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BK Virus Transformation of BHK-21 Cells

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by

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VITA

The author, Martha A. Lampert is the daughter of Elmer G. Lampert and Mary (von der Heidt) Lampert. She was born February 3, 1953 in Aurora, Illinois. Her elementary and secondary education were obtained in the West Aurora public schools. She graduated in 1971. She then attended the University of New Hampshire where in May 1975 she received the degree of Bachelor of Science in Medical Technology.

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1980 and "Analysis of BK virus early gene products using somatic cell hybrids" presented at the Imperial Cancer Research Fund Tumor Virus Meeting, 1979.

TABLE OF CONTENTS

LIST OF TABLES

LIST OF FIGURES

LIST OF FIGURES

ABBREVIATIONS

BHK	Baby Hamster Kidney
BKV	BK Virus
CS	Calf Serum
DMSO	Dimethylsulfoxide
DME	Dulbecco's Modified Eagle Media
EDTA	Ethylenediamine Tetraacetic Acid
FCS	Fetal Calf Serum
FITC	Fluorescein Isothiocyanate
HEK	Human Embryonic Kidney
IFA	Indirect Immunofluorescence
MEM	Minimal Essential Media
m.o.i	Multiplicity of Infection
MBC	Marmoset Brain Cell
PBS	Phosphate Buffered Saline
PEG	Polyethyleneglycol
pfu	Plaque forming unit
PMSF	Phenylmethylsulfonyl fluoride
PPO	2,5 diphenyloxazole
SC	Subclone

ABBREVIATIONS

SDS	Sodium dodecyl sulfate
SV40	Simian virus 40
T	Large T protein
t	Small t protein
TPB	Tryptose Phosphate Broth
ts	Temperature Sensitive

CHAPTER I

INTRODUCTION

This study examines the transformation of BHK-21 cells to anchorage independence by BKV. It follows an observation made in this laboratory that a clone (L29) of BKV transformed BHK-21 cells appeared nuclear large T antigen negative by IFA and immunoperoxidase assays yet was capable of virus rescue (80). This observation stimulated many questions as considerable evidence indicated that the closely related SV40 large T protein is necessary for viral DNA replication, and transformation initiation and maintenance. The current study was undertaken to examine the L29 clone and other clones of anchorage independent BKV transformed BHK-21 cells first for the presence of the BKV large and/or small T proteins as detected by immunoprecipitation of ^{35}S-methionine labeled proteins, then for the parameters which may influence or be influenced by the expression of these viral proteins.

CHAPTER II

LITERATURE REVIEW

The papovaviruses conasitute a group of viruses drawn together by similar physical characteristics. Their unenveloped capsid structure is icosahedral in shape and 40-55 nm in diameter. Their genome is composed of double stranded DNA in a circular, covalently closed, supercoiled conformation. The size of the genome is relatively small, ranging from 3×10^6 daltons to 5.5×10^6 daltons. This small size has made the papovaviruses popular subjects in the study of eukaryotic gene expression and regulation. They are also one of the most widely studied groups of viruses for both their *in vitro* and *in vivo* oncogenic potential.

The papovaviruses can be divided into two genera. These are the papilloma viruses and the polyoma viruses. The papilloma virus group includes a number of different isolates of viruses capable of causing warts in humans and animals. They are separate from the polyoma viruses for two reasons. The first is that the papilloma virion capsid is 55 nm in diameter compared with a 40-50 nm diameter capsid of polyoma viruses. The second is that papilloma viruses cannot successfully be grown in tissue culture while most polyoma viruses can be.

The first DNA tumor virus to be isolated and characterized was a papilloma virus described by Shope in 1933 (104), who studied warts which occasionally became malignant in wild cottontail rabbits. He noticed that cell free extracts made from warts, after passage through

bacteria retaining filters, were capable of causing new warts in rabbits at the inoculation site. Rous (91) reported on the transformation of the cottontail rabbit papilloma wart to a squamous cell carcinoma. Subsequent bovine papilloma isolates were found to be capable of transformation of bovine and murine cells and human isolates were found to be capable of transformation of human cells(5,79).

In most DNA and RNA tumor viruses transformation is accompanied by integration of the viral sequences which are required for transformation maintenance. Among the papovaviruses, papilloma viruses are unique in that integration of viral sequences in tumor cells or in in vitro transformed cells appears to be lacking (2,61,65). The virus exists in these cells as an episome in high copy number. It therefore has afforded investigators a unique cloning vector for the study of eukaryotic gene expression. The mechanisms of transformation by this group of viruses are unknown.

The polyoma genus of papovaviruses consists of viruses of primate and rodent origin. The virus of rodent origin is polyoma virus. Originally, it was detected as a contaminant in cell free extracts of tissues from AK mice being used for studies on murine leukemia (47). The virus was first grown in vitro in mouse embryo cells and the cytopathic effect and productive infection was described by Stewart and Eddy (27,113,114). The virus was found to be capable of inducing multiple types of tumors in hamsters and mice (114,115), thus the name polyoma. It was also found to be capable of transformation of mouse and hamster cells in culture (21,133).

The primate polyoma viruses include the simian virus SV40 and

the human viruses JC (JCV) and BK (BKV). SV40 was originally isolated by Sweet and Hilleman (120) from cultures of rhesus monkey kidney cells which were similar to those being used to produce poliomyelitis vaccines. Eddy et al. (26) first showed that SV40 was oncogenic in newborn hamsters and Shein and Enders (103) first reported the transformation of cultured cells by this virus. In addition to human cells SV40 is also capable of _in vitro_ transformation of mouse, rat and hamster cells (6,103)

The human papovaviruses JCV and BKV were both isolated in 1971. JCV was isolated from brain tissue of a patient with progressive multifocal leukoencephalopathy (PML) (82). The repeated isolation of JCV particles from PML brain tissue makes it the papovavirus most closely associated with human disease. Papovavirus like particles in PML brain tissue had been identified earlier from electron micrographs but Padgett et al. (82), utilizing primary human fetal glial cells, were the first to grow the virus in culture. JCV is highly oncogenic in newborn hamsters when inoculated intracerebrally (134) and is capable of causing cerebral neoplasms when injected into the brains of owl monkeys (67). JCV and JCV DNA can transform primary hamster brain cells in culture (34).

BKV was isolated from the urine of a renal transplant patient by Gardner (36). They observed polyoma-like virus particles by electron microscopic examination of urine sediment from an immunosuppressed kidney transplant patient. They were then able to cultivate the virus in African green monkey kidney cells and determine that it was a newly described virus of the papovavirus group. Several observations estab-

lished BKV as a human virus. BKV was isolated exclusively in humans, it grew preferentially in human cells and antibodies to BKV were detected in 70% of several populations (11,101,122).

In 1973 this human virus was found to be capable of transforming (hamster) cells in culture (69). Subsequently it was shown that BKV can stably transform rat (132), mouse (87), rabbit (87) and monkey (10,87) cells and abortively transform human cells (87). BKV DNA or DNA fragments can also transform cells in culture (86,121).

In some cases cells transformed by BKV *in vitro* are oncogenic *in vivo*. Most hamster cells transformed by BKV *in vitro* induce subcutaneous tumors after injection into weanling hamsters (24). However, mouse and rabbit cells which were transformed by BKV *in vitro* did not produce tumors in the homologous host (87).

BKV is oncogenic when injected directly into hamsters. Studies have shown that it is weakly oncogenic when injected subcutaneously into newborn hamsters (77) and strongly oncogenic when injected intravenously or intracerebrally (18). The in vivo tumor production is an obvious way of determining oncogenic potential and selecting transformed cells. For the *in vitro* study of transformation certain characteristics of transformed cells must be considered for their selection and study.

When cells are infected by papovaviruses one of three things may occur, depending on the species of infected cell and/or the state of the infecting genome. If the cell is permissive the complete viral genome will be replicated, infectious virus will be produced and the cell will be lysed. If the cell type is semi-permissive only a few cells will become lytically infected and virus in the culture will be

produced at a slow rate such that the cell culture survives. If the cell type is non-permissive for the virus transformation may result.

Cells transformed by papovaviruses can exhibit a number of properties which distinguish them from normal cells. Some of these properties may be utilized as a means of selecting transformants. One of these properties is that transformed cells lack normal contact inhibition and thus have a high or indefinite saturation density. Transformed cells loose their dependence on anchorage and can grow in agar suspension. Another property that transformed cells exhibit is tumor formation upon injection into susceptible animals. However, this property is not absolute as some continuous cell lines such as 3T3 cells and BHK-21 cells, can not only grow indefinitely in culture but can also be tumorigenic under certain circumstances (8,117). Transformed cells also usually have a reduced requirement for growth factors present in serum. Thus, they can continue dividing in low serum concentrations which would limit the growth of non-transformed cells. Generally, transformed cells grow in a less ordered manner where they pile up on each other instead of maintaining an almost geometric, even monolayer.

Transformed cells also may exhibit certain characteristic surface and intracellular changes. One of these is increased agglutinability by lectins which was described by Burger and Goldberg (13) and another is increased rate of nutrient transport. Another characteristic cell surface change that accompanies transformation is the appearance of virus specific transplantation antigen (TSTA). This was first indicated by the fact that animals immunized with SV40 or polyoma were resistant to subsequent challenges by transplantable tumors induced

by the respective virus (49,57). Intracellularly, transformation may be accompanied by a disruption of the cytoskeleton, in particular the disruption of the actin cables. This disruption of the organization of actin cables may be the direct cause of other phenotypic changes seen in transformation.

One method of determining the presence of viral DNA in transformed cells is by the rescue of the transforming genome from the cells. Although the transforming genome or parts of it becomes covalently integrated into the cell's chromosome, under certain circumstances it can be rescued as a freely replicating genome. Gerber and Kirchstein (39) reported that when SV40 transformed cells were co-cultivated with permissive cells SV40 could be detected in the culture. If the two cell types were artificially fused with inactivated Sendai virus, infectious SV40 appeared in the culture fluid earlier and in much higher titers (38). Cells could also be successfully fused and virus rescued using polyethylene-dimethylsulfoxide (78) instead of inactivated Sendai virus.

The mechanism of virus rescue from transformed cells is not clearly understood. Results suggest that rescue depends on a diffusable factor supplied by the cytoplasm of the permissive cell rather than on the transfer of viral DNA from the transformed cell nucleus to the permissive cell nucleus. Weaver et al. (135) fused SV40 transformed hamstercells with African green monkey kidney (CV-1) cells and then separated the two types of nuclei in a sucrose gradient. They then determined the presence of infectious virus in the nuclei from different fractions by an infectious center assay. They found that infectious virus first appeared in the transformed cell nucleus and later was pre-

sent in both types of nuclei. Croce and Kaprowski (20) and Poste et al. (88) fused SV40 transformed cells with enucleated permissive cells and obtained infectious SV40.

Other evidence of virus in cells transformed by papovaviruses includes the demonstration of virus specific antigenic proteins, or viral messenger RNA (mRNA). Evidence for virus also includes the detection of virus specific DNA sequences.

Assays for transformation employ the phenotypic changes of transformation as a basis for selection. One such method developed by Stoker and MacPherson (118), involves exposing sub-confluent cultures of cells to virus and then plating them at low cell densities. The untransformed and the transformed cells both form colonies that can be distinguished by their characteristic density and morphology. Another assay involves exposing monolayers of cells to virus and after 2-3 weeks harvesting the transformed cells which appear as dense foci of cells growing out from the monolayer of untransformed cells. In another assay (109) cells are infected with virus and plated at low densities in medium containing low concentrations of serum, insufficient to support the growth of untransformed cells. Cells that are stably transformed are able to divide continuously in very low concentrations of serum. The method of selection that most closely correlates with <u>in vivo</u> tumorigenicity is the selection of transformants in soft (0.33%) agar suspension (68). In this method semi-confluent cultures of cells are harvested and infected with virus. The cells are then plated in 0.33% agar on a 0.9% agar cushion. The transformed cells form large colonies in 2-4 weeks. Transformed cells selected for by growth in soft agar most

closely correlate with *in vivo* tumorigenic potential.

Of the well studied papovaviruses (SV40, BKV, Polyoma) productive infection begins with the adsorption and penetration of the cell by the virus, then proceeds to uncoating of the nucleic acid in the nucleus or cytoplasm. The lytic cycle can be divided into two phases separated by bidirectional replication. These phases are called early and late. These phases correspond to the half of the genome that is transcribed before DNA replication and the half that is transcribed only after DNA replication. The early region codes for non-structural proteins that are involved in transforming infections and in viral DNA replication. The late region codes for structural proteins which compose the capsid structure. Mature virions are assembled in the nucleus and released when the cell lyses.

Of the primate papovaviruses SV40 is the most widely studied. For reference purposes the genome is divided into map units with the single restriction endonuclease Eco R1 cleavage site designated as 0 map units. The early mRNA sediments at 19S in neutral sucrose gradients. It is transcribed from one DNA strand mapping from 0.658 units to 0.153 units. Early studies indicated that there was a single 19S species of mRNA that coded for the early protein. This protein was called the large T protein, molecular weight 90,000-100,000 daltons. Subsequent studies, including *in vitro* translation of the 19S species of mRNA (83) found that the existence of a single early mRNA was inconsistent with the characterization of proteins found. An additional protein of molecular weight 17,000-22,000 daltons, the small t protein was also coded from this 19S class of mRNA. The question that this

finding posed was how a region of DNA with the capacity to code for a 100,000 dalton protein could also code for a 20,000 dalton protein. This question was answered when two distinct mRNAs, each of which were approximately the size of the entire early region were identified. Berk and Sharp (4) first mapped the mRNAs and determined that they could be distinguished by sequences that were spliced out. They did this by treating hybrids between cytoplasmic mRNA and labeled viral DNA with single strand specific S1 nuclease. They then analyzed the protected DNA products on neutral and alkaline gels. The larger mRNA was 2,500 nucleotides and coded from DNA mapping from 0.67 to 0.54 and from 0.54 to 0.14. The smaller mRNA was 2,200 nucleotides long and was coded from DNA mapping from 0.67 to 0.60 and from 0.54 to 0.14. Included in the spliced out region from the smaller mRNA was a translation termination codon. The smaller mRNA coded for large T protein and the larger mRNA coded for small t protein (109).

After the onset of DNA replication the pattern of mRNA synthesis changes. In addition to early region transcription the late region is also transcribed. There are at least two species of late mRNA, one 16S species coding for viral capsid protein 1 (VP1) and one 19S mRNA coding for VP2 and VP3. VP2 and VP3 are translated from different reading frames of the 19S mRNA. Recently, there was an additional late region protein identified (54). This is the 7,900 dalton agnogene product coded from the only open reading frame in the late region. The protein is a DNA binding protein and may prove to have a role in virus assembly.

The organization of the genome of BKV is very similar to that of SV40. Yang and Wu (137,138), by direct sequence analysis showed o-

ver 80% sequence homology between late regions and over 70% homology in the early region of the two viruses. This extensive homology was also demonstrated by Seif et al. (99). The early region of BKV, like that of SV40 codes for two proteins, T and t. This region extends from .67-.16 map units and the late region extends from 0.93-0.14 map units. Manaker et al. (70) examined the early and late mRNAs of BKV by the S1 technique of Berk and Sharp (4). They found that the mRNA coding for large T extends from 0.66-0.16 map units with a spliced out region from 0.59-0.53 map units. The late region of BKV also codes for 3 structural proteins VP1, VP2, and VP3. The map positions of the major RNA segments in the late BKV genomic region were also found to be analogous to those of SV40. There is also an agnogene open reading frame in the late region of BKV but the gene product has not yet been identified.

The genome organization of the early region of the murine papovavirus polyoma as well as functional properties of early proteins are substantially different from those of BKV and SV40. The single Eco R1 cleavage point is still designated 0 map units and the circumference is 100 units. The region from map unit 75 clockwise to map unit 37 contains the information for the early mRNAs. Unlike SV40 and BKV there are 3 known early mRNAs coding for 3 known tumor proteins (110). One RNA extends from map unit 75-25 with 48 bases spliced out at map unit 87. The messenger is translated through to the stop codon at 89 map units and gives rise to small t protein, molecular weight 22,000 daltons. Another messenger extends from map units 75-28 with 61 bases spliced out at map unit 87. The splice removes the small t stop codon and the message is translated through to map unit 99, giving rise to

middle T protein, molecular weight 55,000 daltons. The third messenger extends from 75-27 map units and has a region of 385 bases spliced out from map units 80-87. The stop codons at map positions 87 and 99 are bypassed in a different reading frame and the message is translated through to map position 27 giving rise to large T protein, molecular weight 100,000 daltons.

The reports of the molecular weights of the BKV tumor proteins have been few and somewhat varied. Using the method of immunoprecipitation from labeled cell extracts with anti-tumor serum followed by sodium dodecyl sulfate (SDS) gel electrophoresis and autoradiography Farrell et al. (31) immunoprecipitated a protein of 86,000 daltons from both BKV infected monkey kidney cells and BKV transformed hamster cells. Rundell et al. (96) isolated from BKV infected human embryonic kidney (HEK) cells a ^{32}P labeled protein that on SDS gels was indistinguishable from the SV40 large T protein. From BKV infected HEK cells Simmons et al. (107) isolated ^{35}S-methionine labeled proteins with molecular weights 97,000 daltons and 94,000 daltons. By immunoprecipitation with anti-tumor serum from BKV infected HEK cells Simmons et al. (105) isolated a 17,000 dalton protein which they identified as the BKV small t protein. Olive et al. (81) precipitated a 22,000 dalton protein from BKV transformed BHK-21 cells which may be the BKV small t protein.

Of the primate papovaviruses the SV40 tumor proteins are the most widely studied. The large T protein can be detected in the nucleus of lytically infected and transformed cells by indirect immunofluorescence with anti-tumor serum and can be immunoprecipitated with anti-tumor serum. Large T protein has also been detected on the surface of

infected cells by lactoperoxidase-catalyzed radioiodination of surface proteins (112), by immunoprecipitation of large T antigen from purified plasma membranes obtained from SV40 transformed cells (111) and by immunofluorescence using antiserum prepared against purified large T protein to stain the surface of cells (111).

Functional aspects have also been widely studied. Studies with temperature sensitive (ts) mutants have shown the SV40 T protein to be essential for transformation by this virus. Stimulation of cellular DNA synthesis, transformation initiation and maintenance and initiation of viral DNA replication have all been attributed to the large T protein (12,41,59,124).

Rundell et al. (94) with the use of a constructed deletion mutant lacking part of the SV40 A gene from the early region were able to show that the T protein is a product of the A gene. Previous to that Tegtmeyer (125) found that ts mutants in the A gene failed to initiate stable transformation of cells at the restrictive temperature. After establishment of transformation at the permissive temperature some cell types reverted to a normal phenotype at the restrictive temperature. Martin et al. (72) studied the ability of ts mutants of SV40 to transform Chinese hamster lung cells. They found that only mutants in the A region failed to transform at the restrictive temperature when subconfluent Chinese hamster lung cell monolayers were used. Transformed cell lines then lost the transformed phenotype at the restrictive temperature. In addition, Brugge and Butel (12) studied the A gene function in the maintenance of SV40 transformation in mouse, hamster and human cells. Cells transformed by the tsA mutants exhibited the phenotype

of transformed cells at the non-restrictive temperature. However, when they were grown at the restrictive temperature they were similar to normal untransformed cells. These early studies established the link of the A gene and A gene product to the transformed phenotype.

SV40 large T antigen also has a stimulatory effect on DNA synthesis. Tjian et al. (127) isolated SV40 large T protein and microinjected it into the cytoplasm of cultured cells. They found that the T protein rapidly accumulated in the nucleus and that it stimulated DNA synthesis in quiescent cells. Kriegler et al. (59) loaded red cell ghosts with purified large T protein and fused them with cells infected with the SV40 tsA mutant defective in viral DNA synthesis. They found that by in situ hybridization that the microinjection of purified large T protein resulted in an increase in the amount of viral DNA sequences in the monolayer. Finally, Mueller et al. (76) microinjected different early viral DNA fragments and studied the effects. They found that as little as 70% of the early region including sequences coding for the amino terminal end was required for stimulation of cellular DNA synthesis.

Lanford and Butel (64) utilized an SV40-adenovirus hybrid (PARA) mutant which induced the synthesis of SV40 large T antigen that is not transported to the nucleus, but accumulates in the cytoplasm. Cells transformed by this mutant (PARA cT) exhibited decreased colony forming ability in suspension both in 10% and 1% serum as compared to cells transformed by the wild type hybrid. Both grew to high saturation densities in 10% serum. In 2% serum the saturation density of the wild type hybrid was not altered but that of the mutant was 3 to 5 fold low-

er. In 2% serum the wild type transformants increased in cell number more rapidly than the PARA-ct mutant transformants. The mutant transformants were also less tumorigenic than wild type transformants.

Studies on the small t protein function in SV40 infections have been made utilizing mutants deleted in the genome region unique to small t protein. This is the region between 0.54-0.59 map units which is a coding sequence for small t protein and an intervening sequence for large T protein (84). Deletion mutants within the region produce truncated forms of small t protein. Bouck et al. (9) transformed rat cells and Chinese hamster lung cells with the 0.54-0.59 deletion mutant and selected the transformants in soft agar. Using this method of transformation selection they found that 0.54-0.59 mutants were only 1-2% as efficient in transformation as the wild type parent. Another difference between the 0.54-0.59 mutant transformed cells and wild type fully transformed cells is that cells transformed by the mutant fail to loose their organized actin cable network (42). In studies of oncogenicity investigators found that the 0.54-0.59 mutant required a longer latent period for tumor induction in hamsters than did the wild type virus (22). This study did report suggestive evidence that tumors induced in hamsters by the 0.54-0.59 deletion mutant tended to metastasize whereas tumors induced by wild type SV40 rarely did so. In this study 30 hamsters were injected with wild type virus. All 30 developed tumors and none metastasized. Fifteen hamsters were injected with mutant virus. Thirteen hamsters developed tumors of which 6 metastasized. Metastatic potential is a characteristic that is not usually associated with papovavirus oncogenesis. From these studies it is obvious that the

small t protein contributes to the transformed phenotype.

Further studies demonstrate that the state of the cell prior to and during transformation, in addition to the expression of viral proteins influence the outcome of infection. For the contribution of small t to transformation this was demonstrated in studies by Martin et al. (71). They studied the same 0.54-0.59 mutant as Bouck et al. (9) and found that it could transform actively growing Chinese hamster lung cells nearly as efficiently as wild type virus. However, when these cells were not actively growing at the time of infection the transformation frequency relative to wild type dropped 50 fold.

In regards to the state of the host cell and the contribution of large T to transformation it was discovered that both SV40 and polyoma could generate two types of transformed rat or hamster cells depending on the method of selection of the cells. The transformants selected for by growth in agar were designated A transformants and those selected for by focus formation were designated N transformants (98). When tsA mutants of SV40 and polyoma were used for transformation they found that A transformants were able to maintain the transformed phenotype at the restrictive temperature while the N transformants could not (37,90,98, 100). From these studies it appeared that transformation was totally under control of the A gene in N transformants. In the A transformants however, an additional factor from the host or virus was utilized to maintain the transformed phenotype at the restrictive temperature. In these studies the A transformants were those cells in a resting state (agar selection) at the time of exposure to virus and for the 4 or 5 days immediately following. The N transformants were those actively

growing (focus formation selection) at the time of exposure to virus. More recently it was determined that in rat cells A transformants tend to be produced by exposure to virus at high multiplicities of infection and N transformants are found after exposure to virus at low multiplicities of infection (89). In these rat cells the A transformants have multiple copies of integrated SV40 and the N transformants have a single integrated copy (16). Thus, at least in rat cells gene dosage may be responsible for the relation of multiplicity of infection to A or N transformant type. In Chinese hamster lung cells however, this relationship was not seen (16).

The involvement of the SV40 large T protein in viral DNA replication and in transformation initiation and maintenance is clear. How the large T protein functions to effect these changes is not so well known. One way in which SV40 large T protein may mediate some of the changes directly or indirectly is through its DNA binding capabilities. SV40 large T protein binds both specifically and non-specifically to viral or cellular DNA (14,55,102). It was shown that T antigen has a highly specific binding affinity to three DNA sequences which lie in the vicinity of the origin of replication at 0.67 map units (55,102). Recent studies by Khandjian et al. (56) suggest that SV40 large T protein is also capable of binding RNA. They found that small RNA fragments, apparently derived from high molecular weight RNA, remain bound to RNAse treated gel purified SV40 T antigen. It is possible that SV40 T antigen as an RNA binding protein might be involved in the regulation, maturation or translation of cellular mRNAs.

Other findings which may be important in understanding how SV40

large T protein functions in transformation include the complex of large T protein with cellular proteins. Lane and Crawford (62) first reported that T antigen from a line of SV40 transformed mouse cells formed an oligomeric complex with a specific cell coded protein. When they immunoprecipitated from an SV40 transformed mouse cell line with antiserum raised against purified T, two polypeptides were precipitated. One was large T protein, molecular weight 94,000 daltons and the other was a cell coded protein, molecular weight 53,000 daltons. This same pattern was seen when monoclonal antibody specific for the mouse 53,000 dalton protein was used (48). Lane and Crawford (62) went on to prove that the large T and 53,000 dalton cellular protein existed in solution as a complex. Others reported that the cellular 53,000 dalton protein was present in small amounts in uninfected cells and the amounts increased up to 50 fold upon infection when a functional A gene was present (66).

Lane and Hoeffler reported on a 68,000 dalton nuclear protein present in extracts of mouse L cells and other cell lines including human, monkey, and rat cells, which shared an antigenic determinant with large T protein. This 68,000 dalton protein could be detected by indirect immunofluorescence with monoclonal antibody in the nuclei of cells growing exponentially but to a lesser extent in cells in a quiescent state.

There are a number of other reports of proteins in the 48,000-84,000 dalton range precipitated from SV40 transformed cells by antitumor serum. These may be non-specific background precipitation, proteins complexed with T, proteolytic breakdown products of T, or proteins which share an anitigenic determinant with T (63,74,106,116). The as-

sociation of these cellular proteins in specific complexes or by shared antigenic determinants with T antigen suggests that they may be important for the cellular functions required for transformation.

Two enzymatic activities of SV40 large T protein may be involved in the phenotypes of transformation. They are a protein kinase activity and an ATPase activity. Conflicting evidence exists as to whether the protein kinase activity is inherent in large T protein or simply co-purified with it. Griffin et al. (46) reported that incubation of SV40 tumor antigen containing immunoprecipitates with ^{32}P-ATP resulted in the incorporation of radioactive phosphate into large T antigen. They also reported that highly purified preparations of large T antigen were able to catalyze the phosphorylation of a known phosphate acceptor. Others (128) reported that the protein kinase activity associated with large T protein was reduced as T purification increased indicating that the protein kinase activity was not actually a part of the large T protein. In contrast however, Griffin et al. (115) showed that the kinase activity may be intrinsic in that the activity was reduced at the restrictive temperature in extracts from cells infected with ts mutants. This question has not been resolved.

The ability to hydrolyze ATP to ADP is also associated with purified large T protein (40,129). Rigorous purification of large T protein did not lead to separation of ATPase activity from large T as protein kinase activity was separated from large T protein (129). Clark et al. (17) utilized monoclonal antibodies as probes of the activity in SV40 large T protein. They found that monoclonal antibodies directed to the large T protein were capable of inhibiting ATPase activity. Spe-

cifically, antibodies directed to the region of T antigen coded from between 0.37 and 0.29 map units inhibited ATPase activity. These results strongly suggest that the ATPase activity is intrinsic to T antigen. The ATPase activity of SV40 large T antigen has not been linked to any of the known biological functions of T antigen.

There is substantial evidence that there are different forms of SV40 large T protein. These forms may be the result of fusion products of the virus and cellular genome (15,58) or subtle antigenic differences (48) but often they are the result of varying levels of phosphorylation. Greenspan and Carroll (43) found that SV40 large T protein labeled with ^{3}H-methionine and ^{32}P-orthophosphate isoelectric focused as four distinct species distributed in a highly reproducible pattern. The fact that they differed in their $^{32}P/^{3}H$ ratios suggested that they were heterogeneous in their phosphate content. Fanning et al. (30) characterized the multiple forms of simian virus 40 T antigen. They separated forms of T antigen by zone velocity sedimentation in sucrose density gradients and found a 5S to 6S form, a 14S to 16S form and a 23S to 25S form. Differential labeling indicated that the 5S to 6S form was less highly phosphorylated than the faster sedimenting forms. Interestingly, monkey cells infected with a tsA mutant of SV40 at the restrictive temperature contained only the 5S to 6S form. After shift to the permissive temperature faster sedimenting forms were seen. Montenarh and Henning (75) studied the phosphorylation and DNA binding of SV40 T. They found that the higher phosphorylated forms of T antigen bound more strongly to both dsDNA and ssDNA. This observation is consistent with the hypothesis that the binding affinities of SV40 T antigen to DNA may

be regulated by its phosphorylation. The addition and removal of phosphate groups of chromatin associated proteins of eukaryotic cells has been suggested as a control mechanism for regulation of DNA binding properties (93). Greenspan and Carroll (44) reported that the 48,000 dalton nonviral T associated protein is preferentially complexed with the maximally phosphorylated form of large T protein. They also found that mutation in the A gene caused a decrease in T antigen phosphorylation and a marked decrease in the binding to the 48,000 dalton nonviral T associated protein. These studies provide evidence for a functional inter-relatedness between phosphorylation of T antigen, DNA binding and interaction with cellular proteins.

Reports of the physical and/or funcional aspects of the BKV large and small T proteins have been limited. Of the two viruses, SV40 and BKV, the research emphasis has consistently been placed on SV40. A number of facts are responsible for this. The first is that SV40 was isolated prior to BKV. Another is that the host range of SV40 provides a more accesible supply of cells for growing the virus than does BKV. Thirdly because of the first two reasons and because of the known extensive functional and compositional similarities the emphasis has remained with SV40.

The BKV T antigens are closely related to those of SV40. Dougherty (23) studied the relatedness of the papovavirus T antigens using immunoperoxidase assay , different sources of anti-T sera and immunoadsorption. He found that BKV anti-tumor serum reacted with other papovavirus T antigens but that these T antigens also exhibited some unique antigenic properties. BKV T antigen can be immunoprecipitated

with anti-SV40 tumor serum and SV40 T antigen can be immunoprecipitated with anti-BKV tumor serum (81,96). Tryptic peptide analysis has shown that SV40 and BKV T proteins share 7 pairs of tryptic peptides out of 20 SV40 and 21 BKV specific peptides (108). The small t proteins of BKV and SV40 also cross-react immunologically and share tryptic peptides (105).

One naturally occurring variant of BKV is BKV strain MM (BKV-MM). BKV-MM was isolated from the brain tumor and urine of a patient with Wiskott-Aldrich syndrome (123). The complete sequence of BKV-MM was reported by Yang and Wu (138). The regions from map positions 0 to 0.512 and 0.699 to 1.0 on BKV-MM are identical to those of 0 to 0.489 and 0.711to 1.0 on BKV-WT. On BKV-MM the region between 0.614 and 0.554 codes for small t antigen. Within this region is a deletion which would yield a 100 amino acid small t instead of a 172 amino acid wild type small t. However, Seif et al. (99) have reported that because the deletion removes a donor site for small t mRNA intervening sequences, no small t or truncated form of small t is produced. BKV-MM is capable of tumor induction in nude mice and stable transformation of growing cells (19,73). It is not known if BKV-MM can transform cells as efficiently as the wild type virus. Further studies of the functional aspects of the BKV tumor proteins in transformation are lacking.

CHAPTER III

MATERIALS AND METHODS

Buffers and Solutions

The composition of buffers and solutions is listed in Table 1.

Cells and Cell Culture

Cells were grown in Dulbecco's modified Eagle medium (DME) (1) (Flow Laboratories) at 37°C in a moist 7% CO_2 atmosphere. DME was supplemented with fetal calf serum (FCS) or adult calf serum (CS).

Baby hamster kidney (BHK-21) cells (119), BK virus (BKV) transformed BHK-21 cells and BKV transformed hamster fibroblast (RF-194) cells (23) were grown in DME supplemented with 10% CS and 19% tryptose phosphate broth (TPB), (Difco). TPB was a solution of 29.5% TPB in distilled H_2O. SV40 (simian virus 40) transformed human cell line SV80 (130,131) was grown in DME plus 10% CS. Human embryonic kidney (HEK) cells were grown in DME plus 10% FCS. SV40 transformed hamster fibroblast line THE_2 (95) was grown in DME plus 10% CS. Marmoset brain cells (MBC) established by E.O. Major were grown in DME plus 10% FCS. Hybridoma cells clones 7, 122, and 412 (7) and 108 from E. Gurney were grown in DME plus 20% FCS.

For routine culture (except for hybridoma cells), cells were grown to confluency and washed once with DME. Then 2.0 ml of 0.5% trypsin in versene buffer (pH 7.1) containing 137 mM NaCl, 0.6 mM EDTA, 2.7 mM KCl, 8.1 mM Na_2PO_4, 1.7 mM KH_2PO_4 was added. Once the cells detached

Table 1 BUFFERS USED

Buffer Used	pH	Contents
TD	7.0	137 mM NaCl, 5.0 mM KCl, 0.7 mM N_aHPO_4, 8.2 mM Tris
Versene	7.1	137 mM NaCl, 0.6 mM EDTA, 2.7 mM KCl 8.1 mM Na_2HPO_4, 1.7 mM KH_2PO_4
PBS (IFA)	7.1	137 mM NaCl, 2.6 mM KCl, 1.6 mM Na_2HPO_4, 0.1 mM KH_2PO_4, 0.4 mM $MgCl_2$, 0.9 mM $CaCl_2$
PBS (cell washing)	7.4	9 mM Na_2HPO_4, 1 mM NaH_2PO_4, 140 mM NaCl
TBS (cell washing)	9.0	20 mM Tris, 137 mM NaCl, 1 mM $CaCl_2$, 1 mM $MgCl_2$
Extraction buffer	9.0	20 mM Tris, 137 mM NaCl, 1 mM $CaCl_2$, 1 mM $MgCl_2$, 10% glycerol, 0.75% NP 40, 1 µl/ml of a 50 mg/ml solution of PMSF in 100% ethanol
Bead swelling buffer	9.0	20 mM Tris, 137 mM NaCl
Bead washing buffer	9.0	100 mM Tris, 500 mM LiCl, 1% 2-mercapthoethanol
Bead elution buffer	9.0	20 mM Tris, 20% glycerol, 5% SDS, 5% 2-mercaptoethanol, 0.001% bromophenol blue
Separating gel buffer	8.7	375 mM Tris, 0.1% SDS

Table 1

Buffer Used	pH	Contents
Electrode buffer	8.1	25 mM Tris, 192 mM glycine, 0.1% SDS
DNA cellulose column washing buffer	6.0	5 mM K_2HPO_4
DNA cellulose column elution buffer	9.0	20 mM Tris
TES	7.1	50 mM Tris, 100 mM NaCl, 10 mM EDTA
TNE	7.4	10 mM Tris, 100 mM NaCl, 1 mM EDTA
Agarose gel electrophoresis buffer	8.1	89 mM Tris, 89 mM boric acid, 25 mM EDTA
Restriction endonuclease reaction buffer	7.4	10 mM Tris, 50 mM NaCl, 10 mM $MgSO_4$

they were washed once in DME, counted, and except where indicated plated at 3 X 10^5 cells/60 mm or 100 mm tissue culture plate, or they were frozen at 4 X 10^6 cells/vial in 70% DME, 20% serum, 10% dimethylsulfoxide.

When hybridoma cells reached densities of approximately 9 X 10^6 cells/100 mm tissue culture dish they were harvested, counted and plated at 3 X 10^5 cells/100 mm tissue culture dish or they were frozen at 4 X 10^6 cells/1.5 ml vial in 40% DME, 50% FCS, and 10% DMSO.

Virus Growth and Purification

The BKV stock used in these studies was prepared in the following way. HEK cells were grown to semi-confluency and were infected with the Gardner strain (36) of BKV (plaque isolate 18), at a multiplicity of infection (m.o.i) of 0.1 plaque forming units (pfu)/cell. The virus was allowed to adsorb for two hours. The cells were then refed with DME plus 1% FCS. BKV plaque isolate 18 was harvested 10 days post-infection and BKV-MM was harvested 12 days post-infection.

Virus was purified according to the method of Wright and di Mayorca (136). After scraping the infected cells into the media the material was subjected to six cycles of freezing and thawing in a dry ice-acetone bath. The cellular debris was removed by centrifugation at 10,000 X g for 5 min at 4°C. The supernatant was then centrifuged at 64,000 X g for 3 h at 20°C over a cushion of 10.0 ml of saturated KBr solution in TD buffer (pH 7.0) containing 137 mM NaCl, 5.0 mM KCl, 0.7 mM Na_2HPO_4, 8.2 mM tris(hydroxymethyl) aminomethane (Tris). The visible band of virus was collected and dialyzed against 3 changes of TD buffer. The volume of the dialyzed material was brought to 7.5 ml with TD buffer

added to 3.45 g of CsCl and placed in a cellulose nitrate centrifuge tube. The solution was centrifuged at 74,000 X g for 18 h at 20°C. The virus band formed near a density of 1.3451. It was removed with a syringe and 21 gauge needle, dialyzed against 3 changes of TD buffer and stored at -70°C.

Plaque Assay for Determination of Virus Titer

HEK cells were plated onto 60 mm tissue culture plates at 3 X 10^5 cells/plate. They were allowed to grow to confluency. The media was removed from the plates and the cells were washed once with DME. The virus sample was diluted in 10 fold serial dilutions. After washing the cells with DME 0.4 ml of each dilution was plated onto cell monolayers in triplicate. The virus particles were allowed to adsorb for 1 h. Then 9.0 ml of 0.9% agar in DME plus 1% FCS was added. Plaques were allowed to develop for 12 days. At the end of 12 days the monolayers were overlayered a second time with 5.0 ml of 0.9% agar in DME plus 1% FCS and 0.0005% neutral red dye. The neutral red dye stained only the viable cells leaving the plaques as clear areas which could easily be visualized.

Virus Transformation Assay

BHK-21 cells were treated with virus in suspension and plated in soft (0.33%) agar based on the method of MacPhearson and Montagnier (68). BHK-21 cells were harvested with 0.5% trypsin in versene buffer and resuspended in TD buffer supplemented with 1% CS previously filtered through a 0.45 μM filter to a concentration of 1 X 10^7 cells/ml. A 0.05 ml aliquot of this suspension containing 5 X 10^5 cells was placed

in each tube for virus transformation and in one control tube with no virus added. To each tube for virus transformation 100 pfu/cell of virus was added. The volume in all tubes was adjusted to 0.5 ml with TD buffer supplemented with 1% filtered calf serum. A 5.0 ml long stirring bar was added. The tubes were securely sealed to prevent evaporation and they were incubated for 1 h, 37°C with constant, gentle stirring.

Following incubation any large clumps of cells were broken up by drawing the cells up and down through a pasteur pipette drawn out to capillary size. A 0.2 ml portion of the cell-virus mixture was then added to 4.8 ml Eagle's diluent consisting of 10% filtered calf serum, 10% TPB, and 80% 1X DME. 10 ml of top agar containing 10% calf serum, 10% TPB, 40% 2X DME and 40% 1.275% agar was added to this and 1.5 ml was pipetted onto plates containing 5 ml bottom agar. Bottom agar contained 10% calf serum, 10% TPB, 40% 2X DME, and 40% 1.275% agar.

Colonies could be visualized macroscopically after 3-4 weeks of incubation at 37°C. When the colonies were approximately 1 mm in diameter they were counted and the efficiency of transformation was calculated. This was done by dividing the number of visible colonies/plate by the total number of cells palted/plate and multiplying by 100. The average value from 10 plates was taken. Colonies were then taken from agar with a pasteur pipette. They were dispersed and plated in 60 mm tissue culture plates. When the plates were semi-confluent the cells were harvested and transferred to 100 mm tissue culture plates. They were then studied immediately or stored for later use. BKV transformed BHK-21 cell clones were distinguished by a letter and a number.

Plating Efficiency of Transformed Cells in Soft Agar

Cells were harvested, counted and resuspended to 1.0 X 10^6 cells/ml in DME. A 0.1 ml portion of this suspension was added to 1.9 ml DME to give 5 X 10^4 cells/ml. To 4.8 ml Eagle's diluent was added 0.2 ml of the diluted suspension. This suspension was then added to 10.0 ml of top agar consisting of 40% 2 X DME, 40% 1.275% agar, 10% filtered calf serum and 1.5 ml was plated onto each of 10 plates containing 5.0 ml bottom agar. Bottom agar consists of 40% 1.275% agar, 40% 2X DME, 10% TPB and 10% CS. Colonies were counted when they were approximately 2 mm in diameter. The plating efficiency was calculated by dividing the number of colonies counted/plate by the total number of cells and multiplying by 100. The values represent an average from 10 plates.

Subcloning

BK-BHK C7 passage 23 (TC 23) was harvested, counted, and diluted to 1 cell every 0.2 ml in DME with 10% CS and 10% TPB. Cells were plated into 4, 96 well cloning plates, 0.2 ml/well. Immediately after plating wells were checked microscopically to determine which had received single cells. Fifteen days past plating 56 clones originating from single cells were harvested and plated into 30 mm tissue culture cloning dishes. Of these subclones 10 were randomly selected for further study . They were designated as subclones by the letters SC.

Growth Curves

Cells were plated at 3 X 10^5/60 mm tissue culture plate in DME plus 1% CS, 1% TPB, or in DME plus 10% CS, 10% TPB. They were not refed. Cells were harvested and counted 1, 2, 3, and 7 days past plating. Val-

ues represent the average of two plates counted.

Cell Fusion

Cell fusion with polyethylene glycol (PEG) and dimethyl sulfoxide was done based on the method described by Norwood et al. (78). HEK cells were plated at 3 X 10^5 cells/60 mm tissue plate and allowed to grow for 48 h or until semi-confluent. BK-BHK transformants were plated on top at 5 X 10^5 cells/plate and allowed to grow for 24 h. For fusion 8.0 g of PEG, molecular weight 6,000 were autoclaved and immediately mixed with 10.0 ml of DME plus 15% DMSO. The media was removed from the plates and the cells were washed once with DME. A 2.0 ml portion of the PEG-DMSO-DME mixture was applied to the cells for 1 min and then removed by aspiration. The cells were washed four times with DME plus 15% DMSO and twice with DME and refed with DME plus 2% FCS. After three days the cells were harvested by scraping them off the plate with a rubber policeman and frozen and thawed in a dry ice-acetone bath. Cellular debris was removed by centrifugation at 10,000 x g for 5 min at 4°C. The presence of virus in the supernatant was determined by plaque assay.

Antiserum Production

Antiserum was produced to BKV and SV40 transformed cells using RF194 and THE_2 cell lines. RF194 cells were harvested in versene buffer, washed with PBS (pH 7.1) containing 137 mM NaCl, 2.6 mM KCl, 1.6 mM Na_2HPO_4, 0.4mM $MgCl_2$, and 0.9 mM $CaCl_2$, and counted. Female Syrian Golden weanling hamsters were injected with 2.0 X 10^4 or 2.0 X 10^5 cells in 0.5 ml PBS, subcutaneously over the right or left shoulder. Tumors were allowed to develop until they were 3-4 cm in diameter. This was from 3-4 weeks. Hamsters were anesthetized with an intraperitoneal in-

jection of sodium pentobarbitol (50 mg/ml) and exsanguinated by cardiac puncture. From each hamster approximately 3.0 ml of blood could be obtained. Serum was collected and stored at -70°C.

THE_2 cells were grown to semi-confluency, harvested in versene buffer and counted. Hamsters were injected with 1×10^5 or 1×10^6 cells in 0.5 ml PBS subcutaneously over the right or left shoulder. The tumors were allowed to develop until they were approximately 4.0 cm in diameter. This was different for each hamster and ranged from 4-15 months. Blood was collected as described for RF-194 cell antiserum.

Hybridoma cells were fused, isolated, and supplied by E. Gurney Balb/c mice were immunized with SV40 transformed Balb/c mouse cells. Spleen cells from these mice were then fused with mouse myeloma NS-1 cells. Hybrids secreting antibody to the SV40 T proteins were selected for further study.

Where indicated culture fluid or ascites fluid was used as a source of monoclonal anti-T antibody. Culture fluid was harvested from cell cultures with approximately 9×10^6 cells/100 mm tissue culture plate. Cells were pelletted by low speed centrifugation and culture fluid was stored at -70°C.

For ascites production young adult Balb/c mice were given intraperitoneal injections of 0.5 ml of 2,6,10,14-Tetramethylpentadecane (pristane). Six weeks later each mouse was injected intraperitoneally with 1×10^7 cells in 0.5 ml DME. Ascites fluid was drained with a 22 gauge needle and syringe 2-3 weeks after the cell injections. Fluid was centrifuged at 40,000 x g for 30 min, 4°C. Supernatant was removed and stored at -70°C.

Indirect Immunofluorescence

Indirect immunofluorescence (IFA) was done based on a method described by Pope and Rowe (85). Cells were grown on 20 X 20 mm coverslips placed in 60 mm tissue culture dishes. When the cells were semiconfluent the coverslips were removed and washed twice in PBS (pH 7.1) for 5 min. They were then fixed in acetone for 10 min. Following this they were washed twice in PBS for 5 min. Coverslips were then overlaid with the indicated dilution of antiserum and incubated at 37°C for 1 h. After incubation coverslips were washed three times in PBS for 5 min. Coverslips were then overlaid with the indicated dilution of either fluorescein isothiocyanate (FITC) conjugated rabbit anti-hamster IgG (Cappel Laboratories) or FITC conjugated goat anti-mouse IgG (Cappel Laboratories). They were then incubated for 1 h at 37°C. Following incubation they were washed three times in PBS, 5 min each, and mounted in buffered glycerol. The cells were examined with a Zeis fluorescent microscope and photographed with a Nikon camera on Kodak Ektachrome ASA 400 film at 160X or 400X magnification. Positive and negative cells were counted in random fields at 400X magnification.

Where indicated the cells were prepared for IFA with the use of a Shandon Cytospin 2. Cells were harvested with trypsin, washed twice in PBS, counted, and diluted to approximately 1 X 10^7 cells/ml. A 0.1 ml portion of this was placed in each cytospin funnel and the cells were spun onto slides at 65 x g for 5 min. Slides were allowed to dry and then were immediately fixed. IFA was then done as described for cells grown on coverslips.

^{35}S-Methionine Cell Labeling and Extraction

Except where indicated cells were labeled and extracted according to the following method. Cells were grown to a semi-confluent state in 100 mm tissue culture plates, and washed once with Earle's minimal essential media (MEM) without methionine (Gibco). They were then refed with 2.0 ml of MEM without methionine for 30 min. The medium was removed. The cells were labeled with 0.1 mCi ^{35}S-methionine (1200 Ci/mMol) in 1.0 ml MEM without methionine for 3 h, 37^{o}C.

Cells were then extracted based on methods described by Schaffhausen et al. (97) and Griffin et al. (45). After the 3 h labeling period the medium was removed and the cells were washed twice in phosphate buffered saline containing 10 mM sodium phosphate (pH 7.4), 100 mM NaCl at 4^{o}C. They were then washed three times with Tris buffered saline (TBS) cell washing buffer containing 20 mM Tris, (pH 9.0), 137 mM NaCl, 1 mM $CaCl_2$, 1 mM $MgCl_2$ at 4^{o}C. Cells were then extracted with 1.0 ml of extraction buffer containing 20 mM Tris-HCl (pH 9.0), 137 mM NaCl, 10% glycerol, 0.75% NP-40, 1 mM $CaCl_2$, 1 mM $MgCl_2$ and 1 µl of a 50 mg/ml solution of phenylmethylsulfonyl fluoride (PMSF) in 100% ethanol. Extraction was carried out for 30 min at 4^{o}C. The extracts were then centrifuged at 40,000 x g for 30 min at 4^{o}C and the supernatants were removed.

Immunoprecipitation

Cell extracts were immunoprecipitated according to the methods of Griffin et al. (45) and Schaffhausen et al. (97). Immunoprecipitattion was carried out either in silicone coated glass tubes or in 1.5 ml plastic microfuge tubes. The cell extracts were incubated with 15 µl

of hamster antiserum or normal hamster serum for 30 min at 4°C. Protein A-Sepharose beads (Pharmacia) (2 mg protein A/ml of swollen gel) were swollen in bead swelling buffer consisting of 20 mM Tris-HCl (pH 9.0) and 137 mM NaCl for 30 min at 4°C. They were then washed twice in this buffer. A volume of 80 µl of swollen beads (22 mg dry weight) was added to the extracts and incubated at 4°C for 1 h with mixing. The beads were then washed three times in bead washing buffer consisting of 100 mM Tris-HCl (pH 9.0), 500 mM LiCl and 1% 2-mercaptoethanol, 4°C. Washing was performed using low speed centrifugation in a Sorvall model RC5 at 4°C or in a Fisher microfuge at 4°C. Immune complexes were eluted from the beads with 50 µl of bead elution buffer containing 20 mM Tris-HCl (pH 9.0), 5.0% sodium dodecyl sulfate (SDS), 20% glycerol, 5% 2-mercaptoethanol, and 0.001% bromophenol blue at 85°C for 5 min.

Polyacrylamide Gel Electrophoresis

Polyacrylamide gel electrophoresis was done based on the method of Laemmli (60). Except where indicated the separating gel consisted of 13% acrylamide in separating gel buffer, 0.375 M Tris-HCl (pH 8.7), 0.1% SDS. Acrylamide and methylenebisacrylamide (bis) were added in a 37:1 ratio. Ammonium persulfate was added to 0.025% and the acrylamide solution was deaerated under vacuum for 5 min. Following deaeration 0.025% N,N,N^1,N^1-tetramethylethylenediamine (TEMED) was added. The separating gel acrylamide was then poured between 2, 19X19 cm acid cleaned glass plates separated by two 1.5 mm thick spacers on both sides. The bottom was plugged with 1% agarose in separating gel buffer. The separating gel was then overlaid with distilled H_2O and allowed to polymerize. After polymerization the H_2O was removed and the surface was washed with

stacking gel solution. The stacking gel consisted of 5% acrylamide and Bis in a 20:1 ratio in stacking gel buffer. Stacking gel buffer consisted of 125 mM Tris-HCl (pH 6.8), 0.1 % SDS. Ammonium persulfate was added to 0.25% and the solution was deaerated for 5 min under vacuum. Following deaeration 0.25% TEMED was added. The stacking gel was then poured on top of the separating gel to a depth of 1.5 cm and the sample well former was inserted.

Following polymerization the gel was placed in position between the top and bottom buffer chambers. The electrode buffer, consisting of 25 mM Tris, 192 mM glycine, and 0.1% SDS was added to each chamber. Each sample (20 µl) was applied to the gel. Except where indicated gels were electrophoresed at 7 mA constant current overnight, 12-16 h.

Polyacrylamide Gel Staining

Gels were fixed and stained in a solution containing 50% methanol, 9.2% glacial acetic acid and 0.25% Coomassie Brilliant Blue R 250 (Bio Rad) for 6 h. Insoluble material in the solution was removed by filtration before staining. Gels were destained overnight in a solution containing 5% methanol and 7.5% glacial acetic acid. Destaining was enhanced by the addition of diethylaminoethyl (DEAE) cellulose to the destaining solution.

Polyacrylamide Gel Fluorography

Fluorography was done based on a method described by Bonner and Laskey (7). After staining and destaining the gel was rinsed briefly in H_2O, soaked in 3 changes of DMSO 30 min each, and then immersed in 200 ml of 22.2% 2,5 diphenyloxazole (PPO) for 3 h. The PPO was then preci-

pitated in the gel by immersing the gel in H_2O for 1 h. The gel was then dried in a gel slab dryer (Bio Rad) for 6 h. Kodak Royal X-Omat R film or Kodak Royal X-Omat XAR-5 film was then exposed by the gel at $-70^{o}C$ for the desired length of time (usually 1-2 weeks).

Molecular weights of proteins were determined by comparison with those of known protein markers which were visualized by staining. Protein molecular weight markers (Bio Rad) were phosphorylase B-94,000 daltons, bovine serum albumin-68,000 daltons, ovalbumin-43,000 daltons, carbonic anhydrase-30,000 daltons, soybean trypsin inhibitor-21,000 daltons and lysozyme-14,300 daltons.

DNA Cellulose Chromatography

DNA cellulose chromatography was done based on methods described by Alberts and Herrick (1) and by methods described by K. Rundell (personal communication). Cellulose was prepared by washing 50 g cellulose 410 (Bio Rad) twice with 300 ml of boiling 95% ethanol and once with 200 ml of 0.1 M NaOH, 1.0 mM EDTA. Cellulose was then washed once with 200 ml of 10 mM HCl followed by washing 3 times in distilled H_2O. DNA-cellulose was prepared by dissolving 60 mg calf thymus DNA (Calbiochem) in 30 ml of 10 mM Tris-HCl (pH 7.4), 1 mM EDTA, then slowly adding 10 g of prepared cellulose. The resulting paste was spread on the sides of a beaker and allowed to dry slowly for several days. After drying the DNA cellulose was ground to a powder with a mortar and pestle.

The chromatography was done by first washing 5.0 g DNA-cellulose 3 times in 5 mM K_2HPO_4 (pH 6.0). DNA cellulose was poured into a pasteur pipette plugged with glass wool, and allowed to pack to a length of

2.5 cm. A 1.0 ml sample (cell lysate from one 100 mm tissue culture plate) was adjusted to pH 6.0 with 0.2 ml of 200 mM sodium phosphate and then applied to the column. The column was then washed twice with 1.0 ml of 5 mM K_2HPO_4 (pH 6.0). Any proteins capable of binding at pH 6.0 and eluting at pH 9.0 were released from the DNA-cellulose by adding 1.5 ml of 20 mM Tris-HCl (pH 9.0). The pH 9.0 final sample was collected and the fractions were immunoprecipitated. To precipitate from a pH 6.0 sample the pH was first adjusted back to pH 8.0-9.0 with 0.2 ml of 1 M Tris-HCl, pH 9.0.

Hirt Extraction of Viral DNA

HEK cells were infected with a m.o.i. of 1 pfu/cell of either wild type BKV or BKV-MM. DNA was extracted 7 days after infection for wild type BKV and 12 days after infection for BKV-MM, according to the method of Hirt (51). Monolayers were washed 3 times in TES buffer containing 50 mM Tris-HCl (pH 7.1), 100 mM NaCl, 10 mM EDTA. To each plate 1.0 ml of TES with 0.6% SDS was added and incubated 10 min, 37°C. The lysed cells were gently scraped into a Corex tube number 8445. A volume of 5.0 M NaCl was added so that the final concentration of NaCl was 1.0 M. The tubes were covered, mixed gently and refrigerated overnight at 4°C. The samples were then centrifiged at 40,000 x g for 15 min, 4°C. The supernatants were poured into rinsed Corex tubes number 8445, 2 volumes of 100% ethanol at -20°C were added and the tubes were mixed gently. They were then refrigerated overnight at -20°C followed by centrifugation at 27,000 x g for 1 h, 4°C. The supernatant was removed, 2.0 ml of TES plus 0.1 ml of a 1 mg/ml solution of pronase was added and

the tubes were placed in a 37°C waterbath overnight. Following pronase digestion 1 volume of phenol was added and the tubes were gently mixed and then centrifuged at 3,000 x g for 5 min, 4°C to separate the phases. The aqueous layer was extracted. Then 1 volume of chloroform-isoamyl alcohol (24:1 v/v) was added, tubes gently mixed and then centrifuged at 3,000 x g for 5 min, 4°C. The top layer was removed. The DNA was then precipitated with 100% ethanol, -20°C, then refrigerated at -20°C overnight. It was then centrifuged at 27,000 x g for 1 h, 4°C. The supernatant was removed and the DNA dissolved in 0.3 ml of TES buffer.

Restriction Endonuclease Cleavage of Viral DNA

Viral DNA was cleaved by restriction endonuclease Hind III according to the following method. The digestion was carried out with 20 µl reaction buffer, 3 µg DNA, and 15 units enzyme for 60 min 37°C. Reaction buffer contained 10 mM Tris (pH 7.4), 50 mM NaCl, 10 mM $MgSO_4$. The reaction was stopped by the addition of 10 µl of tracking dye. Tracking dye contained 10% SDS, 5% glycerol, and 0.001% bromophenol blue.

Agarose Gel Electrophoresis

The DNA cleavage fragments were separated by electrophoresis in a 1.2% horizontal agarose gel. The gel and chamber buffer consisted of 89 mM Tris, 89 mM boric acid (pH 8,1), and 25 mM EDTA. Samples were electrophoresed overnight at 10 mA, constant current. The gel was stained in a solution of 2 µg of ethidium bromide/ml of H_2O for 3 h and destained in H_2O for 3 h. The gel was phorographed on an ultraviolet transilluminator with a Polaroid MP-4 Land camera and Polaroid Type 52 Land film. Molecular weights of the DNA fragments were determined by

comparison with those of known DNA fragments from a Hind III digest of lambda DNA (New England Biolabs).

Cell Cycle Synchronization

Cells were synchronized with 1 mM hydroxyurea based on a method first described by Dubbs and Kit (25) and modified by Lanford and Butel (64). Cells were plated at 3 X 10^5/60 mm tissue culture plate in DME plus 0.1% CS and 0.1% TPB and allowed to grow 24 h. After 24 h the medium was replaced with DME plus 10% CS, 10% TPB, 1 mM hydroxyurea. Cells were incubated another 18 h, the medium was removed and the cells washed once in DME. The medium was replaced with DME plus 10% CS, 10% TPB. At various times after release from the block cells were pulse labeled for 30 min in DME containing 10% CS, 10% TPB, and 2.5 μCi/ml of ^{3}H-thymidine (67 Ci/mmol) (ICN). The pulse was terminated by putting the cultures on ice. Cells were then scraped into the media and washed 3 times in 10 mM Tris (pH 7.4), 100 mM NaCl, 1 mM EDTA. Cells were resuspended in 3.0 ml of TNE and lysed by adding 75 μl of 10% SDS for 10 min, 25°C. Samples were deproteinized by treatment with 60 μl of a solution of 1 mg/ml pronase at 37°C for 30 min. A 100 μl sample was then spotted and dried in duplicate on GF/A filter discs (Whatman). They were then washed once in 10% trichloracetic acid (TCA), once in 5% TCA, and once in 95% ethanol for 5 min/wash. Filters were dried and counted in 5.0 ml of scintillation fluid containing 25% triton X-100 (Beckman Scientific), 2% liquid Spectrafluor PPO(Amersham), 73% scintillation grade toluene (Beckman Scientific) with a Beckman LS 8000 scintillation counter. Fifteen minutes into each pulse cells were har-

vested, prepared for IFA with the Shandon cytospin and IFA was done as described.

CHAPTER IV

RESULTS

Characteristics of BKV Transformed BHK-21 Cells

For these studies BHK-21 cells were exposed to BKV and the resulting transformants were selected for their ability to grow in soft 0.33% agar suspension. Cells exposed to virus grew in 0.33% agar at a transformation frequency of 0.70% whether they were exposed to 10^1, 10^2, or 10^3 pfu/cell of virus. The control sample of cells not exposed to virus grew in 0.33% agar at 0.04% frequency.

Twelve of these clones were taken from the 0.33% agar and studied further. One clone (L29) was generated previously (80). The results of some of these studies are shown in Table 2. The transformed phenotype of these clones was shown by increased plating efficiency in 0.33% agar. BHK-21 cells not exposed to virus plated in 0.33% agar with an efficiency of 0.6%. Cells exposed to virus, selected in soft agar then grown up and replated in soft agar plated with efficiencies ranging from 2.6% to 15.0%. The association of virus with these cells was shown by virus rescue. The virus rescue titer/ml of lysate was determined for each clone. Since only 1.0 ml of lysate was tested for virus clones with titers of 1.0×10^0/ml were rescue negative in this assay. Rescue positive clones had titers ranging from 5.8×10^2/ml to 6.0×10^3/ml. Only transformed cells fused with permissive HEK cells rescued virus. Transformed cells fused with themselves did not yield detectable virus. The nuclear T antigen reactivity as reported in Table 2

Table 2 CHARACTERISTICS OF BKV TRANSFORMED BHK-21 CLONES

Clone	Plating Efficiency in Soft Agar [b]	Virus Rescue Titer/ml Lysate [b]	T Antigen Reactivity[a]
BK-BHK A_1	12.9%	5.8×10^2	Positive
BK-BHK A_4	4.6%	4.5×10^3	Positive
BK-BHK A_7	9.9%	$< 1.0 \times 10^0$	Negative
BK-BHK A_8	12.7%	$< 1.0 \times 10^0$	Negative
BK-BHK B_2	8.6%	$< 1.0 \times 10^0$	Negative
BK-BHK B_8	11.5%	2.8×10^3	Positive
BK-BHK C_1	2.6%	4.0×10^3	Positive
BK-BHK C_2	6.4%	$< 1.0 \times 10^0$	Positive
BK-BHK C_4	5.9%	4.5×10^3	Positive
BK-BHK C_7	12.0%	6.0×10^3	Positive
BK-BHK C_8	10.2%	$< 1.0 \times 10^0$	Negative
BK-BHK C_{10}	10.7%	1.8×10^3	Positive
BK-BHK L29	15.0%	1.0×10^3	Positive
BHK-21	0.6%	$< 1.0 \times 10^0$	Negative

[a] T antigen detection was carried out by IFA with anti-THE_2 tumor serum. Positive or negative reactivity as reported here was determined by the presence or absence of positive nuclei regardless of the number of positive nuclei or the tissue culture passage number.

[b] Plating efficiency in soft agar and virus rescue titer were done as described in Materials and Methods.

was determined by IFA with anti-THE_2 antiserum. Clones were identified as positive if any positive nuclei were seen regardless of the number of positive nuclei or the tissue culture passage number. Four out of thirteen clones were negative. These 4 were also rescue negative. One clone, BK-BHK C2 was rescue negative but T antigen positive. The cells of a positive clone were never uniformly positive. As compared with the SV40 transformed human cell line SV80 shown in Figure 1 which is 100% T positive BK-BHK clones were usually less than 10% positive. Photographs of the C1 clone and the A4 clone are shown in Figures 2 and 3. Two negative clones are shown in Figure 4. Positive cells often appeared in clusters. Controls included with each assay included BHK-21 cells reacted with anti-THE_2 serum and SV80 cells with anti-THE_2 serum. BHK-21 cells were never positive and SV80 cells were always 100% positive. The transformed clones were also reacted with normal hamster serum and were always completely negative.

The relationship between tissue culture passage number and the percentage of positive nuclei within each clone was examined. In 5 out of 6 clones the number of positive nuclei increased with passage in culture. The sixth clone C8 did not show an increase through 10 passages. The C8 clone was rescue negative. In Figure 5 are photographs of clone L29 passage 20 and passage 59. Both were assayed at the same time. The cells were reacted with anti-THE_2 serum and stained with fluorescein conjugated rabbit anti-hamster IgG as described for IFA. At passage 20 clone L29 was $<0.1\%$ positive and at passage 59 it was 9.4% positive. The passage at which the percentage of positive nuclei began to increase varied with each clone. For L29 this was after passage 20, for A1 af-

Figure 1. IFA assay of SV80 cells reacted with anti-THE_2 serum. Initial magnification 160X.

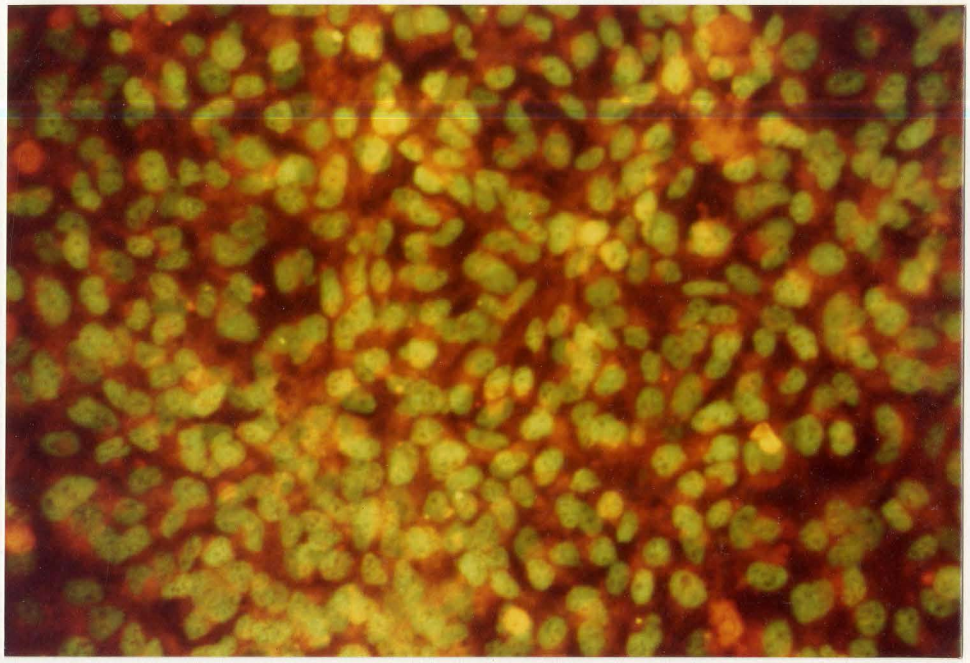


Figure 2. IFA of BK-BHK clone C1 TC13 reacted with anti-THE_2 serum. Initial magnification 160X.

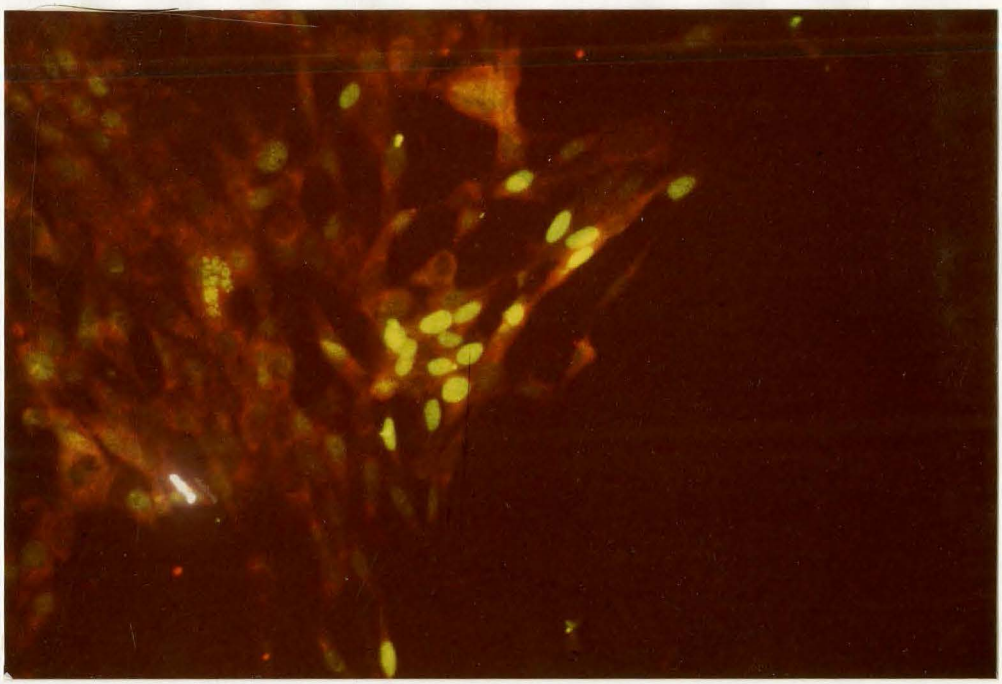
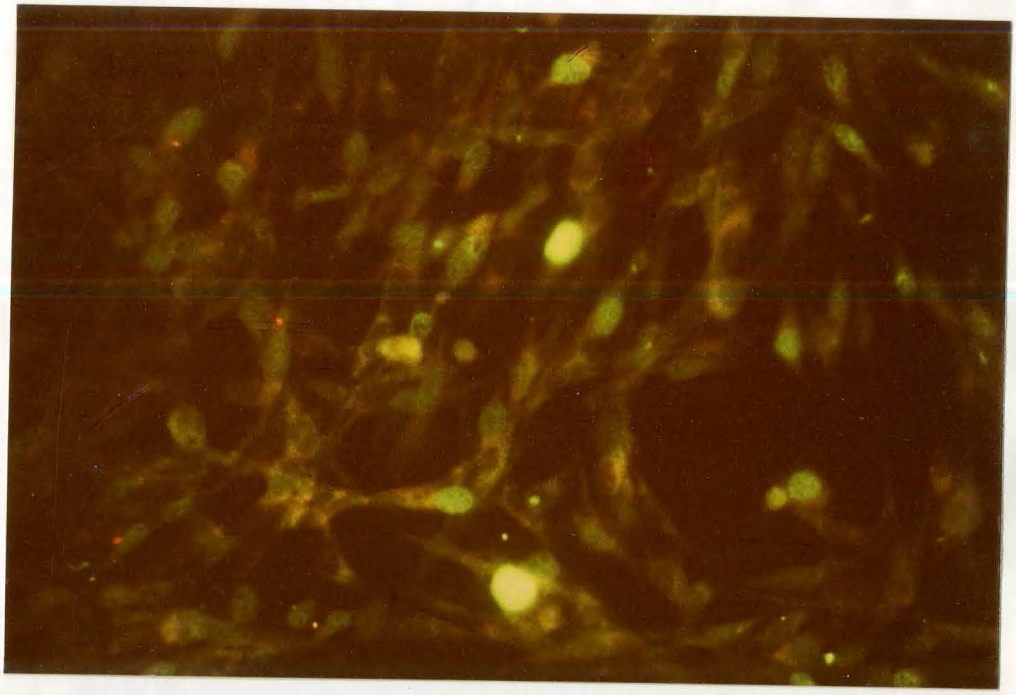


Figure 3. IFA of BK-BHK A4 TC7 reacted with anti-THE_2 serum. A, Initial magnification 160X. B, Initial magnification 400X



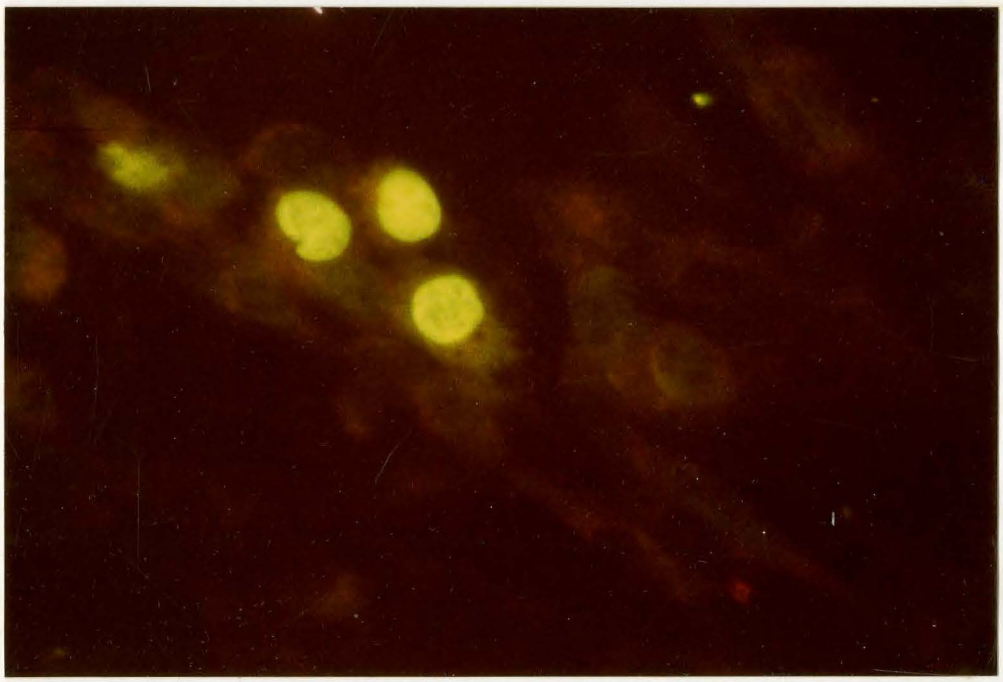
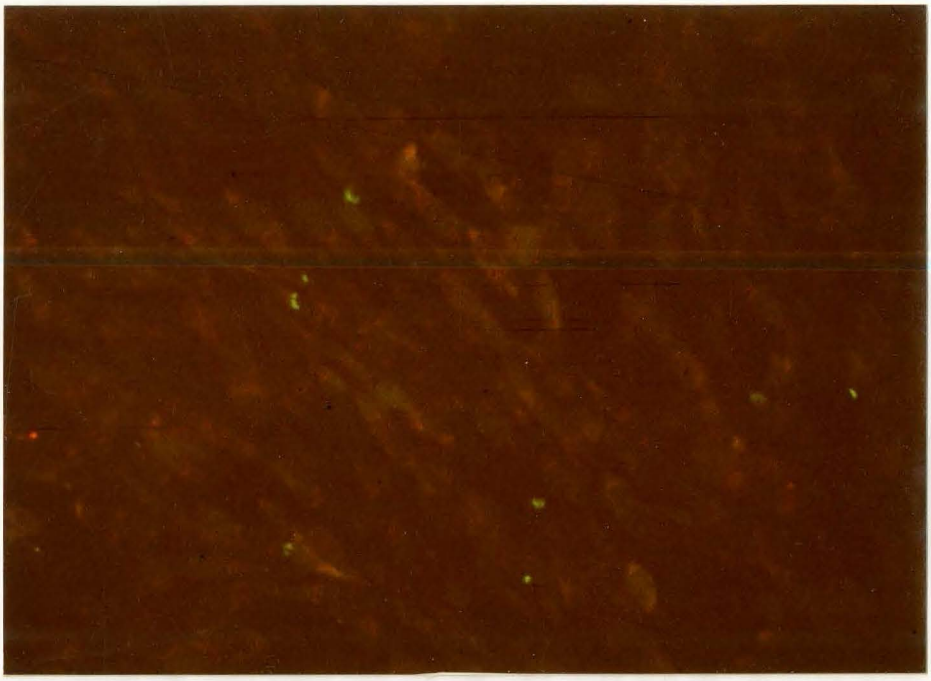


Figure 4. A. IFA of BK-BHK clone C8 TC6 reacted with anti-THE_2 serum. B. IFA of BK-BHK clone A7 TC6 reacted with anti-THE_2 serum. Initial magnification for both 160X.



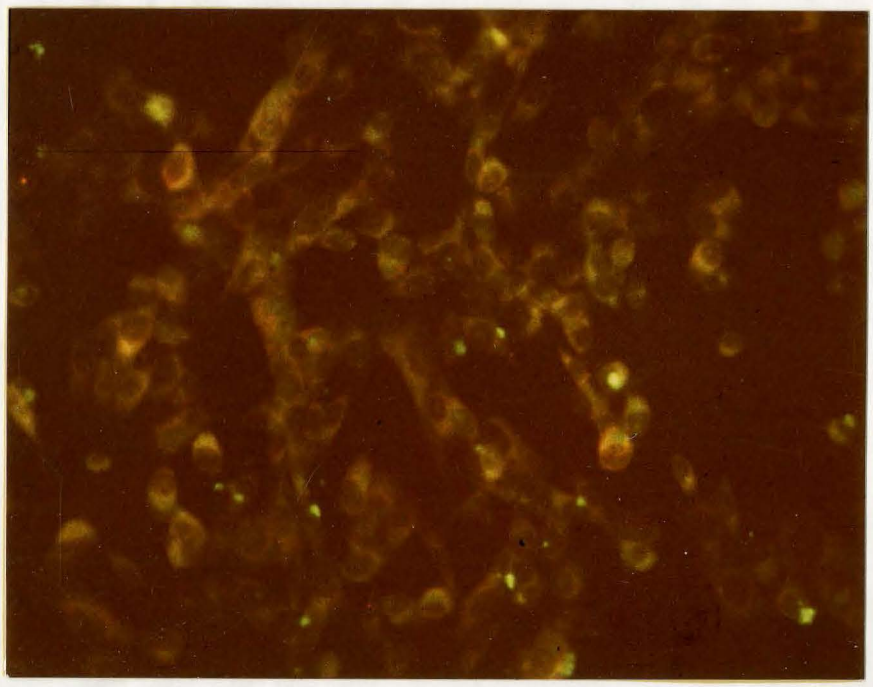
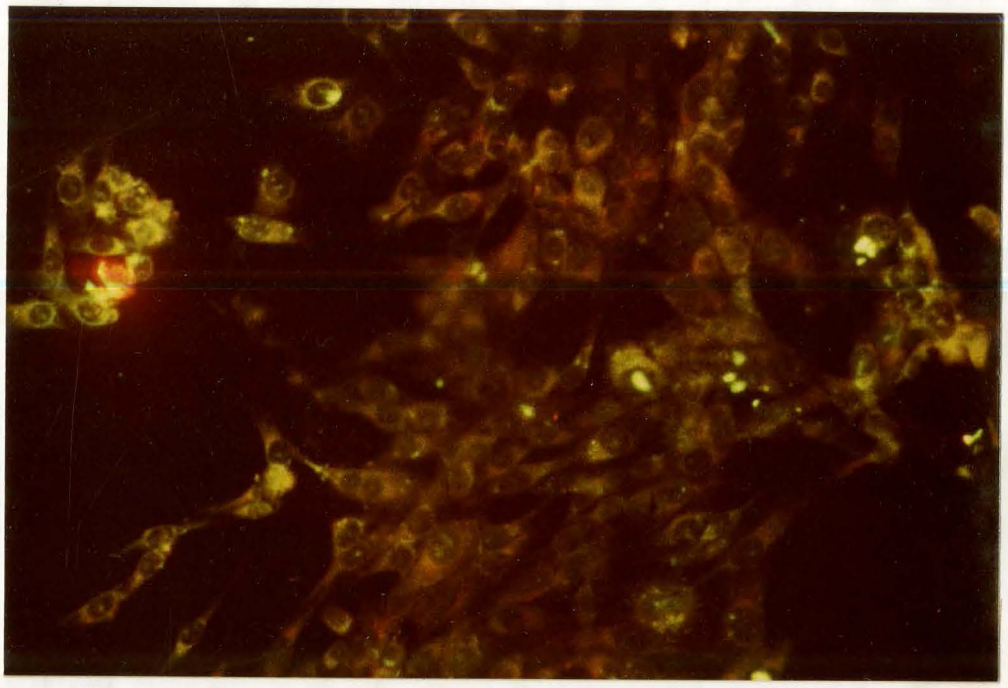


Figure 5. A. IFA of BK-BHK TC20 reacted with anti-THE_2 serum. B. IFA of BK-BHK L29 TC59 reacted with anti-THE_2 serum. Initial magnification for both 160X.



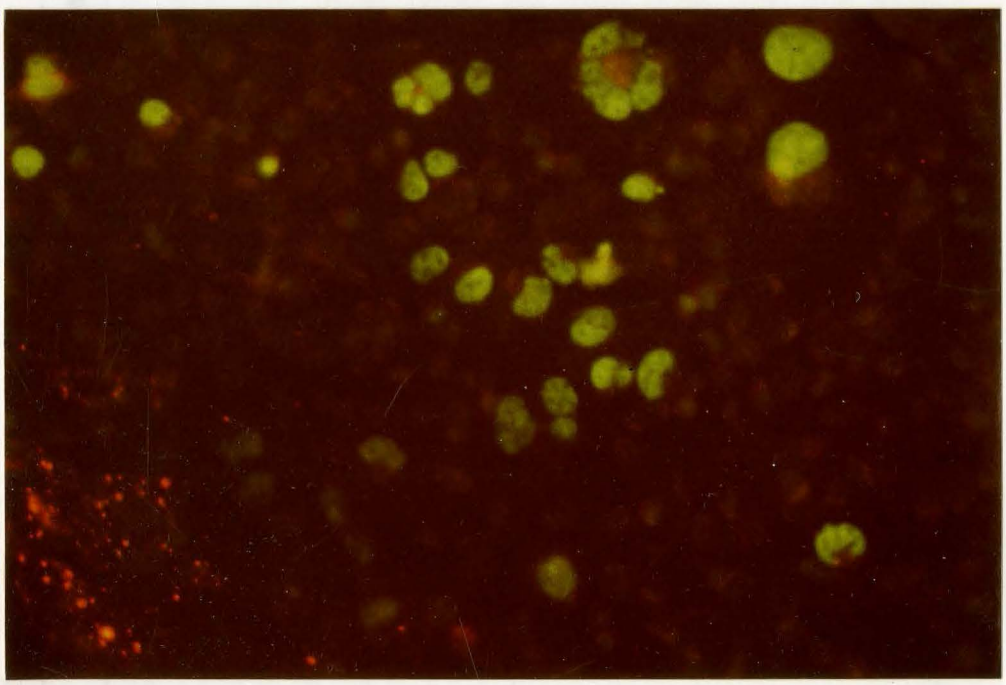


Table 3 CONTINUAL PASSAGE DETECTION OF TUMOR PROTEINS

BK-BHK Clone	Passage Number	Percent Positive[a]
L29	16	< 0.1
	20	< 0.1
	24	0.7
	32	1.0
	59	9.4
A_1	3	0.1
	9	0.2
	10	0.6
	14	2.0
C_4	8	< 0.1
	10	3.3
	13	2.6
	15	23.0
	16	2.0
C_8	5	< 0.1
	6	< 0.1
	9	< 0.1
	10	< 0.1
A_8	6	< 0.1
	10	2.0
	11	5.0
	14	6.5

Table 3

BK-BHK Clone	Passage Number	Percent Positive[a]
C_7	15	<0.1
	16	<0.1
	24	10.0
	28	15.7
	29	10.0

[a] Tumor proteins were detected by IFA with anti-THE_2 serum. The percent positive reflects the number of positive nuclei counted in a total of 1000 cells.

Antiserum and Detection of BKV Tumor Proteins from Lytically Infected Cells

Antiserum was first made in hamsters against BKV transformed BHK-21 (RF-194) cells. When 2 X 10^5 cells were injected hamsters were exsanguinated 4-7 weeks after injection as shown in Table 4. The capacity of this antiserum to detect the BKV large and small T proteins was tested by immunoprecipitation from BKV infected HEK cells. The results are shown in Figure 6. This antiserum precipitated a 94,000 dalton protein which was not precipitated by normal serum. This 94,000 dalton protein comigrated with the SV40 large T protein precipitated from SV80 cells (Figure 13) and is probably the BKV large T protein. The anti-RF194 serum did not precipitate a protein in the 17,000-22,000 dalton range which would be consistent with a small t protein. When 2 X 10^4 RF194 cells were injected (Table 4) hamsters were exsanguinated 6-7 weeks after injection. This antiserum also could not precipitate a protein of a molecular weight consistent with estimates for the molecular weight of BKV small t protein.

When SV40 transformed hamster cells were used to immunize hamsters the tumor development was much slower and hamsters were exsanguinated 21-67 weeks after inoculation as shown in Table 4. Antiserum to THE_2 cells immunoprecipitated a 94,000 dalton protein from BKV infected HEK cells as shown in Figure 7. It also precipitated a 22,000 dalton protein which was not precipitated with normal serum. This 22,000 dalton protein comigrated with the SV40 small t protein from SV80 cells (see Figure 13).

When BKV infected marmoset brain cells were immunoprecipitated

Table 4 ANTISERUM PRODUCTION

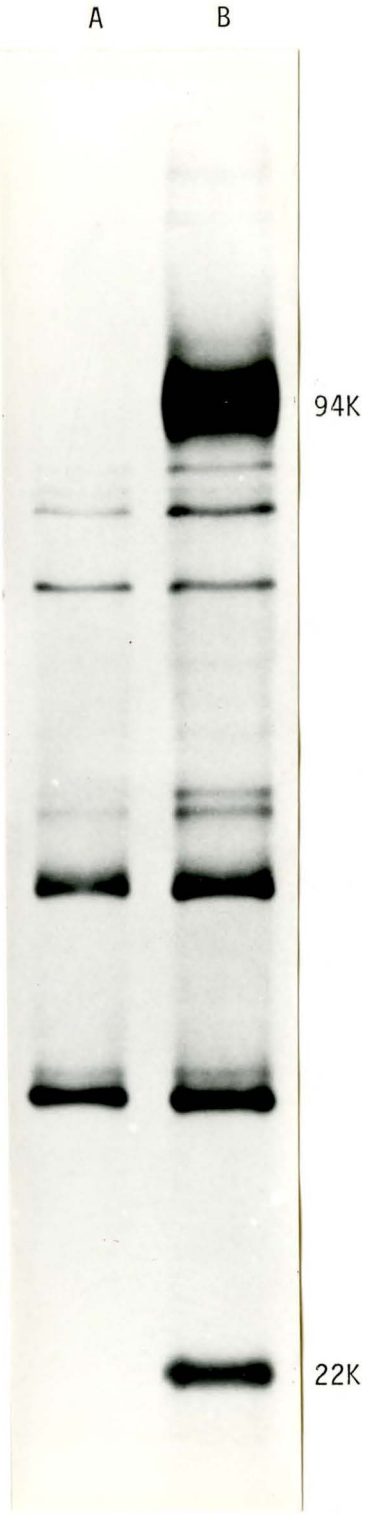
Cell Type	Cell Number/Hamster	Number of Hamsters	Time Course[a]
RF194	2×10^4	7	6-7 weeks
RF194	2×10^5	28	4-7 weeks
THE_2	1×10^5	5	32-61 weeks
THE_2	1×10^6	6	21-67 weeks

[a] The period of time from inoculation, through tumor development to exsanguination. The time course range reflects individual differences between hamsters.

Figure 6. Polyacrylamide gel autoradiograph of immunoprecipitation from BKV lytically infected extracts of ^{35}S-methionine labeled HEK cells with anti-RF194 serum (T.S.) and normal hamster serum (N.S.). Cells were infected with 10pfu of BKV/cell for 5 days then extracted and immunoprecipitated. Electrophoresis was at 75 mA for 5 h.


N.S.
T.S.
—94K
—22K

Figure 7. Polyacrylamide gel autoradiograph of BKV lytically infected ^{35}S-methionine labeled HEK cell extract immunoprecipitated with anti-THE$_2$ serum. Cells were infected with 10 pfu/cell of BKV for 3 days then immunoprecipitated. Electrophoresis was at 75 mA for 4.5 h. Lane A, precipitated with notmal hamster serum. Lane B, precipitated with anti-THE$_2$ serum.


A
B
94K
22K

with anti-THE_2 serum a 94,000 dalton protein was present which was not precipitated with normal hamster serum. This is consistent with the BKV large T protein. Two proteins which migrated to a molecular weight range consistent with small t protein were precipitated with anti-THE_2 serum. One protein migrated at 22,000 daltons and another at 18,000 daltons. Neither were precipitated with normal hamster serum. These results are shown in Figure 8.

Hybridoma cells producing monoclonal antibodies directed against SV40 tumor proteins were obtained from E. Gurney. These were clones 7, 412, 122, and 108. Clones 7, 412 and 108 were specific for SV40 large T protein and clone 122 was specific for the 53,000 dalton nonviral protein which co-precipitated the large T protein (7). Clones 7, 412, and 122 were used to generate ascites fluid in mice. Clone 7 and 412 ascites fluids were titered on SV80 cells using indirect immunofluorescence. Nuclear SV40 T protein reactivity titered at 1:3000 for both ascites fluids. Clone 7,412, and 122 ascites fluids were then used to immunoprecipitate from BKV infected HEK cells. The results are shown in Figure 9. Clone 412 was capable of precipitating the SV40 large T protein from SV80 cells as shown in lane E. Clone 412 could not precipitate the BKV large T protein as shown in lane C. Clone 7 could not precipitate the BKV large T protein as shown in lane B. Clone 122 ascites fluid did not precipitate theBKV large T protein as shown in lane A. Lane D, BKV infected HEK cell extract precipitated with anti-THE_2 serum demonstrates the presence of BKV large T protein in these extracts. Clone 108 was tested separately. The clone 108 culture fluid was incapable of immunoprecipitating BKV large T protein. When clone 108 culture fluid was

Figure 8. Polyacrylamide gel autoradiograph of immunoprecipitation from extracts of BKV infected ^{35}S-methionine labeled marmoset brain cells with anti-THE$_2$ serum (lane A) and normal hamster serum (lane B). Cells were infected with BKV at 10 pfu/cell. After 8 days they were labeled, extracted and immunoprecipitated. Electrophoresis was at 20 mA, overnight.

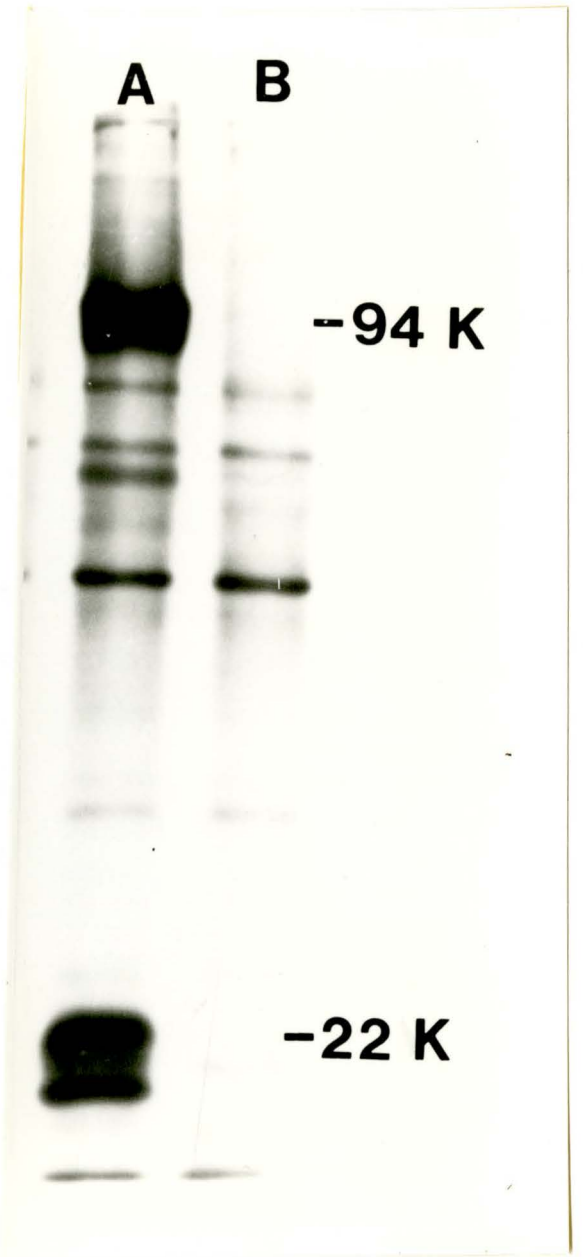
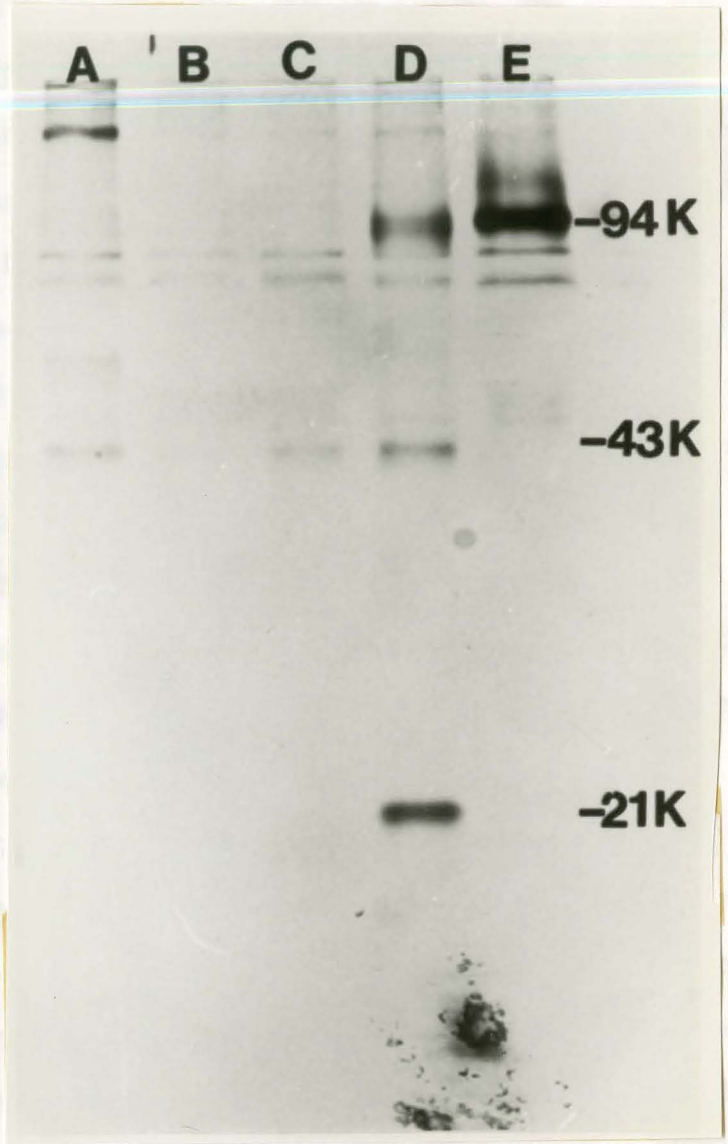

A
B
-94 K
-22 K

Figure 9. Polyacrylamide gel autoradiograph of ^{35}S-methionine labeled, monoclonal antibody precipitated extracts. Lane A, BKV lytically infected HEK cell extract precipitated with 200 µl clone 122 ascites fluid. Lane B, BKV lytically infected HEK cell extract precipitated with 200 µl clone 412 ascites fluid. Lane C, BKV lytically infected HEK cell extract precipitated with 200 µl of clone 7 ascites fluid. Lane D, extract after precipitation with clone 412 antibody, precipitated with 20 µl anti-THE_2 serum. Lane E, SV80 cell extract precipitated with 200 µl of clone 412 ascites fluid. HEK cells were infected with 1 pfu/cell of BKV for 7 days before labeling. Electrophoresis was at 7 mA overnight.


A
B
C
D
E
-94K
-43K
-21K

used for IFA detection of large T protein in BKV infected HEK cells the results were negative. Clone 108 culture fluid was capable of detecting SV40 large T protein in SV80 cells.

Immunoprecipitation from BKV Transformed BHK-21 Cells

BK-BHK clone L29 TC13 was immunoprecipitated with anti-THE_2 serum and normal hamster serum. The results are shown in Figure 10. At passage 13 clone L29 was 0.1% nuclear T protein positive. As can be seen in this figure all the proteins precipitated with anti-THE_2 serum were also precipitated with normal hamster serum. The only difference was a slightly more intense series of protein bands in lane B at 22,000 daltons. The extent of protein precipitated by normal serum indicated non-specific precipitation and made interpretation of this gel and others like it nearly impossible. This extensive protein precipitation with normal serum was a problem unique to the BHK-21 cell type. It was not experienced with HEK, SV80 or marmoset infected cells.

Several changes were made in the protocol to overcome this problem. The successful changes which were incorporated into the method permanently were the use of plastic microfuge tubes for immunoprecipitation and bead washing, and the changing of tubes after each wash. Even with these improvements non-specific precipitation from BHK-21 cells remained a recurrent and limiting problem.

In Figure 11 is an improved immunoprecipitation from clone L29 TC 16 Faint bands of protein suggestive of the BKV large and small T proteins were present in lane A at 94,000 daltons and 22,000 daltons. These bands were not precipitated with normal serum.

Figure 10. Polyacrylamide gel autoradiograph of immunoprecipitation from BKV transformed, ^{35}S-methionine labeled BK-BHK clone L29 TC13 with anti-THE$_2$ serum (lane B) and normal hamster serum (lane A). Electrophoresis was at 75 mA for 4 h.

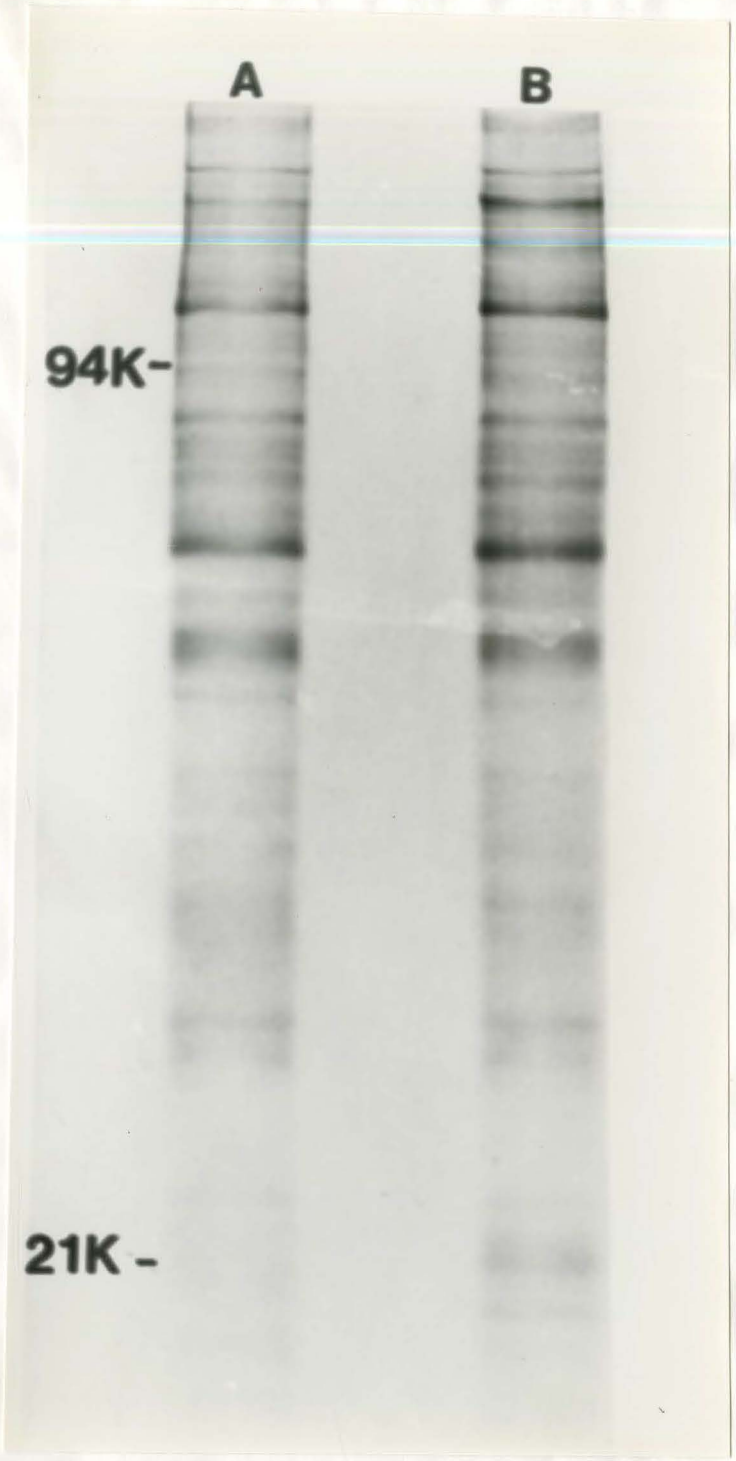
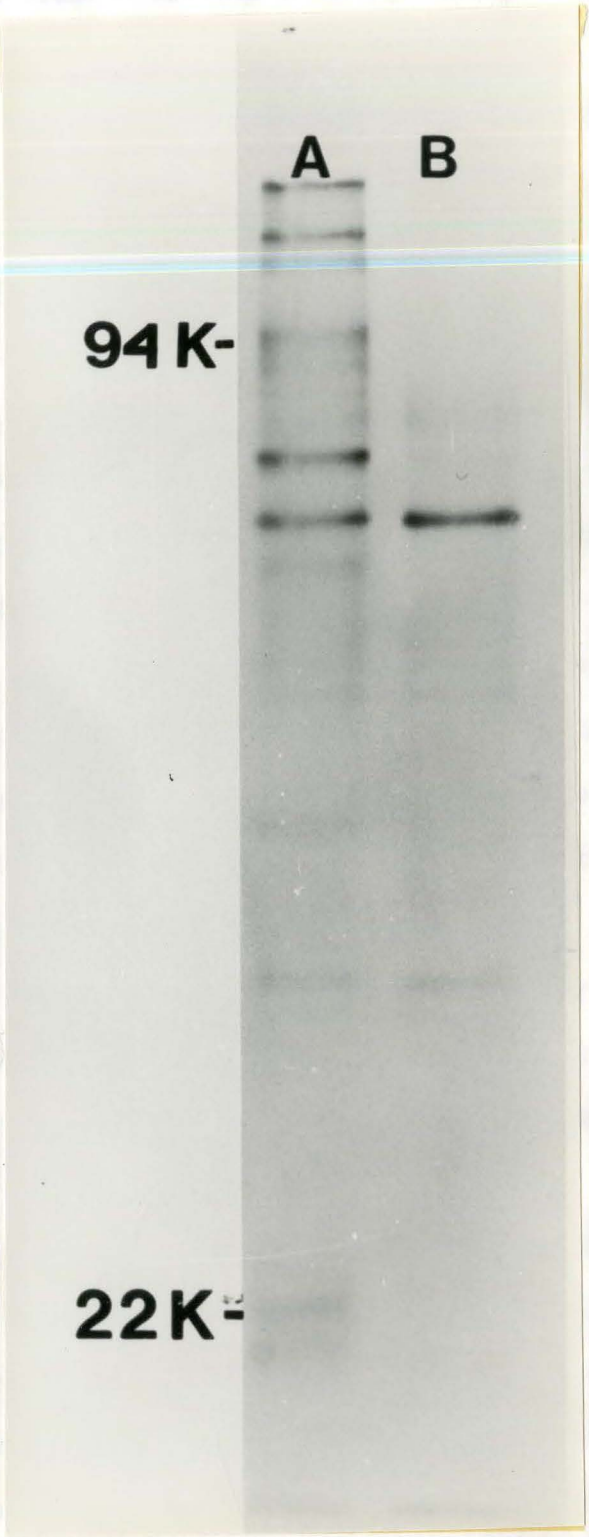

A
B
94K-
21K -

Figure 11. Polyacrylamide gel autoradiograph of immunoprecipitation from ^{35}S-methionine labeled extract of BK-BHK L29 TC16. Lane A precipitated with anti-THE_2. Lane B precipitated with normal hamster serum. Electrophoresis was at 7 mA overnight.


A
B
94K-
22K-

In Figure 12 are the results of immunoprecipitation from clone C7 TC14. Faint bands of protein suggestive of BKV large and small T proteins can be seen at 94,000 daltons and 22,000 daltons. These bands were not precipitated with normal hamster serum.

DNA Cellulose Chromatography

In Figure 13 are results of immunoprecipitation from DNA cellulose column eluates. In lane E is an SV80 cell extract precipitated with anti-THE_2 serum. In lane C is the SV80 cell extract after washing at pH 6.0 and elution at pH 9.0. As can be seen the SV40 94,000 dalton large T protein eluted at pH 9.0. In lane D is the pH 9.0 eluate precipitated with normal serum. No 94,000 dalton protein was precipitated with normal serum. Lanes A and B demonstrate the immunoprecipitation pattern from an extract of BKV infected HEK cells for comparison. In lane H is the pH 6.0 column eluate precipitated with anti-THE_2 serum. In lane F is the pH 9.0 column eluate precipitated with anti-THE_2 serum. As can be seen the 94,000 dalton large T protein was precipitated after elution from the column at pH 9.0. Results in lane G demonstrate that normal serum did not precipitate the 94,000 dalton protein from the pH 9.0 column eluate.

In Figure 14 are the results of immunoprecipitation from DNA cellulose column eluates of a BK-BHK C7 TC23 extract. In lane A is the C7 TC23 extract precipitated with anti-THE_2, demonstrating the presence of the 94,000 dalton protein. In lane D is the pH 6.0 column eluate precipitated with normal hamster serum. In lane E is the pH 6.0 column eluate precipitated with anti-THE_2 serum. A faint band of 94,000 dalton

Figure 12. Polyacrylamide gel autoradiograph of immunoprecipitation from ^{35}S-methionine labeled BKV transformed BHK-21 clone C7 TC14 extract. Lane A, precipitated with normal serum. Lanes B and C, identical samples precipitated with anti-THE_2. Electrophoresis was at 25 mA overnight.


A
B
C
-94K
-21K

Figure 13. Polyacrylamide gel autoradiograph of ^{35}S-methionine labeled proteins immunoprecipitated from DNA cellulose column fractions. HEK cells were infected with 10 pfu/cell of BKV and extracted 10 days later. Lanes A and B, lytically infected HEK cell extract precipitated with 2 different lots of anti-THE$_2$ serum for comparison. Lane C, pH 9.0 column eluate of SV80 cell extract precipitated with anti-THE$_2$ serum. Lane D, pH 9.0 column eluate of SV80 cell extract precipitated with normal hamster serum. Lane E, pH 6.0 column eluate of SV80 cell extract precipitated with anti-THE$_2$ serum. Lane F, pH 9.0 column eluate of BKV infected HEK cell extract precipitated with anti-THE$_2$ serum. Lane G, pH 9.0 column eluate of BKV infected HEK cell extract precipitated with normal serum. Lane H, pH 6.0 column eluate of BKV infected HEK cell extract precipitated with anti-THE$_2$ serum. Electrophoresis was at 7.5 mA overnight.

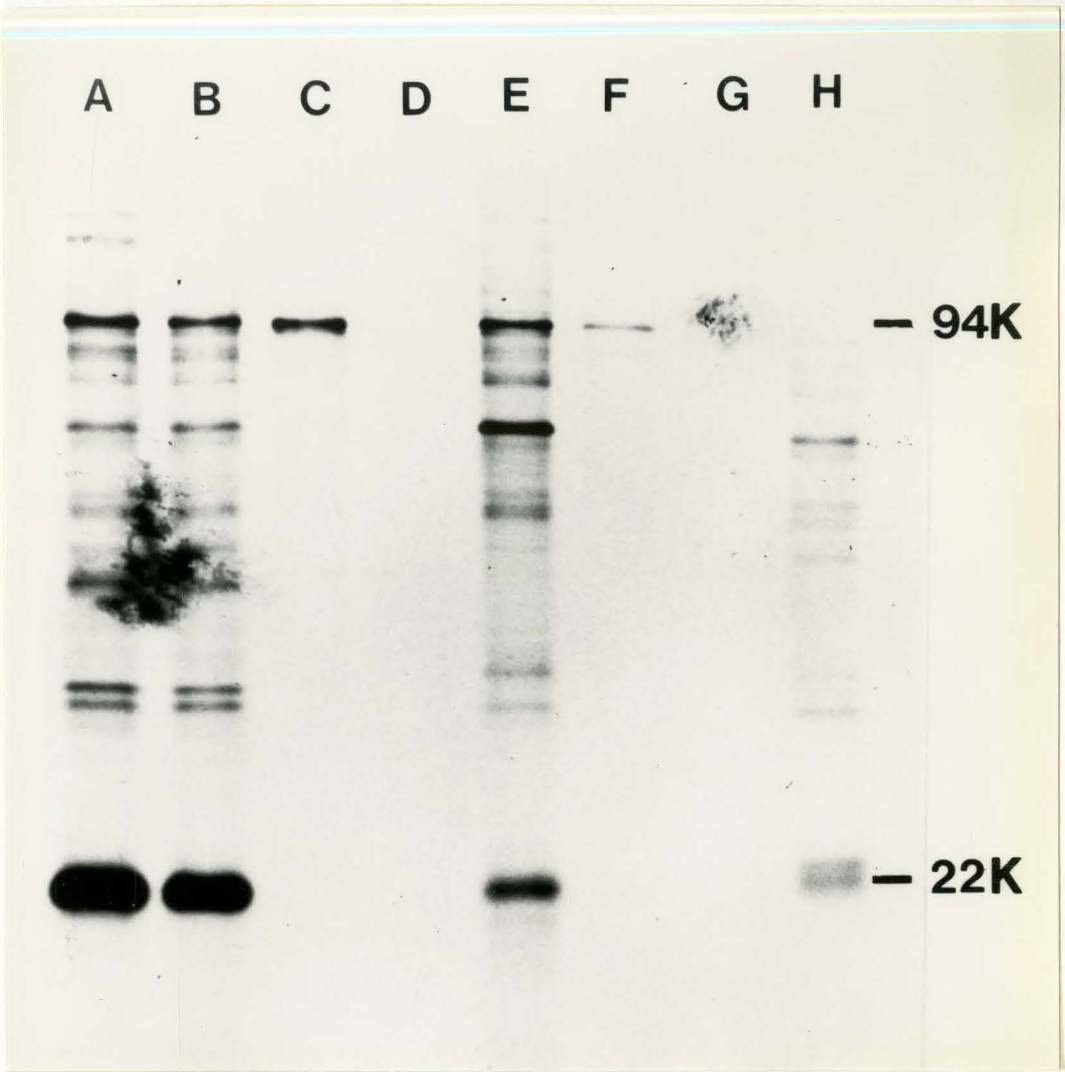
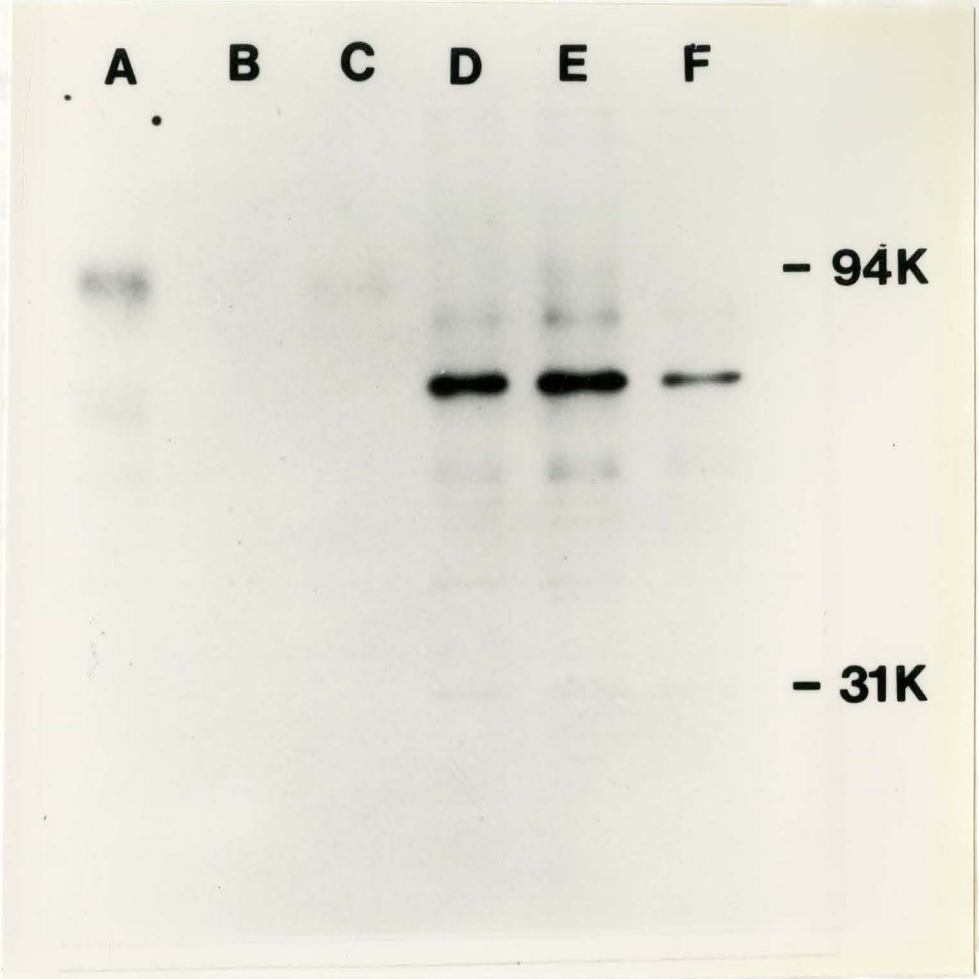

A
B
C
D
E
F
G
H
94K
22K

Figure 14. Polyacrylamide gel autoradiograph of ^{35}S-methionine labeled proteins immunoprecipitated from DNA cellulose column fractions of BK-BHK C7 TC23. Lane A, C7 TC23 precipitated with anti-THE_2 serum. Lane B, pH 9.0 column eluate precipitated with normal hamster serum. Lane C, pH 9.0 column eluate precipitated with anti-THE_2. Lane D, pH 6.0 column eluate precipitated with normal hamster serum. Lane E, pH 6.0 column eluate precipitated with anti-THE_2. Lane F, pH 6.0 column wash number 1 precipitated with anti-THE_2. Electrophoresis was at 7 mA overnight.


A
B
C
D
E
F
- 94K
- 31K

protein is present. In lane F is the first wash at pH 6.0 precipitated with anti-THE_2 serum. No 94,000 dalton protein is present. Results in Lane C demonstrate that the 94,000 dalton large T protein is eluted from the column at pH 9.0. This protein is not precipitated with normal serum as shown in lane B.

The Contribution of BKV Small t Protein to Transformation

The contribution of BKV small t protein to transformation was studied with BKV-strain MM (BKV-MM). This strain of BKV cannot make small t protein because it is missing an essential sequence for mRNA splicing. The two strains can be distinguished by the loss of a Hind III restriction site in BKV-MM. This generates 3 BKV-MM Hind III fragments instead of 4 and is shown in Figure 15. BKV-MM and the wild type (Gardner strain) of BKV were used to transform BHK-21 cells. Two transformants were then immunoprecipitated with anti-THE_2 serum. One BHK colony from the control sample was also immunoprecipitated. The results are shown in Figure 16. As can be seen from this figure no 22,000 dalton small t protein was precipitated from either BKV-MM transformed clone. It was precipitated from the BKV-wild type transformed clone. BKV-MM transformed cells lacked small t but were capable of virus rescue.

The transformation frequencies of BKV-wild type and BKV-MM were compared. These results are shown in Table 5. In this assay wild type BKV transformed BHK-21 cells at an efficiency of 0.90%. BKV-MM transformed BHK-21 cells at an efficiency of 0.91%. BHK-21 control cells not exposed to virus plated at 0.03%.

Figure 15. Comparison of wild type BKV and BKV-MM DNA. Lane A, BKV-MM form I DNA. Lane B, wild type BKV form I DNA. Lane C, BKV-MM Hind III digest. Lane D, wild type BKV Hind III digest.

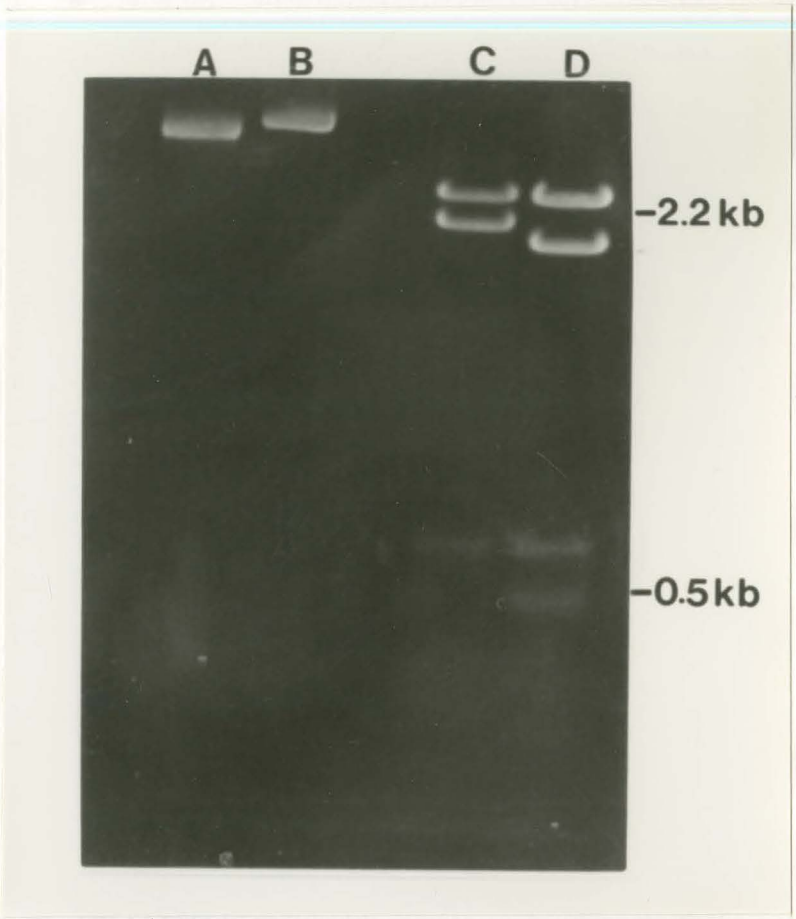

A
B
C
D
~2.2 kb
~0.5 kb

Figure 16. Polyacrylamide gel autoradiograph of immunoprecipitates comparing wild type BKV transformed BHK-21 cells and BKV-MM transformed BHK-21 cells. From left to right BHK-21 cells precipitated with anti-THE_2 serum, wild type BKV transformed cells precipitated with anti-THE_2 serum, BKV-MM transformed BHK-21 clone 5 precipitated with anti-THE_2 serum, BKV-MM transformed BHK-21 clone 6 precipitated with anti-THE_2 serum. Electrophoresis was at 10 mA overnight.

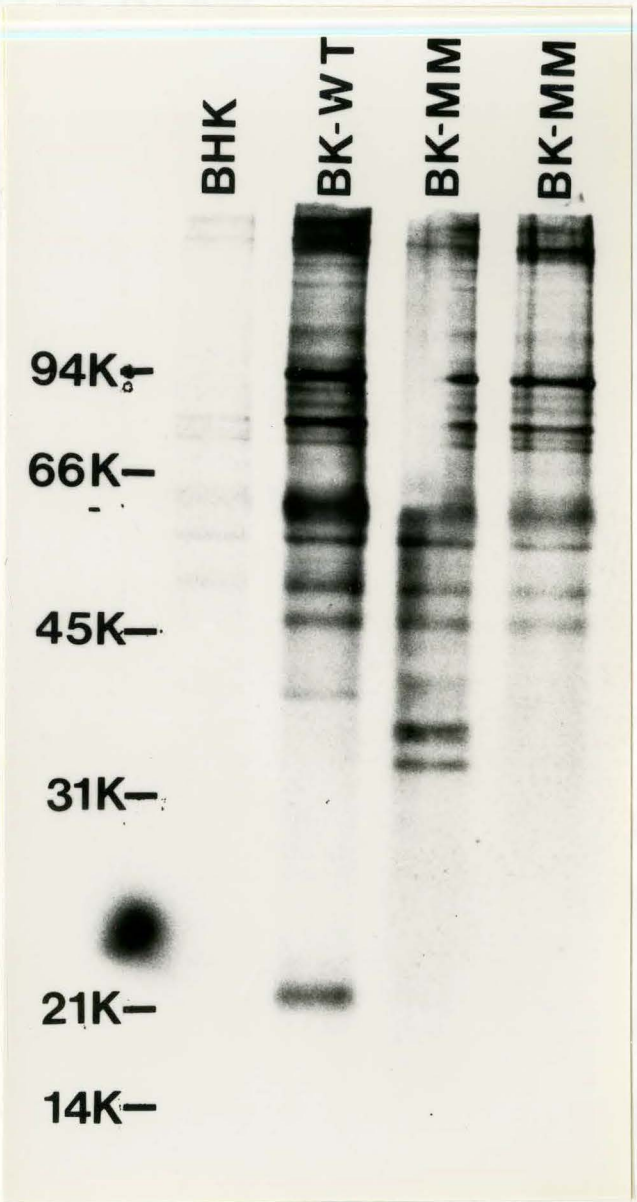

BHK
BK-WT
BK-MM
BK-MM
94K
66K
45K
31K
21K
14K

Table 5

TRANSFORMATION FREQUENCY OF WILD TYPE BKV
COMPARED TO MUTANT BKV-MM

Cell Type	Virus	Transformation Frequency[b]
BHK-21	BKV-WT[a]	0.90%
BHK-21	BKV-MM	0.91%
BHK-21	------	0.03%

[a] Wild type BKV
[b] Transformation frequency was determined as described in Materials and Methods.

T Antigen Expression After Cell Fusion

BK-BHK L29 TC20 cells were fused with permissive HEK TC3 cells using PEG-DMSO. At timed intervals after fusion IFA was done with anti-THE_2 serum. The number of positive cells, both single and multinucleated, in a total of 1,000 cells were counted at each time. The results are shown in Table 6. From an unfused population of L29 TC20 cells only 2 cells out of 1,000 were positive. A photograph of these cells is shown in Figure 17. After fusion the number of postive multi-nucleated cells remained low until the 10 h sample. At this time the number of positive multi-nucleated cells increased 38 times. In Figure 18A is a photograph of cells 5 h after fusion. Faintly staining nuclei are present in the center of the photograph. This faint fluorescence could only be seen in photographs. The proportion of positive multinucleated cells remained high after 10 h past fusion and reached a peak at 24 h past fusion. In Figure 18B and C are photographs of single and multinucleated cells 12 h past fusion. The number of positive cells with a single nucleus remained low until 24 h past fusion when the number increased approximately 10 fold. This apparent increase may be the result of nuclear fusion. At 48 h past fusion the distinction between single and multinucleated cells could not be made. The proportion of positive cells to negative cells decreased at 48 h past fusion as illustrated in Figure 18D.

BK-BHK L29 TC20 cells were fused without the permissive HEK cells. The results are shown in Table 7. At 0.5 h past fusion 2 out of 1,000 cells were positive. Cells 0.5 and 5 h past fusion are shown in Figure 19A and B. The number of positive multinucleated cells remained low until 10 h past fusion when the number increased 25 times. Cells 10

Table 6 T ANTIGEN EXPRESSION AFTER CELL FUSION

BK-BHK-L29TC20-HEKTC3

Hours Past Fusion	Cell Type Counted Single(s) or Multinucleated(m)	Number of Cells Positive[a]	Number of Cells Negative[a]
0.5	----[b]	5	995
2	s	2	998
	m	3	997
5	s	6	994
	m	8	992
10	s	10	990
	m	308	692
12	s	8	992
	m	665	345
14	s	6	994
	m	600	400
24	s	52	948
	m	750	250
48	----[b]	248	752
Not fused		2	998

[a] Number of cells positive and negative for nuclear tumor antigen as determined by IFA with anti-THE_2 tumor serum. A total of 1000 cells in random fields were counted.

[b] At 0.5 and 48 hours past fusion the distinction between single and multinucleated cells could not be made.

Figure 17. Unfused L29 TC20 cells. IFA with anti-THE_2 serum.

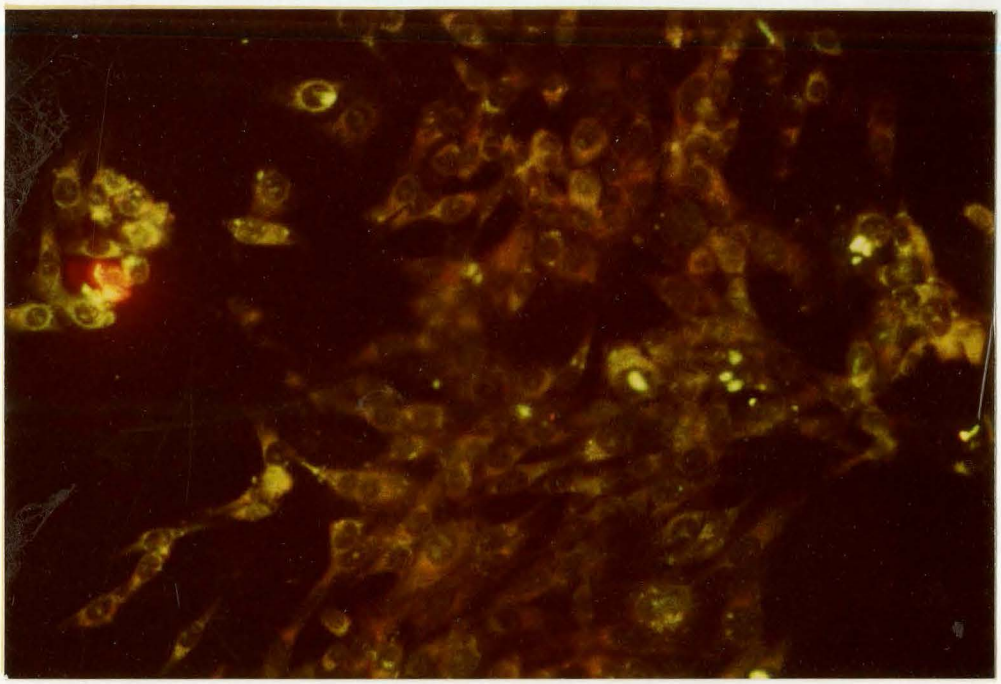


Figure 18. L29 TC20/HEK TC3 fusion.

A. IFA with anti-THE_2 serum 5 h past infection. Initial magnification 160X.

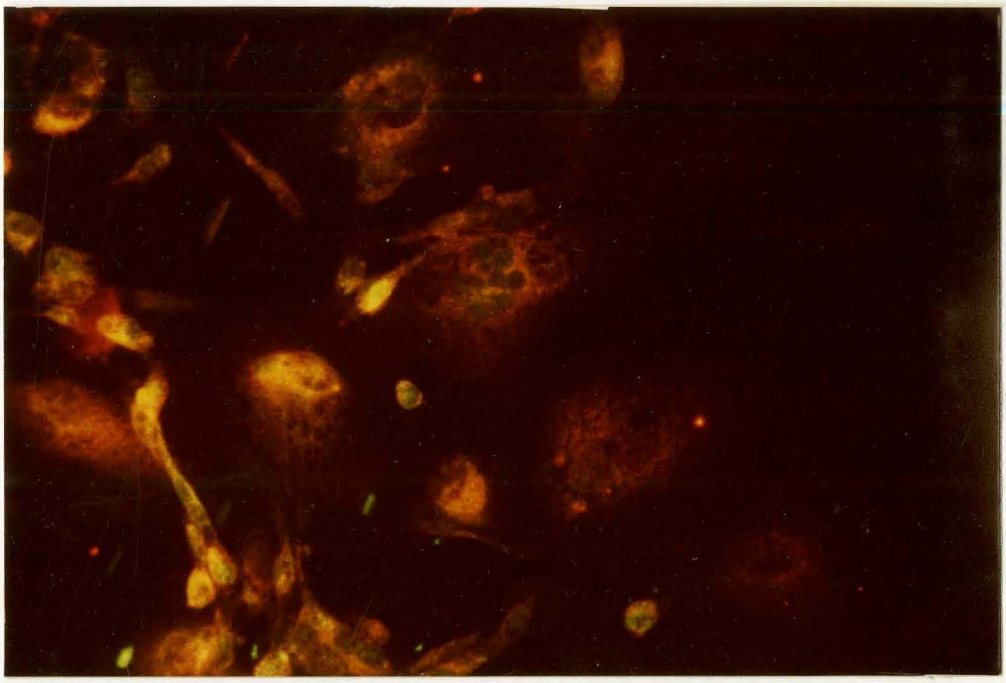
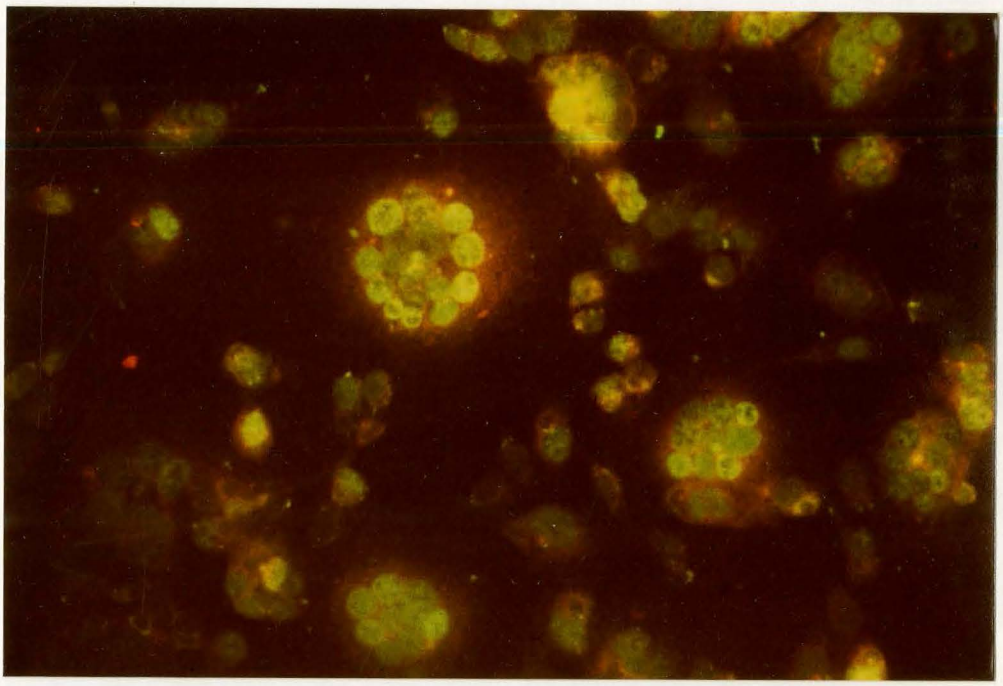


Figure 18 (Continued). L29 TC20/HEK TC3 fusion.

B. IFA with anti-THE_2 serum 12 h past fusion.

C. IFA with anti-THE_2 serum 12 h past fusion.

Initial magnification 160X.



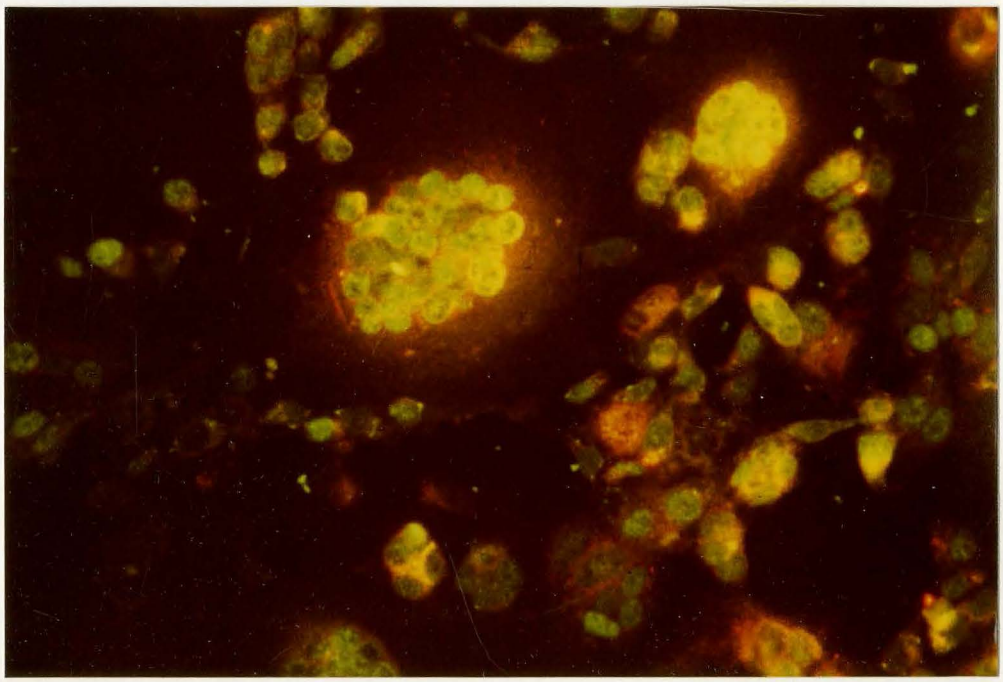


Figure 18 (Continued). L29 TC20/HEK TC3 fusion.

D. IFA with anti-tumor serum 48 h past fusion. Initial magnification 160X.

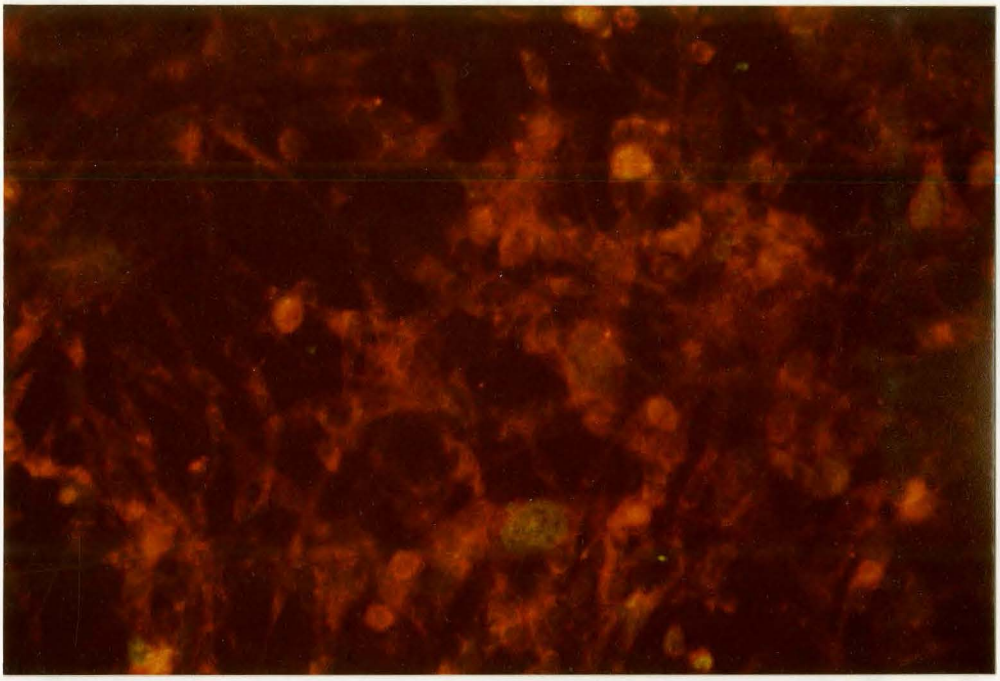


Table 7 T ANTIGEN EXPRESSION AFTER CELL FUSION

BK-BHK L29TC20-BK-BHK L29TC20

Hours Past Fusion	Cell Type Counted Single(s) or Multinucleated(m)	Number of Cells Positive[a]	Number of Cells Negative[a]
0.5	----[b]	2	998
2	s	7	993
	m	4	996
5	s	5	995
	m	3	997
10	s	10	990
	m	101	899
12	s	4	996
	m	205	795
14	s	10	990
	m	360	640
24	s	10	990
	m	99	901
Not fused		3	997

[a] Number of cells positive and negative for nuclear tumor antigen as determined by IFA with anti-THE_2 tumor serum. A total of 1000 cells in random fields were counted.

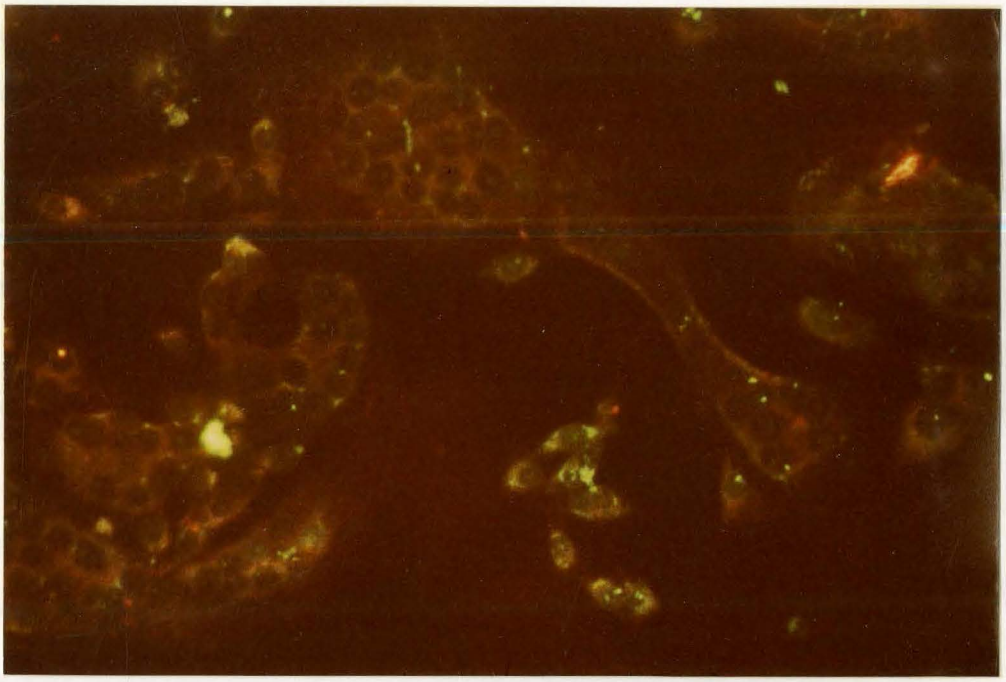
[b] At 0.5 h past fusion the distinction between single and multinucleated cells could not be made.

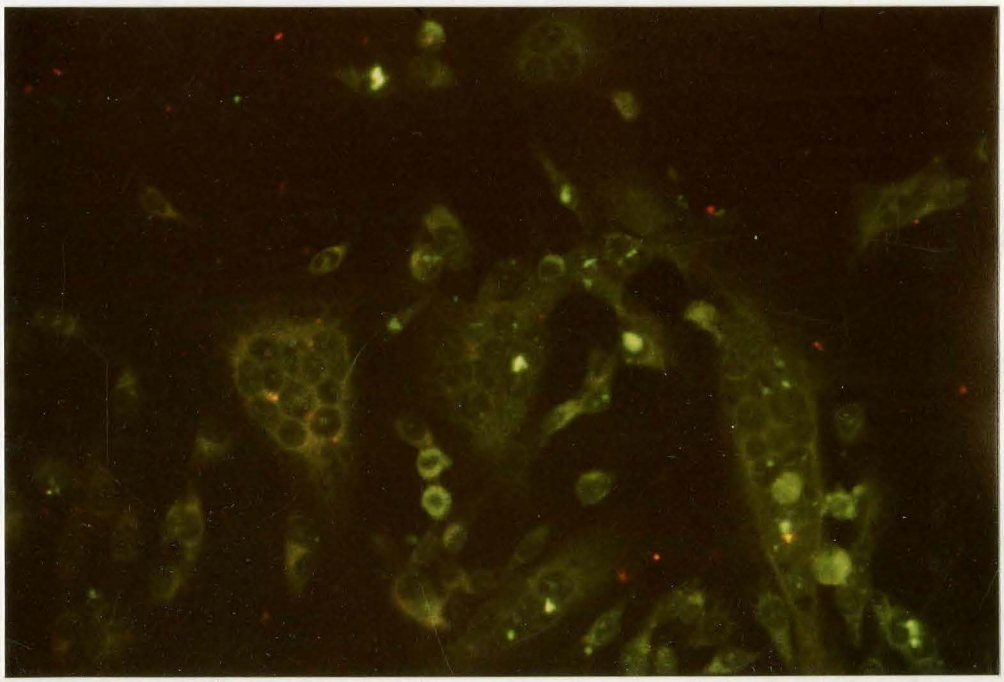
Figure 19. L29 TC20/L29 TC20 fusion.

A. IFA with anti-THE_2 serum 0.5 h past fusion.

B. IFA with anti-THE_2 serum 5 h past fusion.

Initial magnification 160X.





and 12 h past fusion are shown in Figure 19C and D. Note the presence of positive and negative nuclei in the same multinucleated cell. The number of positive multinucleated cells reached a peak at 14 h past fusion and decreased by 24 h past fusion. Cells 14 h and 24 h past fusion are shown in Figure 19E and F. The enlarged nuclei in Figure 19F may be the result of nuclear fusion.

In Table 8 are results of T antigen expression after fusion of BHK-21 cells. At all time periods sampled there were no positive cells seen. A photograph of fused BHK-21 cells 12 h past fusion is shown in Figure 20. Both multinucleated and single cells can be seen in this photograph. No positive cells can be seen.

Factors Influencing the Percentage of T Positive Nuclei in BKV Transformed BHK-21 Cells: Number of Days in Culture.

BK-BHK C7 was chosen for the examination of factors influencing the percentage of T positive nuclei in BKV transformed BHK-21 cells. Clone C7 TC29 is pictured in Figure 21. The number of days in culture past plating and the percentage of nuclear T protein positive cells was examined by IFA with anti-THE_2 serum. BK-BHK C7 TC28 cells were examined at 1, 2, 3, and 4 days past plating and a total of 500 cells were counted each day. At 1 day past plating the cells could not be counted because they detached when fixed. At 2 days past plating 11.6% were positive. At 3 days 13.6% were positive and at 4 days 12.0% were positive.

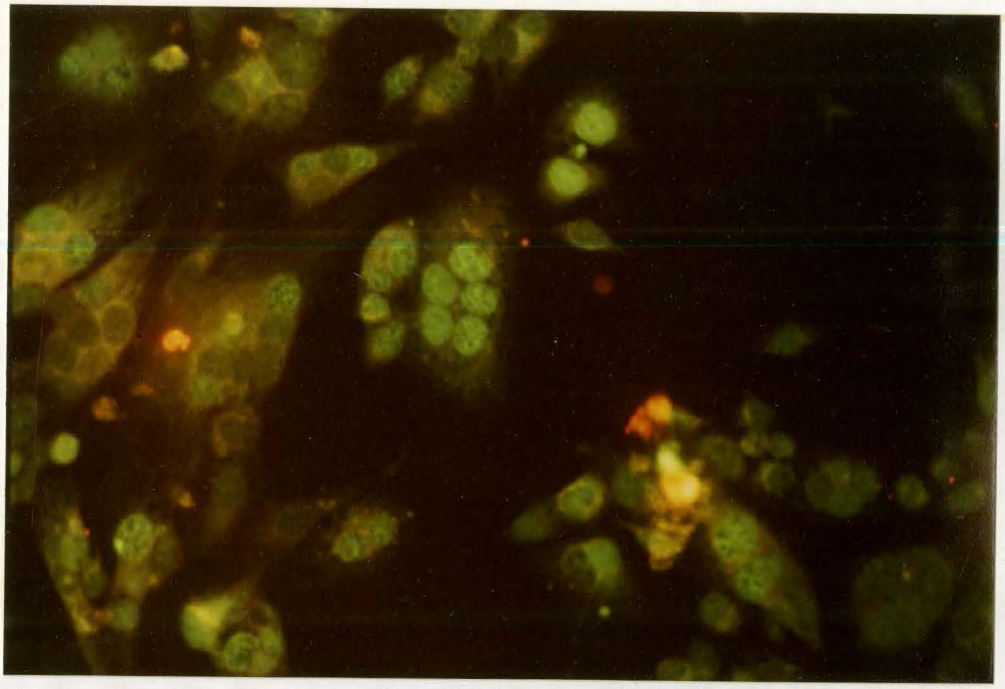
Factors Influencing the Percentage of T Positive Nuclei in BKV Transformed BHK-21 Cells: Cell Cycle and the Expression of T Proteins in Clone C7 TC29.

Figure 19 (Continued). L29 TC20/L29 TC20 fusion.

C. IFA with anti-THE_2 serum 12 h past fusion.

D. IFA with anti-THE_2 serum 12 h past fusion.

Initial magnification 160X.



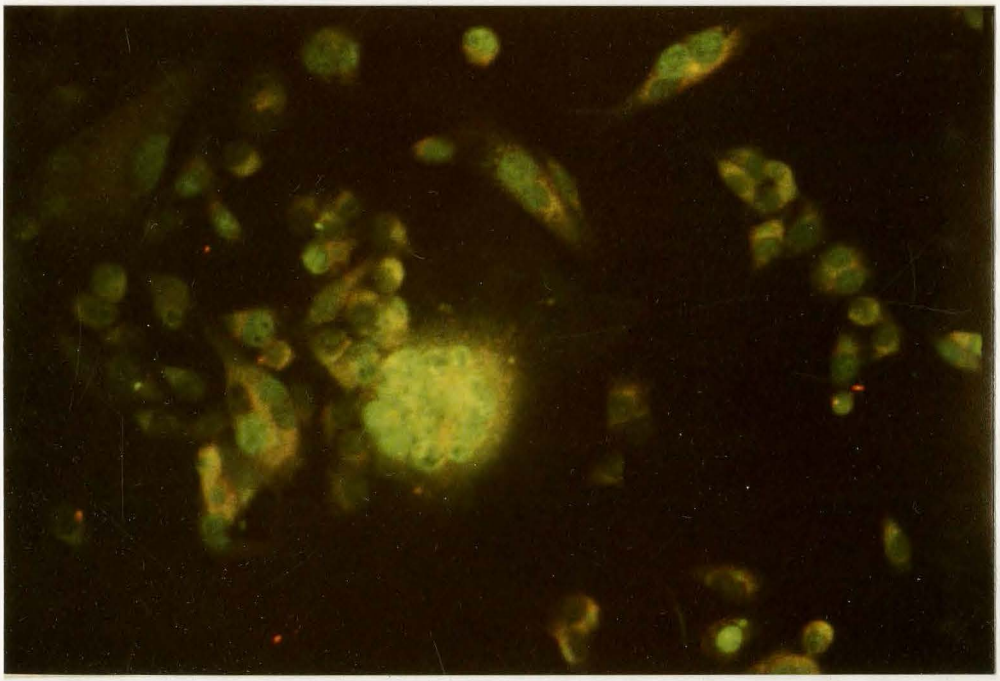
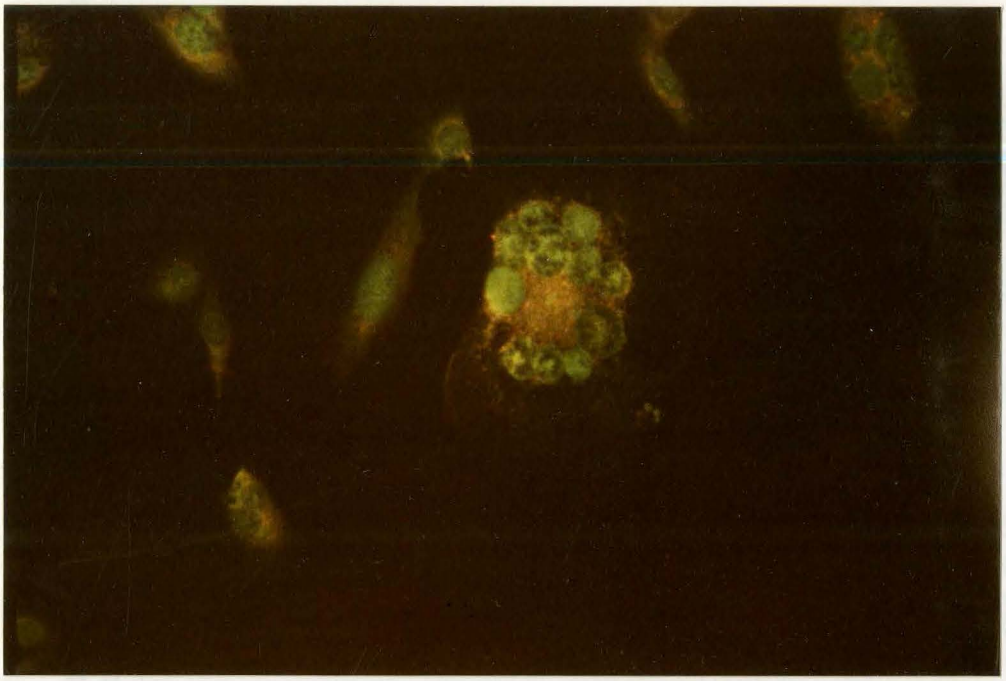


Figure 19 (Continued). L29 TC20/L29 TC20 fusion.

E. IFA with anti-THE_2 serum 14 h past fusion.

F. IFA with anti-THE_2 serum 24 h past fusion.

Initial magnification 160X.



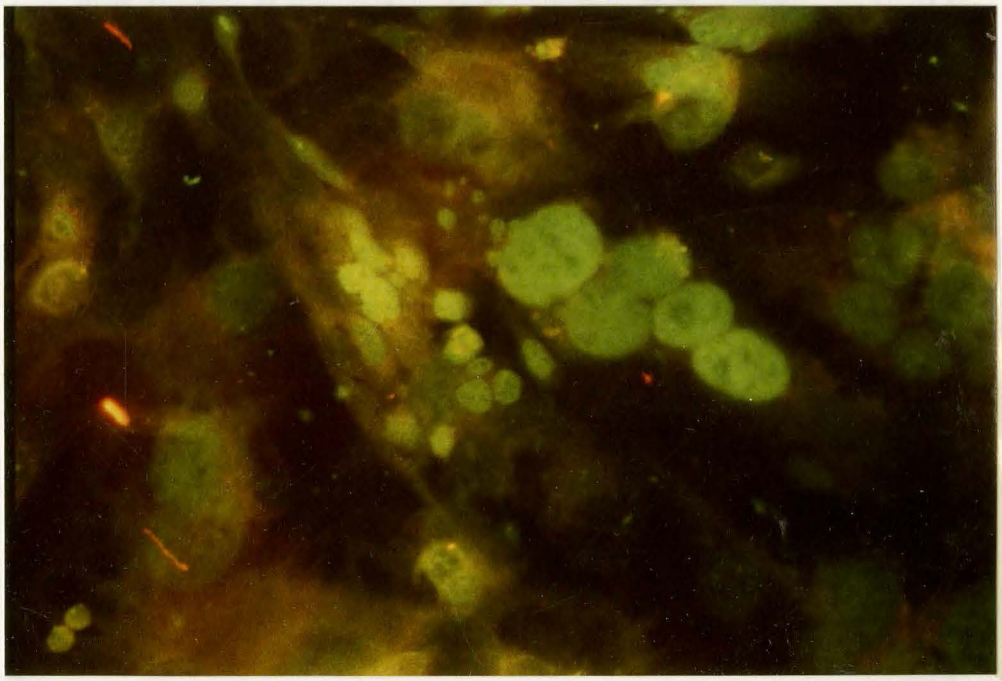


Table 8 T ANTIGEN EXPRESSION AFTER CELL FUSION

BHK-BHK

Hours Past Fusion	Cell Type Counted Single(s) or Multinucleated(m)	Number of Cells Positive[a]	Number of Cells Negative[a]
0.5	----[b]	0	1000
2	s	0	1000
	m	0	1000
5	s	0	1000
	m	0	1000
10	s	0	1000
	m	0	1000
12	s	0	1000
	m	0	1000
14	s	0	1000
	m	0	1000
24	s	0	1000
	m	0	1000
Not fused		0	1000

[a] Number of cells positive and negative for nuclear tumor antigen as determined by IFA with anti-THE_2 tumor serum. A total of 1000 cells in random fields were counted.

[b] At 0.5 h past fusion the distinction between single and multinucleated cells could not be made.

Figure 20. BHK-21/BHK-21 fusion.

IFA with anti-THE_2 serum 12 h past fusion. Initial magnification 160X.

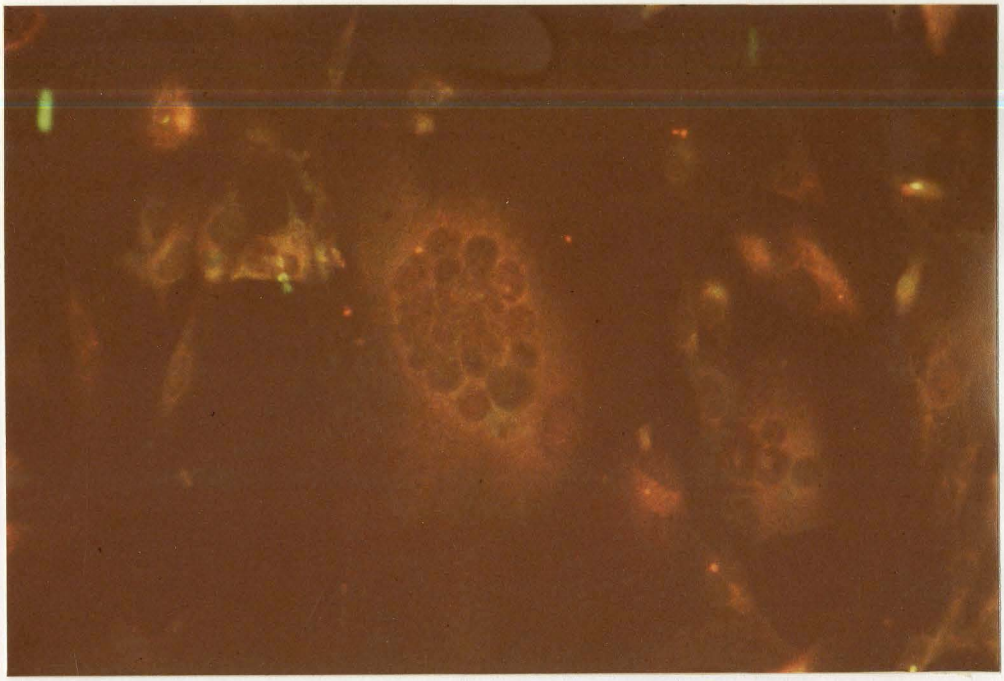
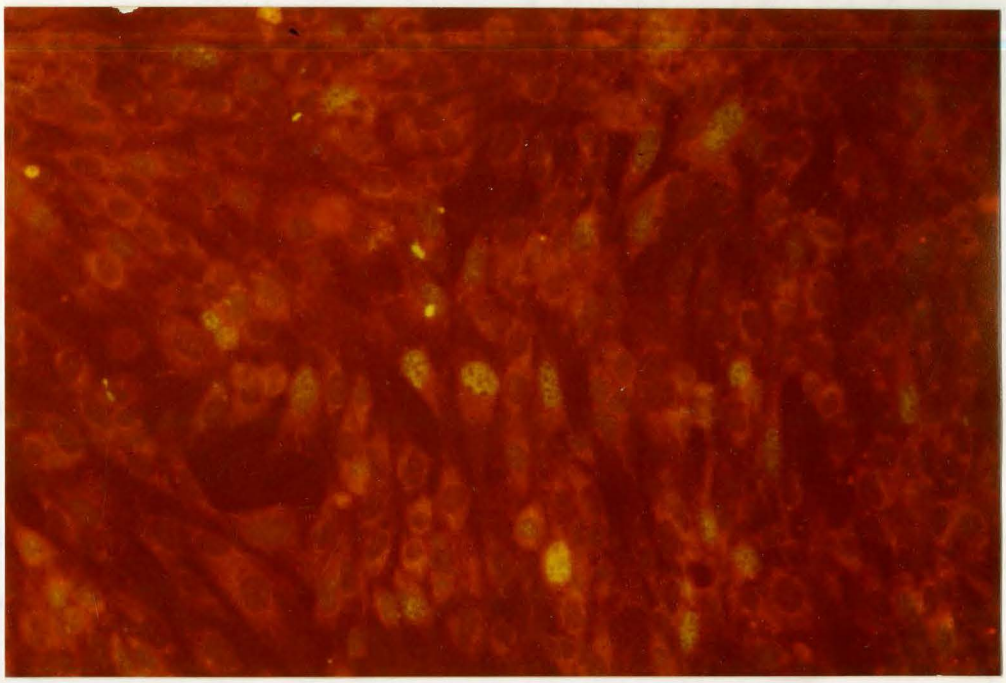


Figure 21. IFA of BK-BHK C7 TC29 with anti-THE_2 serum. Initial magnification 160C.



The cell cycle was blocked in G1 by serum starvation followed by treatement with 1 mM hydroxyurea. The cells were released from the block and at periodic times pulsed with ^{3}H-thymidine. At these same times cells were harvested and prepared for IFA as described for the cytospin. The results of this experiment are shown in Table 9. There was more than a 10 fold increase in counts per minute from 0.5 h after release to 5 h after release from the block. The counts per minute then decreased to a low at 15 h and peaked again at 18-24 h. This indicates a peak in DNA synthesis and a corresponding S phase at 5 h past release from the cell cycle block. This is repeated at 18-24 h after release. The percent nuclear T protein positive did not show a corresponding change with the onset of S phase at 5 h or with the completion of S phase and onset again at 18-24 h. A control sample of unsyncronized cells was included with each timed syncronized sample. The control samples ranged from 25.0-27.5% positive.

Factors Influencing the Percentage of T Positive Nuclei in BKV Transformed BHK 21 Cells: Subclone Analysis.

Subclones of BK-BHK C7 TC23 were isolated by plating single cells in microtiter wells. Ten of these were randomly selected for further study. The percent of nuclei positive for T protein as determined by IFA with anti-THE$_2$ serum in these 10 low passage clones is shown in Table 10. These were as follows: Two of the 10 were <0.1% positive, five subclones ranged between 0.1% positive to 5.4% positive, one subclone was 22.5% positive and two subclones were close to 100% positive.

C7SC21, 97% positive (Figure 22) and C7SC30, <0.1% positive (Figure 23) were examined to determine the relationship between passage

Table 9 CELL CYCLE AND THE EXPRESSION OF TUMOR PROTEINS

Time after Release From Block (Hours)	Counts Per Minute	Percent Nuclear Tumor Protein Positive
1/2	1537	27.0
2	11800	24.5
5	16772	25.5
8	12442	24.4
11	7016	27.0
15	5924	27.0
24	13033	25.8
Not synchronized	n.d.[a]	25.0-27.5[b]

[a]Not done.

[b]The percent nuclear tumor protein positive was determined at each time from an unsynchronized population of cells. This was from 25-27.5%.

Table 10 SUBCLONE T ANTIGEN REACTIVITY

BK-BHK C7 Subclone	Passage[a]	Percent Nuclei Positive for T Antigen[b]
C7SC1	3	2.3%
C7SC2	2	5.4%
C7SC5	3	0.4%
C7SC7	3	22.5%
C7SC11	3	< 0.1%
C7SC12	3	2.0%
C7SC21	4	97.0%
C7SC30	2	< 0.1%
C7SC38	3	92.0%
C7SC44	3	0.1%

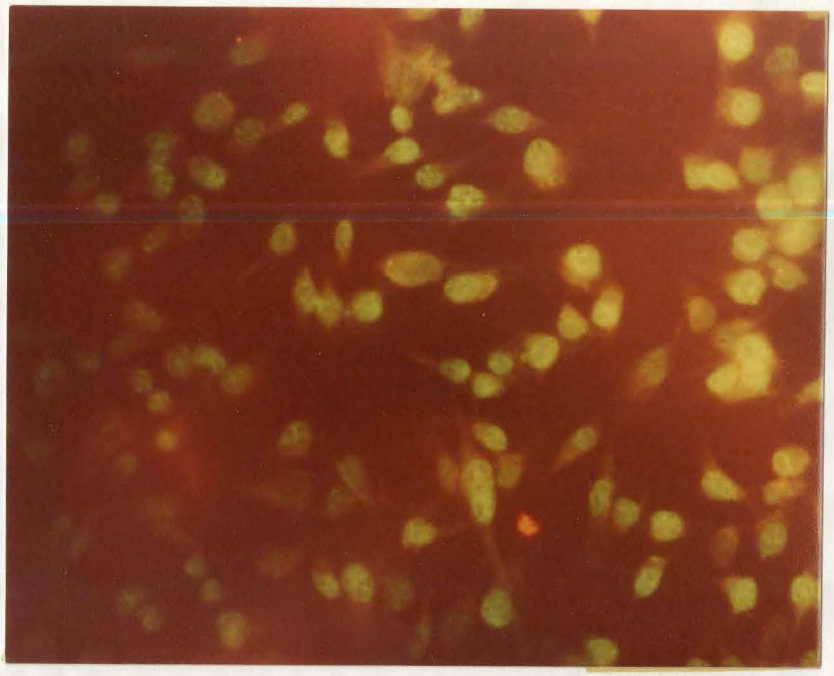
[a] Number of passages in culture past subcloning.

[b] Percent positive nuclei in 1000 total cells counted.

Figure 22. IFA of BK-BHK C7SC21 TC24 with anti-THE_2 serum.

A. Initial magnification 160X.

B. Initial magnification 400X.



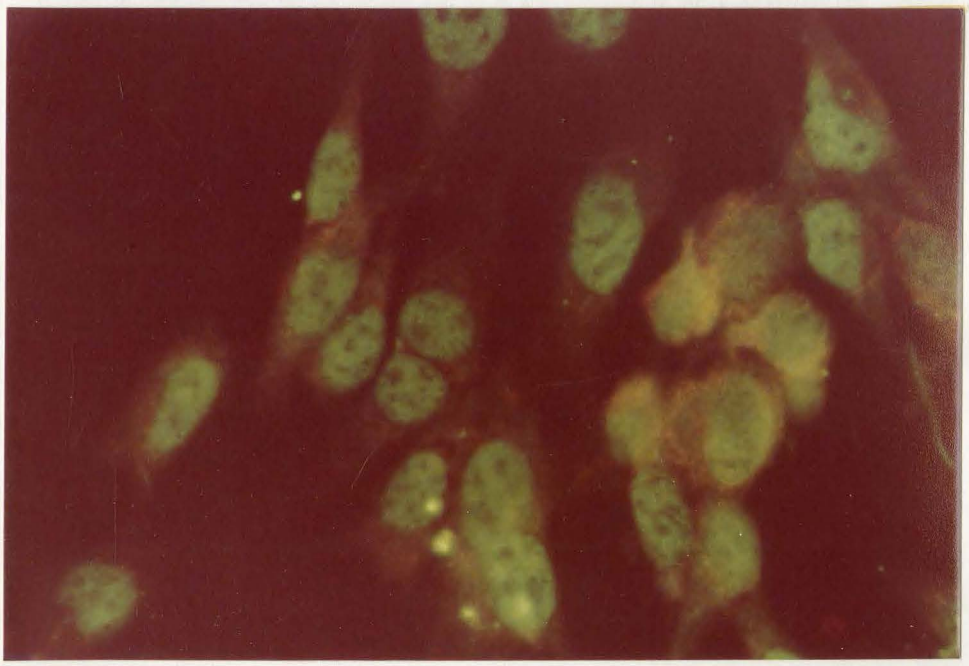
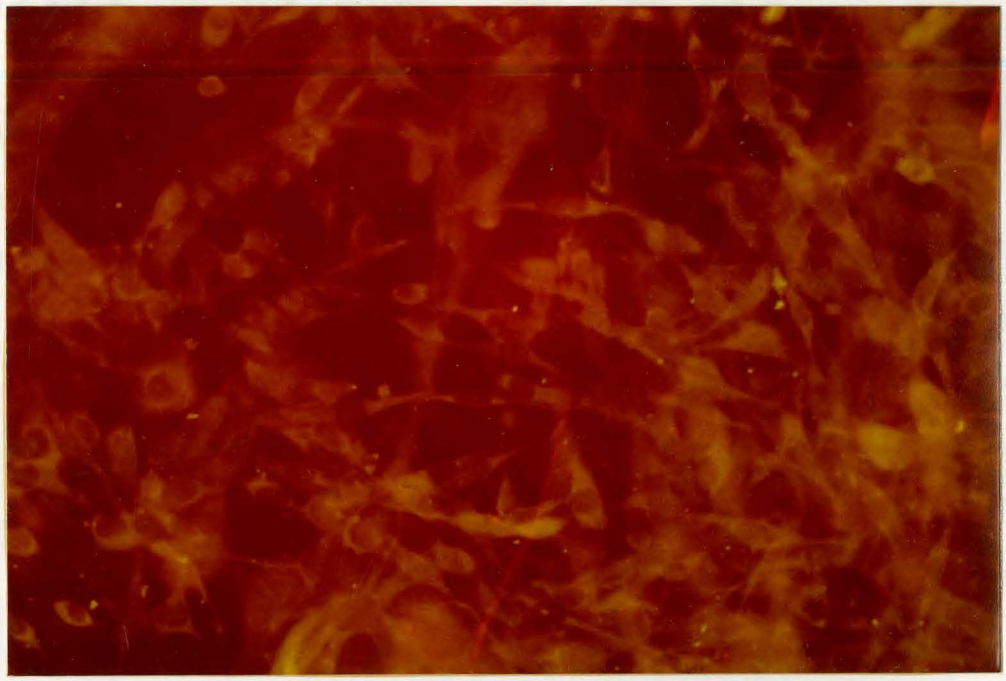


Figure 23. BK-BHK C7SC30 TC24 IFA with anti-THE_2 serum. Initial magnification 160X.



and the percent of cells positive for nuclear T protein. The results are shown in Table 11. C7SC21 remained close to 100% positive through 27 passages. C7SC30 did not remain <0.1% positive. After 14 passages 0.2% of the cells were positive.

Results of immunoprecipitation from C7SC21 TC12 and C7SC30 TC13 are shown in Figure 24. In lanes A and B are C7SC30 and C7SC21 extracts immunoprecipitated with anti-THE_2 serum. Before the addition of Protein A-Sepharose beads these extracts were first incubated with an unlabeled BHK-21 cell extract. Small t protein bands are present in both lanes at 22,000 daltons. Protein bands suggestive of large T protein are present at 94,000 daltons. In lane D is immunoprecipitation from a BHK-21 cell extract. No bands migrating in the range of small t can be seen. Faint bands of protein can be seen where the large T protein would migrate. In lanes E and F are immunoprecipitates from lysates of C7SC30 TC13 and C7SC21 TC12. These lysates were not pre-incubated with an unlabeled BHK-21 cell extract. The protein precipitation patterns were very similar whether or not an unlabeled BHK-21 cell extract was pre-incubated with the labeled extracts. The amounts of 94,000 dalton large T protein in C7SC21 TC11 and C7SC30 TC13 were compared by three successive immunoprecipitations from each lysate. By this method a difference between the amounts of large T protein in C7SC21 and C7SC30 could not be detected (data not shown). When extracted cells from subclones C7SC21 and C7 SC30 were subjected to IFA with anti-THE_2 serum fluorescence was still present.

The plating efficiency and virus rescue titer were compared for C7SC21 and C7SC30. The results are shown in Table 12. The plating ef-

Table 11 SUBCLONE CONTINUAL PASSAGE DETECTION OF TUMOR PROTEINS

BK-BHK Clone	Passage Number	Percent Positive[a]
C_7SC21	4	97.0
	6	98.0
	9	97.7
	11	99.4
	20	98.0
C_7SC30	7	< 0.1
	8	0.3
	9	0.1
	13	0.8
	14	0.2

[a] Tumor proteins were detected by IFA with anti-THE_2 tumor serum. The percent positive reflects the number of positive nuclei counted in a total of 1000 cells.

Figure 24. Polyacrylamide gel autoradiograph of ^{35}S-methionine labeled immunoprecipitated proteins from C7SC21 TC12 and C7SC30 TC13 precipitated with anti-THE_2 serum. Lane A, C7SC30 TC13 precipitated with anti-THE_2 serum. Lane B, C7SC21 TC12 precipitated with anti-THE_2 serum. Lane D, BHK-21 cell extract precipitated with anti-THE_2 serum (lane C 1/10 the sample volume of lane D). Lane E, C7 SC30 TC15 precipitated with anti-THE_2 serum. Lane F, C7SC21 TC11 precipitated with anti-THE_2 serum. The 1.0 ml lysates generating the results in lanes A and B were incubated with 2.3 ml unlabeled BHK-21 cell extracts plus 20 µl anti-THE_2 serum for 30 min before the addition of Protein A-Sepharose beads. Electrophoresis was at 12.5 mA overnight.

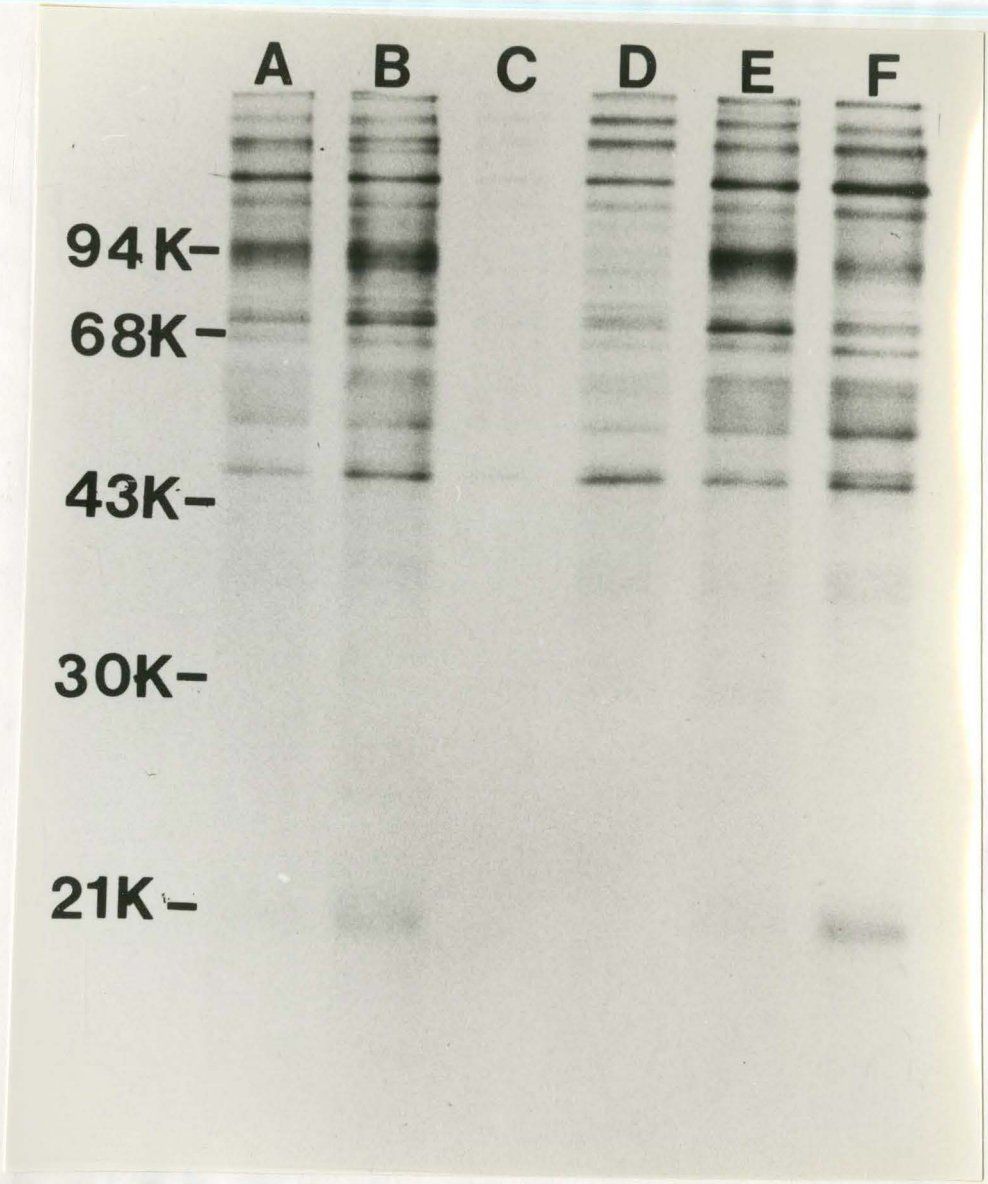

A
B
C
D
E
F
94K-
68K-
43K-
30K-
21K-

Table 12

BK-BHK C7SC30 AND BK-BHK C7SC21 PLATING EFFICIENCY AND VIRUS RESCUE

	BHK-21	BK-BHK C7SC30	BK-BHK C7SC21
Plating Efficiency in 0.33% Agar[a]	0.01%	8.0%	7.3%
Virus Rescue Titer from HEK/BK-BHK Fusion[a]	<1 virion/ml	5.2×10^2/ml	5.7×10^2/ml

[a]Plating efficiency and virus rescue titer were determined as described in Materials and Methods.

ficiencies for C7SC21 and C7SC30 were 7.3% and 8.0% respectively. The virus rescue titers for C7SC30 and C7SC21 were also compared. The rescue titers for C7SC30 and C7SC21 were also compared. The rescue titer for C7SC30 was 5.3×10^2/ml and that for C7SC21 was 5.7×10^2/ml. For these two subclones both the plating efficiencies and the virus rescue titers were very similar.

Growth curves of BHK-21 cells, the parental C7TC25 and the two subclones C7SC21 and C7SC30 were compared in DME plus 10% CS and 10% TPB. The results are shown in Figure 25. As can be seen from this figure C7SC21, 98% T positive did not increase in cell number more rapidly than C7SC30, 0.2% positive. In Figure 26 are growth curves of the same cells in DME plus 1% CS, 1% TPB. The untransformed BHK-21 cells did not increase in cell number under these low serum conditions. All 3 transformed clones did increase in cell number in these conditions. In 1.0% serum as in 10% serum C7SC21 did not exhibit a more rapid increase in cell number from 1-3 days past plating. The parental C7TC25 clone was included here for comparison.

Figure 25. Growth curve comparing growth rates of BHK-21, C7TC25, C7SC30 TC14, and C7SC21 TC13 in DME plus 10% CS, 10% TPB. BHK-21 cells, ■; C7SC30 TC14, ●; C7SC21 TC13, ▲; C7 TC25 ○.

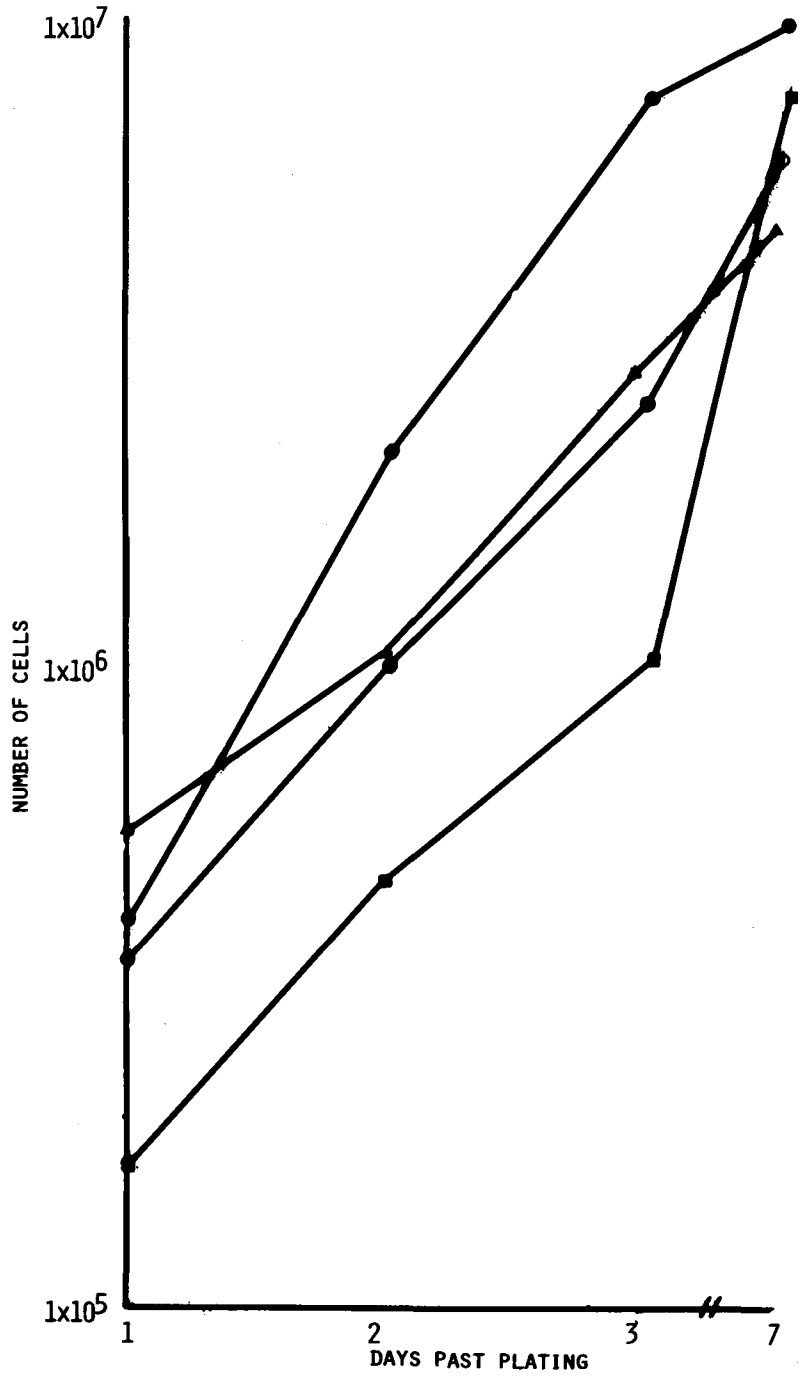
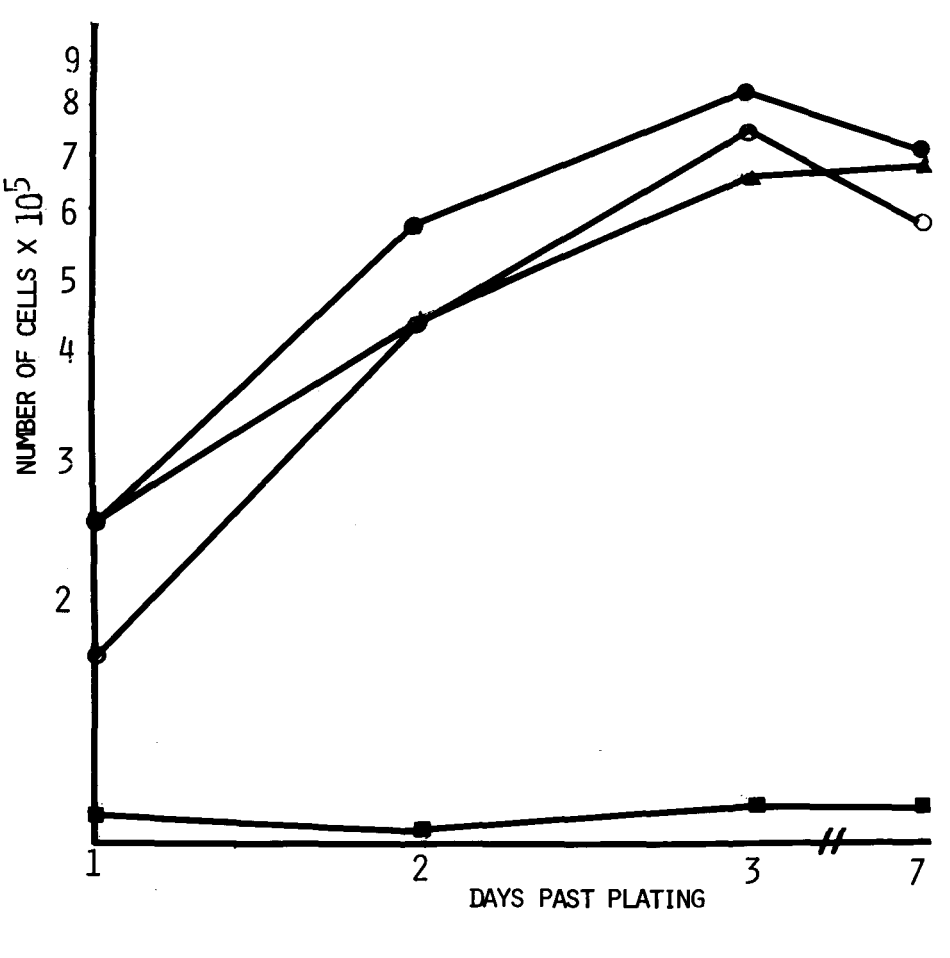

1x10^7
1x10^6
1x10^5
NUMBER OF CELLS
1
2
3
7
DAYS PAST PLATING

Figure 26. Growth curve comparing growth rates of BHK-21, C7TC25, C7SC30 TC14 and C7SC21 TC13 in DME plus 1% CS, 1% TPB. BHK-21 cells, ■; C7 TC25, ○; C7SC30 TC14, ●; C7SC21 TC13, ▲.


NUMBER OF CELLS X 10^5
9
8
7
6
5
4
3
2
1
2
3
7
DAYS PAST PLATING

CHAPTER V

DISCUSSION

This study examined the transformation of BHK-21 cells to anchorage independence by BKV. It follows an observation made in this laboratory that a clone (L29) of BKV transformed BHK-21 cells appeared nuclear T antigen negative by IFA and immunoperoxidase assays yet was capable of virus rescue (80). This observation stimulated many questions as considerable evidence indicated that the SV40 large T protein was necessary for viral DNA replication, and transformation initiation and maintenance. The current study was undertaken to examine the L29 clone and other clones of anchorage independent BKV transformed BHK-21 cells first for the presence of the BKV large and/or small T proteins, then for the parameters which may influence or be influenced by the expression of these viral proteins.

The antiserum used in these studies was made in hamsters to an SV40 transformed cell line, THE_2. For the study of cells transformed by BKV the use of an antiserum directed to BKV transformed cells would have been preferable. However, as shown in Table 4 two attempts were made to produce antisera with RF194 cells. Both times antiserum was produced which was capable of immunoprecipitating from BKV infected HEK cells a 94,000 dalton protein likely to be large T but incapable of precipitating a protein with a molecular weight consistent with a small t protein. This is illustrated in Figure 6. Although the small t protein was present in RF194 cells as detected by immunoprecipitation and

SDS polyacrylamide gel electrophoresis (data not shown) the protein could not stimulate antibody in hamsters capable of immunoprecipitating the protein from cell extracts. There are several possibilities which might account for this. One is that antibody may actually be generated by RF194 cells but be incapable of reacting by immunoprecipitation. Another is that small t protein determinants may be sequestered and therefore unable to elicit an antibory response. One more possibility is that once small t is extracted from cells it may no longer be in a conformation recognizable by the antibody.

When anti-THE_2 serum was used to immunoprecipitate from BKV infected HEK cells prominent bands migrating at 94,000 daltons and 22,000 daltons in SDS polyacrylamide gels were precipitated with anti-THE_2 serum and not normal serum. This is shown in Figure 7. They were also not precipitated from uninfected cells (data not shown). In addition, protein bands at 94,000 daltons and 22,000 daltons were precipitated from BKV infected marmoset brain cells with anti-THE_2 serum and not normal serum. An additional 18,000 dalton band was precipitated. The nature of these two bands migrating in the region of small t is not known. The smaller protein may be a proteolytic product of the 22,000 dalton protein or the result of integration which allows only a truncated form of small to to be made.

These results established that the anti-THE_2 serum was a reagent capable of specifically precipitating proteins migrating at 94,000 daltons and 22,000 daltons from 2 different lytically infected cell types. Along with the characteristic molecular weights in 2 cell types there is sub-

stantial evidence presented throughout this work which further identifies these proteins. Summarized here, this includes the co-migration of these proteins in SDS polyacrylamide gels with SV40 large and small T proteins from SV80 cells. It includes the characteristic DNA-cellulose binding capability of the 94,000 dalton protein at pH 6.0 and elution above pH 8.0. This also includes the lack of an anti-THE_2 precipitated 22,000 dalton protein from BHK-21 cells transformed by BKV-MM.

After the establishment of the apparent molecular weight of BKV large and small T proteins in this SDS-polyacrylamide gel system BK-BHK clone L29 was examined for T proteins by immunoprecipitation with anti-THE_2 serum. Many results of immunoprecipitation from BHK-21 cells were uninterpretable because of non-specific precipitation. The autoradiograph in Figure 10 of immunoprecipitation from clone L29 is an example of this. When immunoprecipitating from BK transformed hamster cells with an antiserum made in hamsters against hamster cells the presence of many non-viral proteins in a precipitate would be expected. The presence of many proteins precipitated by normal hamster serum in Figure 10 however, indicates non-specific precipitation. This is a problem specific to BHK-21 cells and has been reported by others. Hutchinson et al. (53) studied polyoma viral proteins in transformed BHK-21 cells. After immunoprecipitating from these cells with normal serum and anti-tumor serum followed by SDS polyacrylamide gel electrophoresis and autoradiography they found sections of their gels uninterpretable because of extensive precipitation with normal serum. The problem of non-specific immunoprecipitation from transformed BHK-21 cells has also been experienced with the use of monoclonal antibody directed to viral protein

instead of anti-tumor serum (K. Rundell personal communication). Although the non-specific precipitation remained a recurrent problem throughout this work some minor changes in the immunoprecipitation method yielded auroradiographs with interpretable results.

In Figure 11 is an autoradiograph of SDS polyacrylamide gel electrophoresis of immunoprecipitates from BK-BHK L29 TC16. This is a passage where <0.1% of the cells appear nuclear T positive by IFA with anti-THE_2. In this figure bands of protein are present at 94,000 daltons and 22,000 daltons precipitated by anti-THE_2 and not normal serum. This suggests that even though this L29 clone is <0.1% nuclear T positive by IFA that small amounts of large T and small t proteins are produced. This may reflect low levels of synthesis in all or most cells or it may reflect high levels of synthesis in rare cells.

The continual synthesis of BKV large T protein in all cells of transformed BHK-21 clones should not be a requirement for continued growth in culture. Prior to the transformation event BHK-21 cells already exhibit some characteristics of papovavirus transformed cells. They have an infinite lifespan, they do not contact inhibit well and on rare occasions have been reported to form tumors in hamsters. BKV transformed BHK-21 cells however can be selected by anchorage independent growth. When replated in soft agar suspension they exhibit increased plating efficiency over the untransformed BHK-21 cells as shown in Table 2. It is possible that the transformation initiation event itself and growth in suspension require uniform T/t synthesis but that routine growth in tissue culture media does not.

The contribution of BKV small t protein to transformation and

anchorage independent selection was studied with BKV-MM. This mutant carries a deletion which prevents the formation of small t mRNA. Thus, small t protein is not made in cells transformed with this virus as shown in Figure 16. When BKV-MM and wild type BKV were used to transform BHK-21 cells it was determined that they transformed these cells with similar efficiencies (Table 5). These results are in agreement with studies with SV40 small t mutants. These studies found that the lack of small t may or may not influence the transformation efficiency depending on the cell type, the growth state of the cell and the method of selection. In a study by Frisque et al. (34) they examined two cell types, rat embryo fibroblasts (REF) and hamster embryo fibroblasts (HEF). They found that early passage, rapidly growing REF cells transformed by the .54-.59 small t deletion mutant grew in suspension with the same efficiency as wild type virus. When senescent REF cells were used however, the efficiency of growth in suspension for the .54-.59 mutant was significantly less than wild type SV40. When HEF cells transformed by the .54-.59 mutant were examined growth in suspension was not influenced. For both REF and HEF cells transformed with tsA mutants cloning efficiency in suspension was considerably less at the restrictive temperature indicating a dependence on large T for growth in suspension.

In the current study rapidly growing BHK-21 cells were harvested and used for the transformation. The transformation efficiency and growth in agar suspension was not affected by the lack of small t protein. Since there is no BKV large T mutant available the contribution of large T can only be inferred by comparison with SV40. A further study should include a comparison of transformation frequencies of BKV-MM in

rapidly growing and non-dividing BHK-21 cells. A non-dividing population of BHK-21 cells could be obtained by utilizing BHK-21 cells plated in 1% CS (see Figure 26).

Although the BKV large T protein should not be essential for continued growth of BKV transformed BHK-21 cells in routine culture, if evidence accumulated for SV40 can be applied to BKV then the large T protein should be necessary for virus rescue. Virus rescue upon fusion with permissive cells is a very inefficient process. Among approximately 3×10^6 cells exposed to PEG-DMSO the virus rescue titer for L29 cells is 5×10^3 virions in a total of 5.0 ml of lysate. Theoretically, if a virus producing heterokaryon produces 100 virions then one cell in every 6×10^3 could be responsible for the virus rescue. The rare T positive cell seen in clone L29 in early passages (before TC20) could be the cell responsible for virus rescue upon fusion. Alternatively, since large T is necessary but not sufficient for viral DNA replication the fusion process may greatly enhance the production of T protein independent of virus production. To examine this possibility BK-BHK L29 cells were fused with permissive HEK cells and at intervals after fusion cells were examined for the production of nuclear T protein. T protein was detected by IFA with anti-THE_2 serum. After fusion there was an increase in the proportion of T positive cells which peaked at 24 hours past fusion. Most T positive cells were multinucleated. However, at 24 h past fusion there was an apparent increase in positive cells with single nuclei. Nuclear fusion is probably responsible for this increase. By 48 h past fusion the negative single cells overgrow the positive cells, decreasing

the relative number of positive to negative cells. This is illustrated in Figure 18D.

Further results shown in Table 7 indicated that fusion with permissive cells was at least not the only factor responsible for the increase in T positive polynucleated cells seen after fusion. Fusion with the permissive cell type is however, necessary for infectious virus production. When L29 cells were fused without permissive cells an increase in the relative number of positive cells was seen. This began at 10 h past fusion. Figure 19 demonstrates that in a polynucleated cell there can be both positive and negative nuclei. This increase in T positive cells after fusion is a finding not shared with the un-transformed parental BHK-21 cells as shown in Table 8.

Although the permissive cells may contribute to the increase in T positive cells upon fusion with transformed cells they are certainly not the only factor. The fusion process itself is also responsible for inducing the production or the increased production of virally induced or virally coded for protein reactive with the anti-THE_2 serum. Since the antiserum used was raised against transformed cells there is a possibility that the increased reactivity after fusion is due to a cellular and not a viral protein. To resolve this question 4 clones of hybridoma cells secreting antibody to SV40 large T protein were obtained. Monoclonal antibodies to BKV T proteins have not been made. It was unexpected and unfortunate that these antibodies did not cross-react with BKV large T protein. However, even if the monoclonal antibody had cross reacted there is a possibility that the particular specificity may not react with the subclass of BKV T which may be produced after

fusion.

It is not clear what factors are responsible for the results obtained when the L29 clone is exposed to DMSO. It is possible that upon fusion certain factors which allow the expression of previously silent genes are transferred from one cell to another. Perhaps the mRNA for T protein can be transcribed in one cell but not translated and can be translated in the second cell but not transcribed. The defects would compliment each other upon fusion. The finding of both positive and negative nuclei in the same polynucleated cell would support this hypothesis (see Figure 19).

It is also possible that DMSO treatment may be activating previously silent gene expression. It has been reported that DMSO is capable of inducing cellular differentiation (50,126). When cultured Friend leukemia cells are treated with 2% DMSO they begin to accumulate globin specific mRNA and synthesize hemoglobin (50). Since DMSO is capable of changing transcriptional activity in cells it is feasible that it could activate silent viral genes in BKV transformed BHK-21 cells. Experiments studying the separate and collective effects of PEG and DMSO on transcriptional and translational activity in BKV transformed BHK-21 cells should follow.

In Figures 1-5 are examples of detection of T protein by IFA with anti-THE_2 serum. The striking difference between the SV40 transformed human cells in Figure 1 and the BKV transformed BHK-21 cells in Figures 2-5 is that the SV80 cells are uniformly positive and the BKV transformed BHK-21 cells are not. Mouse, rabbit, and monkey cells transformed by BKV were approximately 100% positive for T protein as reported by

Portolani et al. (87). In some clones (Figures 2 and 3) rather than clearly positive and negative nuclei what exists are positive and negative nuclei and varying levels of reactivity in between. In other clones cells are clearly positive or negative (Figure 21).

The percentage of T antigen positive nuclei increased with the cell passage as shown in Table 3. Because of the stimulatory effect of T protein on DNA synthesis the rare positive cells seen at early passages may have a slight growth advantage over the negative cells. The percentage of positive cells would therefore increase with passage. Another explanation for the increased percentage of T protein positive cells with passage involves the inherent heterogeneity of BHK-21 cells. Ehlke (29) studied the karyotype of BHK-21 cells used in this laboratory and found the chromosome number and arrangement to be highly variable. An early study by Fraser et al. (33) followed the accumulation of polyoma virion antigen synthesis in BHK-21 cells by IFA. Their results suggested that cellular heterogeneity, perhaps due to spontaneous mutation, was responsible for the semi-permissiveness of BHK-21 cells.
Folk et al. (32) repeated similar experiments with the use of a polyoma DNA probe and *in situ* hybridization. When 40 pfu/cell of polyoma virus was used to infect BHK-21 cells isolated foci of DNA replication were observed. This indicated that the semi-permissiveness of BHK-21 cells for polyoma virus replication is due to cellular heterogeneity. Previous studies had shown that viruses that replicate in BHK-21 cells do not have detectable genomic alterations (145). In the current study changes in chromosome arrangement and number with increasing generations past the original single cell progenitor may activate silent

genes. Karyotype studies comparing early passage transformants with late passage transformants should be done to examine chromosomal differences.

In one clone (C4) the increase in T positive cells was not gradual. At passage 15 it increased 10 fold and decreased 10 fold by passage 16. It is not clear what may have caused this. One factor which was not controlled for was the effect of storage in liquid nitrogen and then thawing, growing and assaying the cells. Although every effort was made to assay cells at the same state of semi-confluency occasionally there were slight variations in the time of sampling. The T protein synthesis may respond to certain levels of growth hormones present in the growth media at different times. Clone C7 however, was assayed 2, 3, and 4 days past plating and the number of days in culture for this clone had no effect on the percentage of T positive cells in the culture.

Factors which could possibly influence the expression of T protein were further examined with Clone C7. Since the expression of T protein and transport into the nucleus could be cell cycle dependent synchornized populations of cells from clone C7TC29 were examined for T protein. Considering the role of SV40 T protein in the stimulation of cellular DNA synthesis it is possible that T protein might accumulate in the nuclei of a large percentage of cells during S phase. Results presented here however, did not indicate this. This suggets that BKV large T protein may not be involved in the stimulation of BHK-21 cellular DNA synthesis of BKV-transformed cells under the conditions of this experiment.

Another factor which may influence the expression of T protein is the heterogeneity of BHK-21 cells. Subclones of C7TC23 were isolated to determine if the expression of T protein in clone C7 is clonal in origin. In other words, could completely positive or negative subclones be isolated. Previous evidence indicated that there is some clonal influence. This evidence consists of the fact that often clusters of positive cells would be seen in assays for T protein by IFA with anti-THE_2 serum (see Figure 2). Single positive cells however, could also be seen (Figure 21). Out of 10 randomly chosen subclones 2 were almost totally positive, 2 were almost totally negative and 6 were mixtures of positive and negative cells similar to the parental line. These results, especially the isolation of positive or negative subclones indicate clonal influence in the expression of T protein in BKV transformed BHK-21 cells. The 6 subclones containing mixtures of positive and negative cells may have been exclusively positive or negative early in the subcloning and changed by the time sufficient numbers of cells were present to assay.

Two subclones, one negative (SC30) and one positive (SC21) were chosen for further study. When they were subjected to continual passage SC30 became increasingly positive and SC21 remained >96% positive. The isolation of a line of BKV transformed BHK-21 cells almost completely T protein positive is unique and indicates the isolation of an unusually stable population of cells. The isolation of a subclone which is <0.1% T positive and which becomes increasingly positive with passage supports the theory that in most cases the heterogeneity of BHK-21 cells is related to or responsible for the expression of T proteins.

The isolation of subclones which were either >97% T positive or <0.1% T positive provided useful tools for studying the effect of nuclear T protein, as detected by IFA with anti-THE_2, on the phenotype of these transformed cells. The fact that both subclones are capable of similar virus rescue titers is not surprising. Results presented here with clone L29 indicate that the fusion process itself is capable of inducing the production of T protein. In addition, while T protein is necessary for virus DNA replication it is not sufficient.

There are several possible explanations for why the percent of T positive cells did not influence the cloning efficiency in suspension or the growth rate in 10% and 1% serum. One possibility that cannot be overlooked although unlikely, is that the protein(s) responsible for the reactivity in C7SC21 could be viral induced cellular protein(s) reactive with anti-THE_2 serum. Immunoprecipitation from both subclones could not demonstrate an increased amount of large T protein or any precipitable protein in C7SC21. However, after IFA of NP-40 extracted monolayers of SV80 and C7SC21 cells fluorescence remained, indicating that protein(s) reactive with anti-THE_2 serum were still present. Therefore, under these extraction conditions IFA and immunoprecipitation results could not be compared on a quantitative basis, and immunoprecipitation could not confirm the viral origin of the protein(s) responsible for the IFA results. Had the monoclonal antibodies to SV40 cross-reacted with BKV T proteins a definitive answer may have been obtained. A more likely explanation for the subclone plating efficiency and growth curve results is that the low levels of T protein present in C7SC30 cells may be sufficient to account for the plating efficiency in suspension and the growth rate in 10% and es-

pecially 1% serum. The fact that BHK-21 cells already exhibit some aspects of transformed cells may be related to these results.

In conclusion results have been presented which demonstrate the presence of BKV large 94,000 dalton T protein and small 22,000 dalton t protein in cells lytically infected and transformed by this virus. The large T protein from both lytically infected and transformed cells is capable of characteristic binding to a column of DNA cellulose.

The factors which control the unique pattern of large T protein expression in these BKV transformed BHK-21 cells were examined. Results suggested that rather than a cyclic turning on and off of nuclear large T protein expression a clonal influence possibly related to the inherent heterogeneity of BHK-21 cells was responsible.

Results indicated the transformation efficiency and subsequent growth in suspension to be independent of small t protein for these BKV transformed BHK-21 cells. Perhaps the state of growth is as important in BKV transformation of BHK-21 cells as in transformation by SV40.

This work has generated several interesting questions yet to be answered. One is the elucidation of the molecular basis for the expression of T protein after exposure to PEG-DMSO; whether this expression is the result of DMSO treatement or fusion with other transformed cells. Another is the examination of chromosomal differences between early passage and late passage transformants and how these differences might relate to the increased proportion of T positive cells in later passage clones. Thirdly, the effect of the BKV-MM mutation on the transformation of a non-dividing population of BHK-21 cells as compared to a dividing

population has yet to be determined.

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The dissertation is therefore accepted in partial fulfillment of the requirements for the degree of Doctor of Philosophy.

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