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Transfection of Primary Kidney Cells with Mutated SV40 Tag DNA

Containing a CCKCR Intron-Derived Sequence

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ABSTRACT

Cholecystokinin-C receptor (CCKCR) is an alternatively spliced version of cholecystokinin-B receptor (CCKBR) found in pancreatic cells. CCKCR is uniquely expressed by cancer cells, making it an important target for immune therapy. Simian Virus 40 Large Tumor Antigen (SV40 Tag) has proven to be a powerful model in the study of cell-mediated immune responses to tumors. Our goal was to construct cells which express a derivative of SV40 Tag that incorporates the retained intron sequence from CCKCR to use as a tool in the study of cell-mediated immunity targeting this unique sequence. Two variations of plasmids containing mutated Tag DNA with a 20 codon replacement (20mer) from CCKCR intron 4 were prepared: containing either an F408A substitution in epitope IV (pCBKG/F408A) or wild-type (WT) epitope IV (pCBKG/WT). Sequencing confirmed the presence of the 20mer in place of epitope I through II/III, confirmed the appropriate sequence in epitope IV (F408A or WT), and revealed a previously undetected silent mutation within the 20mer. These DNA preparations were purified and used to transfect primary kidney cells harvested from naïve mice. Transfection was performed in four parallel batches: pCBKG/F408A, pCBKG/WT, pSelectESV (WT Tag), and a no DNA control. After several weeks, dense foci of growth were isolated and expanded. Those transfected with WT Tag showed greater numbers of foci and growth rate of isolated cell lines than those transfected with either 20mer replacement mutant. Immunofluorescence revealed successful transfection of the WT, but was inconclusive for pCBKG/WT and negative for pCBKG/F408A.
INTRODUCTION

Pancreatic cancer cells have been found to express a unique version of the gastrin receptor found in normal pancreatic cells. Cholecystokinin-C receptor (CCKCR) is an alternatively spliced version of cholecystokinin-B receptor (CCKBR) found in normal pancreatic cells. CCKCR retains the 69 amino acids of intron 4 of CCKBR (Smith et al. 2012). This is caused by a single nucleotide polymorphism (SNP) in CCKBR (Smith et al. 2002). Expression of CCKCR uniquely in cancerous pancreatic cells makes it an important target for immune therapy.

Simian Virus 40 (SV40) is a polyomavirus closely related to human BK and JC polyomaviruses (Martini et al. 2007). The virus, originating from the rhesus macaque monkey, was discovered around 1960, when it was found in contaminated polio vaccines which had been widely distributed to the public (Butel and Lednicky 1999, Martini et al. 2007). It contains double-stranded, circular DNA and codes for two antigens of particular interest: the large tumor antigen (T ag) and the small tumor antigen (t ag), which are alternately spliced from the same region. Within a cell, the virus undergoes two main stages. In the early stage, T ag and t ag are produced. T ag especially plays an important role in viral DNA replication by binding to the origin of replication, promoting several cellular processes that promote replication such as inducing the S phase in transformed cells, the phase with the proteins and cofactors necessary for DNA replication. T ag also plays a vital role in transforming host cells and causing tumorigenesis because of its integration into host genome (Martini et al. 2007). In a laboratory setting, SV40 T ag provides a powerful model in the study of cell-mediated response to tumors.

Previously, using site directed mutagenesis, an intron replacement mutant of SV40 T ag was constructed in which CTL epitopes I and II/III (and residues separating them) were replaced with a 20mer intron sequence from CCKCR when an F408A substitution mutation was also
introduced into epitope IV (pCBKG/F408A) (Burkett and Mylin 2013). Together, these mutations should inactivate the immune response to the three immunodominant CD8+ epitopes (I, II/III, an IV) while retaining two immunorecessive CD8+ epitopes (V and 295), the 20mer intron sequence, and the three known CD4+ epitopes in order to test immune response while maintaining the immortalizing properties of T ag (Burkett and Mylin 2013). A second variation of this mutant was constructed in this study which contains the 20mer replacement, but has epitope IV restored to wild-type (pCBKG/WT) (Figure 1). The goal of this study was to construct cells which express a derivative of the SV40 T ag that incorporates the CCKCR intron 4 sequence to use as a tool in the study of cell-mediated immunity targeting this unique sequence in pancreatic cancers.
MATERIALS AND METHODS

Bacterial Transformation

Competent DH5α Escherichia coli (E. coli) cells were thawed on ice for approximately 30 minutes. Transformation mixtures were prepared containing chilled 100 µl TMC (10 mM Tris pH 7.0, 10 mM CaCl$_2$, 10 mM MgCl$_2$), 10 µl of 1/200 dilution of each plasmid (equivalent to about 10 ng of DNA) or 10 µl TE (10 mM Tris pH 8.0, 1 mM EDTA) in the no DNA control, and 100 µl E. coli bacteria. Mixtures were mixed on a vortexer and incubated on ice for 25 minutes. Mixture were then rapidly transferred to 37°C water bath for exactly 2 minutes and immediately removed to incubate at room temperature for 10 minutes. A 1 ml aliquot of 2xYT liquid media (16 mg/ml bactotryptone, 10 mg/ml bacto yeast extract, 5 mg/ml NaCl) was added to each mixture and inverted to mix. Mixture were again incubated at 37°C for 45 minutes. Either 100 µl or 10 µl (plus 90 µl 2xYT) aliquots of transformation mixture were spread on 1xYT plates containing ampicillin. Plates were allowed to sit at room temperature until the mixture soaked into the agar, then were inverted and placed in 37°C incubator overnight (12-18 hours). Ampicillin resistant colonies were selected.

Small-Scale Alkaline Lysis

Five ml of sterile 2xYT medium and 30 µl of 1% AMP solution (in methanol) was added to sterile culture tubes following aseptic technique and the tubes were vortexed. A sterile toothpick was used to inoculate each tube with a bacterial clone from the plates of DH5α and the tubes were vortexed. One tube was prepared with just media and AMP but no bacteria and one with just media in order to confirm the sterility of the AMP and the media. Culture tubes were placed in a Rollodrum (New Brunswick) inside an incubator at 37°C overnight.
A 1.5 ml aliquot as removed from overnight culture and centrifuged for 1 minute at full speed in a microcentrifuge. Supernatant was decanted and the tubes re-centrifuged for 10 seconds. The residual media was removed by aspiration. A 100 µl aliquot of Solution I (50 mM glucose, 25 mM Tris-HCL pH 8.0, 10 mM EDTA ph 8.0) was added to each and the bacterial pellets were dispersed by vortex mixing. Then 10 µl of 20 mg/ml lysozyme was added and the tubes were mixed and incubated at room temperature for 5 minutes. A 220 ul aliquot of Solution II (1% (w/v) SDS, 0.2 M NaOH) was then added to the tubes which were mixed by inversion and then incubated on ice for 5 minutes, mixing once during this period. Next, 165 µl of ice cold Solution III (3 M potassium acetate, 2 M acetic acid) was added to the tubes which were mixed by inversion and again incubated on ice for 5 minutes, mixing once during this period. The tubes were then centrifuged for 3 minutes and the supernatants were transferred to 1.5 ml eppendorf tubes.

A 800 µl aliquot of ice cold 100% ethanol was added to each supernatant, the tubes were mixed by inversion, and then incubated at room temperature for 5 minutes. Then the tubes were centrifuged for 5 minutes at high speed and the ethanol supernatants were decanted. The pellets were then washed with 750 µl of ice cold 75% ethanol, mixed, and then centrifuged for 2 minutes. The supernatant was again decanted and the tubes were centrifuged and the residual ethanol was removed by aspiration. Pellets were dried using a Speed Vac rotary concentrator for about five minutes. The DNA pellets were then dissolved in 50 µl TE and 50 µg/ml RNAse A (Sigma) by alternately vortexing and centrifuging in short pulses. Then solutions were incubated at 37°C in a water bath for five minutes. Samples were held on ice until restriction analysis with HindIII and KpnI/BamHI restriction enzymes.
**QIAprep Spin Miniprep**

Seven ml of sterile 2xYT medium and 42 µl of 1% AMP solution (in methanol) was added to sterile culture tubes and the tubes were vortexed. A 10 µl aliquot from liquid cultures as added to each tube. One tube was prepared with just media and AMP but no bacteria and one with just media in order to confirm the sterility of the AMP and the media. Culture tubes were placed in a Rolldrum (New Brunswick) inside an incubator at 37 °C overnight.

To pellet bacterial cells from 5 ml culture, transferred 5 ml overnight culture to 14 ml plastic snap-cap tubes. Tubes were centrifuged for 15 minutes at 4000 rpm at 4°C. Supernatant was decanted and the pellet resuspended in 1 ml TE (pH 8.0). Cell suspension was transferred to 1.5 ml microfuge tubes, centrifuged for 5 minutes at 13000 rpm, and the supernatant decanted. Following another pulse spin, the remaining supernatant was aspirated. Then the “QIAprep Spin Miniprep Kit Using a Microcentrifuge” protocol from the Qiagen manual was followed for the isolation of DNA. DNA was resuspended in 50 µl Buffer EB (10 mM Tris-Cl, pH 8.5). DNA concentration was determined by spectroscopy by measuring 2 µl of sample on a NanoDrop instrument (Thermo Scientific). DNA purity was measured using gel electrophoresis and restriction analysis with *Hind*III restriction enzyme.

**Restriction Digestion**

Premixes were prepared for *Hind*III digestion using the proportions for 1 reaction: 2 µl 10X React Buffer #2, 14.5 µl GDW, and 0.5 µl *Hind*III enzyme. Premixes were prepared for *Bam*HI/*Kpn*I digestion using the proportions for 1 reaction: 2 µl 10X React Buffer #4, 14.5 µl GDW, 0.5 µl *Bam*HI enzyme, and 0.5 µl *Kpn*I enzyme. No enzyme control premixes contained 2 µl 10X React Buffer #2 and 15.0 µl GDW per reaction. A 17 µl aliquot of the appropriate premix was added to 3 µl of DNA on ice in a microfuge tube. Tubes were vortexed then pulse spun in a
microfuge, then immediately placed into 37°C water bath and incubated for at least 1 hour. Each reaction was quenched by adding 5 µl 5X gel loading buffer, vortexing, and centrifuging. Samples were stored at room temperature for immediate gel electrophoresis.

In certain cases of high concentration DNA analysis, alternate proportions were used in restriction digestions. For HindIII digestion, 2 µl 10X React Buffer #2, 16.5 µl GDW, and 0.5 µl HindIII enzyme per reaction was used. A 19 µl aliquot of premix was added to 1 µl DNA.

*Gel Electrophoresis*

Gels were prepared at 1% agarose [1 g/100 ml final volume of agar, 1 ml/10 ml final volume of 10xTAE, 9 ml/10 ml glass-distilled water (GDW), 20 µl/100 ml final volume of 1 mg/ml ethidium bromide (EtBr) stock]. Agarose, GDW, and 10xTAE were added together and microwaved to completely dissolve agarose. The solution was cool to 55°C in a water bath. EtBr was added and mixed. Gel was poured into molds and set at room temperature to solidify.

To electrophorese samples, gels were placed in electrophoresis chambers (Fisher Biotech. Electrophoresis Systems, MidiHorizontal System, 9.62L x 7W x 3.12in.H) and covered in loading buffer 1xTAE to cover the gel to a depth of at least 0.5 cm. A 12.5 µl aliquot of the appropriate samples were loaded to each well. A 3 µl aliquot of 1 Kb Plus DNA Ladder was added to a well for a standard. Gels were run at 100 volts for 1 hour. Each gel was then removed from the chamber, rinsed in distilled water, then photographed using Kodak EDAS 290 camera chamber visualized using UV light and captured with a 2 second exposure. The software Kodak MI was used to optimize the picture.
DNA Sequencing

Following gel electrophoresis, samples were selected for DNA Sequencing. Each DNA sample was diluted with GDW to a concentration of 100 ng/μl to be sequenced. The Applied Biosystems 3130xl Genetic Analyzer at Penn State Hershey Laboratories in the Molecular Genetics Core Facility was used to sequence the plasmids using the fluorescence based cycle sequencing method. Each sample was added to a reaction along with a primer (MYLI507, JTEV27, JTEV03, STEV02, MYLI508, T7 promoter, SP6 promoter) and BigDye Terminator v1.1 Cycle Sequencing Kit. Each reaction was amplified by thermal cycling according to manufacturer’s specifications. The extension products were electrophoresed and analyzed by a laser to produce a printout of the sequence. The computer program NCBI BLAST was used to compare the sequenced plasmids to the original sequence of the SV40 T antigen gene.

Construction of pCBKG/WT

Both WT T ag DNA (#49) and pCBKG/F408A DNA were digested with HpaI restriction enzyme using the formula: 36 μl DNA, 25 μl 10X CutSmart Buffer, 179 μl GDW, and 10 μl HpaI enzyme. A small-scale no enzyme control was prepared in parallel containing 4 μl DNA, 2.5 μl 10X CutSmart Buffer, and 18.5 μl GDW. The digest was performed using the same procedure as other digests. A small-scale gel was run with 12.5 μl sample loaded on 1% agarose gel to ensure digestion before the remaining digested solution mixed with 61.25 μl 5X loading buffer and was run on 1% Prep gel – prepared in a mold with larger wells. Gel was prepared and run as before at 100 volts for 2-3 hours.

The small fragment consisting of the epitope IV region from #49 and the large fragment consisting of the rest of the plasmid (excluding the epitope IV region) were excised from the gel using a razor blade and a UV box. Forceps were used to transfer the gel slice into a pre-weighed
14 ml snap-cap tube. The remaining gel was imaged as before. The QIAquick Gel Extraction Kit Protocol (Qiagen) was followed for extraction of the DNA from the gel matrix.

Dephosphorylation of the 5’ ends of the larger mutant fragment was carried out to prevent re-ligation. Calf intestinal alkaline phosphatase (CIAP, Promega) was diluted to 0.01 U/µl in 1X reaction buffer. CIAP reaction was set up with 25 µl DNA, 10 µl 10X Reaction Buffer, 1 µl CIAP (0.01 U/µl), and 64 µl GDW. A negative control was set up in parallel containing 25 µl DNA, 10 µl 10X Reaction Buffer, and 65 µl GDW. The mixture was incubated at 37°C for 20 minutes, then 56°C for 10 minutes. Another 1 µl aliquot of CIAP was added to tubes with CIAP and they were incubated again at 37°C for 20 minutes, then 56°C for 10 minutes. A 300 µl aliquot of CIAP stop buffer was then added (10 mM Tris-HCl pH 7.5, 1 mM EDTA pH 7.5, 0.5% SDS, 200 mM NaCl).

A phenol:chloroform extraction was performed by adding 0.4 m PCI (TE-saturated phenol:chloroform:isoamyl alcohol 25:24:1) and vortexing for 1 minute. Then tubes were centrifuged for 5 minutes and the upper aqueous phase was transferred to fresh microfuge tubes.

Ethanol precipitation was performed by adding 45 µl 10X TNE to tubes and mixing. An 800 µl aliquot of 100% ethanol was added then the tube mixed and put in -20°C freezer for at least 2 hours. The tubes were then centrifuged for 30 minutes in 4°C microfuge. The supernatant was decanted into tubes immediately after centrifuging. A 750 µl aliquot of ice cold 75% ethanol was added and the tube gently inverted to wash and the tube was centrifuged again for 5 minutes at 4°C. The supernatant was again decanted, the tubes pulse spun, and the remaining supernatant aspirated. The pellet was dried on a Speed Vac rotary concentrator for 2 minutes then air dried for 15 minutes. The pellet was resuspended in 10 µl 10 mM Tris 0.1 mM EDTA (pH 8.0) by 30 minutes of alternately vortexing, flicking, centrifuging and letting stand. The DNA was electrophoresed to confirm the presence of DNA in each sample.
Finally, a ligation reaction was performed to combined the two fragments. Four reactions were carried out in parallel: the “–CIAP –insert” reaction contained 2 µl “–CIAP” reaction, 6 µl diluted elution buffer (EB/5), 2 µl 10X reaction buffer, 9 µl GDW, and 1 µl ligase (T4 DNA ligase, Promega); the “+CIAP –insert” reaction contained 2 µl “+CIAP” reaction, 6 µl diluted elution buffer (EB/5), 2 µl 10X reaction buffer, 9 µl GDW, and 1 µl ligase (T4 DNA ligase, Promega); and two “+CIAP +insert” reactions contained 2 µl “+CIAP” reaction, 6 µl #49 epitope IV fragment, 2 µl 10X reaction buffer, 9 µl GDW, and 1 µl ligase (T4 DNA ligase, Promega). Each reaction was assembled and let stand at room temperature for 4 hours. The reactions were then diluted with 80 µl TE pH 8.0 and placed on 65°C heat block for 10 minutes to heat deactivate the enzyme. The DNA was stored at -80°C for further use. Each preparation was then transformed into *E. coli*, isolation of DNA using QIAprep spin miniprep kit, restriction analysis with *HpaI* and *HindIII*, and DNA sequencing confirmed the desired DNA.

**Large-Scale Transfection-Grade DNA Isolation**

Large cultures were inoculated using 100 ml 2xYT, 600 µl 1% Amp, and 200 µl of small 8 hour culture. Large cultures were incubated overnight on orbital shaker at 250 rpm at 37°C. The “EndoFree Plasmid Maxi Kit” protocol (Qiagen) was followed for isolation of DNA. Centrifugation steps were performed for 60 minutes at 4000 rpm on the table-top centrifuge Jouan RC412 refrigerated centrifuge with the M4 swinging bucket rotor. Samples were resuspended in 500 µl of Buffer TE. Analysis on a NanoDrop instrument determined the concentration of the isolated DNA.
Harvesting Primary Kidney Cells

Kidneys were harvested from C57BL/6 mice (Taconic, aged 4-6 weeks, male), sacrificed by vertebral dislocation and sterilized with ethyl alcohol. Kidneys were placed two per tube containing 10 ml T10x3(no AB) media [Dulbecco’s modified eagle medium supplemented with 10% heat inactivated fetal calf serum (FCS), 20 mM N-2-hydroxyethylpiperazine-N9-2-ethanesulfonic acid (HEPES), 0.15% (w:v) sodium bicarbonate] with no antibiotics. Kidneys were minced in a 60 mm Petri dish using forceps and a scalpel to cut each kidney into very small chunks. After satisfactory mincing, the kidney chunks and cells were pelleted for 5 minutes at 1000 rpm (4°C) in a centrifuge and the supernatant removed.

Trypsinization of Cells

Chunks and cells were each resuspended in 10 ml 1% trypsin-versene (37°C, diluted in trypsin diluent from a 2.5% stock) and mixed thoroughly for 1 minute. The suspension was incubated in a Rollodrum (37°C New Brunswick) for 15 minutes, with inverting and vortexing. The remaining chunks were allowed to settle and the supernatant transferred to a new tube containing 20 ml media to neutralize the trypsin and placed on ice. Chunks were resuspended in 10 ml 1% trypsin and the process repeated for a total of five trypsin washes. Remaining chunks were resuspended in 15 ml media and settled on ice for 5 minutes. The supernatant was transferred to new tubes and all cell suspensions and kidney chunks were pelleted for 5 minutes at 1000 rpm (4°C). After the supernatant was removed, all cell pellets were resuspended in a total of 36 ml T10x3(no AB) media, placed into T175 flasks, and incubated at 37°C and 5% CO₂ with vented caps. All remaining kidney chunks were resuspended in a total of 30 ml media, placed into 100 mm Petri dishes, and incubated under the same conditions.
Cell Expansion and Counting

Cells were allowed to continue growth for 10 days, changing media twice weekly for fresh T10x3(no AB) media (37°C). Cells were then trypsinized for counting. Media was aspirated and flasks washed with sterile phosphate buffered saline (PBS) to remove lingering media. For T175 flasks, 8 ml 2.5% trypsin (37°C) was added to each flask and swirled and struck to dislodge cells, leaving trypsin for no longer than 2 minutes. To each flask was added 12 ml media to quench the trypsin and cells were further disturbed by forcefully pipetting up and down. For T75 flasks and 100 mm Petri dishes, 6 ml trypsin and 8 ml media were used following the same procedure. All cell suspensions were combined in tubes on ice and pelleted at 1000 rpm for 5 mins (4°C). Cells were resuspended in a total of 25 ml T10x3(no AB) media. A small sample was taken for counting, stained by trypan blue and counted on a hemacytometer using a phase contrast microscope. The suspension was plated at approximately 1 x 10^6 cells per flask into T175 flasks, containing T10x3(no AB) media. Cells were allowed to grow in these flasks for 4 days.

Transfection

Cells were trypsinized and counted as before. Cells were plated at approximately 1.5 x 10^5 cells per flask into fresh T75 flasks containing T10x3(no AB) media. Eight transfection reactions were prepared: one with wild-type SV40 T ag pSelectESV-Amp' (#49) plasmids, one with no DNA added, two with pCBKG/WT, and four with pCBKG/F408A. Replicate flasks were prepared for each transfection mixture for a total of 16 transfection flasks. Transfection mixtures were made by adding 8 ml transfection medium [T10x3(no AB) medium] and 20 µl FuGENE®6 (Promega) transfection reagent, mixing using a vortexer, adding the volume of DNA solution that represents approximately 2 µg plasmid DNA for the #49 and 2 mutant preparations, and mixing again. After incubation at room temperature for 30 minutes, each transfection mixture was added dropwise to
the appropriate T75 flasks containing the cell preparations. Transfection flasks were then incubated in the 37°C, 5% CO₂ growth chamber as before, changing media twice weekly. T10x3(no AB) media was switched out for T10x3 (supplemented with antibiotics 100 U penicillin per ml, 100 mg streptomycin per ml, 2 mM L-glutamine, 100 mg of kanamycin per ml).

**Observation and Picking of Immortalized Foci**

Plates were observed for 4 weeks for the appearance of dense patches of growth representing immortalized cells. Once sufficient numbers and sizes of growth foci were reached, foci were picked from each flask using modified disposable glass Pasteur pipets that had their tips stretched over a Bunsen burner and flame polished. A phase contrast microscope was used to visualize the focus and the tip of the pipet was used to scrape around the outside of the focus (Figure 2). Each cell patch was then sucked into the pipet and transferred to its designated well of a 12-well plate containing 4 ml T10x3 media. The individual cell clones were allowed to expand in the 12-well plates and the media was changed twice weekly (Figure 3).

**Propagation of Individual Lines**

When cells covered more than 1/2 to 2/3 of the bottom surface area of the well, they were deemed ready for transfer. When sufficient numbers of lines on each plate were ready, cells were transferred to individual T25 flasks for propagation. Cells were trypsinized like before using 2 ml PBS, 1 ml 2.5% trypsin, and 3 ml T10x3 media to quench the reaction. Each entire suspension was transferred to individual T25 flasks containing 3 ml T10x3 media and incubated at 37°C, 5% CO₂ as previously stated.
**Preparation for Immunofluorescence**

Cell lines were allowed to expand until they reached confluence. Wild-type T ag lines were passed 1 to 6 into 60 mm Petri dishes containing 7 ml T10x3 media and five glass coverslips and passed 1 to 15 back into T25 flasks for propagation and expansion. To pass cells in T25 dishes, cells were trypsinized as before using 2 ml PBS, 2 ml 2.5% trypsin, and 4 ml T10x3 media. The appropriate volume of the cell suspension was transferred to either the dish or the flask to result in 1/6 of the cells going to the dish and 1/15 going to the flask for propagation (any leftover were discarded). Experimental DNA preparations were passed in the same way.

**Ethanol Fixation**

Once cells in the 60 mm Petri dishes reached relative confluence, they were dehydrated to fix them on the glass coverslips. Media was aspirated and each dish was washed with approximately 10 ml PBS three times. A 10 ml aliquot of ice cold 95% ethanol was added to each dish and they incubated with lids on in the -80°C freezer for 30 minutes. After ethanol was aspirated, dishes were set at an angle in a fume hood for several hours until dry, when they were placed in the 4°C refrigerator.

**Immunofluorescence**

Coverslips were transferred cell-side-up to individual 35 mm dishes. Two sets of preparations were prepared in parallel: one without primary antibody as a background and one with either the 419 or 901 primary antibody specific to SV40 T ag. Cells were rehydrated by dipping each coverslip into PBS and blotting off excess PBS. A 125 µl aliquot of primary antibody solution was immediately added directly onto each rehydrated coverslip. They were allowed to incubate at room temperature for 30 minutes, protected from evaporation by keeping lids on the 35 mm dishes. Primary antibody solution was washed off by three approximately 5 ml PBS rinses.
and subsequent aspiration. Immediately after aspirating the third PBS rinse, the coverslip was transferred cell-side-up to the lid of the 35 mm dish and 135 µl of secondary antibody solution [conjugated to Fluorescein isothiocyanate (FITC)] was added directly onto each coverslip. Protected from light and evaporation, they were incubated at room temperature for 30 minutes. The secondary antibody solution was removed by three 5 ml PBS rinses followed by a brief rinse in distilled water. Coverslips were placed cell-side-up in drying racks overnight. The following day, coverslips were mounted to slides cell-side-down using Cytoseal 60 (Richard-Allan Scientific) mounting glue and allowed to dry before visualizing under a fluorescence microscope.

*Growth Test at 5% FCS*

Cells are normally grown in T10x3 media. After splitting cells into flasks for growth and Petri dishes for the start of the IF procedure, the cells were passed 1/15 as described above. Then the following day, the media was switched out with T5x3 media (containing all the same as T10x3 media, but 5% fetal calf serum rather than 10%).

*Cell Expansion & Freezing*

Cells that were determined to be successful transfectant candidates were expanded in culture. To do so, cells were trypsinized as described above. All of the cell suspension was transferred from the T25 flask to a T75 flask containing 5 ml T5x3 media. The cells were allowed to expand and reach confluence. Then they were again passed as described and all of the cell suspension was transferred to a T175 flask containing 24 ml T5x3 media. Cells were again allowed to expand and reach confluence. Once confluent, cells were trypsinized again, centrifuged for 5 minutes at 1000 rpm in 4°C centrifuge, and the supernatant was aspirated. Cell pellets were resuspended in 2 ml freezing media then 1 ml cell suspension was transferred to each of 2 freezing tubes. Tubes were then placed in -80°C freezer for storage.
RESULTS

Preparation and Confirmation of DNA

In order to prepare DNA for transfection into kidney cells, the DNA had to first be purified and confirmed using *E. coli* transformation, isolation via small scale alkaline lysis, and restriction analysis. *E. coli* transformation was successful, with the negative control plates (no DNA added to transformation mixture) showing no growth, while all other transformation mixtures showed the expected growth on both the 10 µl (colony counts 140, 76, 54, 70, and 42 for preparations 1, 2, 3, 4, and 5 respectively) and 100 µl plates, with the 100 µl showing colonies too numerous to count. Colonies were selected for ampicillin resistance, a gene coded for in the plasmid transformed with, and not by the *E. coli* naturally, and 2 successfully growing colonies (A & B) were inoculated into liquid cultures overnight, showing successful sterilization as both the no ampicillin no bacteria and the no bacteria controls did not show growth. Small scale alkaline lysis followed by restriction analysis yielded unexpected results. Figure 4 shows the electrophoresis gel from the *HindIII* digestion. Digestion pattern was as expected for pCBKG, except for the anomaly in the third fragment down (indicated by the arrow), which was smaller in samples 4B, 4A, 1B, and 1A than expected.

All bacteria corresponding to each DNA sample underwent QIAprep spin miniprep protocol to isolate more pure DNA. NanoDrop measured DNA at concentrations 122.8, 226.7, 106.0, 107.7, and 79.2 for 1B, 2A, 3A, 4A, and 5D2 respectively. Samples were again restriction analyzed with *HindIII* and previous results were replicated. Samples were then sent to Penn State Hershey for sequencing using primers JTEV27 and MYLI507. In the meantime, wild-type SV40 Tag pSelectESV-AmpR (#49) was prepared in the same way as the mutants to provide a parallel sample for analysis. Figure 5A shows a large 27 base pair deletion in those samples with the
observed smaller fragment (#4 and #1). This DNA was deemed useless and eliminated from further experimentation. Another mutation was noted in those other samples without the large deletion. Figure 5B shows a silent mutation found within the 20mer sequence, which does not alter the amino acid sequence, but was noted for future reference. Sample 3A was chosen for further use based on purity and was prepared for further sequencing of the entire T ag sequence in parallel to the #49 wild-type T ag DNA. These samples were sent off for sequencing using primers JTEV03, MYLJ508, STEV02, T7 promoter, and SP6 promoter for thorough sequencing results. Sequencing confirmed the previous silent mutation and revealed the expected sequence for the rest of the T ag DNA.

Construction of pCBKG/WT

Both #49 wild-type T ag DNA and pCBKG/F408A mutant T ag were digested with HpaI, which cuts around the epitope IV region in T ag. The preparations were run on gels with large wells to allow for larger fragment bands and the fragments were visualized on a UV box. Using a razor blade, the fragments were excised and the DNA was extracted from the gel matrix. The 5’ ends of the larger fragment (containing all but the epitope IV region) from pCBKG/F408A was dephosphorylated to prevent re-ligation of the ends together without incorporating the epitope IV region from WT T ag. A control reaction minus alkaline phosphatase (CIAP) was prepared in parallel. The DNA was then purified from any excess debris or enzymes and ethanol precipitated to concentrate it. Finally, the ligation procedure combined the epitope IV insert from wild-type with the rest of T ag from pCBKG. The control without CIAP was prepared without the insert to determine the occurrence of re-ligation of the larger fragment compared to a control with CIAP without the insert. The DNA was then transformed into E. coli and yielded unexpected colony counts (Table 1). The non-dephosphorylated reaction seemed to show slightly greater
transformation at 60 colony forming units (cfu) than did the dephosphorylated reaction with 49 cfu. The reactions that were dephosphorylated and contained the insert had 29 and 19 cfu. The no DNA control showed no growth, as expected, and the positive control showed a lot of growth with colonies too numerous to count.

Eight colonies from each of the two reactions with the insert were selected, expanded, and the DNA was isolated using a small scale alkaline lysis procedure. The 16 DNA samples plus #44 (pSelect without T ag) and #49 (pSelectESV with T ag) were digested with HpaI to determine likely candidates. One candidate showed the expected pattern – labelled V25 (Figure 6A). This candidate was then subjected to HindIII digestion to confirm the correct orientation of the epitope IV insert (Figure 6B). The bacteria containing V25 DNA (the candidate for successful transformation with pCBKG/WT) were inoculated into liquid cultures and QIApreps were performed to prepare higher purity DNA which was then measured by NanoDrop, restriction analyzed with HindIII to confirm purity, and sent off for sequencing using primers JTEV27 and MYLI508. Sequencing confirmed the correct placement of the WT epitope IV region into the mutant pCBKG plasmid.

Following confirmation of each DNA sample: WT T ag pSelectESV (#49), pCBKG/F408A (3A), and pCBKG/WT (V25), the Endo-Free Plasmid Maxi Kit protocol was followed to isolate high amounts of transfection-grade, high purity DNA for further use. Restriction analysis with HindIII of each of the finished products confirmed the likely correct identities of each sample.

**Kidney Cell Isolation and Expansion**

Plasmids encoding both intron replacement mutants of SV40 T ag containing the 20mer intron from CCKCR (pCBKG/F408A and pCBKG/WT) were transfected into primary kidney cells harvested from naïve mice. Kidneys were harvested from one mouse, minced, and individual
kidney cells sloughed off using the protease trypsin. These cells were maintained in culture and propagated to sufficient numbers for transfection. The initial cell count after expansion was average 8.25 cells counted in 0.1 µl in a 1/2 dilution of the cell suspension and a total volume of 14 ml. This equates to 2.31 x 10^6 cells total. This suspension was split into 2 T175 flasks, each containing 1 x 10^6 cells. These were allowed to expand and the cells from both flasks were trypsinized and counted. In the first flask, an average of 3 cells per 0.1 µl of a 1/2 dilution were observed, yielding a total of 1.2 x 10^6 cells or 1 x 10^5 cells/ml in 12 ml. A 1 ml aliquot was transferred to each of 12 flasks for 1 x 10^5 cells/flask. In the second flask, an average of 2 cells per 0.1 µl of a 1/2 dilution were observed, yielding a total of 8 x 10^5 cells or 1 x 10^5 cells/ml in 8 ml. A 1 ml aliquot was transferred to each of 4 flasks for 1 x 10^5 cells/flask.

Transfection

Transfection was performed using FuGENE®6 reagent (Promega). Eight transfection mixtures were prepared: four of which contained pCBKG/F408A (labelled 5-8); two contained pCBKG/WT (labelled 3-4); one contained a plasmid encoding the wild-type SV40 T ag as a positive control for immortalization (labelled 1, which represented the #49 plasmid); one mixture contained no added plasmid DNA as a negative control (labelled 2). The flasks were observed for the formation of dense foci over several weeks (Figure 7). Each visible dense focus represented a cluster of immortalized cells that proliferated from a single immortalized cell, which could be a transformant.

Obvious dense foci were observed in cultures transfected with the wild-type #49 plasmid. One denser area of growth was observed in a negative control flask, but this did not appear to expand and was likely a clump of cells from the transfer into transfection flasks. Foci were observed in flasks transfected with both of the mutant T ags at frequencies and intensities less than
those observed in the wild type T ag. Twelve total foci were picked from preparation 1, plasmid #49, the WT T ag transfection. No foci were picked from the negative control preparation 2. Eight total foci from preparations 3 and 4, representing pCBKG/WT, sufficiently expanded in individual culture. Eight total foci from preparations 5-8, representing pCBKG/F408A, sufficiently expanded in individual culture. Overall, more foci from pCBKG/WT grew better and remained alive in culture than those from pCBKG/F408A. After isolating these foci from cultures and expanding them individually in 12-well plates, they were transferred to individual T25 flasks and then also to petri dishes for immunofluorescence analysis.

*Immunofluorescence and Growth Test*

Immunofluorescence (IF) using antibodies specific to SV40 T ag was used to confirm the presence of T ag in immortalized cells. The primary antibody binds T ag, then a secondary body binds the primary and is conjugated to FITC, a compound that glows green under UV light. This would serve to both detect and localize SV40 T ag within cells. Table 2A shows the results of the assay using the 901 primary antibody. Cells that seemed the most promising were again assayed using the 419 primary antibody (Table 2B). Cells transfected with WT T ag were clearly fluorescent with clear and bright localization of fluorescence in the nucleus, as expected. IF was inconclusive for transfection with pCBKG/WT, the cells appearing only slightly more fluorescent than the background control, with no clear localization. IF seemed to indicate unsuccessful transfection with pCBKG/F408A, cells showing fluorescence equal to the background.

At the same time, cells were grown in their flasks with media supplemented with 5% fetal calf serum (FCS), less than the 10% FCS they were grown in normally. Under those conditions, only cells that express T ag are likely to grow and proliferate, while those without T ag are not. Only three distinct lines of cells most notably proliferated – 3A1 and 3B2 from transfections with
pCBKG/WT and 5A2 from transfection with pCBKG/F408A. Given both the growth data and the IF data, these were the most likely candidates to contain the intron replacement mutant of T ag. These three candidates were expanded in culture and stored frozen for further use.
DISCUSSION

Given the results that cells transfected with pCBKG/WT seemed to grow and form foci more readily than those with pCBKG/F408A, it seems that the disruption of the epitope IV region interrupts the ability of T ag to immortalize cells. At the very least, we know that altering the protein sequence in the mutants definitely resulted in a lower transfection efficiency, given that both mutants showed lower numbers and density of foci than the wild-type T ag. Any change in amino acid may alter the overall protein structure and, therefore, the function of the protein, so it is not surprising that it may be the case here.

In addition, the inconclusive IF results may be explained in the same way. The disruption of the epitopes I and II/III region by replacement with the 20mer may interrupt the normal nuclear localization of the T ag protein. Normally, T ag functions within the nucleus, allowing IF to produce clear, bright, nuclear-localized fluorescence when antibodies attach to the protein. However, if that is disrupted, it may result in a spreading out of the protein through the cell, diffusing the fluorescence and making it more difficult to identify the protein using this method. Perhaps future experimentation could use an alternative to IF to detect the protein within these cells to more definitively determine whether that DNA was successfully transfected.

Eventually, cells which are confirmed to carry and express either of the intron replacement mutants will be used in further immunological studies to characterize the cell-mediated immune response to the 20mer of CCKCR. They may be used to stimulate any immune cells generated against the 20mer in another experiment. Or they may be used to immunize mice, then the immune response to the 20mer in the context of the larger protein can be tested and characterized. Results of such studies could have eventual implications in immune therapy against human pancreatic tumors.
ACKNOWLEDGEMENTS

This study was made possible by Dr. Mylin and his advice and guidance through the entire process, by Courtney Burkett by preparing the mutant SV40 DNA, by the Steinbrecher Summer Research Scholarship for providing funding for my research over the summer, by Penn State Hershey for sequencing my DNA, and by Messiah College Department of Biological Sciences and their laboratory facilities and supplies.
REFERENCES


### Table 1. Transformation of E. coli with ligated pCBKG/WT product.
CIAP (calf intestinal alkaline phosphatase). No DNA control was a transformation with no added DNA to render ampicillin resistant. #49 was a positive control for ampicillin resistance (TNTC = too numerous to count).

<table>
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<tr>
<th>Reaction</th>
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<th>100 µl conc.</th>
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<td>53</td>
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<tr>
<td>+ CIAP --insert</td>
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<tr>
<td>+ CIAP + insert (1)</td>
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<td>20</td>
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<td>+ CIAP + insert (2)</td>
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<td>16</td>
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<tr>
<td>#49 WT Tag</td>
<td>3176</td>
<td>TNTC</td>
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### Table 2. Summary of immunofluorescence (IF) and growth data.
Brightness was observed with WT T ag-transfected cells from preparation #1 representing the most bright (+++) and no primary antibody (PA) representing the least bright (-). Localization was observed as being in the nucleus (N) or the cytoplasm (C), or both (N/C), with ~N indicating somewhat localized to nucleus. (A) IF with PA 901. (B) IF with PA 419. (C) Growth at 5% FCS, with (+++) representing the most growth and propagation.

<table>
<thead>
<tr>
<th>Sample</th>
<th>(C) Growth at 5% FCS</th>
<th>(A) 901</th>
<th>(B) 419</th>
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<tr>
<td>1A1</td>
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<td>+++ - N</td>
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</tr>
<tr>
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</tr>
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<td>+++ - N</td>
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<td>+ +/- ~N</td>
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</tr>
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FIGURE LEGENDS

**Figure 1.** Schematics of the wild-type (WT) vs. the two Intron Replacement Mutants. The WT sequence shows CD8+ epitopes I, II/III, IV, and V and their residue numbers. Intron replacement mutant pCBKG/F408A shows the replacement of epitopes I and II/III with the 20mer sequence as well as an F408A substitution in epitope IV. Mutant pCBKG/WT shows the 20mer replacement with WT epitope IV. Diagram is not to scale.

**Figure 2.** Phase microscope-guided harvest of an individual focus from a T75 transfection flask.

**Figure 3.** 12-well plate containing foci harvested from SV40 T ag (wild-type) transfection flask.

**Figure 4.** Electrophoresis gel showing the *Hind*III cutting pattern. The arrow indicates the third fragment which is smaller (further down the gel) than expected in samples 1A, 1B, 4A, and 4B.

**Figure 5.** Sequencing results. (A) Large deletion in samples 4B & A, and 1B & A. (B) Silent mutation in samples 3B & A and 2B & A.

**Figure 6.** Restriction analysis and gel electrophoresis of pCBKG/WT candidates. (A) Restriction analysis with *Hpa*I. (B) Restriction analysis with *Hind*III.

**Figure 7.** Example of growth foci observed. Shown is one of the positive control plates. Note: picture was taken after majority of foci picked out of flask and cells allowed to continue growth. Original foci were circled in black.
FIGURES

Figure 1.

WT SV40 T ag

206-215  223-231  404-411  489-497

I  II/III  IV  V

708

pCBKG/F408A

CCKCR Intron Seq.

F408A subs.
“404-411” “489-497”

1

20mer  X  20mer

IV  V

“708”

pCBKG/WT

CCKCR Intron Seq.

“404-411” “489-497”

1

20mer

IV  V

“708”

Figure 2.
Figure 3.

Figure 4.
Figure 5.

A) Expected

```
TTTGTACAGACTTGCTGGCTAGTTTCGTCAAGATTTCAAGCAGAATTGTGGAGTGA
```

Observed

```
T---------------------TCAAGATTTCAAGCAGAATTGTGGAGTGA
```

27 bp (9 aa) deletion

B) Gly Ala Gly Pro Arg Glu Gln Asn Leu Gly Glu Ala Glu Leu Trp Arg Ala Thr Gly Pro

Expected

```
GGG GCT GGA CCA CGT GAG CAA AAT CTG GGC CAG GAG CCT
```

Observed

```
GGG GCT GGA CCA CQA GAG CAA AAT CTG GGC CAG GAG CCT
```

Gly Ala Gly Pro Arg Glu Gln Asn Leu Gly Glu Ala Glu Leu Trp Arg Ala Thr Gly Pro

Figure 6.

A) HpaI

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B) HindIII

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Figure 7.