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Selection of Somatic Cell Hybrids between Baby Hamster Kidney Cells and Human Embryonic Kidney Cells to Study BK Virus Early Gene Expression

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SELECTION OF SOMATIC CELL HYBRIDS BETWEEN BABY HAMSTER KIDNEY CELLS AND HUMAN EMBRYONIC KIDNEY CELLS TO STUDY BK VIRUS EARLY GENE EXPRESSION

by

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VITA

The author, Sara Lynn Ehlke, is the daughter of Edward O. Ehlke and Norma (Lidicker) Ehlke. She was born on October 19, 1955 in Oconomowoc, Wisconsin.

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INTRODUCTION

BK virus (BKV) is a member of the papovavirus family. This group of viruses is capable of the in vitro transformation of cells and possesses oncogenic activity in animals (Howley, 1980). The family consists of two genera, the papillomaviruses and the polyomaviruses, which physically differ primarily in particle size and amount of nucleic acid in their genomes (Fareed and Davoli, 1977; Howley, 1980). The Shope rabbit papilloma virus and the human wart virus belong to the papilloma subgroup. These viruses are capable of inducing benign tumors in their respective hosts (Howley, 1980). The polyoma subgroup includes the rodent polyoma virus, Simian virus 40 (SV40), and the human papovaviruses JCV and BKV. The rodent polyoma virus was first isolated by Gross in 1953 from inbred strains of mice (Gross, 1953) and has the ability to induce a variety of tumors in mice and hamsters (Stewart et al., 1957). SV40 was first isolated in 1960 by Sweet and Hilliman from rhesus monkey kidney cultures being used to propagate poliovirus for vaccine production (Sweet and Hilliman, 1960; Eddy et al., 1962). SV40 has the ability to malignantly transform human, rat, mouse, and hamster cells in vitro (Koprowski et al., 1962; Black and Rowe, 1963; Butel et al., 1972). JCV and BKV, the human papovaviruses, were isolated from immunosuppressed hosts in 1971 (Gardner et al., 1971; Padgett et al., 1971). JCV was first isolated by Padgett et al. from the brain of a patient with progressive multifocal leukoencephalopathy (PML). JCV is capable of inducing tumors in hamsters and owl monkeys

(Padgett et al., 1971; London et al., 1978). BKV was first isolated by Gardner et al. from urine of a renal transplant recipient. Although the significance of BKV in human pathology has not been established, it has been shown that the virus can lytically infect permissive cells such as human embryonic kidney (HEK), muscle, brain, and lung; a human lung fibroblast cell line (WI38); and African green monkey kidney cells (BSC-1) in tissue culture (Major and diMayorca, 1973; Seehafer et al., 1978). Infection of these cells results in the production of infectious virus particles. BKV has also been found to cause the in vitro malignant transformation of certain nonpermissive cells such as baby hamster kidney (BHK), primate, rabbit, and mouse cells (Major and diMayorca, 1973; Portoloni et al., 1975; Mason and Takemoto, 1977; Bradley and Dougherty, 1978; Portoloni et al., 1978). Human cells may become abortively transformed if the viral lytic genes are repressed (Portoloni et al., 1978; Manaker et al., 1979).

BKV is an icosahedral shaped virus with cubic symmetry and a nonenveloped, protein capsid composed of 72 skewed capsomers (Fareed and Davoli, 1977; Howley, 1980). The virus has a diameter of 40.5 to 44 nm. It is composed of 88% protein and 12% DNA. The DNA has a molecular weight of 3.3×10^6 daltons, contains 5196 base pairs, and is a circular, duplex, covalently closed, superhelical molecule (Howley, 1980). The virus is capable of coding for only 5 or 6 proteins because of the small genome size. Host cell functions must therefore be utilized for viral replication and transcription.

The BKV genome is divided into 100 map units for reference purposes. The unique restriction endonuclease Eco R1 cleavage site is

designated 0 map units (Yang and Wu, 1978). The region extending from .74 to .14 map units is transcribed clockwise from the L strand into RNA late in infection, following DNA replication (Howley, 1980). This region codes for the major structural protein VP1 and the minor structural proteins VP2 and VP3, which form the virion capsid (Howley, 1980). The region extending from .67 to .17 map units is transcribed counterclockwise from the opposite or E strand into RNA early in infection, prior to viral DNA synthesis (Howley, 1980). These BKV early genes encode two proteins having molecular weights of 94,000 and 22,000 daltons (Rundell et al., 1977; Olive et al., 1980) by producing spliced messenger RNAs (mRNA) (Manaker et al., 1979). The synthesis of these nonstructural, viral proteins, the large and small tumor or T proteins, occurs in permissive cells early in lytic infection and also in nonpermissive cells which usually become malignantly transformed (Major et al., 1977). The functions of the large T protein in transformation and the necessity of its continuous synthesis to initiate and maintain transformed cells is uncertain. Initiation and maintenance of transformation, stimulation of cellular DNA synthesis, and initiation of viral DNA replication as a DNA binding protein are the primary functions associated with the large T protein (Butel et al., 1974; Carroll et al., 1974; Osborn and Weber, 1974; Jessel et al., 1975; Kimura and Itagaki, 1975; Reed et al., 1975; Spillman et al., 1975; Frisque et al., 1979; Griffin et al., 1979; Martin et al., 1979; Tjian et al., 1979). The functions and importance of the small T protein in transformation are not certain. It may be essential for transformation maintenance, but may not be capable of establishing cells as transformed (Bouck

et al., 1978; Sleigh et al., 1978; Frisque et al., 1979).

The expression and functions of the early gene products are being studied in order to establish the relationship between BKV and nonpermissive hosts resulting in malignant transformation. Experiments involving the use of viral and host cell mutants have dominated the field, but to date have not clearly defined the exact role of these viruses in transformation. The SV40 system has been most extensively studied. Temperature sensitive (ts) mutants, failing to function in transformation or infection at high temperatures, deletion (dl) mutants, missing specifically defined viral DNA regions, and host range (hr) mutants, able to grow only on specific host cells, were utilized in attempts to define T protein functions.

An SV40 dl mutant lacking a portion of the early region "A gene" was used to show that the large T protein was specified by the SV40 A gene (Rundell et al., 1977). Stable transformation of cells and maintenance of growth characteristics of some transformed cell lines were shown to be dependent on A gene function (Brugge and Butel, 1975; Kimura and Itagaki, 1975). Specific SV40 ts mutants were incapable of stably transforming host cells during incubation at the restrictive temperature (Tegtmeyer and Rundell, 1977). Results were less consistent when cells transformed by ts A mutants at the permissive temperature were shifted to the restrictive temperature to determine if continuous function of the A gene was required to maintain the transformed state. In some transformation events, A gene function seemed necessary for initiation but not maintenance of transformation. A gene expression in SV40 mutants influenced the behavior of cells long after stable

transformation, and many new growth properties characteristic of transformed cells were affected by a shift to restrictive temperatures. Specific deletions in the SV40 early gene region resulted in mutants incapable of initiating transformation although the large T protein was expressed (Sleigh et al., 1978). The use of SV40 ts mutants demonstrated that the large T protein could directly or indirectly regulate host cell DNA synthesis in SV40 transformed cells (Butel and Soule, 1978). Deletions between .54 and .59 map units resulted in normal production of the large T protein and alterations in or the absence of the small T protein (Crawford et al., 1978). Mutants with deletions between .50 and .54 map units transformed cells with 1/100 the efficiency of wild type SV40 (Bouck et al., 1978). Actively growing Chinese hamster lung (CHL) cells were transformed by a mutant with a deletion between .54 and .59 map units as effectively as by wild type SV40. Resting CHL cells were transformed at a much lower frequency, indicating that the physiological state of the host cell was also important in the transformation process (Martin et al., 1979).

As evident from the conflicting genetic data, the use of SV40 mutants has not clearly established the functions of the T proteins. Functions seem to depend in part on the properties and physiological state of the host cell. Host cell contributions must be determined for the above results to be explained in order to define the role of the T proteins in transformation.

Another approach to this study is the development of a host cell system which regulates the expression of the early genes, allowing determination of the functions of the gene products along with factors

affecting their synthesis. Cell hybrids between permissive and nonpermissive cells in which one species of chromosomes usually predominates have been developed (Ozer and Jha, 1977; Barrett et al., 1978). In hybrids between human and rodent cells, for example, preferential loss of human chromosomes has been demonstrated (Weiss, 1970; Croce and Koprowski, 1974; Ozer and Jha, 1977; Croce, 1980). The rate of chromosome loss may not be constant throughout hybrid cell propagation. Also, the mechanism of chromosome loss has not yet been elucidated. The rapid elimination of one species of chromosomes has allowed localization of genes on definite chromosomes and the study of specific gene functions (Ozer and Jha, 1977).

The most extensively studied cell hybrid systems in vitro involving gene expression in virally transformed cells have utilized SV40 and polyoma (Ozer and Jha, 1977). The dominance or recessiveness of the transformed phenotype has been determined by constructing cell hybrids between virus transformed and nonpermissive or nontransformed permissive host cells (Weiss, 1970; Basilico and Wang, 1971; Marin, 1971; Croce et al., 1973; Wiblin and Macpherson, 1973). Somatic cell hybrids between animal papovavirus transformed cells such as SV40 transformed human cells and nontransformed cells such as mouse cells have been found to maintain the transformed phenotype and to express the large T protein (Marin, 1971; Croce et al., 1974, 1975; McDougall, 1975). Expression of the large T protein and rescuability of the SV40 genome has been shown to correlate with the presence of human chromosome number 7, which was necessary for the expression of the transformed phenotype (Croce et al., 1974, 1975, 1976). It has been shown that only ribosomal RNA

(rRNA) genes of the dominant species were expressed in hybrids between rodent and human cells, although rRNA genes of both species were present (Soprano et al., 1979). SV40 infection was capable of inducing the expression of the silent rRNA genes, and results have indicated that this reactivation required the presence of the large T protein (Soprano et al., 1980, Soprano et al., 1981).

Another set of experiments attempted to define primate factors that influenced cell permissiveness to viral infection (Garver et al., 1980). Cell hybrids between cercopithecoid monkey and Chinese hamster cells differing in primate chromosome content also differed in their susceptibility to SV40 viral replication. The presence of rhesus monkey chromosome 11 or African green monkey chromosome 12 correlated with elevated SV40 viral replication. The presence of either chromosome in hybrid cells allowed SV40 virus rescue from transformed rodent cells.

Investigations have shown that infection of most murine cells with SV40 leads to the production of early gene products. The large and small T proteins are not produced by murine embryonal carcinoma cells, the stem cells of teratocarcinomas, when infected with SV40. The nature of host cell range restriction to SV40 early gene expression in embryonal carcinoma cells has been studied by constructing hybrids between SV40 transformed cells expressing T protein and embryonal carcinoma cells (Balint et al., 1980). All clones expressed the large T protein. Host range, therefore, was shown to be a recessive property. Embryonal carcinoma cells appeared to lack cellular functions required for SV40 early gene expression.

This laboratory has observed that in BKV transformed BHK cells,

clones can be isolated in which only the small T protein can be detected. The entire BKV genome is present as demonstrated by virus rescue. It is possible that BHK cells inhibit the synthesis of the large T protein. Both T proteins are produced during BKV infection of HEK cells, indicating that synthesis of the T proteins is not inhibited in these cells. The main objective of this work has been the development of hybrid cells which could be used to test whether the expression of BKV early genes was under host control. Through the use of this somatic cell hybridization system, hybrids between BHK and HEK cells could be constructed, infected with BKV, and tested for their susceptibility to either lytic or transforming infection.

In order to accomplish the stated objectives, a selection system that would eliminate all parental cells had to be designed because the efficiency of fusion of BHK and HEK cells is only 20 to 30%. This was estimated by light microscopic examination to determine the percentage of multi-nucleated cells. By use of this selection system, somatic cell hybrids could be cloned and propagated. The following narrative briefly describes the development of this selection system, based on properties of the parental cells.

HEK cells are highly susceptible to BKV lytic infection during which both the large and small T proteins are produced. For this reason, HEK cells were selected as one parental cell type for the fusion studies. HEK cells can only be passaged five times after primary culture establishment and fail to clone at low density. Therefore, drug selection markers could not be induced in these cells. HEK cells maintain a normal phenotype in culture, are anchorage dependent for growth,

and are able to propagate in Dulbecco's Modified Eagle Medium (DME) supplemented with 10^{-5}M hypoxanthine, 10^{-6}M aminopterin, and 10^{-5}M thymidine (HAT) (Ozer and Jha, 1977) because of the presence of a functional thymidine kinase enzyme (TK+). However, these cells are sensitive to ouabain even at concentrations as low as 10^{-7}M (Kucherlapati et al., 1975). The use of ouabain at 10^{-5}M along with continual passage in culture allowed for selection against HEK cells.

BHK cells were selected as the other parental cell type because they are capable of being transformed by BKV. BKV transformed BHK cells are characterized by anchorage independent growth in agar suspension and can be selected on the basis of this phenotypic trait. BHK-B1 cells are a thymidine kinase deficient (TK-) cell line established by continual passage in 5-bromodeoxyuridine (BrdU) (Littlefield and Basilico, 1966), a thymidine analog, and have been used in the cell hybridization experiments. BHK-B1 cells are resistant to 10^{-5}M BrdU and 10^{-5}M ouabain but are sensitive to HAT because they lack a functional thymidine kinase enzyme. Cells that are TK- cannot utilize the thymidine in HAT. BHK-B1 cells, like HEK cells, can be propagated in conventional growth medium because of their de novo pathways of thymidylate and purine nucleotide synthesis. Inhibitors of folate reductase, such as aminopterin, a folic acid analog, block the primary pathways of thymidylate and purine nucleotide synthesis. Therefore, the salvage pathways must be utilized, and the thymidine kinase enzyme becomes essential for growth. The absence of this enzyme is recessive to its presence, so hybrids between BHK-B1 (TK-) cells and HEK (TK+) cells are phenotypically TK+ and are viable in HAT. Propagation in HAT therefore allowed for selection

against the BHK-B1 cells.

This thesis details the development of a cell hybridization and selection system based on properties of the parental cells. The system has been developed in order to provide another method for studying the regulation of the expression of the early genes and the functions of the BKV early gene products.

MATERIALS AND METHODS

A. Materials.

Cell Culture and Medium Requirements. Human embryonic kidney (HEK) cells were established in this laboratory from primary cultures and passed up to 5 times. These cells were propagated in Dulbecco's Modified Eagle Medium (DME) supplemented with 10% fetal calf serum (FCS). Baby hamster kidney (BHK) cells (Stoker and Macpherson, 1964), subline B1, deficient in thymidine kinase (Littlefield and Basilico, 1966), were propagated in DME supplemented with 10% calf serum (CS) and 10% tryptose phosphate broth (TPB). The BHK-B1 cell line was a generous gift from Dr. Harriett Meiss. Hybrid cells between BHK and HEK cells were cultivated in DME supplemented with 10% FCS plus 10^{-5}M hypoxanthine, 10^{-6}M aminopterin, 10^{-5}M thymidine (HAT) (Ozer and Jha, 1977), and 10^{-5}M ouabain (Kucherlapati et al., 1975). Stock solutions of HAT were prepared in 1.0 ml volumes at concentrations of 10^{-2}, 10^{-3}, and 10^{-2}M respectively. Hypoxanthine was prepared in 20% 1N HCL and 80% DME, aminopterin in 20% 1N NaOH and 80% DME, and thymidine in DME. A 10^{-3}M ouabain stock was prepared in 10.0 ml of DME. Stock solutions were filtered using 0.45 Millipore filters, stored at 4°C, and used only for periods of 1 week. Cells were passaged at a density of 3.5 x 10^5 cells per 60 or 100mm culture dish and incubated at 37°C in a humidified, 8% CO_2 atmosphere.

Virus. To propagate BK virus (BKV) or to determine the response to infection with BKV, HEK, BHK-B1, or BHKxHEK hybrid cells were grown

to semi-confluency in 100mm culture dishes (approximately 1 to 3 x 10^6 cells per 100mm plate). The BKV stock was diluted in DME to obtain the desired multiplicity of infection (moi) (1.0 or 0.1 pfu per cell for HEK cells and 10.0 pfu per cell for BHK-B1 and BHKxHEK hybrid cells) (Seehafer et al., 1978). Exisiting medium was aspirated from plates, and cells were washed twice with DME. Cells were inoculated with 0.5 ml of the virus suspension. Control cells were inoculated with 0.5 ml of DME. Cells were incubated at 37°C for 90 minutes with tilting of the plates every 15 minutes to allow even distribution of the virus suspension. The HEK and BHK cells were refed with 10 ml of DME plus 1% FCS or CS respectively, and the BHKxHEK hybrid cells were refed with the appropriate selective medium supplemented with 1% FCS. Cells were incubated at 37°C until cytopathic effects (CPE) were evident.

Sera. Antisera developed against SV40 virions in horses and normal horse sera were obtained from Flow Laboratories. Antisera raised against BK virions in rabbits and normal rabbit sera were provided by Dr. E. O. Major. Anti-BKV tumor sera were raised against RF194 cells, hamster embryo fibroblast cells transformed by a BKV isolate, RF194, in Syrian golden weanling female hamsters. Hamsters were inoculated subcutaneously in the upper back with 1 x 10^6 cells per 0.5 ml sterile Dulbecco's phosphate buffer solution (PBS) (137mM NaCl, 3mM KCl, 8mM Na_2HPO_4, 1.5mM KH_2PO_4, 0.5mM $MgCl_2$, 0.9mM $CaCl_2$), pH7.2. When tumors grew to 2 to 3 cm in diameter, hamsters were exsanguinated by cardiac puncture. The blood was centrifuged for 10 minutes at 800 rpm at room temperature and the sera collected and pooled. The anti-BKV tumor sera

were tested for activity against the BKV T proteins by immunofluorescense. Normal hamster sera were obtained by cardiac puncture and centrifugation as described.

B. Routine Procedures.

Plating Efficiencies (PE) and Clone Isolation in Liquid Medium. Cells were harvested by trypsinization, counted using a hemacytometer, and plated at 500 or 1000 cells per 60 or 100mm culture dish in the appropriate liquid medium. Media including DME, DME supplemented with 10^{-5}M ouabain or 10^{-5}M bromodeoxyuridine (BrdU), HAT, and HAT plus ouabain were used in PE studies. At least 10 60mm plates were used for each test. After visible colonies appeared in 1 to 2 weeks, the percentage of cells forming colonies was determined. Colonies with the characteristics of the particular cell type were selected for cloning. For example, BHK cells should demonstrate a swirling pattern of growth with flat fibroblastic cells. Existing medium was aspirated from the plates. Sterile aluminum cylinders 5mm in diameter (cloning chambers) dipped in sterile vaseline were placed over the selected colonies and a few drops of 0.5% trypsin (w/v) in versene buffer (137mM NaCl, 3mM KCl, 8mM Na_2HPO_4, 6mM Na_2EDTA), pH7.10, were added with pasteur pipets. Following a 5 to 10 minute incubation period at 37°C, cells were transferred with pasteur pipets to 35mm cloning dishes containing 2 ml of the appropriate medium. Clones reaching confluency in 35mm cloning dishes were trypsinized and transferred to 60 or 100mm dishes for expansion of the cultures.

Virus Purification. BKV was purified according to the protocol

described by Wright and diMayorca (1975). After virus infection had been established (3+ to 4+ CPE), the infected culture plates were allowed to stand at room temperature for 2 to 3 hours until the medium became alkaline. This aided release of virus from the cells. Virus lysates were scraped into flasks, frozen-thawed for 3 to 5 cycles, and clarified by centrifugation at 5000 rpm for 10 minutes at 20°C using the Sorvall (RC5) GSA rotor. Supernatant fractions were collected, and virus was concentrated onto a 10.0 ml cushion of saturated KBr (50mM Tris, 10mM EDTA), pH7.95, by centrifugation of the lysate in a SW25.2 rotor at 24,000 rpm at 20°C for 3 hours. Virus was removed from the gradient by pumping from the bottom of the tube using a peristaltic pump and capillary tube system. After 12 to 16 hours of dialysis against TD buffer (137mM NaCl, 5mM KCl, 0.7mM Na_2HPO_4, 25mM Tris Base), pH6.8 to 7.0, (1 ml material to 1 l buffer) at 4°C, virus was resuspended in CsCl (ρ=1.3649) and centrifuged for 16 to 18 hours at 20°C to equilibrium in a Type 50 fixed angle rotor at 36,000 rpm. Virus was removed from the gradient by side puncture of the tube with a 21 gauge needle, dialyzed against TD buffer 12 to 16 hours at 40°C, and stored at -70°C until use. Cellular debris resulting from the first low speed centrifugation was treated with 0.25% deoxycholate sodium salt (DOC) in DME. After 1 hour of incubation with stirring at 37°C, the suspension was centrifuged at 10,000 rpm for 10 minutes at 20°C in a Sorvall GSA rotor. The supernatant was underlayered with 5.0 ml of 15 or 20% cold sucrose in TD buffer to prevent precipitation of the DOC with the KBr, followed by underlayering with 10.0 ml of saturated KBr, and centrifuged at 24,000 rpm for 4 hours at 20°C in the SW25.2 rotor. Virus was collected,

dialyzed, and centrifuged to equilibrium as previously described.

Plaque Assay. The plaque assay was performed according to the method of Major and diMayorca (1973). HEK cells at passages 3 to 5 were grown to confluency in 60mm dishes. Serial 10-fold dilutions of BKV samples to be titrated were made in DME. Existing medium was aspirated from the plates and cells were washed twice with DME. Cells were inoculated with 0.4 ml of the appropriate virus dilution, with dilutions being titrated in triplicate. Cell controls were inoculated with 0.4 ml of DME. Cells were incubated for 90 to 120 minutes at 37°C with tilting of the plates every 15 minutes to allow even distribution of the virus suspension. Cells were then overlaid with 9.0 ml of 0.9% agar in DME supplemented with 1% FCS and 50 units of mycostatin per ml and incubated at 37°C for 2 weeks. Plaque development was visualized by staining cells with neutral red (0.017% neutral red in distilled water), a vital dye, incorporated into agar. Cells were overlaid with 5.0 ml of 0.9% neutral red agar in DME plus 1% FCS. After a 24 hour incubation period at 37°C, plaques were counted and virus titers determined on the basis of the number of plaques and the virus dilution factor.

Determination of the Cell Phenotype of Anchorage Independence by Growth in Agar Suspension (Transformation Assay). The procedure for cell transformation was adapted from the method of Macpherson and Montagnier (1964) and modified by Major and diMayorca (1973). One 100mm plate of each cell type was harvested by trypsinization, counted using a hemacytometer, and resuspended in TD buffer supplemented with 1% FCS at a concentration of 10^7 cells per ml. Each test tube with a small stir bar contained 5×10^5 cells (0.05 ml of cell suspension) and 50

pfu of virus per cell. Volumes were adjusted to 0.5 ml with TD buffer. Virus was not added to control tubes, and TD buffer was used to adjust the volumes to 0.5 ml. Suspensions were incubated with stirring at 37°C for 1 hour. Clumps of cells were broken apart using pasteur pipets drawn out to capillary size after adsorption of the virus. Top agar (0.33%: 10% filtered FCS or CS, 10% TPB, 40% 2xDME, 40% of a 1.275% agar solution) in a 10.0 ml volume was added to 0.2 ml of virus-cell or cell suspension and 4.8 ml of Eagle's diluent (10% filtered FCS or CS, 10% TPB, 80% DME). The virus-cell or cell suspension in volumes of 1.5 ml was plated onto 60mm plates containing 5.0 ml of bottom (feeder) agar (0.5%: 10% FCS or CS, 10% TPB, 40% 2xDME, 40% of a 1.275% agar solution), resulting in 2×10^4 cells being added to each plate. Top and bottom agar were supplemented with selective medium when appropriate. Agar was allowed to set for 5 to 10 minutes before incubating the cells at 37°C. Visible colonies appeared after 2 weeks of culturing. The percentage of cell transformation was determined by counting the number of colonies and comparing this to the total number of cells plated multiplied by 100.

Clone Isolation from Agar Suspension. Cells were cloned from colonies growing in agar suspension (0.33%) by picking them with pasteur pipets and plating them into 35mm cloning dishes containing 2 ml of the appropriate medium. The cells were well dispersed by pipetting. Clones reaching confluency in 35mm dishes were trypsinized and transferred to 60 or 100mm plates for expansion of the cultures.

Immunofluorescence Assay (Fluorescent Antibody Technique). The assay was adapted from the protocol of Pope and Rowe (1964). Cells were

grown to confluency on coverslips in 100mm culture dishes and washed twice for periods of 5 minutes with PBS. Cells were fixed for 10 minutes in cold (-10°C) acetone and washed 3 times for periods of 5 minutes with cold PBS. A 1 to 5 dilution in PBS of hamster anti-BKV tumor serum was added so that the cells were completely covered. Cells were incubated at 37°C for 1 hour and then washed 3 times for periods of 5 minutes with PBS. Fluorescein conjugated rabbit anti-hamster IgG (Cappel) was added at a 1 to 10 dilution in PBS, and the cells were incubated at 37°C for 1 hour. Cells were washed 3 times for periods of 5 minutes with PBS. Coverslips were mounted in glycerol, and the cells were examined under a fluorescent microscope. Cells were graded on a scale from 0 (negative control) to 4+ (positive control).

Hemagglutination and Hemagglutination Inhibition Assays (HA and HI). The HA and HI were adapted from the methods described by Casals (1967) and Major et al. (1977). Human type 0 erythrocytes in Alsever's buffer (110mM d-glucose, 27mM sodium citrate·$2H_2O$, 72mM NaCl), pH6.5, were centrifuged 3 to 5 minutes at 1250 rpm at room temperature until the supernatant appeared clear. The supernatant was aspirated, and a 0.4% solution of erythrocytes ($\sim 10^9$ erythrocytes per ml) in Alsever's buffer was prepared. Round bottom microtiter plates containing 96 wells were used, with 12 wells being used per sample. Samples were run in duplicate. For the HA, 0.05 ml of Alsever's buffer was added to each microtiter well using a micropipet. To the first well of each series was added 0.05 ml of the virus sample. Serial 2-fold dilutions were made using microdiluters. A micropipet was used to add 0.05 ml of the 0.4% erythrocyte suspension to each well. A control series of 0.05 ml

of the 0.4% erythrocyte solution plus 0.05 ml of Alsever's buffer per well was included. Microtiter plates were incubated for at least 1 hour at 4°C, until hemagglutination was visually evident. The last well where hemagglutination was observed was taken to be the virus titer. For the HI, 0.05 ml of Alsever's buffer was added to each well with a micropipet. To the first well of each series was added 0.05 ml of the test serum. Serial 2-fold dilutions were made using a microdiluter. A micropipet was used to add 0.05 ml of virus to each well. The test serum was challenged with the appropriate concentration of virus (8 HA units) as determined by the HA. The microtiter plates were allowed to stand at room temperature for 30 to 45 minutes to permit virus and antibody to combine. A micropipet was used to add 0.05 ml of the 0.4% erythrocyte solution to each well. The last well that did not show agglutination indicated the highest dilution of serum that had the ability to prevent agglutination. Control series of 0.05 ml of Alsever's buffer plus 0.05 ml of the 0.4% erythrocyte suspension; 0.05 ml of Alsever's buffer, 0.05 ml of the 0.4% erythrocyte suspension, and 0.05 ml of serum (serially diluted); and 0.05 ml of Alsever's buffer, 0.05 ml of the 0.4% erythrocyte suspension, and 0.05 ml of virus (serially diluted) were included. Normal horse and rabbit sera, anti-BK virion sera, and anti-SV40 virion sera were used. Test sera were treated prior to use in the assays to eliminate nonspecific inhibitors of agglutination such as mucopolysaccharides and mucoproteins. The addition of 0.1 ml of 10mM KIO_4 to 0.1 ml of serum resulted in oxidation of glycol, aldehyde, and ketone groups and the breakage of bonds between vicinal hydroxyl groups. Following incubation at 56°C for 30 minutes, 0.8 ml of 1% glycerol in

distilled water was added to terminate oxidation and cleavage reactions.

Virus Density Determination. Virus samples were centrifuged in CsCl (ρ=1.3649) as described. Collection of virus samples was performed by side puncturing the tubes or fractionating the gradients into 0.2 ml aliquots by bottom puncturing the tubes and counting drops. Optical density readings were taken at 260 nm using a spectrophotometer (Beckman Recording Quartz). The refractive index was measured with a refractometer (Zeiss). The density of the fractions containing the virus bands was determined using the formula $\rho_{25°}=a\eta_D^{25°}-b$ (Ifft et al., 1961) where a and b are coefficients (a=10.8601, b=13.4974) based on density range (1.25 to 1.90) of the CsCl gradient, and η is the refractive index of the fraction.

C. Experimental Methods.

Cell Fusion. The procedure for cell fusion followed Norwood et al. (1976), and was adapted for use in this laboratory by Major et al. (1980). HEK cells at passage 3 or 4 were allowed to grow 48 to 72 hours until confluent. The existing medium was then aspirated, and the BHK-B1 cells were plated onto the HEK monolayers at a concentration of 5×10^5 cells per 100mm plate. Cells were allowed to settle in 1.0 ml of DME during a 30 to 40 minute incubation period at 37°C, gently refed with 9.0 ml of DME plus 1% FCS, and allowed to grow for 24 to 48 hours until the BHK-B1 cells formed a visible layer on the HEK cells. The existing medium was aspirated, cells were washed once with DME, and 1 to 2 ml of a solution of 44% polyethylene glycol (PEG-6000) in DME plus 15% dimethylsulfoxide (DMSO) was added to the top of a tilted plate. The PEG-

DMSO solution was allowed to run over the cells until it collected at the bottom of the dish. Approximately 15 to 30 seconds were required for the PEG-DMSO to cover the cells. Residual PEG-DMSO was aspirated from the plate, and the cells were immediately washed 4 to 5 times with DME plus 15% DMSO and then 2 times with DME. Cells were fed with 10 ml of DME plus 1% FCS and then refed with selective medium 24 hours following PEG-DMSO treatment. Cells were passaged twice at 1 week intervals and then plated at 500 or 1000 cells per 60mm plate in HAT medium plus ouabain. Selected colonies were cloned as previously described.

Karyotype Analysis. Karyotyping procedures were adapted from the techniques used by the clinical genetics laboratory at Northwestern University, Chicago, Illinois and from procedures learned at the Chromosome Banding Techniques seminar held at the W. Alton Jones Cell Science Center, Lake Placid, New York. Cells were cultured in 25 cm^2 T-flasks and subcultured 48 hours prior to harvest. When adequate cell growth was obtained and high mitotic indices evident as determined by light microscopic examination, the existing medium was replaced with 10.0 ml of fresh medium. Cells were allowed to incubate at 37°C for 12 to 18 hours. One to 4 hours prior to cell harvesting, 0.02% colchicine in Hank's balanced salt solution (5×10^{-5}M) was added to each flask, giving a final colchicine concentration of 2×10^{-7}M. An alternate method was to add 0.1 ml of a stock solution (10 µg per ml) of Colcemid in Hank's balanced salt solution (Grand Island Biological Company) to every 10.0 ml of medium, giving a final Colcemid concentration of 0.01 µg per ml.

The following procedures were used for BHK-Bl and HEK cells. The existing culture medium was aspirated and centrifuged at 1500 rpm for

5 minutes at room temperature in plastic centrifuge tubes to collect cells and to minimize mitotic cell loss. Supernatants were decanted and cells gently resuspended in the remaining liquid. To each flask was added 2.5 ml of 0.7% sodium citrate, and to each tube was added 0.5 ml of 0.7% sodium citrate. Flasks and tubes were incubated for 6 to 8 minutes at 37°C, and flasks were monitored by light microscopy for removal of cells from surfaces. Flasks were tapped gently with a large rubber cork to aid removal of cells from surfaces. Cell suspensions were transferred from flasks to the tubes containing the previously collected 0.5 ml cell suspensions and centrifuged at 1500 rpm for 5 minutes at room temperature. Supernatants were decanted and cells gently resuspended in the small amount of remaining liquid by tapping. Fixative, 1 part glacial acetic acid to 1 part absolute methanol prepared and chilled at 4°C prior to use, was added dropwise very slowly to the tubes, with the first drops being allowed to run down the sides of the tubes. Suspensions were thoroughly mixed by gentle tapping. Fixative was then added dropwise with mixing until 0.5 ml had been added to each tube. Additional fixative was added more rapidly with mixing to give a final volume of 3 ml per tube. The cells suspended in fixative were refrigerated for 30 minutes. Cell suspensions were then centrifuged, supernatants decanted, and cells gently resuspended in the remaining liquid. Fixation was repeated with 3 ml of fixative being added more rapidly than before with thorough mixing of cell suspensions. Suspensions were centrifuged at 1500 rpm for 5 minutes at room temperature, supernatants decanted, and cells gently resuspended in the remaining liquid. Cold fixative was added to a total volume of 0.5 ml to give adequate cell

suspensions for preparation of 2 or 3 slides per cell suspension. Acid or alcohol cleaned glass slides stored in distilled water at 4°C were placed in the freezer for 30 minutes to chill prior to use. Slides were used when a thin layer of ice crystals formed. Four to 8 drops of cell suspension from a pasteur pipet were dropped onto a wet slide from a height of approximately 6 inches. Slides were briefly held level, then slanted to allow the liquid to drain, and blotted along the edges. Slides were air-dried, briefly placed on a warm surface, and then steamed for 10 seconds. Microscopic examination of the slides under low power was performed to evaluate the number and quality of the metaphase spreads. Slides were then aged for at least 3 days, dried in a 50 to 55°C drying oven for 1 to 2 hours, and individually stained for G banding, using a trypsin-Giemsa method. Slides were agitated 11.5 seconds in 0.1% trypsin (1:250) in isotonic saline, pH6.8, briefly drained, quickly dipped 5 to 7 times in isotonic saline supplemented with 6% FCS, drained briefly, dipped 5 to 7 times in isotonic saline, and quickly drained. Slides were stained for 7.5 minutes in a 2% Giemsa solution (GT Gurr's Giemsa R66 Stock stain in Gurr buffer, pH6.8) and then dipped quickly once or twice in Gurr buffer, pH6.8. The backs of slides were blotted with paper towel, and slides were placed on end until dry. Slides were then dried for 1 to 2 hours in a 50 to 55°C drying oven, and smears were mounted in Protex (Scientific Products) with coverslips.

The following procedures were used for the BHK-B1xHEK hybrid cells. Cells were collected by shaking the flask or pipetting the medium over the cell monolayer several times. The medium containing the cells was poured into a conical plastic centrifuge tube, and the cells

were centrifuged at 800 rpm for 7 minutes at room temperature. Cells were gently resuspended in 5 ml of hypotonic 75mM KCl and left at room temperature or at 37°C for 5 minutes. Cells were centrifuged at 800 rpm for 7 minutes at room temperature and resuspended in freshly prepared 3 to 1 methanol to acetic acid fixative. A small amount of hypotonic 75mM KCl was left in the tube to prevent cells from clumping. Cells were left at room temperature for 1 hour. Fixative was changed 2 to 3 times as previously described before slides were prepared. Cells were stored in fixative in pellet form at 4°C if slides were not immediately prepared. Slides were individually cleaned with soap and water, rinsed thoroughly in running tap water, rinsed twice in distilled water, and chilled at 4°C in distilled water. Cells were resuspended in 0.5 ml of fixative following the final wash in fixative or after storage at 4°C. A chilled slide was removed from the distilled water and the excess water drained, leaving a thin film of water on the slide. One to 2 drops of cell suspension from a pasteur pipet were dropped on the slide from a height of 6 inches. The cell suspension was gently blown over the slide, and slides were allowed to dry at room temperature. The slides were stored at 4°C until stained. Slides were incubated in a 60°C drying oven for 16 to 18 hours prior to staining and then incubated in 25mM KH_2PO_4, pH6.8, in a 56°C water bath for 10 minutes. Slides were gently flooded with the Giemsa-trypsin mixture at room temperature for 10 minutes. The Giemsa stock solution was prepared and aged. One gram of Giemsa powder (Fisher G-146) was added with stirring to 66 ml of glycerin, and the container was covered and placed in a 56°C water bath for 2 hours. The mixture was stirred 2 or 3

times. After addition of 66 ml of methanol, the mixture along with the sediment was transferred to an amber bottle. The stock Giemsa solution was stored at 4°C and allowed to age at least 2 weeks before use. The Giemsa-trypsin mixture was prepared by combining 36.5 ml of 25mM KH_2PO_4, pH6.8; 12.5 ml of methanol from a fresh bottle; 1.0 ml of the Giemsa stock solution; and 0.25 ml of the trypsin-EDTA-10X solution (Grand Island Biological Company). The amount of Giemsa stock solution required for optimal chromosome banding varied from 0.8 to 2.0 ml depending on the age of the solution. After staining, slides were rinsed and dipped once in distilled water and allowed to air dry. Slides were examined under low power using bright field microscopy to determine the quality of staining.

Slides were scanned using low power bright field microscopy to evaluate the chromosome spreads, and high power oil immersion bright field microscopy was used for chromosome counts. Well spread metaphases with optimal banding were photographed using high power oil immersion bright field microscopy. An Olympus Vanox Research Microscope with a narrow-band pass interference filter with maximum transmission at 546 nm (Zeiss 467808) was used. Kodak Panatomic-X or panachromatic high contrast copy film was used. Developing procedures were those suggested by the manufacturer. Photographed metaphase spreads were cut apart so that chromosomes could be identified according to species by the banding pattern, size, and centromere location.

RESULTS

The main objective of this work, as previously described, was the development of a model cell culture system in which the regulation of BKV early gene expression could be evaluated. The system would provide a means for studying the functions of the early gene products along with factors affecting their synthesis. The approach this laboratory utilized was to construct somatic cell hybrids between BHK cells, a nonpermissive host capable of being transformed by BKV, and HEK cells, a permissive host for BKV multiplication. The major portion of the investigation reported in this thesis involved development of a selection system based on parental cell characteristics that would eliminate the parental cells and allow for isolation of these hybrid cells. Hybrid cell cultures could then be infected with BKV and tested for their susceptibility to either lytic or transforming infection.

A. Selection of BHK-B1 x HEK Hybrid Cells.

1. Parental Cell Sensitivity to Selective Media.

The selection system for obtaining somatic cell hybrids was developed by utilizing properties of the parental cell types. The parental cells, HEK and BHK-B1 cells, were first tested for their ability to grow in selective media. Since HEK cells fail to clone at low density and can only be passaged up to five times following establishment of primary cultures, selection of these cells using biochemical markers cannot be done. Therefore, experiments were designed to utilize existing HEK cell properties. Since selection of the cells must begin at the time of cell fusion, HEK cell sensitivity studies were performed in a manner that mimicked the conditions established for cell hybridization. To test HEK cell sensitivity to various selective agents, the cells were first grown to confluency. This correlated with cell fusion being performed with PEG-DMSO when the HEK cells reached confluency. Cell cultures were then refed with selective medium, which paralleled the cultures being refed with selective medium following cell fusion. Cell sensitivity to the selective medium was determined by performing cell counts at various times during the study. Cell counts were done on specific days that paralleled events in the hybridization procedure, such as refeeding the cells with selective medium and passage.

Since BHK-B1 cells are able to clone at low density, they were tested for their ability to propagate in various selective media by performing plating efficiencies. The percentage of cells plating in selective medium compared to the percentage of cells plating in growth

medium determined cell sensitivity to the various selective agents.

The response of HEK cells to medium containing HAT was first studied. HEK cells were plated at 3.5×10^5 cells per 60mm plate in growth medium and allowed to grow for 4 days until monolayers were confluent. One set of cultures was refed with growth medium plus HAT and the other set refed with growth medium, as diagrammed in Figure 1. Cell counts were performed in triplicate on days 4, 5, 12, and 19 following plating and the resulting counts averaged. HEK cells were able to propagate in medium containing HAT, as shown in Figure 1. Cells reached confluency on day 4 at 3×10^6 cells per 60mm culture dish. The gradual decrease in the number of cells per culture dish was probably due to senescence of the cells. This observation can be consistently made in various HEK derived cell cultures.

HEK cells were then tested for their ability to propagate in growth medium containing ouabain. Cells were plated at 3.5×10^5 cells per 60mm culture dish in growth medium and allowed to grow for 4 days until confluency was reached at 3×10^6 cells. Cultures were refed with growth medium supplemented with 10^{-5}, 10^{-6}, or 10^{-7}M ouabain. One set of cultures was refed with growth medium, as diagrammed in Figure 2. Cell counts were performed in triplicate on days 4, 5, 12, and 19 following plating and averages taken. HEK cells were unable to survive in medium containing ouabain, as shown in Figure 2. Cells were sensitive to ouabain even at concentrations as low as 10^{-7}M. By day 12, cell counts were reduced to 1.5 to 2×10^5 cells per 60mm plate. There were too few cells to count using a hemacytometer by day 10. Therefore, 10^{-5}M ouabain along with continual passage in culture could

Figure 1. HEK cell sensitivity to 10^{-5}M hypoxanthine, 10^{-6}M aminopterin, and 10^{-5}M thymidine (HAT). One set of HEK cell cultures was refed with growth medium containing HAT when monolayers reached confluency. The time of refeeding is indicated by the arrow. Cell counts were performed at various times during culture to determine HEK cell sensitivity to HAT.

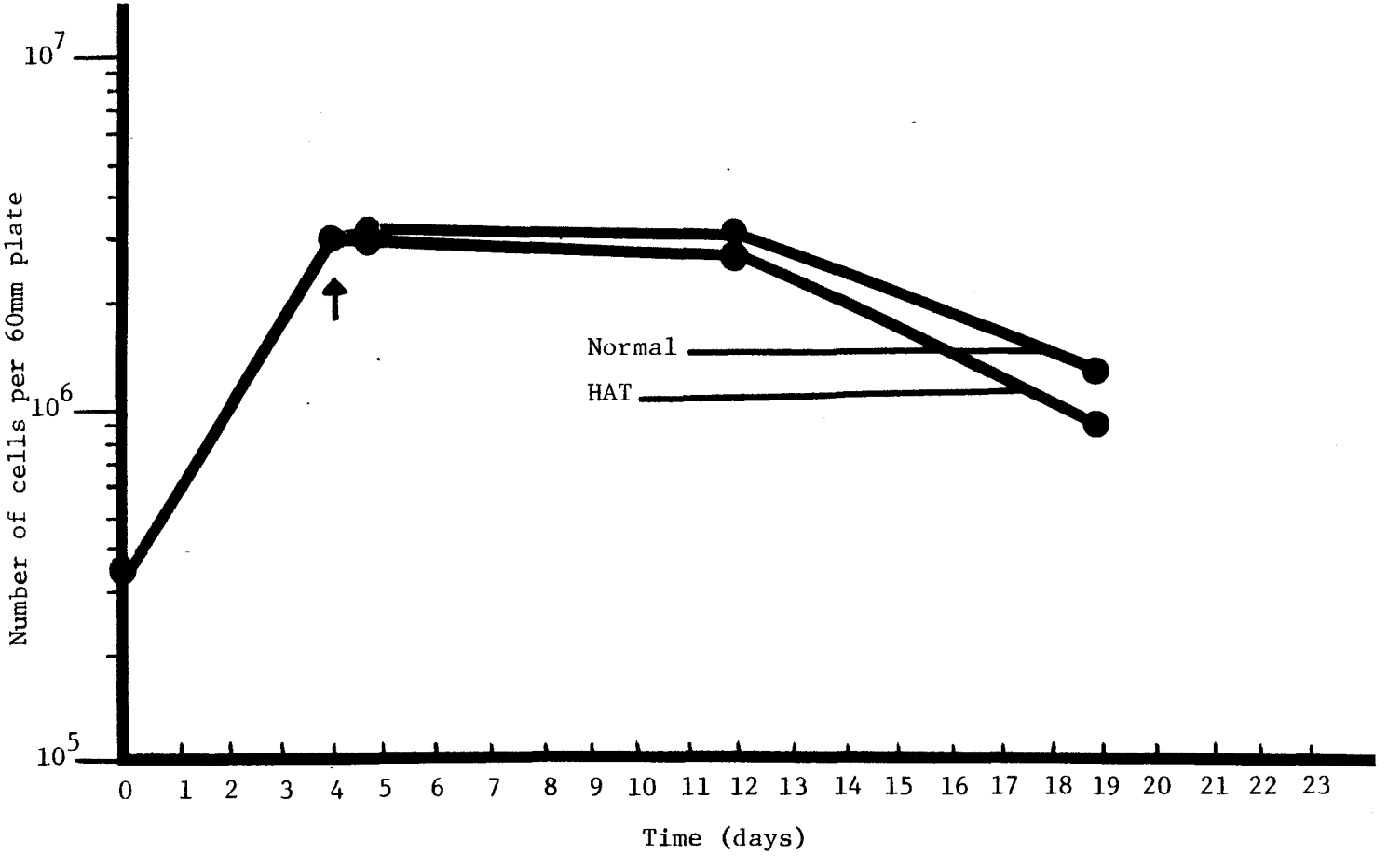
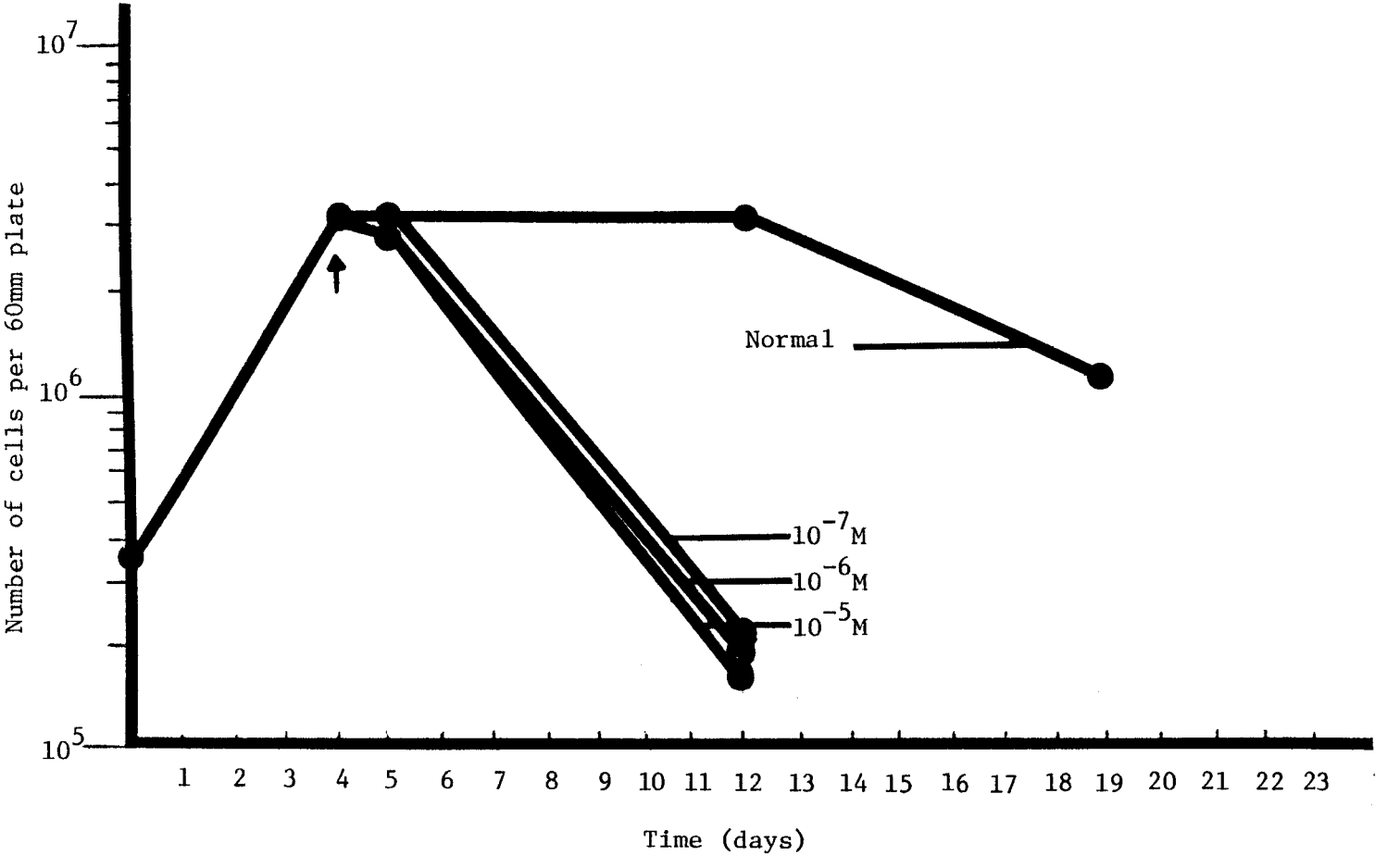

10^7
10^6
10^5
Number of cells per 60mm plate
Normal
HAT
0 1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16 17 18 19 20 21 22 23
Time (days)

Figure 2. HEK cell sensitivity to ouabain. HEK cultures were refed with growth medium containing 10^{-5}, 10^{-6}, or 10^{-7}M ouabain when monolayers reached confluency. The time of refeeding is indicated by the arrow. Cell counts were performed at various times during culture to determine HEK cell sensitivity to ouabain.


10^7
10^6
10^5
Number of cells per 60mm plate
Normal
10^{-7}M
10^{-6}M
10^{-5}M
1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16 17 18 19 20 21 22 23
Time (days)

be used to select against the parental HEK cells.

Cell counts performed on days 4 and 5 paralleled, respectively, cell fusion with BHK cells using PEG-DMSO and refeeding the fused cells with selective medium. Cell counts done on days 12 and 19 corresponded to passage of the hybrid cells at weekly intervals.

BHK-B1 cells were then tested for their ability to grow in various selective media. Cells were plated at 500 cells per 60mm culture dish in multiples of 10. Colonies were counted 10 days following plating. Table 1 illustrates the ability of the BHK-B1 cells to grow in the selective media. In experiment 1, cells were plated in growth medium, growth medium supplemented with 10^{-5}M BrdU, or HAT medium. The percentage of cells plating in growth medium (28.8%) and growth medium plus BrdU (30.4%) was approximately equal. No cells survived in HAT medium. In experiment 2, the percentage of cells growing in growth medium (11.6%) and growth medium plus 10^{-5}M ouabain (9.6%) was approximately the same. BHK-B1 cells were able to propagate in growth medium supplemented with 10^{-5}M BrdU or ouabain but were sensitive to HAT medium. These results demonstrated the lack of a functional thymidine kinase enzyme in the BHK-B1 cells. Therefore, selection against the parental BHK-B1 cells could be achieved by propagating the cells in HAT medium.

2. Hybridization of BHK-B1 and HEK Cells.

Initial work in this laboratory involved fusion of BKV transformed BHK cells, resistant to high concentrations of BrdU, with HEK cells (Major et al., 1980). The main objectives of this work were to

Table 1. BHK-B1 plating efficiencies[1] in liquid culture.

	Experiment 1			Experiment 2	
	DME	BrdU	HAT	DME	Ouabain
BHK-B1	28.8%	30.4%	<0.02%	11.6%	9.6%

1. 500 cells per 60mm culture dish were plated in multiples of 10. BrdU was at 10^{-5}M, ouabain was at 10^{-5}M, and HAT was at 10^{-5}M hypoxanthine, 10^{-6}M aminopterin, and 10^{-5}M thymidine final concentration per plate. Colonies were counted 10 days after plating.

determine if the transformed phenotype was dominant in these interspecies hybrids and to study the synthesis of the BKV T proteins. BHK cells were selected against by propagation in HAT medium. Anchorage independent growth, the most stringent criteria for malignant transformation of cells, was used to select against HEK cells. Following fusion of BKV transformed BHK cells and HEK cells with PEG-DMSO, the cells were plated in agar suspension in HAT medium. This selection system did not allow for isolation of BKV-BHK x HEK hybrid clones expressing phenotypes other than anchorage independent growth. It was also difficult to obtain BHK cells that remained resistant to high concentrations of BrdU and sensitive to HAT medium. Therefore, the selection system was modified.

As in the previous work, somatic cell hybrids between normal and transformed cells were utilized. In order to isolate hybrid clones with the potential of expressing various transformed phenotypes, selection of the cells was not performed utilizing anchorage independent growth in soft agar. At this time, BHK-B1 cells, lacking a functional thymidine kinase enzyme, were obtained from Dr. Harriett Meiss. The modified selection system involved propagating the cells in HAT medium immediately following fusion. It was felt that continual passage of the cells in culture would eliminate the parental HEK cells. BHK-B1 cells would not grow in HAT medium. Therefore, hybrid clones could be isolated after several passages in HAT medium. Hybrid clones between BKV transformed BHK-B1 clones and HEK cells were characterized.

BHK-B1 cells were transformed by BKV prior to fusion of the cells with PEG-DMSO. BKV transformed BHK-B1 clones were selected from

colonies growing anchorage independently in soft agar suspension and expanded in culture. Two clones, BKV-BHK-B1 Clone 13 and BKV-BHK-B1 Clone 16, were selected for use in the fusion studies. Following fusion, cells were propagated in HAT medium through several passages. The hybrid cells were then plated at 500 cells per 60mm culture dish in HAT medium. BKV-BHK-B1 Clone 13 x HEK cells did not plate at low density whereas BKV-BHK-B1 Clone 16 x HEK cells plated at low density. BKV-BHK-B1 Clone 13 x HEK cells were positive for virus rescue, with all cells displaying CPE and undergoing lysis. BKV-BHK-B1 Clone 16 x HEK cells were negative for virus rescue. The culture supernatants were tested for the presence of virus by plaque assay using confluent HEK cell monolayers. Virus was present in the BKV-BHK-B1 Clone 13 x HEK cell culture supernatant. T protein expression could not be studied in BKV-BHK-B1 Clone 13 x HEK cells by indirect immunofluorescence because all cells underwent viral induced lysis. BKV-BHK-B1 Clone 16 x HEK cells were negative for T protein expression. These results are summarized in Table 2.

Whenever virus transformed cells are fused with nontransformed, permissive cells, there is the possibility of virus rescue occurring. This was evident when BKV transformed BHK-B1 cells were fused with HEK cells. Therefore the selection system was further modified in order to obtain hybrid clones between two normal cell types having the potential of expressing various transformed phenotypes.

Ouabain was introduced into the system as a more definite way of selecting against the HEK cells (Baker et al., 1974; Mankovitz et al., 1974; Kucherlapati et al., 1975; Corsaro and Migeon, 1978).

Table 2. Properties of two BKV transformed BHK-B1 clones and these BKV transformed BHK-B1 clones fused with HEK cells.

Cell Derivative		Plating Efficiency[1] in Liquid Culture	Virus Released[2]	T Protein Expression[3]
BKV-BHK-B1	Clone 13	NT*	<1 pfu/0.13 ml	(-)
	Clone 16	NT	<1 pfu/0.13 ml	(-)
BKV-BHK-B1xHEK	Clone 13xHEK	<0.02%	2.3×10^6 pfu/ml	NT
	Clone 16xHEK	4%	<1 pfu/0.13 ml	(-)

*NT = not tested

1. Plating efficiency is determined as the number of colonies per number of cells plated x 100. 500 cells were plated per 60mm culture dish in HAT medium.

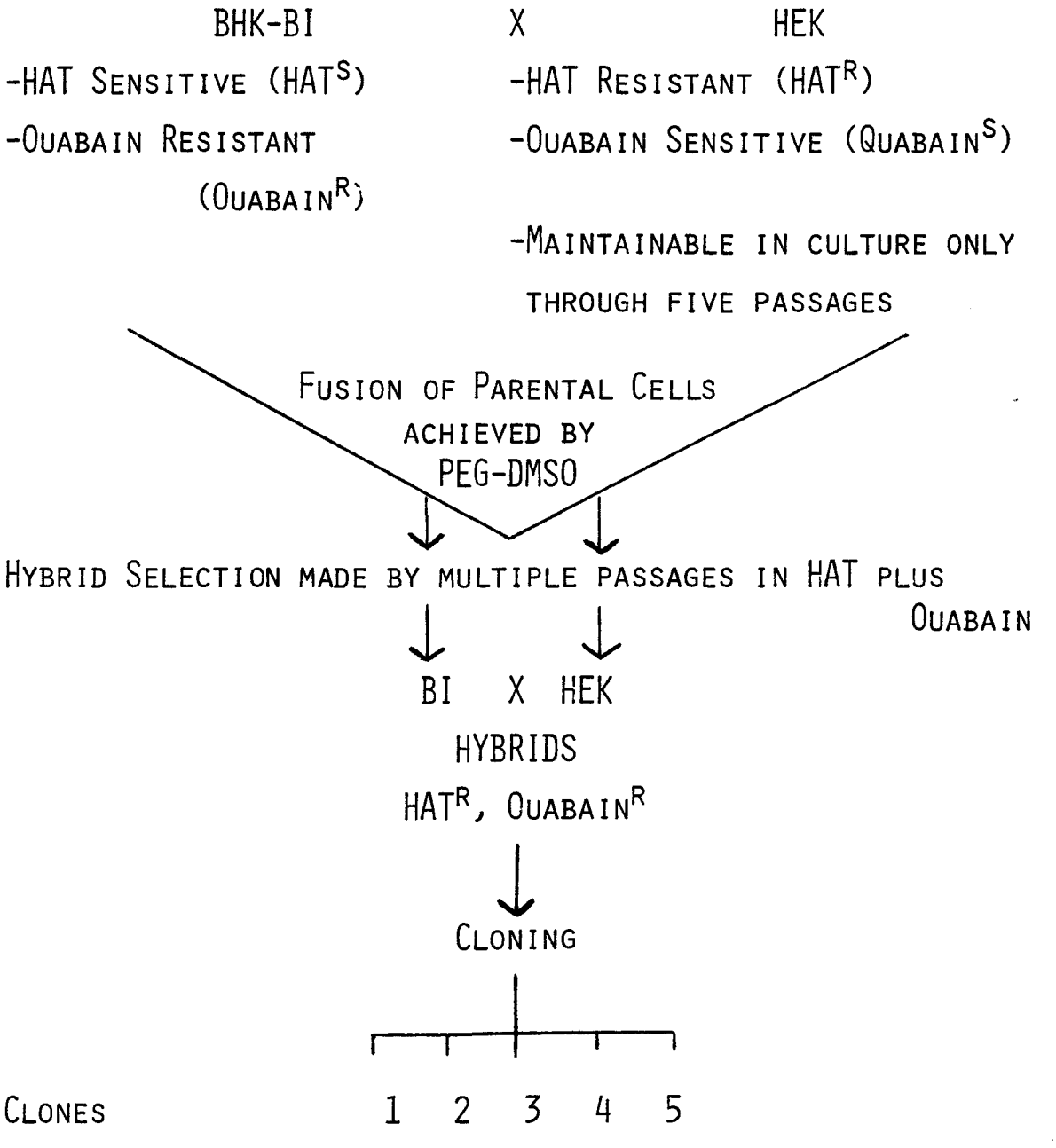
2. CPE was present and virus was released during cell propagation. Virus titer is determined as pfu per ml of cell culture lysate by plaque assay method.

3. Positive indicates the presence of cells demonstrating fluorescence by indirect immunofluorescence.

Ouabain at 10^{-5}M along with continual passage in culture would eliminate the parental HEK cells. Growth in HAT medium would select against the BHK-B1 cells. These modifications improved the effectiveness of the selection system. It was hoped that this would allow for isolation of clones expressing a wider range of phenotypes as a result of BKV infection or transformation. Therefore, an improved system for studying BKV-host cell interactions would have been developed.

The generalized experimental protocol for cell hybridization and clone isolation is diagrammed in Figure 3. Fusion of the parental cells was achieved by use of PEG-DMSO. No toxicity was observed when PEG-DMSO was applied to the cells for 15 to 30 seconds under the established experimental conditions. This fusion technique effectively produced hybrid cells as evidenced by the presence of multi-nucleated cells during light microscopic examination. The ability of the cells to survive propagation in the selective medium, HAT plus ouabain, substantiated the effectiveness of this fusion technique. Following fusion, cells were refed with growth medium for 24 hours and then refed with growth medium supplemented with HAT and ouabain. Cells were passaged twice at 1 week intervals and then plated at low density for cloning. A total of 1.7×10^5 cells were plated at 500 or 1000 cells per 60mm culture dish in HAT medium plus ouabain. Five colonies were successfully cloned and expanded in culture, resulting in a cloning frequency of 3×10^{-5}. To the best of our knowledge, this is the first time interspecies hybrids have been successfully isolated between human embryonic kidney cells and Syrian hamster cells.

Figure 3. Generalized experimental protocol for cell hybridization and hybrid clone isolation.


BHK-BI
-HAT Sensitive (HAT^S)
-Ouabain Resistant (Ouabain^R)
X
HEK
-HAT Resistant (HAT^R)
-Ouabain Sensitive (Quabain^S)
-Maintainable in culture only through five passages
Fusion of Parental Cells achieved by PEG-DMSO
Hybrid Selection made by multiple passages in HAT plus Ouabain
BI X HEK
HYBRIDS
HAT^R, Ouabain^R
Cloning
Clones
1 2 3 4 5

B. Analysis and Characterization of BHK-B1 x HEK Hybrid Cells.

BHK-B1 x HEK hybrid cells were analyzed by several methods in order to characterize the clones and verify the hybrid nature of the cells. The effectiveness of the selection system was substantiated by these techniques. BHK-B1 x HEK hybrid Clone 1 was chosen as the prototype clone in some experiments.

1. Phenotypic Characteristics of Hybrid Cells.

The five isolated clones were passaged twenty-five times in selective medium, HAT plus ouabain. Phenotypic characteristics were studied. The phenotypes of the BHK-B1 x HEK hybrid clones differed from those of the parental BHK-B1 and HEK cells. BHK-B1 cells are fibroblastic and display a swirling pattern of growth. HEK cells are mostly epithelial in morphology and exhibit no structured growth pattern. The hybrid clones appeared more epithelial than fibroblastic and displayed no structured pattern of growth. Hybrid cells within a clone were irregularly shaped, and cell size varied with the hybrid cells generally appearing larger than the parental cells. The clones appeared to be contact inhibited, which is more characteristic of HEK cells than of BHK-B1 cells. Saturation densities of the clones (4 x 10^6 cells per 100mm culture dish) were higher than that of HEK cells (2 to 3 x 10^6 cells per 100mm culture dish) but were lower than the saturation density of BHK-B1 cells (6 x 10^6 cells per 100mm culture dish). Hybrid clones grew poorly in HAT medium plus ouabain supplemented with 10% CS or newborn calf serum (NCS) but grew well when 10% FCS was used. This indicated a FCS dependence which is characteristic of HEK cells.

These phenotypic characteristics helped to establish the hybrid nature of the five isolated clones and are summarized in Table 3.

2. Karyotype Analysis of Hybrid Cells.

One species of chromosomes usually predominates in cell hybrids between permissive and nonpermissive cells (Ozer and Jha, 1977; Barrett et al., 1978). Preferential loss of human chromosomes most often occurs in hybrids between human and rodent cells (Weiss, 1970; Croce and Koprowski, 1974; Croce, 1980). The mechanism of chromosome loss has not yet been elucidated, but the rate of chromosome loss is not constant throughout hybrid cell propagation (Bernhard, 1976; Labella et al., 1976; Schall and Rechsteiner, 1978).

SV40 and polyoma have most frequently been utilized in hybrid cell systems to study gene expression in virally transformed cells (Ozer and Jha, 1977). As previously explained, our laboratory is studying BKV early gene expression by utilizing BHK-Bl x HEK hybrid cells. One approach we are using is to identify the chromosome complements of the hybrid clones and relate these to the permissiveness or nonpermissiveness of the cells to BKV infection.

Karyotype analysis of other somatic cell hybrid models correlated the presence of specific chromosomes with the response of the cells to SV40 lytic or transforming infection. For example, the transformed phenotype and expression of the large T protein was maintained in somatic cell hybrids between SV40 transformed human cells and mouse cells (Marin, 1971; Croce et al., 1974, 1975; McDougall, 1975; Croce, 1977). The presence of human chromosome number 7 correlated with the

Table 3. Phenotypic characteristics of parental and hybrid cells.

Cell Identification	Sensitivity to HAT[1]	Sensitivity to Ouabain (10^{-5}M)	Unlimited Generation Number in Culture	Morphology and Growth Pattern	Contact Inhibition	Saturation Density (number of cells per 100mm plate)	FCS Dependence
BHK-B1	Yes	No	Yes	Fibroblastic, Swirling	No	$6x10^6$	No
HEK	No	Yes	No	Epithelial, Non-structured	Yes	$2-3x10^6$	Yes
BHK-B1xHEK Hybrid Clones	No	No	Yes	Epithelial, Non-structured	Yes	$4x10^6$	Yes

1. HAT was at 10^{-5}M hypoxanthine, 10^{-6}M aminopterin, and 10^{-5}M thymidine final concentration.

expression of the large T protein and rescuability of the SV40 genome. This chromosome was necessary for the expression of the transformed phenotype (Croce et al., 1974, 1975, 1976). The SV40 integration site in hybrids between another SV40 transformed human cell line and mouse cells was assigned to human chromosome 17 (Croce, 1977). This chromosome was responsible for T protein expression and correlated with tumorigenicity of the hybrid cells in vivo.

Susceptibility of cell hybrids between cercopithecoid monkey and Chinese hamster cells to SV40 infection was dependent on the chromosome complement. Elevated SV40 viral replication occurred when rhesus monkey chromosome 11 or African green monkey chromosome 12 was retained (Garver et al., 1980).

The karyotypes of the five BHK-B1 x HEK hybrid clones were analyzed to verify the hybrid nature of the cells. The presence of human and hamster chromosomes had to be demonstrated. Emphasis was placed on locating human chromosome number 17, the chromosome that carries the gene for the soluble thymidine kinase (Boone et al., 1972). This was the only chromosome specifically selected for by use of HAT medium. It was possible that only a small fragment of chromosome 17 remained in the hybrid cells due to chromosome pulverization (Wullems et al., 1977). The presence of the same human chromosome or chromosomes in the hybrid clones would have indicated a specific retention pattern.

It was important to first karyotype the parental HEK and BHK-B1 cells. Chromosomes were identified according to trypsin-Giemsa G banding pattern, size, and centromere location. This identified the chromosome complements of the parental cells before fusion. Normal Syrian

hamster cells contain a 44 chromosome complement, distributed into 21 pairs of autosomes and 2 sex chromosomes, X and Y (Lehman et al., 1963; Migeon, 1968; Popescu and DiPaolo, 1972). BHK-B1 cells, an established cell line, may contain diploid, pseudodiploid, aneuploid, or tetraploid cells. Numerous chromosomal rearrangements occur, with translocations of entire arms of chromosomes being common. The chromosomal banding patterns of established cell lines may vary somewhat from the banding patterns of the standard karyotype due to continual passage of the cells in culture.

The BHK-B1 cells fixed well and lysed easily as evident from light microscopic examination of the preparations. Numerous metaphase spreads banded well. The modal chromosome number for the BHK-B1 cells was 39, with a range of 26 to 87 (Table 4). The karyotype of a BHK-B1 cell containing 39 chromosomes is shown in Figure 4. The normal male Syrian hamster chromosome complement was fairly well preserved, with most chromosomes being present in pairs. Chromosome numbers 12, 13, 16, 18, and 21 were each present in a single copy. The p arm of the X chromosome and the q arm of the Y chromosome fused. A q arm of chromosome number 2 fused with a chromosome number 6, and one chromosome number 11 contained a chromosome number 5 q arm and centromere region translocation. Many chromosomes had slightly altered banding patterns when compared to the standard trypsin-Giemsa G banded karyotype of the Syrian hamster. This and the chromosomal rearrangements were probably due to continual passage of the BHK-B1 cells in culture. It was also possible that treatment of the BHK cells with BrdU in order to establish the TK^- BHK-B1 cell line resulted in slight banding pattern alterations

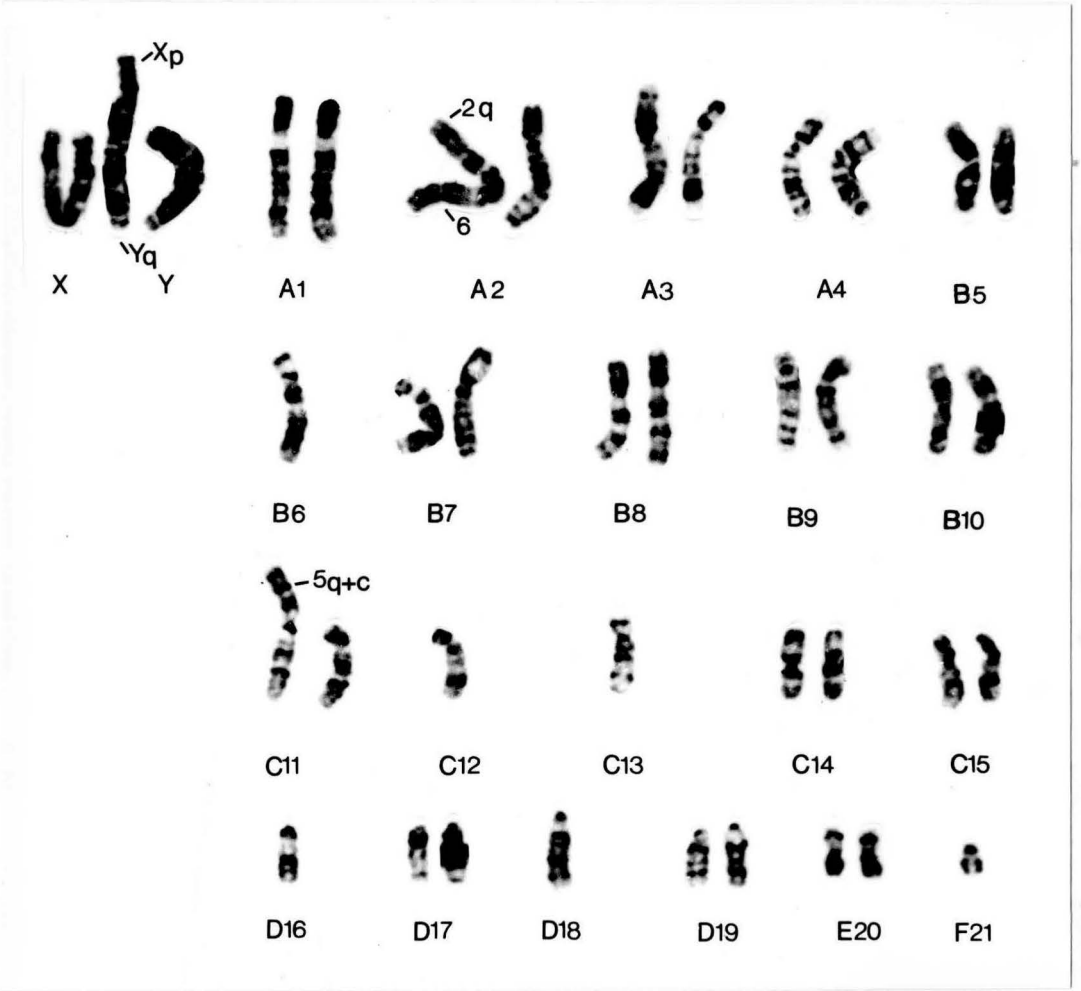
Table 4. Karyotypic characteristics of parental and hybrid cells.

Cell Identification	Chromosome Number		Chromosome Number[3]			Human Chromosome Identification
	Range[1]	Mode[2]	Marker	Hamster	Human	
BHK-B1	26-87	39	0	NA	NA	NA
HEK	44-46	46	0	NA	NA	NA
BHK-B1 x HEK Hybrids						
Clone 1	64-89	78	8	64	6	3,6,8,17*,18
Clone 2	57-81	68	8	57	3	11,16,17
Clone 3	63-90	77	12	57	8	6*,11,16*,17*,18
Clone 4	59-88	78	7	67	4	8,11,17,22
Clone 5	51-83	62	8	52	2	3,17

NA = not applicable
* indicates chromosome pair

1. Range was determined from chromosome counts of 25 metaphase spreads.
2. Modal chromosome number was determined from chromosome counts of 25 metaphase spreads.
3. Number of hamster, human, and marker chromosomes refers to the number of these chromosomes in the karyotypes shown in Figures 6 through 10.

Figure 4. Karyotype from trypsin-Giemsa G banding preparation of the parental BHK-B1 cells.


Xp
Yq
X
Y
A1
2q
6
A2
A3
A4
B5
B6
B7
B8
B9
B10
5q+c
C11
C12
C13
C14
C15
D16
D17
D18
D19
E20
F21

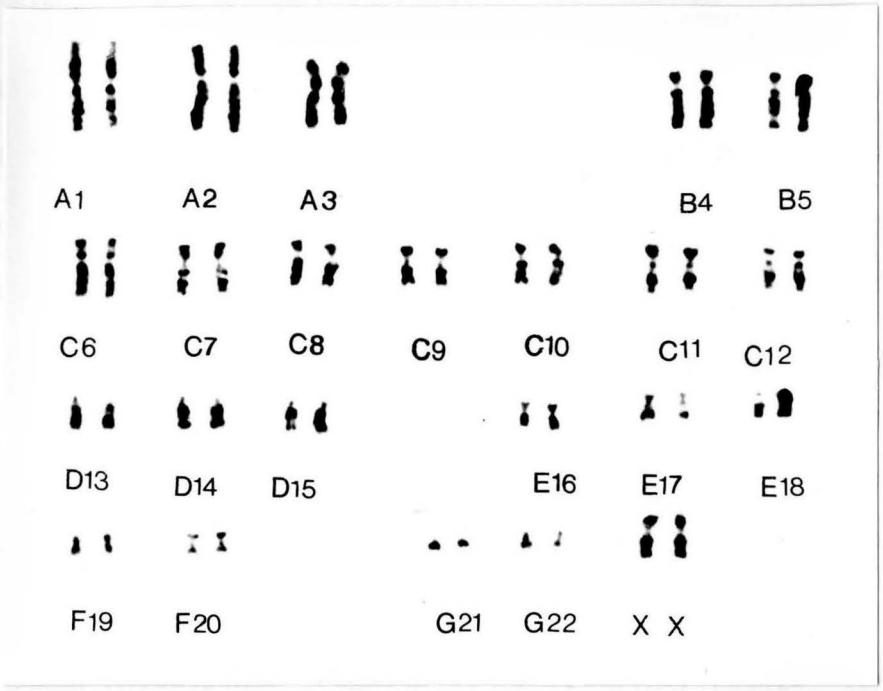
and chromosomal rearrangements.

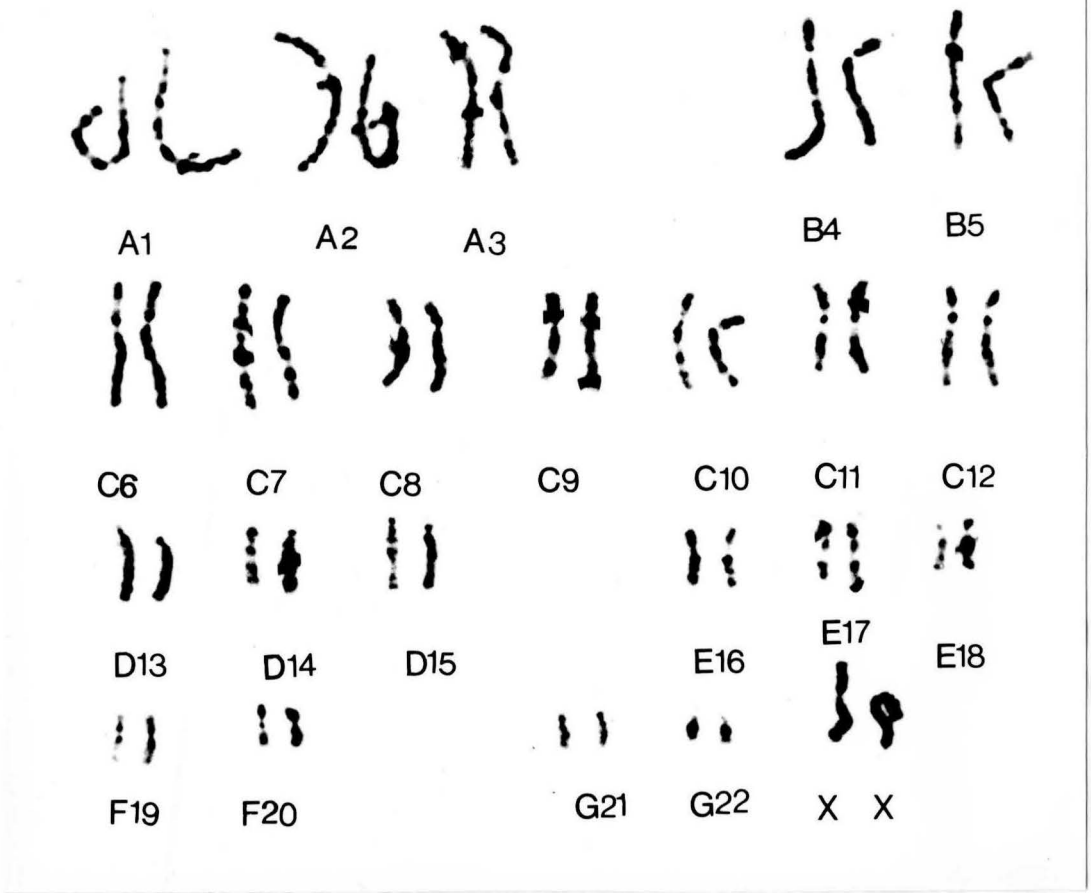
Normal human diploid cells have a complement of 46 chromosomes distributed into 22 pairs of autosomes and 2 sex chromosomes, X and Y (Tjio and Levan, 1956; Drets and Shaw, 1971; Aula and Saksela, 1972). The diploid nature of the HEK cells was well preserved. All chromosomes were present in pairs with the sex chromosomes being X, X. The modal chromosome number was 46, with a range of 44 to 46 (Table 4). The loss of chromosomes during metaphase spread preparation would most likely account for the slight variation from the diploid state. No chromosomal rearrangements were evident. Karyotypes of HEK cells are shown in Figure 5.

HEK cells were well fixed, underwent lysis easily, and banded well, although only a limited number of metaphase spreads were evident. HEK cells have a long generation time of approximately 36 hours. This made it more difficult to obtain enough metaphase cells for spread preparation. Therefore, a longer Colcemid treatment time was used to increase the yield of metaphase cells. Colcemid was added to the cultures for 5 hours following 40 hours of cell propagation. An increased number of metaphase cells was obtained. Addition of Colcemid for long periods of time can be toxic to cells and often results in extreme condensation of the chromosomes. This results in the obliteration of banding patterns. Therefore, cells were carefully monitored by light microscopic examination during the Colcemid treatment. Two HEK cell karyotypes are shown to demonstrate the appearance of the banding patterns of chromosomes at different stages of condensation (Figure 5).

The presence of human and hamster chromosomes was demonstrated

Figure 5. Karyotypes from trypsin-Giemsa G banding preparations of the parental HEK cells.


A1
A2
A3
B4
B5
C6
C7
C8
C9
C10
C11
C12
D13
D14
D15
E16
E17
E18
F19
F20
G21
G22
X X


A1
A2
A3
B4
B5
C6
C7
C8
C9
C10
C11
C12
D13
D14
D15
E16
E17
E18
F19
F20
G21
G22
X X

in the BHK-B1 x HEK hybrid clones by karyotype analysis. Chromosomes were again identified by trypsin-Giemsa G banding pattern, size, and centromere location. The BHK-B1 chromosomes in the hybrid clones were identified and read out of the karyotypes as background. Unidentifiable or marker chromosomes were also present in the hybrid clones. The species of origin of the marker chromosomes could not be determined, but they were probably altered Syrian hamster chromosomes.

Results of the karyotype analysis of the five BHK-B1 x HEK hybrid clones at an intermediate passage level demonstrated that all clones retained human chromosomes (Figures 6, 7, 8, 9, 10) (Table 4). Chromosome number 17 was the only human chromosome retained in every clone. This chromosome was specifically selected for by use of HAT medium. The number of human chromosomes per clone ranged from 2 to 8.

BHK-B1 x HEK hybrid Clone 1 modal chromosome number was 78, with a range of 64 to 89 (Table 4). There were 8 marker chromosomes present (Figure 6). Sixty-four hamster chromosomes were identified, with at least one copy of every chromosome in the complement being present. One hamster chromosome number 11 contained a chromosome number 5 q arm and centromere region translocation. Human chromosome numbers 3, 6, 8, and 18, and a pair of human number 17 chromosomes were identified.

The modal chromosome number of BHK-B1 x HEK Clone 2 was 68, with a range of 57 to 81 (Table 4). Eight marker chromosomes were present, along with 57 hamster chromosomes (Figure 7). At least a single copy of every hamster chromosome was identified. Three human chromosomes, numbers 11, 16, and 17, were identified.

BHK-B1 x HEK hybrid Clone 3 had a range of 63 to 90 with a modal

Figure 6. Karyotype from trypsin-Giemsa G banding preparation of the BHK-B1 x HEK hybrid Clone 1.

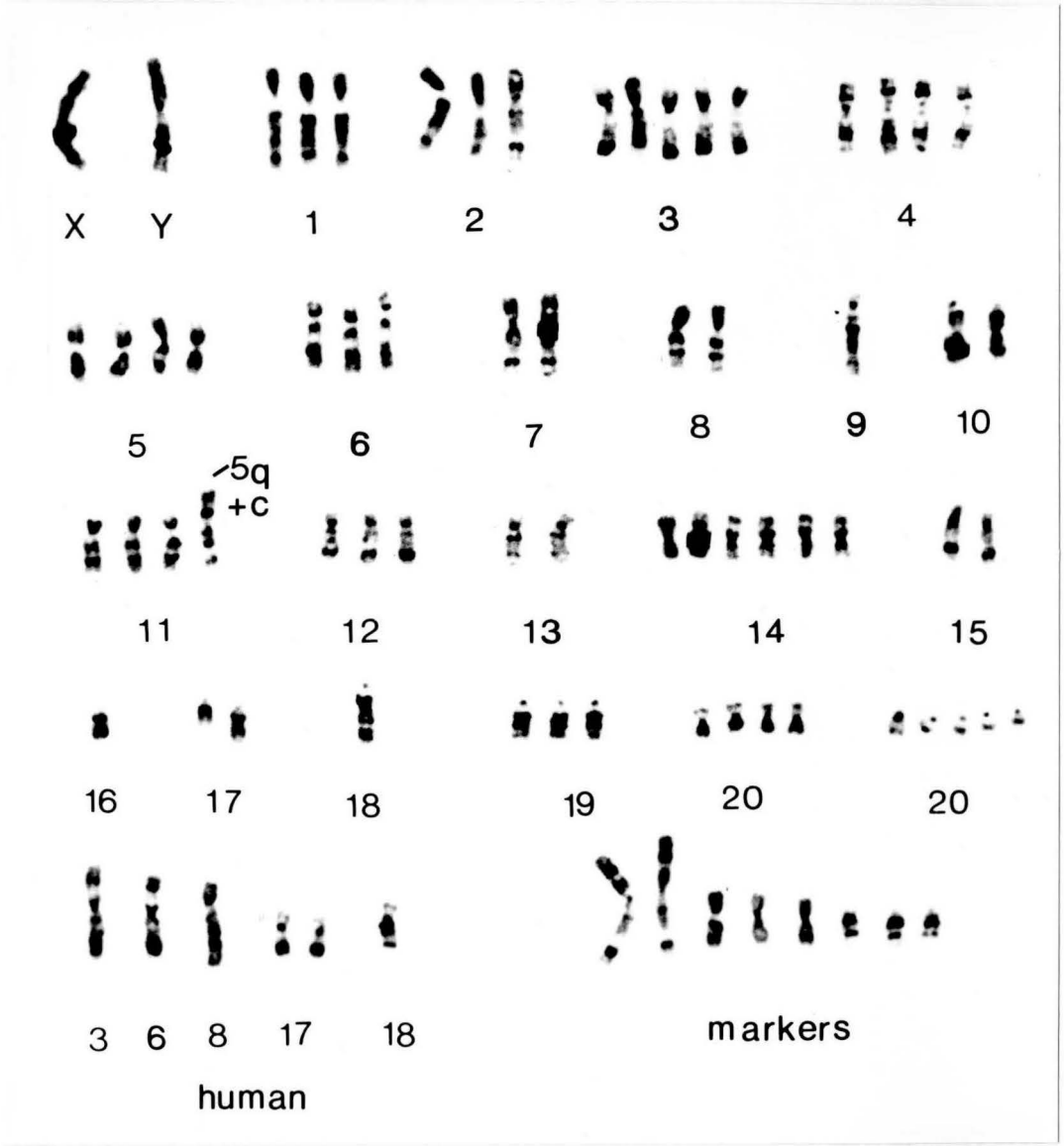

X
Y
1
2
3
4
5
6
7
8
9
10
5q
+c
11
12
13
14
15
16
17
18
19
20
20
3
6
8
17
18
human
markers

Figure 7. Karyotype from trypsin-Giemsa G banding preparation of the BHK-B1 x HEK hybrid Clone 2.


X
Y
1
2
3
4
5
6
7
8
9
10
11
12
13
14
15
16
17
18
19
20
21
11
16
17
human
markers

Figure 8. Karyotype from trypsin-Giemsa G banding preparation of the BHK-B1 x HEK hybrid Clone 3.

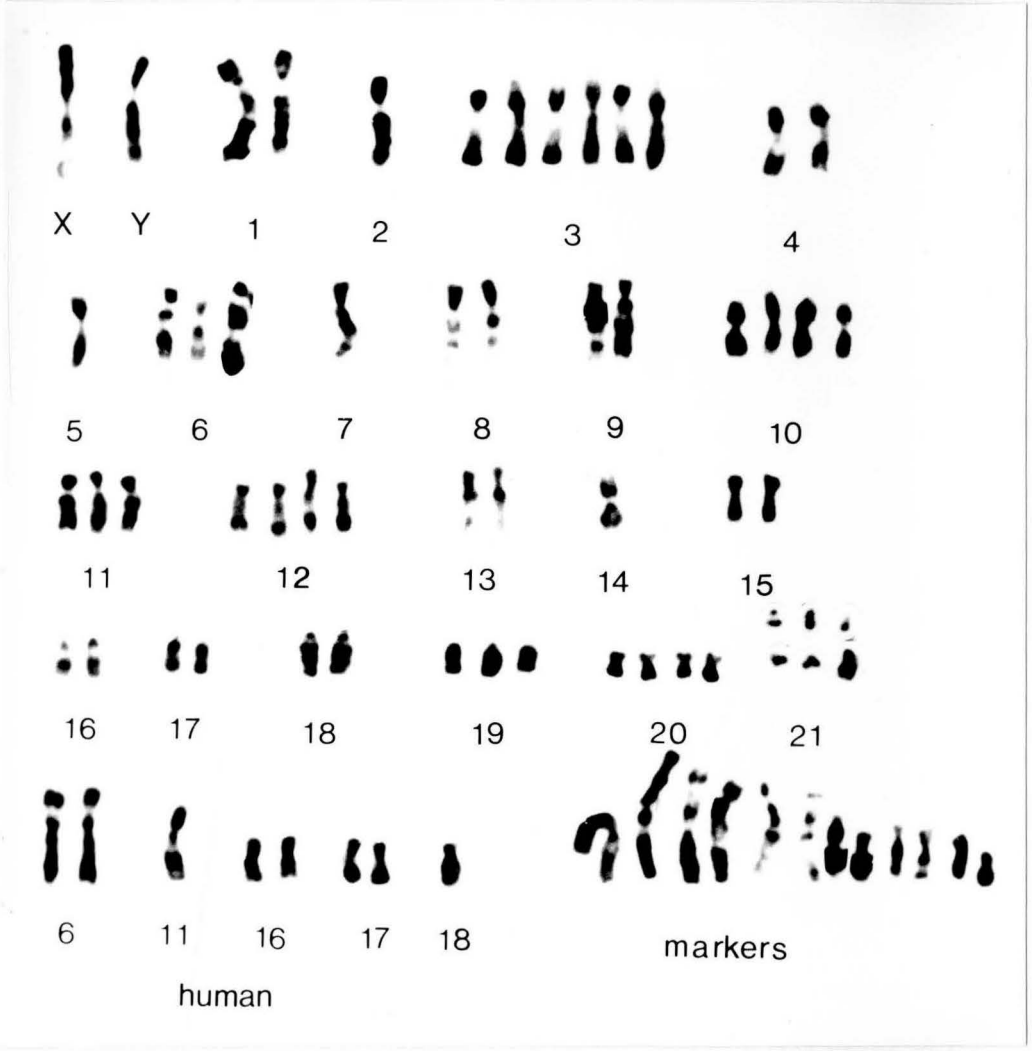

X
Y
1
2
3
4
5
6
7
8
9
10
11
12
13
14
15
16
17
18
19
20
21
6
11
16
17
18
human
markers

Figure 9. Karyotype from trypsin-Giemsa G banding preparation of the BHK-B1 x HEK hybrid Clone 4.

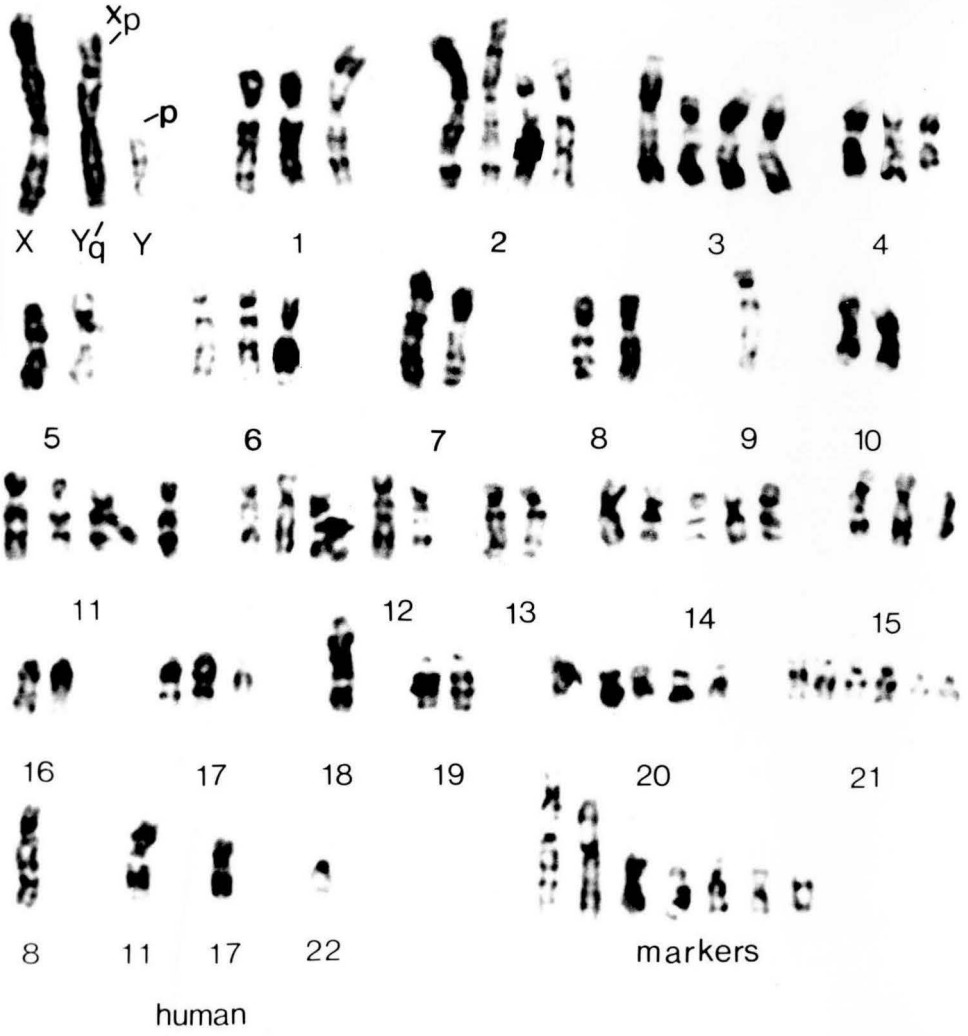
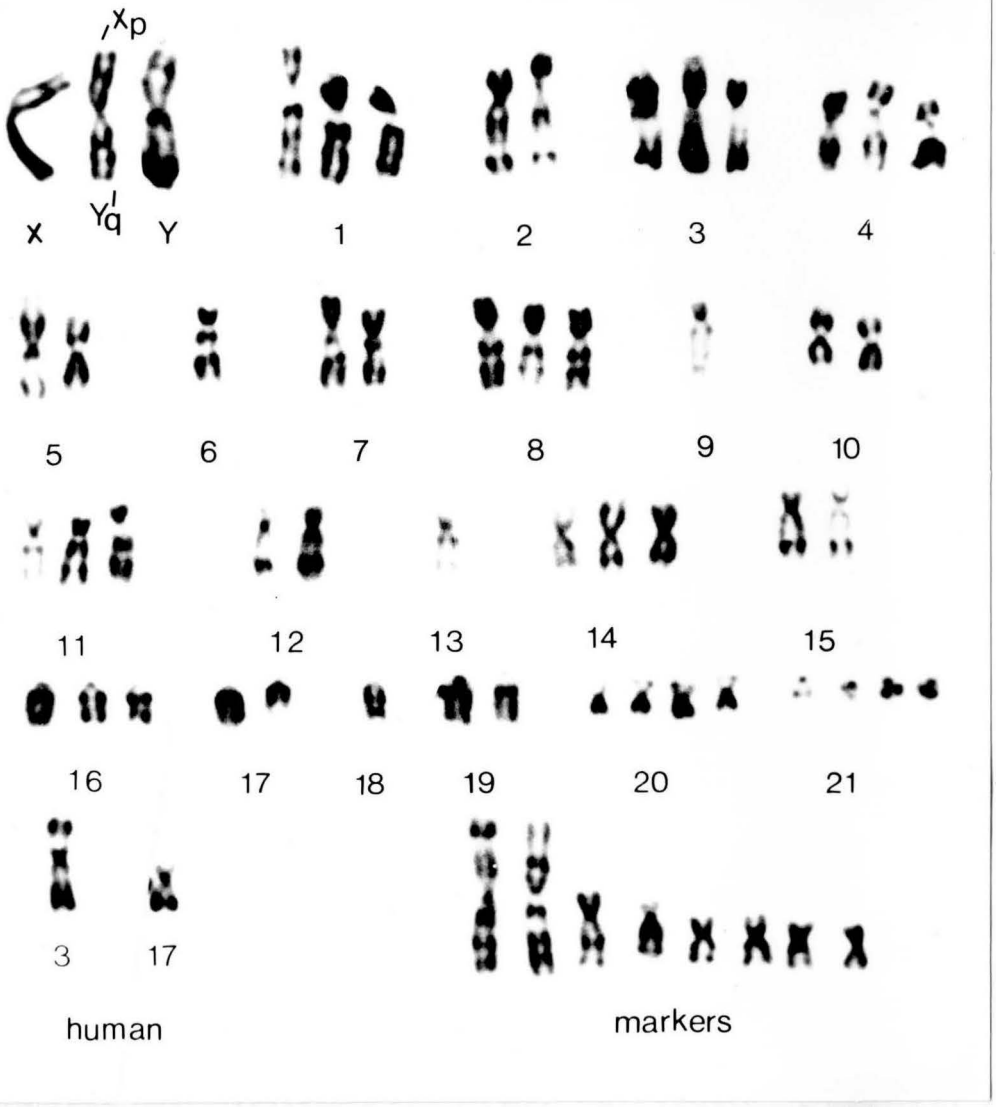

Xp
p
X
Yq
Y
1
2
3
4
5
6
7
8
9
10
11
12
13
14
15
16
17
18
19
20
21
8
11
17
22
markers
human

Figure 10. Karyotype from trypsin-Giemsa G banding preparation of the BHK-B1 x HEK hybrid Clone 5.


Xp
X
Yq
Y
1
2
3
4
5
6
7
8
9
10
11
12
13
14
15
16
17
18
19
20
21
3
17
human
markers

chromosome number of 77 (Table 4). Twelve marker chromosomes were present (Figure 8). Fifty-seven hamster chromosomes were identified, with every chromosome being present in at least one copy. Eight human chromosomes were present. Chromosome number 11 and 18 were identified, along with pairs of human chromosome numbers 6, 16, and 17.

The modal chromosome number of BHK-B1 x HEK hybrid Clone 4 was 78, with a range of 59 to 88 (Table 4). Seven marker chromosomes were present (Figure 9). Sixty-seven hamster chromosomes were identified, with at least one copy of every chromosome being present. The X chromosome p arm and Y chromosome q arm fusion product was also present. Four human chromosomes, numbers 8, 11, 17, and 22, were identified.

BHK-B1 x HEK hybrid Clone 5 modal chromosome number was 62, with a range of 51 to 83 (Table 4). Eight marker chromosomes were present, along with 52 hamster chromosomes (Figure 10). At least one copy of every chromosome was identified. The X chromosome p arm and Y chromosome q arm fusion product was also present. Two human chromosomes, numbers 3 and 17, were identified.

The BHK-B1 x HEK hybrid cells were more difficult to lyse. Intact nuclei were often present in the metaphase spread preparations. Cell membrane characteristics different from those of the parental cells due to cell fusion could have accounted for the difficulty in lysing the cells. Therefore, the hypotonic treatment time was doubled to increase the number of cells undergoing lysis. Since the chromosome complements of the hybrid cells contained approximately twice as many chromosomes as those of the parental cells, it was difficult to find metaphase cell preparations that were well spread with few overlapping

chromosomes and at the same time, well banded.

C. Response of BHK-B1 x HEK Hybrid Clones to BKV Adsorption.

Once the clones were verified as being hybrid cells, response of the clones to BKV adsorption was studied. It was hoped that a range of phenotypic expression would occur as a result of cell interactions with BKV. Following infection with BKV, cells were plated for productive infection in monolayers or for transformation in agar suspension.

1. Lytic Infection.

Hybrid clones were infected with BKV to determine if BKV could multiply in this system. BKV infection of the clones at 10 pfu per cell in HAT medium plus ouabain resulted in productive infection (Table 5). Lytic infection occurred at low and high passage levels, with CPE characteristic of BKV appearing 4 to 5 days post infection. Virus resulting from productive infection of hybrid Clone 1 was capable of reinfecting HEK cells. Neutralization by viral specific antisera of virus isolated from the clones occurred in the HI (Table 6). Virus samples for buoyant density determination were collected by side puncturing the tubes. Purified virus from the hybrid clones had buoyant densities in CsCl at 25°C consistent with that of wild-type BKV, 1.34 g per ml (Table 7). Purified virus from hybrid Clone 1 was collected by gradient fractionation. Optical density readings of the fractions at 260 nm and density readings in CsCl at 25°C of the virus-containing fractions were used to further characterize this virus (Figure 11). Virus lysates from BKV infections of the clones at both passage levels were tested by plaque assay using HEK cells. Undiluted lysates and 10^{-1} dilutions lysed the cell monolayers. These results provided evidence that BKV propagated efficiently

* Following cell hybridization, the hybrid clones were isolated and propagated in selective medium. Hybrid clones were infected with BKV at low and high passages. BKV infected cells were plated for transformation in agar suspension or for productive infection in monolayer culture.

1. Transformation frequency is determined as the number of colonies which grow to 0.5mm or greater in 2 weeks following virus treatment divided by the number of cells plated per 60mm plate x 100. 2×10^4 uninfected or BKV infected cells were plated per 60mm culture dish in HAT medium plus ouabain.

2. CPE was noted in cultures 4 to 5 days post infection. Cultures were harvested 7 days following BKV infection. Virus was isolated by equilibrium sedimentation and buoyant density centrifugation.

Table 5. Response of BHK-B1xHEK hybrid clones to BKV infection.*

Clone Identification	Low Passage			High Passage		
	Transformation[1] Frequency		Productive[2] Infection	Transformation Frequency		Productive Infection
	uninfected	50 pfu/cell	10 pfu/cell	uninfected	50 pfu/cell	10 pfu/cell
1	$<2.5\times10^{-4}$%	$<2.5\times10^{-4}$%	(+)	$<2.5\times10^{-4}$%	0.64%	(+)
2	$<2.5\times10^{-4}$%	$<2.5\times10^{-4}$%	(+)	0.44%	1.02%	(+)
3	$<2.5\times10^{-4}$%	$<2.5\times10^{-4}$%	(+)	0.08%	0.43%	(+)
4	$<2.5\times10^{-4}$%	$<2.5\times10^{-4}$%	(+)	0.01%	0.58%	(+)
5	$<2.5\times10^{-4}$%	$<2.5\times10^{-4}$%	(+)	0.01%	0.30%	(+)

Table 6. Serologic properties of virus produced from BKV infection of BHK-B1xHEK clones.

	Clone Identification									
	1	2	3	4	5	1	2	3	4	5
	Low Passage					High Passage				
HA titer[1] of cell lysate per ml of sample	256	256	512	128	128	512	256	1024	256	256
HI[2] of purified virus using a 1 to 5 dilution of rabbit hyper-immune anti-BK virion antisera	(+)	(+)	(+)	(+)	(+)	(+)	(+)	(+)	(+)	(+)
Titer of Normal Rabbit Serum	<2									
Titer of Normal Horse Serum	<2									
Titer of Anti-SV40 Specific Horse Antisera	16									

1. Reciprocal of dilution.

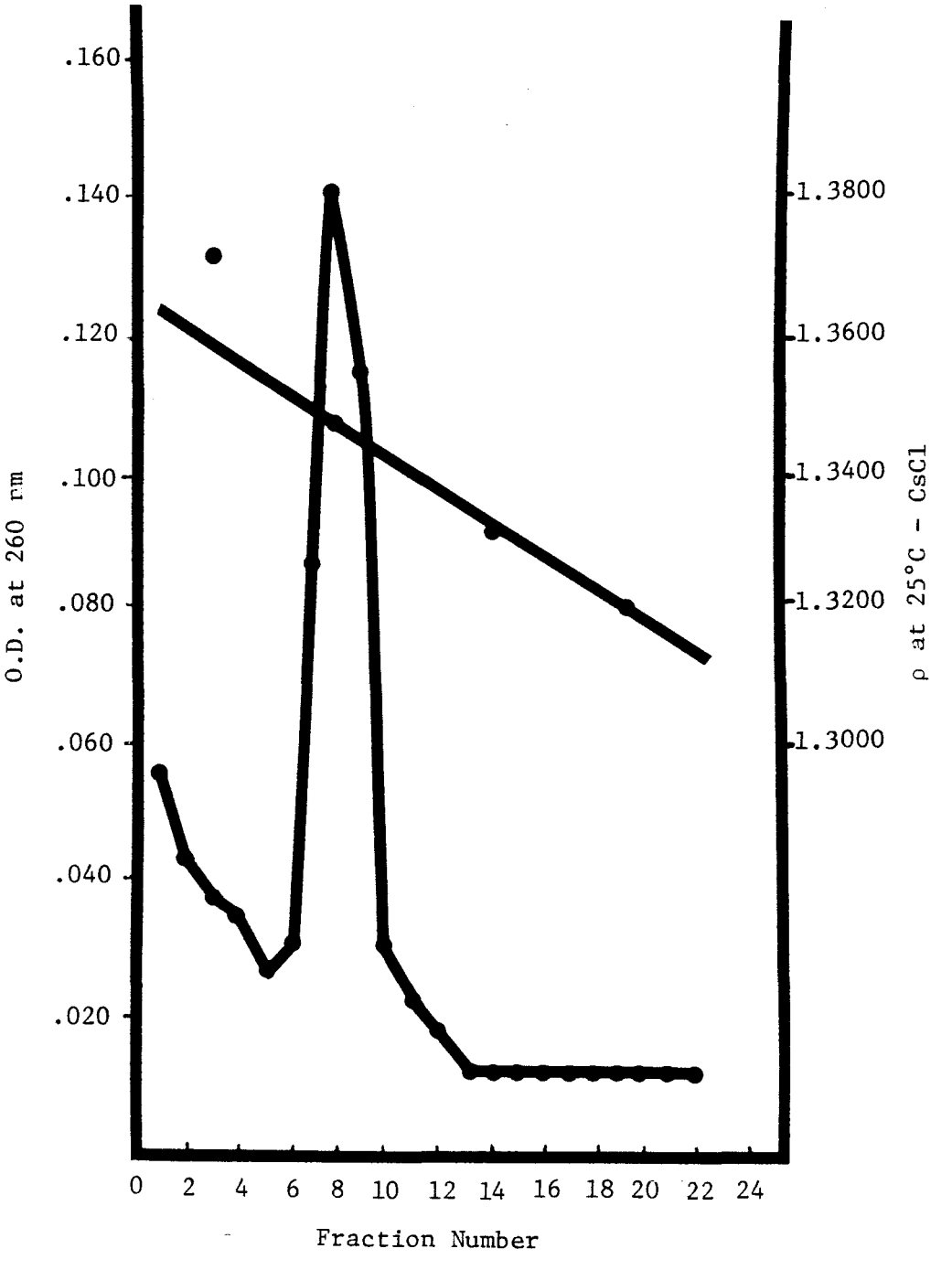
2. HI assay performed using 8HA units of CsCl purified virus based on above HA titers.

Table 7. Buoyant density at 25°C in CsCl of virus[1] isolated from BHK-B1xHEK hybrid clones infected with 10 pfu of BKV per cell.

Hybrid Clone Identification	Buoyant Density (ρ)	
	Low Passage	High Passage
1	1.3484	1.3494
2	1.3484	1.3429
3	1.3473	1.3462
4	1.3462	1.3505
5	1.3483	1.3484

1. Collection of virus samples for density determinations performed by side puncture of tubes.

Figure 11. CsCl equilibrium centrifugation of virus isolated from BHK-B1 x HEK hybrid Clone 1 infected with 10 pfu of BKV per cell. Collection of virus was performed by gradient fractionation. Optical density readings of the fractions at 260 nm and density readings in CsCl at 25°C of virus-containing fractions were used to characterize the virus. This virus was established as being similar to wild-type BKV based on its buoyant density at equilibrium.


.160
.140
.120
.100
.080
.060
.040
.020
O.D. at 260 nm
1.3800
1.3600
1.3400
1.3200
1.3000
ρ at 25°C - CsCl
0 2 4 6 8 10 12 14 16 18 20 22 24
Fraction Number

in the hybrid clones, and that wild-type BKV was purified from the lysates following infection of the BHK-B1 x HEK hybrid clones with BKV.

2. Transforming Infection.

Anchorage independent growth is considered the most important in vitro criteria when determining malignant transformation of cells. Growth in agar suspension correlates with in vivo oncogenicity. Transformation assays are based on the observation that malignantly transformed cells are anchorage independent for growth whereas normal cells maintain anchorage dependent growth (Macpherson and Montagnier, 1964; Shin et al., 1975).

Transformation assays demonstrated that BHK-B1 and BHK-B1 Clone 6 cells, like BHK-21 cells, could be stably transformed (Table 8). BHK-B1 Clone 6 was unable to grow in agar suspension and was selected for use in the assay because of this property. Normal BHK cells are anchorage dependent for growth whereas virus transformed cells are anchorage independent for growth. BKV transformed BHK-B1 cells were cloned from agar suspension and tested for their ability to grow in selective media. Cells were able to propagate in medium containing BrdU but were unable to grow in HAT medium. This indicated that the BHK-B1 cells remained TK$^-$ following transformation by BKV.

Transformation of the hybrid clones did not effectively occur at the lower passage level (Table 5). An approximately equal percentage of background colonies appeared when BKV infected or uninfected cells were plated in agar suspension. The colonies were less than 0.5 mm in diameter. Colony diameters of 0.5mm or greater indicate stable

Table 8. BKV transformation frequency[1] of BHK-B1 cells in agar suspension.

Cell Line	uninfected	50 pfu/cell
BHK-21	$<2.5 \times 10^{-4}$%	0.18% (ave 35 colonies per plate)
BHK-B1	$<2.5 \times 10^{-4}$%	0.19% (ave 38 colonies per plate)
BHK-B1 Clone 6	0.03% (average 6 colonies per plate)	0.16% (ave 32 colonies per plate)

[1] Transformation frequency is determined as the number of colonies which grow to 0.5mm or greater in 2 weeks following virus treatment divided by the number of cells plated in 60mm culture dishes x 100. 2×10^4 virus infected or uninfected cells were plated per culture dish.

transformation of the cells. Small background colonies are not uncommon in transformation assays utilizing BHK cells (Marin, 1980). When cloned in liquid medium, these clones failed to plate in 35mm cloning dishes containing HAT medium plus ouabain. Therefore, stable viral transformation of the BHK-B1 x HEK hybrid clones did not occur at the lower passage level.

BKV transformation of the five hybrid clones occurred at the higher passage level (Table 5). Colonies growing in agar suspension had diameters greater than 0.5mm and appeared stably transformed. These colonies were cloned and expanded in liquid culture containing HAT medium plus ouabain. Isolated clones remained viable and phenotypically transformed, growing to confluency in 35mm dishes containing liquid medium supplemented with HAT and ouabain. Upon reaching confluency, these clones were passed into 60mm plates containing the selective medium. Approximately half of these clones failed to plate following passage. Other clones remained viable but did not reach confluency. Cells grew anchorage dependently, underwent a few cell divisions, and then detached from the surface of the culture dishes before reaching semiconfluency. Clones that reached confluency in 60mm culture plates were transferred to 100mm dishes containing HAT medium plus ouabain. These clones could not be passaged more than one time following transfer to 100mm plates, and none of these clones reached semiconfluency. Cells grew anchorage dependently, underwent a few cell divisions, and then detached.

To further study the observed growth characteristics, colonies were picked from soft agar, dispersed in selective medium, and plated

both in liquid culture and agar suspension. All clones grew in agar suspension, with colony diameters being greater than 0.5mm. The size of the colonies indicated that the cells were stably transformed. To reach this size, cells had to undergo many divisions while growing anchorage independently. Cells also grew to confluency in liquid culture in 35mm cloning dishes. Once again, these clones could not be successfully passaged after transfer to 60 or 100mm plates containing selective medium. Cells attached, underwent a few cell divisions, and then detached from the surfaces. Cells remained viable for 24 to 48 hours following detachment as indicated by their ability to exclude trypan blue dye.

These observations were unexpected and puzzling. Other experiments were performed in order to better understand the above results. An attempt was made to grow the detached clones in suspension culture to determine if the BKV transformed hybrid clones were constitutively anchorage independent for growth. Due to laboratory limitations, it was difficult to establish these cultures. Suspension medium, Minimum Essential Medium (MEM) lacking magnesium and calcium ions, was unavailable. HAT medium requiring an 8% CO_2 concentration was utilized. Therefore, the Spinner culture flask had to be adapted to allow CO_2 to pass through the medium while cells were being maintained in suspension. The experimental conditions were not optimal for establishment of the cells in suspension culture.

It is possible that the BHK-B1 x HEK hybrid cells could be semi-permissive for BKV infection. A transitory state of cell transformation could be established. Virus could later be released, resulting

in lysis of the cells. This has been shown to occur in polyoma and SV40 systems. Populations of BHK cells have been shown to be semi-permissive rather than absolutely nonpermissive for polyoma (Fraser and Gharpure, 1963; Bourgaux, 1964; Folk, 1973). Semipermissiveness for SV40 has been demonstrated in human and Syrian hamster cells (Burns and Black, 1968, 1969; Rothschild and Black, 1970; Dubbs and Kit, 1971; Boyd and Butel, 1972; Butel et al., 1972; Kaplan et al., 1972).

In order to rule out viral lysis of the cells as the reason for detachment, culture supernatants were analyzed for the presence of BKV. Supernatants containing the detached cells were collected. Following centrifugation, cells were lysed by resuspending the pellets in 0.6% sodium dodecyl sulfate (SDS) in TES buffer (10mM Tris, 10mM EDTA, 10mM NaCl), pH7.2. The method of Hirt (1967) was used to extract DNA from the lysed cells. Extracted DNA was separated by agarose gel electrophoresis. No BKV DNA was detected. Culture supernatants were also tested by HA for the presence of BKV. No hemagglutination was observed. These results could rule out cell lysis by activation of the inherent viral genome and virus multiplication as the cause of cell detachment.

3. Presence of T Proteins.

Indirect immunofluorescence was performed on the BKV transformed hybrid clones at the higher passage level to detect the expression of the BKV nuclear T proteins. All hybrid clones tested were negative by immunofluorescence for the T proteins. SV80 cells, human fibroblasts transformed by SV40, were used as the positive control and

exhibited 4+ nuclear fluorescence. Negative controls included BHK cells and hybrid clones not transformed by BKV. These cells displayed no nuclear fluorescence. BHK-B1 cells transformed by BKV exhibited 1+ nuclear fluorescence, which is typical of BKV transformed BHK cells.

The assay was not performed using confluent cell monolayers. Clones were not successfully grown to confluency, as discussed previously. Assays were performed when the cells had plated and undergone a few cell divisions, although cells were beginning to detach. The cells remaining attached to the coverslips appeared well fixed by the acetone.

In summary, the results presented above report the successful isolation of interspecies hybrids between human embryonic kidney and Syrian hamster cells. The selection system utilizing HAT medium plus ouabain was based on properties of the parental BHK-B1 and HEK cells. Cell fusion was achieved with PEG-DMSO, and five hybrid clones were successfully expanded in culture. Phenotypic characteristics and karyotype analysis were used to verify the hybrid nature of the clones. BKV lytic infection of the hybrid cells occurred at low and high passage levels with the virus being purified from all clones. Transformation of the hybrid cells only occurred at the high passage level. The biology of the tranformed cells proved to be complicated. The cells could not be propagated in liquid culture, indicating that the transformed clones might be anchorage independent for growth. Although the cells appeared stably transformed when cultured in soft agar, no expression of the T proteins as detected by indirect immunofluorescence was evident. The phenotypic characteristics displayed by the BHK-B1 x HEK hybrid cells in response to BKV must be studied in greater detail to explain the biology of this system.

DISCUSSION

The most significant accomplishment of this thesis is the isolation of unique interspecies hybrids between Syrian hamster cells and human embryonic kidney cells. The selection system utilized in hybrid clone isolation and propagation, the fusion protocol, characteristics of the hybrid cells, and the response of these cells to BKV lytic and transforming infections comprise the component parts of this work.

The biology of established cell lines and their use in constructing somatic cell hybrids is complex. Therefore, experiments utilizing such cells can become difficult to design. In our investigation, for example, a continual cell line such as the BHK-B1 line does not maintain a constant complement of 44 chromosomes. Cells often do not remain diploid, becoming pseudodiploid, aneuoploid, or tetraploid. Numerous chromosomal rearrangements occur, with translocations of entire arms of chromosomes being common. This was evident in our BHK-B1 cells (Figure 4). Chromosome identification by standard banding techniques then is more difficult in established cell lines because standard chromosome banding patterns may be slightly altered in continual cell lines due to passage in culture.

In contrast, human diploid cells in culture maintain a constant chromosome complement of 46, with essentially no variation from the diploid state. No HEK cells had fewer than 44 chromosomes. Loss of chromosomes during preparation of the metaphase spreads would account for the incomplete chromosome complements. Few chromosomal rearrangements occur in human diploid cells. This was evident in our HEK cells

as no translocations were present (Figure 5). In this case, standard banding techniques are very useful in identifying human chromosomes in diploid cells.

Somatic cell hybrids contain a single nucleus composed of functioning chromosomes from both parental cells (Bernhard, 1976; Croce, 1980). Duplication or triplication of one chromosome complement initially occurs after cell fusion. This initial multiplication of one genome is followed by a rapid, extensive, irregular elimination of chromosomes from the other parental cell complement. In hybrids between human and rodent cells, preferential loss of human chromosomes has most often been demonstrated (Bernhard, 1976; Ozer and Jha, 1977). Our BHK-B1 x HEK hybrid clones are an example of this phenomenon, with hamster chromosomes being present in high copy number (Figures 6, 7, 8, 9, 10). As many as six or eight copies of some hamster chromosomes were present in the hybrid clones. Loss of human chromosomes occurred in the BHK-B1 x HEK hybrid clones during propagation, with few human chromosomes being retained in these cells (Figures 6, 7, 8, 9, 10). Hybrid Clone 1 retained single copies of human chromosome numbers 3, 6, 8, and 18 along with a pair of human number 17 chromosomes. Single human chromosome numbers 11, 16, and 17 were identified in Clone 2. Clone 3 retained single copies of human chromosome numbers 11 and 18 and pairs of chromosome numbers 6, 16, and 17. Clone 4 retained single copies of human chromosome numbers 8, 11, 17, and 22. Single copies of chromosome numbers 3 and 17 were identified in Clone 5.

Chromosome pulverization in these interspecies hybrids could account for the extensive loss of human chromosomes. Pulverization

results from premature chromosome condensation of the interphase genome (Kato and Sandberg, 1968; Johnson and Rao, 1970; Rao, 1977) and occurs due to initial mitotic asynchrony of the nuclei of multi-nucleated cells (Johnson and Rao, 1970; Rao and Johnson, 1972, 1974; Sperling and Rao, 1974). The small elements of chromosomal material coding for essential gene products, such as the human thymidine kinase in the BHK-B1 x HEK hybrid cells, would be maintained during cell propagation in selective medium. Detection of these chromosomal fragments by standard banding techniques would be impossible as they would not be evident by karyotype analysis.

Chromosome loss may also be directed by spindle formation in the somatic cell hybrids. Retention of one species of chromosomes would occur because of the prevalence of that parental cell's spindle fibers. The failure of the other genome to replicate or interact correctly with the spindle apparatus would result in extensive chromosome loss (Bernhard, 1976).

As is evident by analysis of the BHK-B1 x HEK hybrid cell karyotypes, clones containing different chromosome complements can be isolated. The copy number of the BHK-B1 chromosomes varied greatly. The copy number of eight hamster chromosomes, numbers 7, 8, 9, 13, 15, 16, 18, and 19, was fairly consistent within the hybrid clones and with the copy number of these chromosomes present in the BHK-B1 modal chromosome complement. Six hamster chromosomes, numbers 3, 11, 12, 14, 20, and 21, were generally present in high copy number in the hybrid clones. Three of the hybrid clones had one hamster chromosome, number 5, 10, or 17, present in a much higher copy number when compared to the modal

chromosome complement. The specific chromosomes were present in the other clones in copy numbers similar to those of the BHK-B1 modal chromosome complement. Hamster chromosome numbers 1, 2, 4, and 6 were present in slightly elevated copy number. The copy numbers of the X and Y chromosomes were similar to those in the BHK-B1 modal chromosome complement. The human chromosome complement retained differed in each of the five clones. Unfortunately, there is little known about the gene map of the Syrian hamster. Most gene products of these chromosomes have not been identified. The five hybrid clones did not retain the same human chromosomes, except number 17, as previously discussed (Table 4). Chromosome number 11 was identified in three of the five clones, chromosome numbers 3, 6, 8, 16, and 18 in two clones each, and chromosome number 22 in one clone. This allows study of individual chromosome functions and determination of chromosome retention patterns. Isolation of several BHK-B1 x HEK hybrid clones containing different chromosome complements would allow evaluation of host cell contributions to the regulation of BKV early gene expression. Permissiveness and nonpermissiveness of the hybrid clones to BKV infection could then be related to chromosome content.

A possible reason why the BHK-B1 x HEK hybrids retained specific human chromosomes was that these chromosomes may have conferred selective growth advantages on the cells. Human chromosome number 17 carrying the gene coding for the soluble thymidine kinase was selected for by use of HAT medium. This gene was vital for maintaining hybrid cell viability under conditions imposed by this selection system. As expected, this chromosome was present in all five clones as demonstrated

by karyotype analysis. Only those human chromosomes or fragments of chromosomes necessary for maintaining viability and conferring growth advantages may have been retained. Loss of the other nonselected human chromosomes would not have been lethal to the hybrid cells because hamster vital genes were present in high copy number. The gene maps of the human chromosomes identified in the BHK-B1 x HEK hybrid cells are listed in Appendix B (Sandberg, 1980). It is possible that the human chromosomes retained in the hybrid clones, but not specifically selected for, conferred growth advantages on the cells. These chromosomes in conjunction with a specific hamster chromosome complement may be important in maintaining the viability of the hybrid cells. These chromosomes may somehow increase the potential for growth of the hybrid cells.

The identification of different combinations of human chromosomes in numerous hybrid clones under identical selective pressure would indicate that the chromosomes were not retained due to the influence of the selection system. These chromosomes probably would not confer selective growth advantages on these cells. The mechanism of cell hybridization could be responsible for retention of these chromosomes. Consistent combinations of chromosomes identified in numerous clones could be attributed to the selection system or to the nature of the hybrid cells. If these chromosomes were retained because of the conditions imposed by the selection system, then changes in selection conditions should result in alteration of the human chromosome complements maintained. Retention of the same combinations of chromosomes under different selective conditions would implicate the nature of the

hybrid cells in regulation of chromosome elimination. In order to attribute the patterns of chromosome retention in the BHK-B1 x HEK hybrid clones to the conditions imposed by the selection system or to the mechanism of cell hybridization, more clones would have to be isolated and the combinations of human chromosomes present identified. The selection system using HAT medium plus ouabain would have to be altered, and the combinations of human chromosomes retained under various conditions determined.

It is interesting to note that human chromosome number 17 was found to carry the integration and transformation sites for SV40 in GM-54 VA cells, a human cell line transformed by SV40, when these cells were fused with mouse cells (Croce, 1977). As discussed previously, human chromosome number 17 was the only chromosome retained by all the hybrid clones, due to continual selective pressure. It would be interesting to specifically study this chromosome in relationship to BKV lytic and transforming infections.

Once the hybrid nature of the cells had been established, primarily by karyotype analysis, the preliminary investigations of BKV early gene regulation were initiated. The system utilized somatic cell hybrids between a permissive host capable of BKV multiplication and a nonpermissive host capable of being transformed by BKV. The response of the BHK-B1 x HEK hybrid cells to BKV adsorption was studied. Lytic infection occurred at the low and high passage levels, whereas transforming infection occurred only at the high passage level (Table 5). BHK-B1 x HEK hybrid Clone 1 was also lytically infected with BKV at intermediate passage levels. It was possible that specific combinations

of hamster and human chromosomes determined the response of the cells to BKV adsorption. The majority of human chromosomes may not have been segregated at the lower passage level. Therefore, the influence of these permissive host chromosomes may have allowed lytic infection to occur. The cells that had segregated most of the human chromosomes at this time would have been in very low concentration within the population. Transformation of these cells may not have been detected by our assay procedure. At the higher passage level, some cells may have segregated enough human chromosomes to allow BKV transformation to occur. This would have resulted from the influence of the BHK-B1 chromosome complement present in the hybrid cells. The concentration of cells within the hybrid population containing fewer human chromosomes would have greatly increased by the high passage level. Therefore cell transformation would have been detected utilizing our assay system.

Another explanation for the occurrence of lytic infection at both passage levels and transforming infection only at the high passage level may be that BHK-B1 cells, and therefore, the BHK-B1 x HEK hybrid clones, are semipermissive for BKV infection. As discussed previously, semipermissiveness exists in polyoma and SV40 systems. Our laboratory has found that some BHK-B1 cells can be lytically infected using 10 pfu of BKV per cell. Evidence for this was the presence of viral CPE and cell lysis 4 to 5 days post infection. Virus was purified from these cultures by equilibrium sedimentation and buoyant density centrifugation. This purified virus was neutralized by virus specific antisera in the hemagglutination inhibition assay and had a buoyant density in CsCl at 25°C characteristic of wild-type BKV. Therefore, we feel that the

purified virus was BKV. Our laboratory also demonstrated that BHK-B1 cells could be transformed at approximately the same frequency as BHK-21 cells (Table 8). The cells were stably transformed as shown by their ability to propagate in liquid medium when cloned from soft agar suspension. Defective virus particles were not responsible for BHK-B1 cell transformation. This was demonstrated by rescue of infectious BKV from BKV-BHK-B1 Clone 13 following cell fusion with HEK cells. Therefore, due to these experimental results it was felt that our BHK-B1 cells contained subpopulations of cells permissive or nonpermissive for BKV infection. The permissive BHK-B1 cells probably were present in low concentration within the population. It may therefore be possible to correlate the BHK-B1 karyotype with the permissiveness or nonpermissiveness of the cell to BKV infection. Specific factors such as chromosome copy number, the loss of certain chromosomes, or chromosomal rearrangements may have influenced the BHK-B1 cell's response to BKV infection.

It should therefore be possible to establish subclones of BHK-B1 cells and determine their permissiveness or nonpermissiveness to BKV infection. Clones transformed by BKV should not allow BKV lytic infection to occur. Extensive subcloning would have to be performed to isolate and characterize such clones. Once these subpopulations of BHK-B1 cells were established, they could be utilized in future cell hybridization experiments to study BKV early gene expression. The absolute permissiveness or nonpermissiveness of the BHK-B1 cells would be established by subcloning and determining each clone's response to BKV adsorption.

Assuming that the BHK-B1 cells did contain subpopulations of nonpermissive and permissive cells, then fusion of these cells with HEK cells permissive for BKV infection would result in subpopulations of cells expressing different phenotypes. Hybrids between two permissive cells or between a nonpermissive and permissive cell would be present. This would result in different responses of the cells to BKV infection. Cell hybrids between two permissive cell types such as permissive BHK-B1 and HEK cells would lytically infect at low and high passage levels. It may be an inherent property of some of these hybrid cells to undergo transformation at some point in passage (Marin, 1980). BHK-B1 x HEK hybrid Clone 2 may be an example of a clone expressing this phenotype. A high frequency of transformation occurred both in BKV infected and noninfected cells at the high passage level (Table 5). Some alteration in the BHK-B1 chromosome complement with passage may have resulted in the cells being able to undergo viral transformation.

If fusion between a nonpermissive BHK-B1 cell and permissive HEK cell occurred, then it would be important to consider the influence of the chromosome complement on the response of the hybrid cells to BKV infection. The karyotypes of the cells would have to be examined at every passage level to determine which human chromosomes segregated and to identify the hamster chromosome complement. A loss of most human chromosomes from some cells in the population may have occurred at the higher passage level. This could have resulted in these cells becoming transformed at this passage level. The contribution of both parental genomes would be important in determining the cell's response to BKV lytic or transforming infection.

It would also be important to study the karyotypes of the hybrid cells during lytic and transforming infections. This may identify which chromosomes were responsible for the permissiveness or nonpermissiveness of the cells to BKV infection. It may be possible to subclone these populations of cells. Permissive and nonpermissive hybrid clones would result. Therefore, it is critical to our experiments to be able to propagate the BKV transformed BHK-Bl x HEK hybrid cells. It may be that once transformed by BKV, these hybrid cells are constitutively anchorage independent for growth. Therefore suspension cultures would have to be utilized. Once the karyotypes of the transformed and lytically infected hybrid clones are determined, it may be possible to subclone populations of cells on the basis of karyotype before infection with BKV. Subcloned hybrid cell populations would each be tested for response to BKV lytic and transforming infections. Either transformation or permissive infection should occur if subpopulations of cells were successfully cloned. The response of BHK-Bl x HEK hybrid subclones to BKV infection would be evaluated on the basis of chromosome complement. The use of karyotype analysis provides another approach to the study of the regulation of BKV early gene expression.

The valuable insight into viral gene regulation provided by such an experimental approach warrants continuing our study of BKV transforming and lytic infections in the BHK-Bl x HEK hybrid cells. A brief discussion of other investigations and the information obtained from similar systems will focus attention on what experiments could be performed using our somatic cell hybrids.

1. Expression of the large T protein and rescuability of the SV40 genome in somatic cell hybrids between SV40 transformed human cells and mouse cells, segregating human chromosomes, correlated with the presence of human chromosome number 7 (Croce et al., 1974, 1975, 1976). This chromosome was also responsible for expression of the transformed phenotype *in vitro* and for the ability of the hybrid cells to form tumors *in vivo*. The SV40 integration site was assigned to human chromosome number 17 in cell hybrids between another SV40 transformed human cell line and mouse cells (Croce, 1977). T protein expression and tumorigenicity *in vivo* correlated with the presence of this chromosome.

2. Cell hybrids between mouse cells and spontaneous yielder SV40 transformed hamster cells, segregating hamster chromosomes, were isolated to investigate the maturation of SV40 (Suarez et al., 1978). Hybrid cells were positive for T protein production, but infectious SV40 DNA was only occasionally detected due to the inability of the hybrid cells to correctly activate the integrated SV40 genome. This result could not be attributed to loss of essential hamster chromosomes or to the presence of inhibitory mouse components. Superinfection of the hybrid cells with SV40 did not result in virus assembly although capsid proteins were synthesized. This system is being utlilized to more thoroughly investigate the dependence of SV40 maturation on cellular functions.

3. Somatic cell hybrids between embryonal carcinoma cells (ECC) and SV40 transformed murine cells were utilized to elucidate the nature of the host cell range restriction to SV40 early gene expression in ECC

(Balint et al., 1980). The hybrid cells were positive for T protein production, indicating that the host range restriction to SV40 in ECC was a recessive property, with the required cellular functions for SV40 early gene expression not being present. The regulation of viral transforming genes during cell differentiation and development is being evaluated. This system should provide a means of determining if the block in the expression of the SV40 early gene products occurs at the transcriptional or post-transcriptional level.

4. The role of the SV40 A gene product in initiation of cellular DNA synthesis was investigated by isolation of somatic cell hybrids between mouse kidney cells transformed by a SV40 tsA mutant and dormant chick erythrocytes (CE) (Dubbs and Kit, 1977; Dubbs et al., 1978). SV-40 transformed mouse cells, depleted of T protein by growth in medium containing a reduced serum level, fused with CE activated CE DNA synthesis before the CE nuclei became positive for T protein production. These results indicated that the SV40 A gene product probably was not the direct initiator of cellular DNA synthesis.

5. Primate factors involved in determining susceptibility to SV40 lytic infection were studied by use of somatic cell hybrids between cercopithecoid monkey and Chinese hamster cells (Garver et al., 1980). The cell hybrids differed in their primate chromosome complement and in their susceptibility to SV40 infection. The presence of rhesus monkey chromosome 11 or African green monkey chromosome 12 correlated with elevated SV40 replication in the hybrid cells. The ability of the hybrid cells to rescue virus from SV40 transformed rodent cells was correlated with the presence of these chromosomes.

6. In hybrids between mouse and human cells, rRNA genes of both species are present although only the rRNA of the dominant species is expressed (Soprano et al., 1979, 1980). Infection of these hybrids with SV40 resulted in reactivation of the silent rRNA genes. SV40 reactivation of the silent rRNA genes required the presence of the large T protein, although sequences of the A gene from 0.27 to 0.17 and from 0.67 to 0.39 were not necesary for reactivation (Soprano et al., 1981). This investigation indicated that certain cellular functions were suppressed in somatic cell hybrids. The mechanism of cell transformation is being studied in this system since the transforming genes of SV40 can activate silent genes.

All of the systems discussed utilized somatic cell hybrids to evaluate the regulation of viral gene expression, factors influencing expression, and functions of the gene products. The rationale for hybridization of BHK-B1 and HEK cells as another means of studying the expression of the BKV T proteins and the regulation of this expression is strongly supported by the results of these investigations.

What is necessary for continuation of our study is the determination of which human chromosomes remain in the BHK-B1 x HEK hybrid cells and which preferentially segregate in relationship to the hamster chromosome complement. The presence of specific human chromosomes along with the multiple copies of hamster chromosomes may allow productive infection of the hybrid cells to occur. Certain combinations of human and hamster chromosomes or specific hamster chromosome complements may determine the permissiveness or nonpermissiveness of the cells to BKV infection. The preferential loss of human chromosomes during

cell passage may correlate with the ability of the hybrid clones to become transformed at higher passage levels. More fusions of BHK-B1 and HEK cells must be performed, and more hybrid cells must be isolated, subcloned, and infected with BKV. In this way, clones retaining different numbers and combinations of chromosomes will be analyzed for their response to BKV adsorption. Viral gene regulation will be studied by infecting the subcloned hybrid cells with BKV and determining which parental cell predominates, resulting in lytic or transforming infection. Karyotype analysis of the hybrid cells must be performed at every passage in order to determine if there is a consistent pattern of chromosome loss during propagation or if chromosome loss is a random event. It should be possible to determine specifically which chromosome complements allow productive infection and which allow transformation to occur. Phenotypes of the BKV infected hybrid cells will be correlated with the preferential segregation of human chromosomes and the hamster chromosome complements. The dominance or recessiveness of the transformed phenotype will be determined and correlated with the presence of specific human chromosomes. Permissiveness and nonpermissiveness of cells to BKV infection will be related to human and hamster chromosome complements.

Evidence to date suggests that our BHK-B1 cells transformed by BKV express the small T protein but lack large T protein expression, as demonstrated by immunoprecipitation, gel electrophoresis, and autoradiography. HEK cells synthesize both T proteins during BKV lytic infection. Therefore, hybridization of BHK-B1 and HEK cells appeared to be the ideal system for studying BKV early gene expression. The introduction

of human chromosomes into BHK-Bl cells through cell hybridization may allow evaluation of the regulation of expression of both the large and small BKV T proteins during BKV transformation. Karyotype analysis of the hybrid clones at every passage should permit identification of those human chromosomes in relationship to the hamster chromosome complements that allow transformation and those that allow lytic infection to occur. We feel that correlation of BKV cell transformation with the presence or absence of the large and small T proteins should be possible. This correlation could then be related to the presence or absence of one or a combination of human chromosomes.

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APPENDIX A

Appendix A Cell Identification	Derivation	Source of Cells
HEK	-Human embryonic kidney -Primary and secondary cell strains	-Lab derived
BHK-21	-Baby hamster kidney -Male Syrian hamster fibroblasts -Continuous cell line	-Stoker and Macpherson (1964)
BHK-B1	-Subline of BHK cell line -Thymidine kinase negative -Established by growth in BrdU medium	-Meiss (personal communication) -Littlefield and Basilico (1966)
BKV-BHK-B1 Clone 13 Clone 16	-BKV transformed BHK-B1 cells -Anchorage independent growth	-Lab derived -Virus transformed
BKV-BHK-B1 Clone 13xHEK Clone 16xHEK	-BKV-BHK-B1 Clones x HEK fusions -Selection in HAT medium	-Lab derived -Somatic cell fusions
BHK-B1xHEK Hybrid Clone 1 Clone 2 Clone 3 Clone 4 Clone 5	-BHK-B1 x HEK fusions -Selection in HAT medium plus ouabain	-Lab derived -Somatic cell fusions

APPENDIX B

Appendix B — The gene map of each human chromosome present in the karyotypes of the BHK-B1 x HEK hybrid clones.

Chromosome #3	β-Galactosidase
	Glutathione peroxidase
	Herpes virus sensitivity
	Temperature-sensitive complement
Chromosome #6	Glyoxylase-1
	Major histocompatibility complex HLA-A, B, C, D
	C2, C4, C8
	Rogers blood group
	Chido blood group
	Properdin factor B
	Phosphoglucomutase-3
	Malic enzyme (soluble)
	Superoxide dismutase (mitochondrial)
	Glutamic oxaloacetic transaminase (mitochondrial)
	Pepsinogen
Chromosome #8	Glutathione reductase
Chromosome #11	Lactate dehydrogenase-A
	Lethal antigen
	Acid phosphatase-2 (tissue)
	Esterase-A_4
	Species antigen
Chromosome #16	Adenine phosphoribosyl transferase
	Thymidine kinase (mitochondrial)
	Cholinesterase (serum)
	α-Haptoglobin
	α-Hemoglobin
	Lecithin-cholesterol acyltransferase
Chromosome #17	Thymidine kinase (soluble)
	Acid α-glucosidase
	Galactokinase
	Adenovirus-12 chromosome modification site-17
	Collagen-1
	SV40 integration site
	SV40 transformation site
Chromosome #18	Peptidase-A
Chromosome #22	Ribosomal RNA
	Arylsulfatase-A
	Aconitase (mitochondrial)
	NADH diaphorase
	α-Galactosidase B

APPROVAL SHEET

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The final copies have been examined by the director of the thesis and the signature which appears below verifies the fact that any necessary changes have been incorporated and that the thesis is now given final approval by the Committee with reference to content and form.

The thesis is therefore accepted in partial fulfillment of the requirements for the degree of Master of Science.

4-28-81
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