidium bromide stained gels were photographed using one of two camera systems with UV illumination, supplied by a transilluminator, or at either 254nm or 304 nm supplied by a UV lamp. Kodak Wratten gelatin orange filters were used to filter out orange fluorescence from the DNA-ethidium bromide complexes, and UV light was filtered out by a specific Kodak UV filter. Ilford film, ASA 400, was exposed at an aperture of 2,8 for 0,5 to 10 seconds, developed for 6,5 minutes at 20° using Paterson Acutol developer, and MayBaker Amfix fixer for 2 minutes. Polaroid type 665 film was exposed for between 10 and 20 seconds at an aperture of 2,8 using appropriate filters on a Hasselblad camera. Film was then developed according to the accompanying technical intructions.

5.2.5 Extraction of plasmid DNA from bacteria

Cultures of bacteria harbouring plasmid DNA were initiated from single

colonies obtained by streaking on selective, antibiotic-containing agar plates.

5.2.5.1 Bulk extraction of plasmid pBR322 DNA was performed essentially according to the methods of Clewell and Helinski 1972, and Guerry et al, 1973, but modified as follows: E.Coli were grown to late logarithmic phase of growth in 10ml Luria broth containing bacteriostatic levels of ampicillin and tetracycline at 37°. A 1:500ml dilution was then made into Luria broth, and incubated at 37° with vigorous magnetic stirring for aeration until OD_{600} = 1,5, i.e. late log phase. Plasmid DNA was then amplified in preference to chromosomal DNA for 12 hours or overnight at 37° with 150µg/ml final concentration of chloramphenicol. (Lewin, 1977, Clewell and Helinski, 1972). Whilst techniques were being developed, preferential labelling of plasmid DNA was achieved with 0,5 µCi/ml ^{3}H-thymidine for 5 hours during amplification.

Bacteria were harvested by spinning at 7000g for 10minutes at 10°. The pellets were resuspended in 250-500ml cold fresh 10mM Tris HCl, 1mM Na_2EDTA pH8,0, and spun at 7000g, for 10 minutes at 4°. This pellet was then resuspended in 10ml per 250ml starting material, of cold 0,05M Tris HCl pH8,0; 25% sucrose. Bacteria were lysed with 2ml of cold fresh 10ug/ml lysozyme solution in 0,05M Tris HCl pH8,0; 25% sucrose, and incubated on ice with gentle stirring for a minimum of 5 minutes, or until the solution cleared. 0,2ml cold 0,5M Na_2EDTA pH8,5 was then added to inhibit DNAse activity. Cellular lysis was completed by addition of 20ml cold fresh detergent solution to obtain a cleared, highly viscous "cleared lysate". 100ml detergent solution consisted of 1ml 10% Triton X100; 12,5ml 0,5M Na_2EDTA pH8,5; 5ml Tris HCl pH8,0; and 81,5ml distilled water. After no more than 10 minutes swirling on ice, chromosomal DNA was precipitated by centrifugation at 20 000rpm for 45 minutes at 4°. Cleared lysates were further purified by various methods. (See section 5.2.9)

Varying the lysozyme concentration was investigated since it is held that the ratio of lysozyme concentration to the number of cells is critical for high yields of plasmid DNA. However, decreasing the lysozyme concentration by half (by doubling the reaction volume) as well as using a fresh stock as opposed to frozen and thawed crystals of lysozyme, made no significant difference to plasmid yields. (See section 3.1.1)

5.2.5.2 Screening colonies for recombinant plasmid DNA was performed using either of two methods;

1) The rapid alkaline extraction procedure described by Birnboim and Doly, 1979. Selected clones were grown in 2,5ml L-broth containing 15ug/ml tetracycline for 18 hours, after which 1/5 was extracted whilst the rest was frozen down at -20° in 4% glycerol. After a 15 second spin, the supernatant was carefully removed and the pellet resuspended in 100ul freshly prepared 2mg/ml lysozyme, 50mM glucose, 10mM Na_2EDTA; 25mM Tris HCl pH 8,0 and left at 0° for 30 minutes. 200ul 0,2N NaOH; 1% w/v SDS was then added and the tube gently vortexed to clear the suspension, which becomes viscous. After 5 minutes at 0°, 150ul of 3M sodium acetate pH4,8 was added to denature high molecular weight DNA. 60 minutes at 0° allowed most protein and high MW RNA and chromosomal DNA to precipitate. The supernatant was cleared by centrifugation for 5 minutes, after which 4/5 of the supernatant was ethanol precipitated, and the plasmid DNA was re-suspended in 100ul 0,1M sodium acetate; 0,05M Tris HCl pH8,0. This solution was then reprecipitated and resuspended in 40ul distilled water for immediate electrophoretic analysis.

2) The alternative second method used to screen colonies for recombinant DNA, by gel analysis, is described by Sherratt, 1979. Single colonies with antibiotic sensitivity profiles expected of recombinant colonies were screened by resuspension in 200ul 0,04M Tris HCl pH8,2, 0,02M NaAc, 1mM Na_2EDTA, and 50ul lysing buffer i.e. 10% Ficoll-Paque, 5% SDS, and 0,05% bromophenol blue. Solutions were heated at 65% for 45 minutes then vortexed for 10-15 seconds before immediately being applied to 1% horizontal agarose gels for electrophoretic analysis.

5.2.6 <u>Infection of human cells in culture with BK (MM) virus</u>

Human embryonic brain glial cells were obtained from biopsy material. Secondary and tertiary cultures were grown to form monolayers in Falcon flasks or in roller tubes, with flying cover-slips. Virus stock was diluted in MEM without serum but containing penicillin, streptomycin and reomycin, and buffered with Hepes and $NaHCO_3$. Monolayers, (approximately 10^5 cells/ml),

were drained before 0,5ml/flask or 0,2ml/tube of diluted virus stock (10^{-2}, 10^{-3}, and 10^{-4}) was added and allowed to adsorb at 37° for 90 minutes. Control cells were not exposed to BK(MM)virus. The cells were then incubated at 37° and monitored for cytopathic effects by light microscopy and staining of flying coverslips for intranuclear inclusions. Cells and media were harvested once 75 - 100% of cells showed intranuclear inclusions and/ or cytopathic effects.

5.2.7 Extraction of BK viral DNA from glial cells infected in tissue culture

Viral DNA was extracted essentially according to the procedure described by Hirt et al., 1967. This differential salt precipitation of cellular DNA, and SDS denaturation and precipitation of proteins, requires a very gentle technique since any pipetting, vortexing or shearing causes denaturation of cellular DNA which then remains in solution and does not separate from viral DNA in the supernatant.

Cells were extracted separately from the media, by the addition of 1ml 0,6% w/v SDS in 0,01M Na_2EDTA pH7,5 at 20°. Cells slid easily off the dish after 30 minutes and were transferred to a centrifuge tube and brought to 1M (final concentration) NaCl. The tube was inverted gently 10 times then left overnight at 4°. Cellular DNA and the protein-SDS precipitate were separated from viral DNA by centrifugation at 17000g for 30 minutes at 4°. Viral DNA in the supernatant was then purified by treatment with 10µg/ml heated RNAse A at 25° for an hour, followed by two phenol and two chloroform extractions and alcohol precipitation of the DNA.

5.2.8 Extraction of human polyoma viral DNA from urine

After a low speed spin of the urine (5000g, 10 minutes, 4°) to remove debris, viral DNA was precipitated at 30 000 rpm in a SW 50.1 rotor at 4° for 60 minutes and resuspended in a minimal volume (1-2 drops) of distilled water. 2ml (.1N) sodium perchlorate, 1% w/v SDS was added, the suspension vortexed and incubated at 37° for 20 minutes. Two chloroform extractions preceded the addition of a further 10ml of 1N sodium perchlorate, 1% w/v SDS solution and incubation at room temperature for three hours. Five more chloroform extractions were performed, after which the solution was made 0,5M with

respect to NaCl, and the DNA alcohol precipitated prior to gel analysis and further purification, etc. (Harley and White, 1973a).

5.2.9 Purification of DNA

5.2.9.1 Phenol extractions were performed as described by Meyers et al., 1976, to remove residual proteins. An equal volume of TES-saturated redistilled phenol containing 0.2% w/w 8 - hydroxyquinolone was added to the cleared lysate, and the phases separated after gentle inversion of the tube, by centrifugation at 20° for 15 minutes at 12 000 rpm. This extraction was repeated until the aqueous-phenol interface was quite clear. Residual phenol was removed by 3 to 4 chloroform extractions using chloroform containing 1% v/v isoamylalcohol, 2% v/v distilled water. The clear aqueous phase was brought to 0,3M sodium acetate, 2 volumes of cold absolute ethanol were added to precipitate the DNA at -20° for 5 to 12 hours. After spinning at 12000g at 2° for 30 minutes, precipitated DNA was vacuum dried and resuspended for 12 or more hours in appropriate volumes of appropriate buffers.

5.2.9.2 Acid phenol extractions were performed as described by Zasloff et al., 1978, to selectively remove chromosomal DNA. Acid catalysed depurination of DNA was minimised by working at 4°. Protein was completely removed from DNA solutions prior to acid-phenol extractions by several phenol-chloroform extractions. DNA in TES buffer at a concentration equal to or less than 500µg/ml was made acidic by the addition of 1/20 volume 1M sodium acetate pH4,0 and 1/20 volume 1,5M NaCl. This solution was shaken vigorously with one volume of redistilled phenol, equilibrated with 50mM sodium acetate pH 4,0, and then was spun at 8000g for 5 minutes at 4°. The aqueous phase was re-extracted and then neutralised with 1/20 volume 1M Tris HCl pH8,0. Phenol was removed by two chloroform-octanol extractions (24:1 v/v) at 20°.DNA was then alcohol precipitated.

5.2.9.3 Caesium-chloride ethidium bromide density gradient centrifugation separated DNA according to molecular conformation and size; i.e. linear, NOC, and CCC molecules were separated from each other. (Guerry et al., 1973, Pikó et al., 1968). DNA dissolved in TES buffer pH8,0 was added to CsCl to give a refractive index of 1,394 ± 0,001. EtBr solution was added to a final concentration of 0,76 µg/ml. The solution was overlaid with paraffin

oil and spun at 23 000 rpm at 20° for 64 hours in SW 27.1 rotor or at 37 000 rpm in a SW 50.1 rotor. Bands of CCC DNA were removed by piercing the tube with a sterile syringe under UV illumination and collecting the drops in fractions, or by fractionation of the gradient using an Imsco fraction collector. EtBr was removed by extraction with TES-saturated amylalcohol. CsCl was removed by extensive dialysis against TES buffer at 4°. DNA containing fractions were detected by agarose gel electrophoresis or by determination of tritium-radioactivity of aliquots of the fractions. Alternatively large amounts of DNA were detected by OD_{260} measurements.

5.2.9.4 Polyethyleneglycol (PEG) 6000 concentration of large volumes of DNA-containing solutions was performed by addition of 1/10 volume of 5M NaCl to DNA solutions. 1/10 volume of PEG 6000 was added and the solution left for 12-18 hours at 4°. The precipitate was spun down at 4-5000g for 10-15 minutes at 4°. After resuspension of the pellet in 10mM Tris HCl pH8,0; 1mM Na_2EDTA, PEG was removed into the supernatant with an equal volume of chloroform 2% v/v octanol. (Humphreys et al., 1975). The concentrated DNA solution was then further purified by phenol extractions or by CsCl-EtBr density gradient centrifugation.

5.2.9.5 RNA was digested by treatment with 20µg/ml RNAse A at 37° or 25° for 1 hour. DNAse activity in the RNAse A solution was destroyed prior to use by boiling for 2 to 3 minutes. Dialysis of small volumes was performed using a perspex unit which allows continuous passage of dialysis buffer from a peristaltic pump past the stationary DNA-containing solution. All dialysis membrane was pretreated by boiling in distilled water containing 10mM Na_2EDTA and 0,12M Na HCO_3 to inhibit DNAse activity and to neutralise the membranes, which were then rinsed in 50% ETOH and TES buffer containing 100µg/ml tRNA to prevent DNA adherence to the surface of the membrance.

5.2.9.6 DNA was electroeluted from agarose or acrylamide gels in the following manner.

Under UV illumination for a minimum length of time, bands of the particular DNA required were sliced from cylindrical or slab gels using a flame-sterilised blade. Electroelution apparatus was constructed by covering one end of a cut-off sterile plastic pipette tip with treated dialysis membrane, and the other end with gauze. The tip was then inserted into one end of a perspex cylindrical tube 5mm in diameter, and all leakage prevented by sealing with

parafilm. Electrophoresis buffer containing 5% glycerol and 50ug/ml tRNA was pipetted into the dialysis chamber and all air bubbles were excluded. The gel slice was placed flat on top of the gauze and electrophoresis carried out for 90 minutes at 75V. Current was reversed for 5 seconds to release DNA from the membrane. DNA electrophoresed from the gel into the chamber was then carefully removed with a Pasteur pipette, the chamber extensively rinsed with buffer which was pooled with the DNA solution prior to removal of any agarose by phenol and chloroform extractions and dialysis and alcohol precipitation. Agarose was removed from the DNA by isoamylalcohol precipitation of DNA in the slice, followed by vortexing and centrifugation at 4 000g for 20 minutes at RT°. Alcohol was then evaporated at 37°, and DNA was alcohol precipitated at -20°. (Tabak and Flavell, 1978; Fuke and Thomas, 1970; Yang et al., 1979c.)

5.2.10 Electrophoresis of DNA

It has been shown by various workers that DNA molecules in different conformations (CCC, NOC, or linear) separate according to their MW as well as their conformations under electric charges in both agarose and acrylamide gels. Mobility is inversly proportion to MW, whilst, when dealing with $2-5 \times 10^6$ dalton molecules in 0,8 - 1,2% agarose or 2-3% acrylamide gels CCC DNA migrates more rapidly than linear DNA which in turn migrates faster than NOC DNA of the same MW (Harley et al., 1973b; Johnson et al., 1977; Aaij and Borst, 1972).

5.2.10.1 Agarose gel electrophoresis of DNA

1% w/v agarose was dissolved by boiling in electrophoresis buffer (Dugaiczyk, 1975). The gel was poured at 47° onto the 10 x 10mm horizontal bed of the perspex electrophoresis apparatus or into 5ml perspex cylinders sealed with parafilm. The gel was set at RT° for 2 hours and then 20 - 50ul samples containing 5% glycerol and bromophenol blue as a marker dye were layered into the slots. Slab gels were run for 10 minutes with migration towards the anode, after which the gel was flooded with buffer and electrophoresis was carried out at 35V, 18mAmp for 16 hours, or at 75V 30mAmp for $2^1/2$ hours at RT°. Cylindrical agarose gels were run for 75V, 42mAmp, for $2^1/2$ hours. Gels were stained with 0,5ug/ml EtBr in electrophoresis buffer for 20 minutes. Examination under short wavelength ultraviolet

illumination allowed detection of less than 10ng DNA in a single band, the precise level being dependent on the molecular size of the DNA.

5.2.10.2 Polyacrylamide gel electrophoresis of DNA

DNA molecules of low molecular weight (e.g. as small as 1 x 10^6 daltons) were resolved by electrophoresis on 3% polyacrylamide gels. 5% glycerol was added to the electrophoresis buffer and to the gel constituents if the gel was to be sliced. Gel solutions consisted of

1) 10ml recrystallised 12% w/v acrylamide containing 0,6% w/v N N' methylenebisacrylamide made up in double distilled water, to give 5% cross-linked gels; 4ml 10 x electrophoresis buffer; 4 ml glycerol; 0,40ml 10% TEMED made up fresh in double distilled water; and 21,20ml double distilled water.
2) 0,40 ml 10% ammonium persulphate, also made up in double distilled water.

Solutions 1 and 2 were separately degassed using a vacuum pump. They were mixed at 4° and 4,5ml aliquots poured into perspex cylinders covered at one end with parafilm and gauze. Gels were overlaid with degassed water and polymerised at 4° for 2 - 3 hours. Gels were pre-electrophoresed at 75V, 20 mAmp for an hour at 20°, after which 10 - 20ul samples containing 5% glycerol and bromophenol blue were layered onto the gel and migration of DNA towards the anode was carried out at 75V, 15mAmp for 4 hours at room temperature.

Pressure from a wide-gauge syringe was used to extrude gels from the cylinders; they were then stained in 0,5ug/ml EtBr in electrophoresis buffer. Gels to be sliced were frozen in dry ice in aluminium foil holders to retain the gel shape. Reproducible slices were obtained using a Mickle gel slicer.

5.2.11 Restriction Endonuclease Digestion of DNA

All restrictions were carried out in gas-sterilised Eppendorf tubes. Solutions were preheated to 37° prior to addition of enzyme and incubation at 37° for an hour. Reactions were terminated by inactivating the enzyme at 65° for 10 minutes. One unit of enzyme activity (that amount of enzyme required to digest completely 1,0ug lambda phage or equivalent DNA in an hour at the appropriate temperature - generally 37° - i.e. a 50ul reaction volume)

was used for all amounts of DNA less than or equal to 1ug, to be digested, (Nathans and Smith, 1975.).

Buffers used for digests were as follows: (based on recommendations made by suppliers of the enzymes)

Hind III: 6mM $MgCl_2$; 50mM Tris HCl pH7,5; 60mM NaCl.

Pst I; EcoR I; Pst I EcoR I simultaneous digests: 60mM Tris HCl pH7,5 6mM $MgCl_2$; 50mM NaCl; 6mM DTT made up freshly on day of use.

BamH I : 6mM $MgCl_2$; 20mM Tris HCl pH7,0; 100mM NaCl; 6mM DTT made up freshly on day of use.

Sal I: 8mM Tris HCl pH7,6; 6mM $MgCl_2$; 0,2mM Na_2 EDTA; 150mM NaCl.

10 x stock solutions of digest buffers were made up weekly (excepting dithiothreitol which was made up freshly on the day of use), filter-sterilised and stored at 4°.

5.2.12 Ligation of restriction endonuclease digested DNA molecules

5.2.12.1 Reaction conditions were based on those discussed by Dugaiczyk et al., 1975, and were carried out in gas-sterilised Eppendorf tubes. Buffers were made with sterilised water and were millipore-filtered before use. Final concentrations of ligation buffer constituents were 66mM Tris HCl pH7,6; 10mM $MgCl_2$; 10mM dithiothreitol, and 1mM ATP. DNA was cleaned and purified before ligation. Agarose was removed as far as possible by phenol and extensive chloroform extractions and/or ethanol precipitation, and washes with 70% ethanol 30% ligation buffer . Ethanol was completely removed by drying with a vacuum pump. Where possible, DNA to be ligated was not electroeluted from gels since constituents thereof, particularly agarose, inhibit ligase. Agarose apparently coats the DNA helix rendering it inaccessible to ligase.

5.2.12.2 Investigations of reaction conditions were performed to optimise ligations. DNA concentrations varied from 0,6 to 32ug/ml in reaction volumes of 20 to 100ul (Modrich et al., 1973). j/i ratios for recombinant circularisation were calculated with respect to the MW of the linear ligated recombinant dimer/multimer molecules concerned and ranged from 0,06 to 7,5. 0,2 - 2,0 Weiss units of enzyme were utilised, depending on the amount of DNA present. (One Weiss unit is the amount of T4 DNA ligase catalysing

the conversion of 1nmol of ^{32}PPi into (α/β ^{32}P) - ATP at 37° in 10 minutes. Recombination ligations were performed with either equimolar proportions of insert and bacterial alkaline phosphatased vector molecules or with three fold excess insert DNA to non-alkaline phosphatased vector molecules. Optimum temperature was found to be 12,5°, a compromise between the ligase's optimum temperature of 37° and the melting temperature of noncovalently bound cohesive ends (i.e. 5 - 6° with EcoR I ends). Eventually, incubations varied from 2 to 5 hours duration after which the DNA was added directly to competent cells, or 20mM EDTA was added to inhibit the enzyme by chelating Mg^{2+} ions. If the ligated DNA was to be used in the transformation of cells, use of EDTA was avoided to prevent a reduction in the frequency of transformation (see section 3.4.4) Alternatively, the solution was heated to 65° for 10 minutes.

5.2.12.3 To prevent religation of Pst I EcoR I digested pBR322 molecules, and thus to favour recombination with untreated BK (MM) DNA or other foreign DNA, digested pBR322 was treated with bacterial alkaline phosphatase to remove 5' phosphoryl groups. (Ullrich et al, 1977). 10ug digested pBR322 in 250ul 6mM $MgCl_2$; 100mM NaCl; 10mM Tris HCl pH7,5 was treated with 12,5 units of nuclease-free bacterial alkaline phosphatase at 65° for 30 minutes. One unit of bacterial alkaline phosphatase is defined as the amount of enzyme which hydrolyzes 1 nmol of (γ- ^{32}P) - ATP in 30 minutes at 37°. However, optimum dephosphorylation requires incubation at 65° (Efstratiadis et al, 1977). The enzyme was removed by phenol and chloroform extractions followed by alcohol precipitation of the DNA. (See section 3.1.2.)

5.2.13 <u>Transformation of E.Coli K-12 with pBR322 or with recombinant pBR322 molecules</u>

5.2.13.1 The transformation procedure for E.Coliχ1776 was first described by Curtiss et al, 1977, but the modifications described by Norgard et al, 1978, were followed in these experiments. Overnight cultures of χ1776 in L-broth supplemented as described (5.1) with diaminopimelic acid and thymidine were diluted 1:100 into prewarmed growth medium and incubated at 37° with shaking to an OD_{550} of 0.28 - 0.32. Cells were harvested by spinning at 2 500g, 10 minutes at 4°. Pellets were washed twice in 25ml cold 0,1M NaCl; 5mM $MgCl_2$ 5mM Tris HCl pH7,6 and left to stand on ice each time for 5 minutes. The third wash was performed in 20ml 100mM Ca Cl_2, 250mM KCl, 5mM $MgCl_2$, and 5mM Tris HCl pH7,6 and the resuspended pellets were left to stand for 25 minutes on ice. The final pellet was resuspended in 0,4ml

of the latter buffer. 100µl of an approximately 30µg/ml solution of DNA in $CaCl_2$ buffer was added to 0,2ml competent cells and left on ice for an hour. Cells were then immediately plated out onto selective plates which were incubated at 37°. It was found that pre-incubation in broth not containing antibiotics,led to cell clumping and lysis before plating. χ1776 grew more slowly than C600 and HB101 and therefore was usually incubated for 48 hours before inspection for colonies. Great care was necessarily taken to avoid all contact of χ1776 with detergents of any kind; even scratched glassware was avoided since residual detergent may stick to such surfaces.

5.2.13.2 The transformation procedure finally adopted after extensive comparisons with other methods for the C600 and HB101 strains, was based on that described by Cohen et al., 1973a, and was modified according to the protocol of Dr. F.Robb (Dept. Microbiology, U.C.T.).

Single colonies from overnight L-agar plates were inoculated into 50ml L-broth containing 2g/l Casamino acids and grown overnight to late logarithmic phase of growth. A 1:100 dilution into supplemented L-broth was made and cultures were incubated in a 37° shaking water bath until OD_{600} = 0,2 to 0,3. Cells were harvested by centrifugation at 2 500g for 10 minutes at 4°. Pellets were gently resuspended in 10ml cold sterile 50mM $CaCl_2$ and spun again at 2 500g for 10 minutes at 4°. The pellet was then gently resuspended in 1ml 50mM $CaCl_2$ and left waiting on ice until the ligation reaction was complete. Transformation frequencies were found to increase as much as 10 fold if DNA was ligated immediately prior to its addition to competent cells. 200µl competent cells were added to 10-150µg ligated DNA in approximately 10 - 20µl reaction volume, and the mixtures left on ice for 60 minutes. This was followed by a 2 minute 45° heat shock after which cells were chilled on ice. 2 - 3 ml warm L-broth (antibiotic free) was added and the cells incubated at 37° for 60-90 minutes in a shaking water bath to allow expression of plasmid vector genes conferring antibiotic resistance to recombinant colonies, before challenging with an antibiotic. 100µl aliquots were plated onto fresh control and selective plates and incubated at 37° for 24 hours before inspection for colony growth. Controls included both nonselective and selective plates onto each of which were plated

1) $CaCl_2$ and competent cells, or
2) DNA ligation mixture.

5.2.13.3 Selective plates were prepared from L-broth pH7,2 added to sterile agar, and they contained the bacteriostatic dose of tetracycline. Particular minimum inhibitory concentrations of the antibiotics for different E.Coli strains were predetermined on agar and in broth using the standard microbiological method.

5.2.13.4 Screening of colonies by antibiotic sensitivity profiles.

pBR322 contains genes coding for resistance to 1) ampicillin and
2) tetracycline.

Insertional inactivation of the ampicillin gene occurs by recombination with foreign DNA after restriction at the Pst I site. Thus, Amp^S Tet^R colonies putatively contained recombinant plasmids. The possibility of ligation of two large Pst I Eco RI fragments of pBR322 raised the likelihood of non-viral-DNA-containing colonies expressing Amp^S Tet^R. Colony hybridisation decreases the possibility of selecting such colonies for DNA screening.

Religated full length pBR322 vectors confer Amp^R to the cells and therefore Tet^R colonies are initially screened by replica plating or toothpicking for ampicillin resistance; such colonies are disregarded for further analysis.

1) Replica plating allows the transfer of many bacteria simultaneously on a velvet cloth on a wooden block, pressed onto the tetracycline plate and then onto an ampicillin plate orientated for colony identification on the original plate. (Hayes, 1965; and Elek and Hilson, 1954).
2) Toothpicking is used for screening smaller numbers of colonies. A sterile toothpick is used to transfer some of a Tet^R colony onto a grid marked on an ampicillin plate for identification of the original colony. (Grunstein and Hogness, 1975).

5.2.14 <u>End-labelling of DNA molecules digested with restriction endonucleases giving 5' hydroxyl groups.</u>

The method described by Maxam and Gilbert (Methods in Enzymology,1980,Part 1) was followed, with the exception that the complete E.Coli DNA polymerase I was used since the Klenow fragment of E.Coli DNA polmerase I was not available (see section 3.6.1) 2ug lambda DNA were digested with Sal I or with EcoRI as a negative control(with no dATP supplied as the nucleotide required.) 10uCi (methyl 1',2' - ^{3}H)- thymidine triphoshate was

dried down under vacuum in a sterile Eppendorf tube to which the digested lambda DNA was added along with 7,2 units E.Coli DNA polymerase I, and 10μg nuclease-free BSA in a final volume of 20ul. After $2^{1}/2$ hours at 37°, the reaction was stopped with 5ul 10% w/v SDS,5% glycerol and bromophenol blue. TCA precipitable counts were determined and the products were electrophoresed through a 1% agarose slab gel. Unincorporated ^{3}H.dTTP was removed by extensive washing of the gel in 5% acetic acid, followed by 12 hours rinsing with distilled water. After this the gel was dried down and autoradiographed, as well as being scanned with the Berthold Dünnshicht scanner.

5.2.15. <u>In vitro labelling of DNA by nick-translation, using single labelled nucleotides.</u>

Labelled and unlabelled nucleotide triphosphates used for nick translation were tested for their state of phosphorylation i.e. mono-,di -,or tri-phospates by high pressure liquid chromatography using a 3-99% gradient of filtered 0,5M $Na_4H_2PO_4$ pH4,3 made up with double distilled water, on an anion exchange column G12 D1S5 CS6 with a flow rate of 4ml per minute. (F.C.on and F.S.D. = 0,1 260nm). Fractions were collected at 30 second intervals and counts of aliquots determined in the Beckman β counter. The majority of nucleotides were present in the triphosphate state and were therefore suitable for use in nick translation experiments. Protocols investigated included 1) Amersham Technical Bulletin 80/3,

2) Rigby et al., 1977 ; Deutscher and Kornberg 1969; Maniatis et al., 1975).

Preliminary development of the technique involved use of Pst I EcoRI digested pBR322, and undigested lambda DNA. Initially 200pg DNase I was incubated with 100ug PstI EcoRI digested pBR322, 8uM 3H.dTTP (12,4 uCi), and a 2 fold excess (16uM) of unlabelled nucleotide triphosphates, and 5,5U E.Coli DNA polymerase I. Labelled nucleotide triphosphates were lyophilized before use to remove all alcohol in the sample. (1 unit is defined as the amount of enzyme that incorporates 10nmol of total nucleotide into acid precipitable form in 30 minutes at 37° using a poly (dA-dT) template-primer). Nick translation buffer used was the same as for simultaneous Pst I EcoR I digests, containing fresh 6mM DTT. Incubation was at 15° for $1^{1}/2$ - 2 hours. Reactions were stopped by the addition of 10mM Na_2EDTA; 0,6% SDS pH7,2.

Accumulation of acid-precipitable counts was monitored throughout the reaction at 30 minute intervals. Unincorporated labelled nucleotides were removed by dialysis against TES buffer. Sizes of labelled DNA products were determined by determination of acid-precipitable counts of fractions of a glycerol density gradient. Centrifugation of the 5% to 30% glycerol and TES gradient, was at 35 000 rpm for 16 hours at 4° in a SW 41 Ti rotor. In addition, fractions were electrophoresed through a 1% agarose slab gel, which was then autoradiographed.

Alcohol-precipitated commercially prepared lambda DNA was electroeluted from 3% polyacrylamide or 1% agarose gels and nick translated at 15° without DNase 1, using (per microgram of DNA) 1,28uM ^{3}H-dTTP (2uCi), 40 uM unlabelled nucleotide triphosphates and 3,7 U E.Coli DNA polymerase I. Acid precipitable counts of aliquots were determined following phenol and chloroform extractions and alcohol precipitation. Aliquots were electrophoresed on cyclindrical 1% agarose gels which were then sliced and counted to obtain the sizes of the labelled products.

BK (MM) DNA was electroeluted from 3% polyacrylamide gels and alcohol precipitated before nick translation, or alternatively was purified by the following steps :

1) Treatment with 50ug/ml heated RNase A for 2 hours at 37° followed by
2) separation of RNA from DNA on a Sephadex G200 column. The Sephadex was boiled for 3 hours before being equilibrated with TES buffer and poured into a 5ml sterile plastic pipette plugged with sterile glass wool.
3) An acid phenol extraction was performed to remove chromosomal DNA, after which viral DNA was
4) alcohol precipitated, washed in 70% ethanol, 3% TES buffer, vacuum dried and resuspended in nick translation buffer.

1200ng BK(MM) DNA were nick translated without DNAse I, using 16 pmols α-^{32}P -dCTP (16 uCi) and 2 fold excess unlabelled nucleotide triphosphates, with 7,2 U E.Coli DNA polymerase I. Acid precipitable counts were determined on an aliquot of material. Unincorporated trinucleotides were separated from the DNA by passage through a 1ml Sephadex G50 column in a sterile Pasteur pipette plugged with sterile glass wool. Beads were swollen by heating at 37° for an hour and were then equilibrated with 3 column volumes of TES buffer. After allowing the column's top 1cm to run dry,

100ul sample was layered on top and 150ul fractions collected in sterile Eppendorf tubes. Aliquots were counted and electrophoresed through a 1% agarose slab gel which was autoradiographed in order to determine the sizes of labelled products. The probe specificity was tested by hybridisation to a Southern gel blot containing pBR322, E.Coli chromosomal DNA, BK (MM) and (WW) DNA and digests thereof, as well as control human brain glial cell chromosomal DNA. The blot was then autoradiographed. (See section 3.6.4)

5.2.16. Southern Transfers of DNA from agarose gels.

DNA transfer to nitrocellulose filters for hybridisation with radioactive DNA probes was carried out essentially as described by Southern, 1975, with modifications described by Southern in 1979. Horizontal 1% slab agarose gels were photographed alongside a ruler for orientational purposes. DNA was denatured by soaking the gel in 250mls 0,5M NaOH; 1,5M NaCl for 3-5 hours with gentle rotatory shaking. Gels were rinsed with distilled water and neutralised for 1-2 hours in 250mls 0.5M Tris HCl, 20 x SSC pH5,5. A photographic tray was half-filled with 20 x SSC pH7,0. A piece of thick filter paper soaked in 20 x SSC was placed flat on a glass plate resting on two sides of the tray, with 2 ends in the 20 x SSC acting as wicks. The treated gel was gently placed on the filter paper, taking care not to trap air bubbles between the two layers. A sheet of Millipore HA 0,45uM nitrocellulose, the size of the gel, and soaked by flotation in 2 x SSC, was placed on the gel, starting at one side and excluding all air bubbles. The filter paper on either side of the gel was covered with cling film to prevent evaporation of SSC and sagging of the filters. 2 pieces of thick filter paper, the size of the nitrocellulose membrane, were soaked in 2 x SSC and gently placed on top of the membrane. A thick wad of paper towels was piled on top of these filter papers and a glass plate laid on top. The whole assembly was covered with cling film and lead pots were positioned on the plate to weigh the towels down evenly. Transfer was carried out at 4° for 48 - 62 hours with replacement of the 20 x SSC at intervals.

After transfer, well positions were marked on the nitrocellulose filter, which was then cut into conveniently-sized pieces and marked with orientation markings, soaked in 2 x SSC for at least 10 minutes, and baked at 80° for 2 hours, or until dry. Before hybridisation, nitrocellulose membranes were stored at 4° in sealed filter paper jackets.

5.2.17. Hybridisation of ^{32}P- labelled BK(MM) DNA to Southern gel transfer filters

This technique was based on that described by Southern, 1975. Nitrocellulose membranes were soaked at 65° in 50 - 150ml of fresh preheated solutions of 3 x SSC for 60 minutes followed by soaking in 3 x SSC; 1 x Denhardt's solution; 0,1% sodium pyrophosphate for one hour. 0,25 - 0,60 g ^{32}P labelled DNA ($6,25 \times 10^6$ - $12,5 \times 10^6$ cpm) was heat denatured by boiling for 5 minutes and the volume made up to 5 ml by addition of hybridisation solution (3 x SSC; 1 x Denhardt's; 0,1% w/v SDS; 50ug/ml herring sperm DNA; 0,1% sodium pyrophosphate). Filters were sealed into a plastic bag containing 5ml hybridisation mix, and excluding all air bubbles, and the bag immersed in water in a Tupperware box ; hybridisation was performed at 65° in a shaking water bath for 48-62 hours. Individual filters were separated from each other in the same bag by blank, washed, nitrocellulose filters.

Post-hybridisation washes of the filters consisted of six 2 minute washes in 50 - 150ml 3 x SSC; 10 x Denhardt's; 0,1% w/v SDS at 65°, followed by two more washes of 30 minutes with shaking. Filters were carefully and individually transferred from one solution to the next using forceps, and sterile rubber gloves were worn at all times. These steps were repeated if background radioactivity was still high, as monitored with a Geiger Counter. 3 stringent washes followed, using 0,1 x SSC; 0,1% w/v SDS at 65°,lasting 30 minutes each. Filters were blotted dry on absorbent filter paper, dried at 37° and then stuck onto cardboard in the correct orientation, wrapped in cling film and autoradiographed for 4 - 24 hours at -20°.

The Denhardt's solution and denatured herring sperm DNA reduce background nonspecific hybridisation of labelled DNA to the filter. Sodium pyrophosphate exchanges with free ^{32}P to reduce background radioactivity.

5.2.18 Colony hybridisation

Selected bacterial clones were screened by DNA-DNA hybridisation to identify those containing specific DNA sequences i.e. containing BK DNA inserts which hybridise to the BK(MM) DNA radioactive probe. A modified method of Grunstein and Hogness, 1975, was followed after investigation of a protocol obtained from G.Hager, NIH, USA.

5.2.18.1 Circles of nitrocellulose filters were cut from sheets (Millipore HA, 0,45um pores) and orientated with pencil marks. The filters were handled with sterile millipore forceps and sterilized gloves were worn at all times to prevent contamination with DNAse. Using sterile toothpicks, A^S T^R bacterial clones were transferred onto the circles which were placed onto L-agar plates containing bacteriostatic levels of tetracycline. Approximately 36 colonies were applied per filter circle. Plates were incubated at 37° for 18 hours, by which time colonies were approximately 1mm in diameter. Filters were then removed from the plates and gently blotted onto absorbant paper to remove excess medium. Colonies were lysed at room temperature without disturbing the colony surface of the filters in any way. Filter papers were equilibrated, by flotation, with 0,5N NaOH. To achieve cellular lysis and denaturation of DNA, membranes were applied face-up to these filters for 7 minutes and then blotted and applied to filters equilibrated with 1M Tris HCl pH7,4 for 1 minute . After blotting, the membranes were neutralised by application to fresh filters equilibrated with 1M Tris pH7,4 for 5 minutes and blotted once more. Membranes were then applied to filters equilibrated with 1,5M NaCl; 0,5M Tris HCl pH7,4 for 5 minutes. Colony residues were dried by application of a strong vacuum for approximately 30 minutes. Identified membranes were then dipped upside down in a minimum volume of a fresh 2mg/ml Proteinase K,1 x SSC solution in a petri dish and left for 20 minutes at room temperature. After blotting the membranes dry, they were transferred to 10ml 1 x SSC for 2 minutes, and completely dried by vacuum. They were floated in 10 ml chloroform for 2 minutes and air dried prior to floating in 10ml 2 x SSC for 2 minutes to remove loose cellular debris, and drying for 30 minutes at RT°. Drying of membranes was completed in a 80° oven for 2 hours in filter paper jackets. These filters could also be stored at 4° while waiting for the ^{32}P- probe DNA preparation.

5.2.18.2 Hybridisation of ^{32}P-labelled BK (MM) DNA to nitrocellulose filters from colony hybridisations ; methods described by Wensink et al, 1974, and Grunstein and Wallis, 1979, were used as guidelines. Dried nitrocellulose filters were washed with gentle shaking at 68° for 60 minutes in 25ml prehybridisation solution made up as follows : 4 x SET buffer,

10 x Denhardt's 50ug/ml sonicated herring sperm DNA, 0,1% sodium pyrophosphate, 0,1% SDS. 2,0 x 10^7cpm of ^{32}P-labelled BK(MM) DNA, at a specific activity of 2,5 x 10^7 cpm/µg in 525ul TES buffer, was denatured by boiling for 5 minutes. 4,465 ml of the prehybridisation buffer was added to the probe solution to bring the volume to 5,0ml. Filters were transferred with sterile forceps to a plastic bag which was heat-sealed after addition of the probe solution and exclusion of all air bubbles. Hybridisation was carried out in a Tupperware box in a 68° water bath with gentle shaking, for 64 hours.

Filters were removed from the bag and the two post-hybridisation washes were performed in a Tupperware box; the first wash was in 50ml preheated 4 x SET, 10 x Denhardt's, 0,1% sodium pyrophosphate, 0,1% SDS, at 68° for an hour with shaking, and the second wash was in 200ml 0,66 x SET buffer at 20° for an hour, with gentle shaking. Filters were blotted dry on Whatmann 3MM filter papers, wrapped in cling film and autoradiographed for 28 hours.

Background radioactivity was almost completely removed by a more stringent post-hybridisation wash in 0,1 x SET at room temperature for an hour. This wash was found to be more suitable to the particular ^{32}P- BK(MM) probe used. Wash stringency is related to the size and complexity of the probe involved and specific ideal wash conditions usually have to be determined by trial and error.

Abbreviations

DNA - deoxyribonucleic acid
RNA - ribonucleic acid
DNAse - deoxyribonuclease
RNAse - ribonuclease
SV 40 - Simian Virus 40
E. Coli - Escherichia Coli
Pst I, Eco RI, Bam HI, Sal I, Hinc II, Hind III - restriction endonucleases
CCC - covalently closed circular conformation of DNA
NOC - nicked open circular conformation of DNA
p.f.u. - plaque forming units
$Amp^{R/S}$ - ampicillin resistant or sensitive
$Tet^{R/S}$ - tetracycline " " "
MW - molecular weight
UV - ultraviolet
BSA - bovine serum albumin, fraction V
HCl - hydrochloric acid
TCA - trichloracetic acid
cpm - counts per minute
dpm - disintegrations per minute
Tris - NH_2 $(CH_2OH)_3$ - (Hydroxymethyl) methylamine
TEMED - N, N, N', N' - Tetramethylethylenediamine
Hepes - (N-2-Hydroxyethylpiperazine-N'-2-ethanesulfonic acid)
DTT - dithiothreitol
CsCl - caesium chloride
EtBr - ethidium bromide
SDS - sodium dodecyl sulphate
TES buffer - 30mM Tris HCl pH8,0; 50mM NaCl; 5mM EDTA
SET buffer - 0,12M Tris HCl pH7,0; 0,6M NaCl; 8mM EDTA
SSC buffer - 0,15M NaCl; 0,015M sodium citrate, made to pH7,0 with HCl
MEM - minimal essential medium
Denhardt's solution - 0,02% Ficoll (Type 400); 0,02% BSA; 0,02% polyvinyl-pyrrolidone (Type 40) in 3 x SSC (Denhardt,1966)
Electrophoresis buffer - 50mM Tris HCl pH8,05; 2mM Na_2 EDTA; 20mM NaAc.
RT° - Room temperature
chr - chromosomal DNA
Ag - antigen
Ab - antibody

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