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Construction of a Novel System to Analyze Recognition of MHC Class II-Restricted Epitopes by CD4\(^+\) T Cell Hybridoma Clones \textit{in vitro}

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Abstract

The Simian Virus 40 large tumor antigen (SV40 Tag) is an excellent model system for studies in cancer immunology because it is strongly immunogenic, but capable of causing tumors when expressed as a transgene in vivo when protected by immune tolerance. The CD8+ T cell response to Tag in the C57BL/6 murine system is directed against four well-characterized epitopes. However, the CD4+ T cell response in this system remains uncharacterized. Recent work using Tag-specific CD4+ hybridoma clones and immune splenocytes to screen a 175 member, overlapping 15-mer peptide library corresponding to the 708 amino acid Tag protein sequence have identified three CD4+ epitopes. In order to complement this peptide-based screening system, we are constructing a system in which candidate epitopes can be amplified by PCR and inserted into a modified pGLO plasmid, forming an amino terminal extension of the green fluorescent protein (GFP). Such plasmids will be transformed into E. coli. Induction of the fusion gene with arabinose should fill the bacteria with glowing fusion protein. Incubation of arabinose-induced (glowing) bacteria with cultured murine dendritic cells (BMDCs) results in uptake which is readily observed as the phagocytes also begin to glow; glowing BMDCs containing E. coli DH5a expressing unmodified GFP do not stimulate hybridoma clones. Antigen processing should release the epitope peptide from the amino terminus of the fusion protein for presentation to added NFAT-lacZ+ CD4+ T cell hybridoma clones. This system should provide an excellent complement to the current methods which rely on incubation of MHC class II-positive APCs with synthetic peptides because it more closely resembles in vivo presentation. Efforts to generate and test the GFP fusion protein system will be described. The system will initially be tested by inserting the LT529-543 epitope and detecting it with appropriate hybridoma clones.
Introduction

One of the major functions of the immune system is to eliminate compromised host cells before they bring further harm to an organism. This includes cells that have undergone an oncogenic transformation. T cells are the major adaptive immune cells involved in this anti-tumor response. They respond to antigenic peptides, called epitopes, which are presented by other host cells on membrane proteins called major histocompatibility molecules, or MHCs. As shown in figure 1A, peptides interact with MHCs via specific anchor residues. A given MHC can bind a range of peptides, provided that they all have similar residues in the anchor locations. T cells interact with these MHC-peptide complexes in a highly specific manner via membrane-bound T cell receptors (TCRs). There are two major types of T cells, each with a corresponding type of MHC molecule. CD8+ T cells recognize peptides presented on MHC-I molecules, which are present on all cells in the body. When activated, they proliferate and differentiate into cytotoxic T lymphocytes, which eliminate cells presenting their cognate epitope by secreting cytotoxic substances. CD4+ T cells recognize peptides presented on MHC-II molecules, which are only expressed on “professional” antigen presenting cells (APCs) like Dendritic cells (DCs). When activated, they proliferate and differentiate into helper T cells, which modulate the activity of other immune cells by secreting soluble factors known as cytokines (1). Although CD8+ cells are tasked with destroying tumor cells, studies have demonstrated that CD4+ cells play a critical role in the anti-tumor response by increasing CD8+ activity and CD8+ presence in the tumor microenvironment, as well as delaying the onset of CD8+ tolerance, as shown in figure 1B (2,3,4).

In order to investigate the immune response to tumors, our lab uses the Simian virus 40 large tumor antigen (SV40 Tag) in C57BL/6 mice. Tag is an excellent model system for such studies, as it is capable of both transforming cells in vitro and forming tumors in vivo when expressed as a tissue-specific transgene (5). In addition, it is strongly immunogenic – Tag tumors are controlled by CD8+ T cells. This response has been well-characterized and is directed against four epitopes (figure 1C, red segments) (6). Less is known about the CD4+ response to Tag in this system, however. Recently, we found that such a response can be observed when the immunodominant CD8+ epitopes are removed from Tag by mutagenesis (7). Using a library of overlapping 15-mer peptides corresponding to the Tag sequence, we identified the approximate locations of three putative CD4+ epitopes and isolated CD4+ hybridoma clones recognizing each
(figure 1C, purple segments). However, the epitopes have not yet been fine-mapped to identify their precise locations and anchor residues.

In order to perform this fine-mapping, we sought to create a system in which putative CD4+ epitopes and epitope variants could be expressed by *E. coli* in a fusion protein with GFP (figure 2A,B). We have already demonstrated that *E. coli* expressing GFP alone can be efficiently phagocytosed by cultured murine bone marrow DCs (figure 2C). Furthermore, DCs which have been fed *E. coli* expressing unmodified pGLO do induce a CD4+ hybridoma response (unpublished data). Thus, if fusion protein-bearing *E. coli* are fed to cultured DCs, the DCs should process the protein, liberating the Tag peptide and presenting it on their MHC-II proteins where it can be probed by T cell hybridomas. This process mirrors the type of epitope processing that occurs in vivo. As *E. coli* expressing GFP without an insertion do not produce a response, any hybridoma reactivity will be the result of the peptide in the fusion protein. Fine-mapping can then be performed by comparing hybridoma reactivity to different epitope variants (figure 2D).

To create such a system, we began with the pGLO plasmid, which expresses GFP under control of an arabinose-inducible promoter (figure 3A). However, there is no way to insert portions of the Tag gene at the 5’ end of the pGLO GFP (5’ fusions are preferable because they guarantee that glowing cells contain the epitope). To remedy this, we selected two restriction sites, *Not*I and *Spe*I, that are not present anywhere in the unaltered pGLO sequence, to insert between the GFP start codon and the rest of the gene. Once these unique sites have been introduced using a site-directed mutagenesis technique, it is easy generate fusion proteins with any portion of Tag desired – as shown in figure 2E, segments of interest can simply be PCR amplified with primers introducing the sites onto the appropriate ends, then digested and ligated into the modified pGLO in an orientation-specific manner. We also included an additional *Hind*III site in the plasmid design between the *Spe*I and *Not*I sites. Because this segment will be lost whenever something is successfully inserted into the fusion site, its absence provides an indicator of a successful insertion event.

We selected PCR-based site-directed mutagenesis to introduce the desired sites into the GFP pGLO gene. This technique uses two mutagenic oligonucleotides that contain the desired insertion in the center flanked by regions complementary to the site where the insertion will be introduced. These oligos are used to prime *in vitro* PCR-like DNA synthesis with a high-fidelity
DNA polymerase, thereby producing linear copies of the plasmid containing the desired insertion. However, several attempts to introduce the 21 bp insertion via this method were unsuccessful, possibly because of the 69 bp size and self-complimentary nature of the oligos used. A second attempt at mutagenesis was made using an alter-site phage-based technique. This method uses an R408 helper phage to generate single stranded copies of a phagemid containing the DNA to be mutagenized. These ssDNA phagemids are then isolated and used for *in vitro* second strand synthesis primed with a single mutagenic oligonucleotides like those described for the PCR-based methods. This avoids the problem of having self-complimentary primers. Before performing this type of mutagenesis, the pGLO GFP gene had to be transferred to pSelect, a phagemid containing the site required for operation of the viral DNA polymerase. After some difficulty, several *E. coli* clones were obtained harboring phagemids which, according to preliminary testing using the restriction enzyme sites introduced, contain all the desired sites. In the future, two of these phagemid candidates will be sequenced to ensure the integrity of the GFP gene and then returned to pGLO. Finally, the system will be tested using by inserting the 529 Tag CD4+ epitope and probing with the corresponding CD4+ hybridoma.
Methods

Preparation of competent bacterial cells
Preparation of small scale saturated cultures
Suspensions of E. coli were prepared by inoculating an Erlenmeyer flask containing approximately 20 ml of sterile 2xYT broth [1.6% (w/v) bactotryptone, 1% (w/v) bacto yeast extract, 1% (w/v) NaCl] with a single colony of the appropriate bacteria obtained from a streaked agar plate, and allowing this culture to grow overnight at 37°C in an orbital shaker.

Preparation and monitoring of exponentially growing cultures
Competent cells were prepared using large scale exponentially growing cultures. In order to obtain such cultures, Erlenmeyer flasks containing 400 ml 2xYT were inoculated with 4, 2, or 1 ml of saturated culture. Concentrations of cultures were periodically determined by using a spectrophotometer to measure their optical densities at 600 nm (OD \text{600}). Before taking readings, the spectrophotometer was first blanked using sterile 2xYT broth. Cultures were harvested when the OD \text{600} was slightly under 0.550, and were then placed on ice for 5 minutes before being used for the remainder of the procedure.

Preparation of competent cultures from exponentially growing cells
After OD \text{600} = 0.550 for any given culture, 50 ml were transferred into a pre-chilled 50 ml screw cap conical centrifuge tube. The culture was then centrifuged at 4000 rpm for 15 minutes in a Jouan RC412 centrifuge that had been precooled to 4°C. After this, the supernatant was decanted, and the bacterial pellet was kept on ice. The pellet was then resuspended with 5 ml ice cold TSS [85% 2xYT (v/v), 10% polyethylene glycol 8000 (w/v), 5% dimethyl sulfoxide (v/v), 50 mM MgCl\text{2} (pH 6.5)]. The suspended cells were maintained at -80°C until needed.

Bacterial transformation and growth
Competent E. coli cells were thawed and held on ice. An appropriate amount of each plasmid to be used for transformation was combined with 100 µl TMC (10 mM Tris pH 7.0, 10 mM CaCl\text{2}, 10 mM MgCl\text{2}) and 100-150 µl of competent bacteria. This mixture was mixed by flicking the microcentrifuge tubes and then incubated on ice for 25 minutes. After this period, the suspensions were again mixed, then rapidly transferred to a 37°C water bath for exactly two minutes. After this heat shock, the suspensions were incubated for 10 minutes at room temperature. 1 ml 2xYT media was added to each tube, and tubes were again incubated, this time at 37°C for 45 minutes. After this period, a portion of each transformation suspension was spread on agar plates containing ampicillin (Amp)-supplemented 2xYT solid media (2xYT media with 15 mg/mL bacto agar and 80 µg/ml Amp) such that individual colonies were visible. These were incubated overnight at 37°C, after which time the colonies on each plate were counted. If liquid cultures of the transformed cells were required, the remainder of each transformation suspension was added to 5 ml 2xYT media containing 80 µg/ml ampicillin. These cultures were grown overnight at 37°C in a rollodrum. Cells were then pelleted from these cultures by centrifugation at full speed in a microcentrifuge for 1 minute. The supernatants were discarded and the pellets were used to acquire DNA quick preps or stored at -20°C for future use.
Plasmid extraction by alkaline lysis
Overnight cultures were vortexed and 1.5 ml from each were pipetted into 1.5 ml Eppendorf microcentrifuge tubes. The bacteria were pelleted by centrifugation for 30 seconds at 14,000 rpm in a room temperature microcentrifuge. The supernatants were decanted and the tubes were centrifuged again for about 10 seconds. The remaining supernatants were aspirated using a micropipette before the collected pellets were loosened by vortexing. Each tube received 100 µl of Solution I [50 mM glucose, 25 mM Tris-HCl pH 8.0, 10 mM EDTA (ethylenediaminetetraacetic acid)]. The pellets were dissolved in the solution using a vortex mixer. Each tube then received 10 µl of a 20 mg/ml hen egg lysozyme solution (Sigma) and was allowed to incubate at room temperature for five minutes. Aliquots of 220 µl of Solution II (1% sodium dodecyl sulphate, 0.2 M NaOH) were added to each tube before immediately inverting at least five times and then placing on ice for five minutes. The tubes were again inverted several times during this period. Each tube received 165 µl of ice cold Solution III (3M potassium acetate, 1.95 M acetic acid) before being mixed through gentle inversions and shaking. The tubes were incubated on ice for about five minutes (and briefly mixed as before once during this time). The tubes were centrifuged for three minutes at 14,000 rpm at room temperature before the supernatants were transferred to new 1.5 ml Eppendorf tubes. Aliquots of 800 µl of freezer cold 100% ethanol were added to each tube containing the supernatants. The tubes were then mixed by inversion, allowed to incubate at room temperature for two minutes, and centrifuged for five minutes at 14,000 rpm at room temperature. The supernatants were decanted and 750 µl of freezer cold 75% ethanol were added to each tube before centrifuging for two minutes at 14,000 rpm at room temperature. The supernatant from each tube was decanted and the tubes were centrifuged again for 10 seconds. Most of any collected supernatant was removed using a micropipette, and then the rest was evaporated using a Speed Vac rotary concentrator for about five minutes. The pellet in each tube was dissolved in 50 µl of T.E. (10 mM TRis pH 8.0, 1 mM EDTA) that contained 50 µl/ml ribonuclease (RNAse). The tubes were then alternately vortexed and centrifuged before being incubated at 37°C for 15 to 20 minutes. The resulting DNA solutions were frozen at -20°C for future use.

Restriction analysis
3 µl aliquots of alkaline lysis products were placed into 1.5 ml Eppendorf tubes. 2 µl of the appropriate digestion buffer, and ~10 units of each restriction enzyme were added to the DNA with GDW on ice such that the final reaction mixture volume was 20 µl. For digestions with large numbers of samples (>5), a premix with was used. All tubes were vortexed, centrifuged for 10 seconds at 14,000 rpm and, and then incubated at 37°C for about 45 minutes.

Agarose gel electrophoresis
Agarose gel preparation
In order to create a 1% agarose gel, a solution of 6 g of agarose and 540 ml of GDW were mixed in a large Erlenmeyer flask. 60 ml 10x TAE (400 mM Tris-acetate, 10 mM EDTA, pH 8.3) was added and the solution was mixed by swirling. The agarose was dissolved and allowed to cool to for 15 minutes in a 56°C water bath before 120 µl of 1 mg/ml ethidium bromide was added (diluting it to a final concentration of 0.2 µg/ml). Any bubbles that formed in the resulting solution were removed with a micropipette before the gel was cast and allowed to cool.
Electrophoresis of DNA samples
Appropriate amounts of purified DNA were combined with GDW and 5x loading buffer [50% (v/v) glycerin, 25 mM EDTA, 1 mg/ml bromophenol blue]. Up to 12.5 µl of these samples were loaded onto an agarose gel containing 0.2 µg/ml ethidium bromide. 3 µl of a 1Kb Plus DNA Ladder (Invitrogen) was also loaded where convenient. The DNA was then electrophoresed at 150 to 200 volts in 1x TAE with 0.2 µg/ml ethidium bromide until the tracking dye had migrated approximately ¾ of the length of the gel. After this time, the gel was removed from the chamber and rinsed in distilled water. Images were obtained by placing the gel into a UV transilluminator box and photograph it with a digital camera.

Preparation of oligonucleotide primers
All oligonucleotides were purchased from the Penn State Hershey Macromolecular Core facility. Dry oligos were diluted to 200 µM using ultrapure glass distilled water (GDW) before further use. Oligos were phosphorylated by preparing kinase reactions consisting of 1µl of the 200µM oligo, 5µl kinase buffer (Invitrogen), 5 µl 10 mM ATP (Gibco), 1 µl T4 polynucleotide kinase (Invitrogen) and 29 µl GDW. Reaction mixtures were incubated at 37°C for 1 hour, followed by 10 minutes of heating at 65°C to inactivate the enzyme. Phosphorylated oligonucleotides were stored at -20°C.

PCR-based site-directed mutagenesis
Mutagenic PCR
PCR-based mutagenesis was performed using a QuikChange Lightning Site-Directed Mutagenesis kit (Agilent Technologies) according to the manufacturer’s specifications, with minor modifications. In short, mutagenic oligonucleotide primers containing the desired mutation were designed using the software available at www.agilent.com/genomic/qcpd and phosphorylated. Mutagenesis reaction mixtures were prepared by combining an amount of each primer corresponding to a final concentration of ~100 pM in the final 50 µl volume, an appropriate amount of plasmid to be mutagenized (corresponding to a molar amount of ~1000-fold less than the primers, although this was varied in order to achieve maximum mutagenic efficiency), 1 µl of the provided dNTP mix, 1.5 µl QuikSolution reagent, 5 µl 10x QuikChange Lightning Buffer, and 1 µl Quikstart Lightning Enzyme. Each reaction was brought up to 50 µl total volume using ultrapure GDW. As a control, similar reactions were prepared using the maximum amount of plasmid without mutagenic primers. Additional controls reactions were prepared using the provided pWhiteScript (pWS) control primer, one with and one without 1.25 µl of the provided oligonucleotide control primers. All reactions were then placed into a thermal cycler, where they were heated to 95°C for 2 minutes, followed by 18 cycles of the following: 95°C for 20 seconds, 60°C for 10 seconds, then 68°C for 2 minutes and 40 seconds (annealing phase parameters calculated using formulas in the Agilent literature). After this, reactions were kept at 68°C for an additional 5 minutes and subsequently cooled to 4°C. A 5 µl aliquot was removed from each reaction for yield estimation by agarose gel electrophoresis. To digest starting plasmid, 2 µl of the provided DpnI was added to each reaction. Tubes where mixed by vortexing, spun down, and incubated at 37°C for 5 minutes. Another 5 µl aliquot was removed from digested samples for yield estimations.
**Transformation of high-competency cells**
The provided XL10-Gold Ultracompetent cells where thawed on ice, and 37 µl aliquots were added to Falcon tubes. These cells were supplemented with 2 µl of the provided BME, swirled gently, and incubated on ice for 2 minutes. 10 µl of each digested DNA sample, as well as 1 µl of a 0.01 ng/µl of the provided pUC18 transformation control, where added to the cell aliquots. These were incubated on ice with periodic mixing for 30 minutes. Samples were then heat-shocked at 42°C for exactly 30 seconds, then quickly returned to ice and incubated for another 2 minutes. After this short incubation, 0.5 ml NZY broth [NZY broth (Gibco) supplemented with 0.4% (w/v) glucose] was added to each tube, and samples were incubated for 1 hour at 37°C in a rollodrum. 10 µl of each incubated sample suspension and 2.5 µl of the pUC18 control suspension were plated onto 2xYT plates supplemented with 80 µg/ml ampicillin.

**Fragment isolation and ligation**

**Fragment isolation**
The fragment of interest was excised by preparing a large-scale digestion consisting of ~2 fmol of plasmid, ~100 units of each restriction enzyme (selected such that they excise the sequence of interest and only cut once in the recipient vector), and the appropriate amounts of buffer(s), brought up to 200 µl with GDW. This solution was incubated at 37°C for 1 hour. Following this incubation, 20 µl was removed from the digested reaction and mixed with 5 µl of 5x loading dye. 10 µl of this mixture was electrophoresed to check the success of the digestion. Meanwhile, ~10 more units of each restriction enzyme were added and the main digestion reaction was incubated for another 30 minutes. Following this extra incubation step, 45 µl loading dye was added and the reaction mixture was split into two aliquots and electrophoresed on a 1% agarose prep gel at 150 V. The band of interest was cut out of the gel with the aid of a UV box, and the DNA was extracted from each band separately using a QIAquick Gel Extraction kit according to the manufacturer’s specifications, including all optional steps. The final product from each band was eluted with 50 µl of a 1:4 dilution of the provided elution buffer.

**Recipient vector cutting and purification**
~1.3 fmol of the recipient vector was digested in a large-scale (300 µl total volume) reaction with ~100 units of each restriction enzyme and the associated buffer(s). In order to purify the cut vector for the ligation reaction, the volume of the sample was brought up to 400 µl with TE. 400 µl phenol:chloroform:isoamyl alcohol (25:24:1) was added, and the sample was vortexed for 1 minute, then centrifuged for 5 minutes. The upper aqueous layer was transferred to a fresh tube, and 45 µl 10x TNE solution (1.5 M NaCl, 100 mM Tris, 10 mM EDTA) was added. After again mixing by vortexing, 800 µl 100% ethanol was added to precipitate the DNA. Ethanol-containing samples were centrifuged for 45 minutes at top speed in a 4°C microcentrifuge. The supernatant was decanted, 750 µl of 75% ethanol was added, and the samples were centrifuged at 4°C for another 10 minutes. The ethanol was decanted and the tube was briefly re-centrifuged to collect residual ethanol, which was subsequently aspirated with a micropipette. The DNA pellet was dried in a Speed Vac rotary concentrator for 10 minutes, then re-dissolved in 25 µl ultrapure GDW by repeatedly mixing, freezing, thawing, and incubating. Yield was estimated by gel electrophoresis and Nanodrop 2000 analysis.
**Ligation**

Ligation reactions were prepared using DNA isolated from each fragment band. Control reactions lacking either the fragment or both the fragment and the ligase enzyme were also prepared. Reaction mixtures consisted of 2 µl 10x ligation buffer (Promega), 2 µl cut vector, 10 µl insertion fragment or 10 µl 1:4 extraction kit elution buffer (for controls), and 2 µl T4 polymerase (Promega). Each mixture was brought up to a final volume of 20 µl with GDW, mixed, and centrifuged briefly. Then, inserts were ligated into the recipient vector by incubating at 16°C for 16 hours. After this time, the reactions were stopped by adding 80 µl TE to each mixture and heat inactivating at 65°C for 10 minutes. Finally, the ligated fragments were transformed into DH5α *E. coli* for maintenance and further manipulations.

**Preparation of single stranded phagemid templates**

**Growth of plasmid-bearing cultures**

Competent JM109 *E. coli* (Promega) to be infected were transformed with a pSelect derivative containing the gene of interest. The transformation was performed as described in the Promega literature. Saturated cultures of transformed *E. coli* were obtained by inoculation into 5 ml TYP broth (16 mg/ml bacto-tryptone, 16 mg/ml bacto-yeast extract, 5 mg/ml NaCl, 2.5 mg/ml K2HPO4) containing 15 µg/ml tetracycline (added as tetracycline in 95% ethanol) followed by overnight incubation in a 37°C roddrum.

**Helper phage infection and phage particle collection**

40 ml of TYP broth containing 15 µg/ml tetracycline was inoculated with 2 ml of the saturated overnight culture. This culture was agitated at 225 rpm and 37°C for 30 minutes. Next, the culture was infected with R408 Helper Phage (Promega) at a multiplicity of infection of ~10. 480 µl of 0.5M K2HPO4 was also added to the infected culture, which was then incubated in a 37°C shaker for at least 6 hours. Following this incubation period, cells were pelleted from the culture by transferring them to 50 ml conical tubes and centrifuging them at 4,000 rpm for 15 minutes in a cooled (4°C) Jouan tabletop centrifuge fitted with an M4 rotor. The resulting supernatant was transferred to a pair of sterile screw cap high-speed tubes and re-centrifuged in a Sorval RC5B centrifuge fitted with an HB6 swinging bucket rotor. After this, the supernatant was again transferred to a new screw-cap tube and placed on ice. Phage particles were precipitated from the supernatant by adding 0.25 volumes of phage precipitation solution [3.75 M ammonium acetate, 20% (w/v) polyethylene glycol 8000], covering securely, mixing by inversion, and incubating on ice for 30 minutes. After incubation, the solution was centrifuged as previously described and the supernatant was thoroughly aspirated. Each remaining pellet was resuspended in 1600 µl TE [10mM Tris (pH8.0), 1 mM ethylenediaminetetraacetic acid (EDTA)] and vortexed vigorously. Like pellets were combined and stored in 800 µl aliquots at -20°C in 1.5 ml microcentrifuge tubes.

**Extraction and precipitation of single stranded phagemid DNA**

800 µl phage particle suspensions were thawed at 37°C, vortexed briefly, and split into two 400 µl aliquots. 400 µl of a chloroform:isoamyl alcohol (24:1) solution was added to each aliquot. This mixture was vortexed at full speed for over 1 minute, then centrifuged at 12,000 g for 5 minutes in a room temperature microcentrifuge. The upper aqueous layer resulting from the centrifugation was transferred to a fresh microcentrifuge tube and the volume was adjusted to 400 µl using TE. This layer was again extracted, this time with 400 µl PCI
(phenol:chloroform:isoamyl alcohol 25:24:1). This mixture was also vortexed at full speed for 1 minute and centrifuged at 12,000 g for 5 minutes. The PCI extraction was repeated 5 times, with each resulting aqueous layer being transferred to a clean 1.5 microcentrifuge tube and adjusted to 400 µl with TE. Phagemid DNA was precipitated from the final aqueous layer by adding 45 µl of 10x TNE solution, mixing by vortexing, and then adding 800 µl of 100% ethanol and vortexing again. This solution was placed in a -80°C freezer for 30 minutes. The precipitated DNA was then recovered by centrifuging at full speed in a refrigerated (4°C) microcentrifuge for 15 minutes and then decanting the supernatant. The resulting DNA pellet was rinsed by pipetting 0.75 ml 75% ethanol in the tube, gently inverting several times, and then centrifuging for another 5 minutes. The ethanol was carefully decanted and residual ethanol was evaporated by placing the tube in a speed vac rotary concentrator for 5 minutes (no added heat). Finally, DNA pellets were dissolved in 20 µl sterile glass distilled water (GDW). This was done by adding the water, and then alternately incubating at 37°C, vortexing, and centrifuging the tubes over 15 minutes. Like DNA solutions were combined and stored at -20°C. In addition, yields were checked by gel electrophoresis.

**In vitro second strand synthesis**

**Oligonucleotide annealing**

Annealing reactions were prepared in sterile microcentrifuge tubes by combining 5 µl of single stranded phagemid DNA, 1 µl ampicillin repair (AmpR) oligonucleotide (Promega), 1 µl of each phosphorylated mutagenic oligonucleotide, and 2 µl annealing buffer (Promega). Reaction mixtures were brought up to 20 µl with GDW, and the tubes were kept on ice. After all components were added, the solutions were vortexed and briefly centrifuged. Oligonucleotide annealing was accomplished by placing the annealing reaction mixtures into beakers with 76°C water. The mixtures were kept in the beaker until the temperature fell to 45°C. After this, the tubes were transferred to a 37°C water bath for another five minutes. Finally, the tubes were allowed to sit at room temperature for five minutes, after which time they were centrifuged to collect condensation and placed on ice.

**In vitro second strand synthesis and BMH71-18 E. coli transformation**

30 µl synthesis reactions were prepared from the 20 µl oligonucleotide annealing reactions. First, 5 µl sterile GDW was added to each annealing reaction. Then 3 µl of 10x synthesis buffer (Promega) was added and the tubes were vortexed. Next, 1 µl of T4 DNA polymerase (Promega) was added and the tubes were again vortexed. Finally, 1 µl T4 DNA ligase (Promega) was added and the tubes were vortexed and then briefly centrifuged to collect solution from the walls. The reaction mixtures were then incubated in a 37°C water bath for 90 minutes and chilled on ice. Mutagenized plasmids were transformed into competent mutS BMH71-18 E. coli cells. 1/10 of the resulting cell suspensions (128 µl) were spread onto 2xYT plates supplemented with 80 µg/ml ampicillin to assess the diversity of mutagenized plasmid. The remainder of each transformation suspension was added to 5 ml 2xYT media containing 60 µg/ml ampicillin. These cultures were grown overnight at 37°C in a rollodrum. Three 1 ml aliquots were taken from each saturated culture. These were then pelleted by centrifugation at full speed in a microcentrifuge for 1 minute. The supernatants were discarded and the pellets were stored at -20°C for future use.
Preparation of Plasmids for Sequencing

Growth of saturated bacterial cultures
Plasmid candidates were used to inoculate 5 ml overnight cultures (2xYT with 50 µg/ml of ampicillin [Amp]). These were grown to saturation overnight on a roldrum at 37°C. 10 µl of cells from this primary culture were added to a solution of 40 ml 2xYT and 200 µl 1% (w/v) Amp and allowed to grow overnight.

Harvesting plasmid candidate cells
40 ml of secondary culture was poured into 50 ml conical tubes. These were centrifuged for 15 minutes at 4000 rpm using the M4 rotor of a Jouan CR412 table top refrigerated centrifuge (precooled to 4°C). The supernatants were decanted and each bacterial pellet was suspended in 5 ml of T.E., after which three 1 ml aliquots were taken from each solution (for a total of nine 1 ml aliquots) and were placed into 1.5 ml Eppendorf tubes. The tubes were centrifuged at room temperature briefly and the supernatant was decanted. The tubes were briefly spun again and the remaining supernatants were removed.

Plasmid purification and column isolation using Roche High Pure Plasmid Isolation Kit
Plasmid purification and isolation were performed using the High Pure Plasmid Isolation Kit (Roche). The kit was used according to the manufacturer’s specifications, except that a 1:5 dilution of the provided elution buffer was used. A bacterial pellet representing 8 ml of saturated culture was used as the starting material. Yields were estimated by gel electrophoresis and Nanodrop 2000 analysis.
Results

Plasmid design and initial testing

As discussed in the introduction, inserting putative epitopes in an orientation-specific manner at the 5’ end of the GFP gene such that the gene will produce a fusion protein when translated requires two unique restriction sites between the GFP start codon and the rest of the gene. The pGLO starting plasmid does not contain any sites at this position (figure 3A). Thus, appropriate sites had to be introduced via a site-directed mutagenesis procedure. The sites selected for mutagenic insertion into pGLO were those of SpeI, which cuts at 5’-A|CTAGT-3’, and NotI, which cuts at 5’-GC|GGCCGC-3’. As discussed previously, neither site is present in either pGLO (figure 3A) or SV40 Tag, so cutting the new sites would only open pGLO, not fragment it, as would occur if the sites were already present in either. Importantly, both restriction enzymes cut well close to DNA ends, a characteristic which is required for cutting terminal sites introduced by PCR primers (9). Finally, both enzymes are functional in New England BioLabs buffer 2.1.

In addition to the unique insertional sites, the recognition site of EcoRI was also selected for introduction between the NotI and SpeI sites. The purpose of this site, which is already found at the end of the pGLO GFP gene, is to provide a convenient initial screen for successful fragment insertion. If an insertion event occurs, the EcoRI site separating the unique insertional sites will be lost. Thus, EcoRI-digested plasmid without an insertion will yield two bands when electrophoresed (two EcoRI sites), while plasmid with an insertion will only be linearized (one EcoRI site). The planned insertion also contains an extra A-T base pair to compensate for the 8 basepair long NotI site and allow for easy in-frame insertion of Tag fragments. The resulting 21 bp insertion sequence is shown in figure 3B.

In order to ensure that pGLO really did not contain the SpeI and NotI sites, the plasmid was digested with those enzymes (Invitrogen), along with EcoRI (New England BioLabs). As a positive control for the SpeI digestion, the pGEM T-Easy vector (Promega), which contains a SpeI site, was also digested with the enzyme. As expected, EcoRI linearized pGLO (figure 4 lane 3; note the shorter run distance vs. uncut pGLO), while NotI and SpeI had no effect (figure 4, lanes 4 and 5). As expected, SpeI linearized the pGEM T-Easy vector (figure 4 lane 7). Thus, the mutagenized pGLO should allow for the insertion of Tag fragments, as planned.
**PCR-based site-directed mutagenesis of pGLO plasmid**

**Primer design**

PCR-based site-directed mutagenesis was selected to introduce the desired sites into pGLO. This method is convenient because, unlike some other techniques for introducing site-directed mutations, it does not require special sequences to be present in the plasmid being mutagenized. 69 bp mutagenic oligonucleotides primers were designed using the web-based primer design software available from Agilent Technologies (10). The sequences of these primers, which are fully self-complimentary as per Agilent’s recommendation, are shown in table 1. They contain the 21 bp insertion flanked on both sides by 24 bp of the pGLO sequence around the GFP start codon. Due to the nature of PCR-based mutagenesis (in which the high-fidelity Pfu polymerase copies the entire plasmid, with the copy being linear rather than circular) and the self-complementarity of the primers, the mutagenized plasmid copies cannot be re-replicated, and “PCR” produces linear rather than exponential expansion.

**PCR-based site-directed mutagenesis of pGLO**

The first round of PCR-based mutagenesis was performed as described in the methods. The gel resulting from electrophoresis of samples taken from the PCR reactions before and after the introduction of the DpnI enzyme is shown in figure 5A. Reactions included the pWhiteScript (pWS) mutagenesis control plasmid without (lanes 2+3) and without (lanes 4+5) the provided pWS mutagenic primers, as well as 100 ng pGLO without primers (lanes 6+7) and 100 ng (lanes 8+9) and 33 ng (lanes 10+11) pGLO with the 69 bp primers Myli 531 and Myli 532. The gel has bands in all pre-DpnI except for that of the no primer pWS reaction. The position of the pWS with respect to the ladder corresponds well to the 4.5 kb plasmid size, while the 5.4 kb pGLO ran surprisingly fast. In the post-DpnI lanes, however, only the pWS still has visible DNA, which is fainter than the pre-DpnI band (the difference in signal intensity corresponding to the methylated and hemimethylated input DNA degraded by the enzyme). Thus, all pGLO appears to have been degraded by the enzyme, which in turn suggests that no mutagenized plasmid was generated in the reactions. This notion was reinforced when the reactions were transformed into the XL10-Gold cells. Only the provided pUC18 (1 µl of 0.01 ng/µl plasmid was used for the transformation) transformation control produced ampicillin-resistant colonies, indicating that all plasmids had been degraded by DpnI (i.e. none were produced in vitro).
**Test mutagenesis of the pWS control plasmid**

Given that undegraded DNA was apparent in the post-digestion pWS control digestion, it was surprising that no amp-resistant colonies were obtained from this reaction mixture. In order to rule out a problem with the kit or the specific techniques used, mutagenesis was repeated using only varying amounts of the pWS plasmid. This included 25 ng of the plasmid without the primers, as well as 25, 5, and 1 ng of the plasmid with primers. In order to increase the amount of mutagenized plasmid produced, the amount of PCR cycles was increased to 25. Aliquots from before and after *DpnI* digestion were electrophoresed as before. The resulting gel is shown in figure 5B. Once again, no DNA is visible in either lane in the reaction cycled without primers, suggesting that the amount of input plasmid in the small aliquot run is simply too low to visualize on a gel. In addition, a post-*DpnI* band is evident in the 25 ng and 1 ng lanes, but not the 5 ng lane. The pre-*DpnI* band is also more intense in the 1 ng lane than in the 5 ng lane, suggesting a possible sample mix-up. When DNA from these reactions was transformed into XL10-Gold bacteria, using 2 µl rather than 10 µl of DNA, large amounts of ampicillin-resistant colonies were obtained for all primer-positive reactions. Additionally, ~100 resistant colonies were obtained from the primer-negative control. However, this number is low enough compared to the others obtained that it does not cast significant doubt on the idea that some successful mutagenesis had successfully occurred.

To further ensure that mutagenized plasmids had successfully been obtained, all pWS plates were replica plated onto 2xYT+ampicillin plates supplemented with 32 µl 5% X-Gal (Promega) and 40 µl of 50 mg/ml isopropylthio-β-galactoside (Promega). With these reagents, bacteria transformed with mutagenized pWS produce blue colonies, but colonies with the original version of the plasmid do not because the β-galactosidase gene responsible for cleaving X-gal to a colored substrate has a premature stop codon (the provided mutagenic oligos repair this codon). Although many colonies were rendered difficult to count by the replica plating procedure, countable colonies on the primer-positive plates averaged 69.5% blue (range 66.8% to 71.4%). In contrast, none of the 81 colonies counted on the primer-negative plate were blue. Thus, this experiment demonstrates mutagenesis of the control plasmid occurring at a reasonably high efficiency, with some leak at the template-elimination step (a result which is somewhat at odds with the previous experiment).
Round 2 PCR-based site-direct mutagenesis of pGLO

Having demonstrated successful mutagenesis of pWS, another attempt was made to introduce the desired mutation into pGLO. As a result of limited reagents, reactions were 1/3 the size of that described in the methods. Reactions were prepared with 33.3, 16.7, and 6.7 ng of pGLO (equivalent to 100, 50 and 20 ng at normal reaction volume). In an attempt to increase the likelihood of obtaining product, reactions were cycled for 13 rounds of PCR using the parameters recorded in the methods, followed by 13 more rounds using 65°C instead of 68°C at the annealing step if the mutagenic oligos were not annealing fully to the template at the higher temperature. When samples were run on a gel to check for DpnI resistant DNA, pre-DpnI but not post DpnI bands were observed for all pGLO concentrations, indicating that very little if any plasmid was produced in the reactions (figure 5C). Very strong low molecular weight bands corresponding to the mutagenic oligos were also observed, reinforcing this notion (these bands ran off the end of the other two gels). The resulting reaction mixtures were digested with 1 µl DpnI and the 33.3 and 16.7 ng reactions were transformed into XL-10 Gold E. coli. A significant number of ampicillin-resistant colonies were obtained from these transformations. A subset of these colonies was transferred to an amp-supplemented patch plate for further manipulations, and successful growth was obtained there as well. However, no candidates grew when transferred to 5 ml 2xYT broth supplemented with 60 µg/ml ampicillin. This suggested that the plates being used did not contain sufficient amounts of the antibiotic. When the plates were replica plated to fresh amp-supplemented 2xYT plates, only one colony on one of the plates grew. This colony was grown up and DNA was extracted via quick prep in hopes that it might contain mutagenized pGLO. However, running the quick prep product on a gel revealed that no plasmid DNA was present.

In a final bid to obtain mutagenized colonies, the remaining DNA from the second round of pGLO mutagenesis was transformed into DH5α and JM109 (Promega) E. coli. The DH5α transformation was performed as detailed in the methods except that the amount of competent cells used was increased to 200 µl, and the JM109 transformation was performed in accordance with the Promega literature, except that NZY broth supplemented with 0.4% (w/v) glucose was used for the recovery incubation. 2-3 µl of each pGLO mutagenesis reaction was used to transform both cell lines. pUC18 and TE were used as positive and negative transformation
controls, respectively. 100 µl of each transformed cell suspension was plated on an ampicillin-supplemented 2xYT plate. Controls behaved as expected, while no resistant colonies were obtained on any of the pGLO plates. Thus, PCR-based site-directed mutagenesis with the primers in table 1 did not introduce the desired mutation, and this failure was most likely due to the primers themselves rather than the kit, technique, or competent cells used.

**Altered sites, phage-based mutagenesis**

**Experimental design and preliminary experiments**

Since the PCR-based mutagenesis method did not successfully introduce the desired restriction sites into pGLO, phage-based altered sites mutagenesis was pursued as an alternative. This technique takes advantage of the single stranded DNA virus R408. Engineered versions of this virus will infect *E. coli* but not reproduce themselves. If the *E.coli* contain a phagemid such as pSelect (figure 6) with the f1 origin of replication recognized by the phage’s DNA polymerase, a single stranded copy of the phagemid will be generated and packaged into phage particles. Synthesis of the second strand for this ssDNA can be carried out *in vitro*. If a partially mismatched oligonucleotide is used to prime second strand synthesis, site-directed mutations can be introduced. Input plasmid is selected against by also using a second mutagenic oligonucleotide primer, AmpR that repairs a broken (frame shifted) ampicillin resistance gene in the phagemid. When selection is carried out on ampicillin-containing media, only clones bearing the duel-mutagenized plasmid will survive.

Because pGLO does not contain the broken ampicillin resistance gene, the GFP gene had to be excised and transferred to the pSelect phagemid, which does have this feature. Unfortunately, no single set of restriction enzymes allowed for the transfer of the GFP 5’ end into pSelect. The only possible way of performing this transfer was to excise the pGLO GFP and some of the material 5’ of the gene via an *EcoRI/EcoRV* double digestion and then ligate it into pSelect cut with *EcoRI* and *SmaI* (both of which are in the MCS of the phagemid). This works because *EcoRV* and *SmaI* are both blunt-cutting restriction enzymes. However, the blunt-blunt ligation produces a hybrid site that cannot be recognized by either enzyme, and this site must therefore be repaired via mutagenesis so the mutagenized GFP gene can be re-excised and returned to pGLO, which contains the machinery required for operation of the arabinose-inducible promoter. A simple map of the product of such a ligation, termed pSel-GFP, is shown...
in figure 7A. The main mutagenic oligo and the hybrid site repair oligo, shown in table 2 were designed to be anneal to the appropriate strand of the pSel-GFP phagemid and introduce the desired 21 bp insertion/6 bp alteration. Because EcoRI was used to excise the GFP fragment, EcoRI could not be introduced between the SpeI and NotI sites, so the cognate site of HindIII, which cuts at 5’-A|AGCTT-3’ and is already found in the middle of the GFP gene and the pSelect MCS, was used instead.

In order to ensure that the enzymes behaved as expected, a small-scale double digestion was performed on pGLO with EcoRI and EcoRV-HF (New England BioLabs, both enzymes are operational in NEBuffer 2). This released a DNA fragment running at about 1800 bp, which is reasonably close to the theoretical EcoRI-EcoRV fragment size of 2296 bp (figure 8A). A similar, but large-scale, digestion was performed on pSelect using EcoRI-HF and SmaI (New England BioLabs). In this case, a 250 µl reaction was prepared using NEBuffer 4 and 50 units of SmaI. After 30 minutes of incubation at 37°C, another 50 units of the enzyme was added. The reaction mixture was incubated for another 30 minutes, after which 20 µl of the reaction was removed to run on a gel. 50 µl of 1x NEBuffer 4 and 100 units of EcoRI were added to the remaining mixture, and incubated at 37°C for another 30 minutes. When this and a parallel small-scale digestion with no enzyme were run on a gel, the majority of the phagemid appeared linearized (the EcoRI and SmaI sites are within 20 bp), although there was also a band corresponding to undigested DNA (figure 8B).

**Creation of the pSel-GFP phagemid**

The previously double-digested pSelect was purified as described in the methods. The sample was then Nanodropped to assess yield. DNA in solution was only 30.3 ng/µl. After addition of 1 mM EDTA, many freeze/thaw cycles, and long-term (>36 h) incubation at 4°C, more of the pellet was dissolved and yield was increased to 104.1 ng/µl. Purity and possible degradation of the vector were assessed by gel electrophoresis (figure 9A), revealing that the vector was pure, intact, and ready to receive an insert. The EcoRI-EcoRV GFP fragment was excised from pGLO as described in the methods. The smaller GFP fragment was excised from the prep gel with the aid of a UV box, and placed into pre-weighed 40 ml tubes for the remainder of the procedure. As shown in figure 9B, the edges of the bands were left intact in order to estimate the efficiency of the extraction procedure. Electrophoresis of a portion of the product
from both bands (figure 9C) revealed that the fragment had been successfully purified. Ligation reactions were then prepared using DNA from each band (reactions 1 and 2) alongside the no-DNA controls described in the methods (3, no DNA with ligase and 4, no DNA without ligase).

Following inactivation of the T4 ligase, 40 µl of all four reactions were transformed into 200 µl of competent DH5α E. coli. 50 µl of each resulting suspension was plated onto 2xYT agar plates supplemented with 72 µl of 0.5% (w/v) tetracycline (the pSelect plasmids contain this secondary antibiotic resistance gene since the ampicillin gene is inactivated). Reaction 1 had around 2956 resistant colonies, reaction 2 had 486, reaction 3 had 1616, and reaction 4 had only 2. The growth on the reaction 3 was unexpected and probably indicates cross-contamination from reaction 1 or 2. However, this was not an issue because the other control reaction did not produce resistant colonies (indicating that the source of contamination was not one of the reagents used) and because cross-contamination of reactions 1 and 2 did not matter since they contained essentially the same thing. 16 total pSel-GFP candidates from reaction plates 1 and 2 were patched onto another tetracycline-supplemented agar plate. Candidates were then grown up in 5 ml 2xYT broth supplemented with 20 µl 0.5% tetracycline. These cultures were grown overnight and used to obtain quick prep DNA from each candidate, which was subsequently electrophoresed on a 1% agarose gel (figure 10A). To further determine the nature of the plasmid in each candidate clone, quick preps were digested with HindIII (Invitrogen) and EcoRI-HF (NEB) and electrophoresed to determine banding pattern. As shown in figure 7A, HindIII should cut at two sites in the 7353 bp pSel-GFP, excising a 1112 bp fragment and from the other 6241 bp piece (figure 10B). In contrast, the plasmid has only one EcoRI site, which was used to insert the GFP fragment, so this digestion should only linearize the phagemid (figure 10B). E. coli candidate clones 7 and 8, which contained phagemid displaying the proper banding pattern on all three gels (substantially shorter run on the undigested gel, corresponding to the increased size from the 2296 bp EcoRI-RV GFP fragment, two bands on the HindIII gel, and decreased run distance corresponding to linearization on the EcoRI gel), were selected for further manipulations.
Phage-based altered-sites mutagenesis of pSel-GFP candidates 7 and 8 (1- and 2-series)

Single stranded phagemids were prepared for each candidate as described in the methods. All phage-based mutagenesis reactions were also performed on unaltered pSelect as a positive control. When DNA extracted from the phage particles was electrophoresed on an agarose gel, no phagemid or phage DNA was visible for any of the three samples. In an attempt to remedy this, a second series of single-stranded phagemid synthesis was performed using the R408 helper phage at a multiplicity of infection of ~800 instead of 10. When the 2-series single stranded phagemids were electrophoresed, a faint band was evident running at about 4.8 kb (figure 11A). When second strand synthesis was performed on the 2-series ssDNA samples, with or without the AmpR oligo, no ampicillin resistant colonies were obtained from any of the mutagenesis reactions upon transformation into mutS BMH71-18 E. coli, although transformation controls behaved as expected. On the contrary, growth was observed in all liquid cultures, even that inoculated with E. coli that had been mock-transformed without DNA. Given the lack of growth on ampicillin-supplemented plates, it seems probable that the ampicillin stock used to supplement the 2xYT broth did not contain a sufficient concentration of ampicillin. As a result, a new 1% ampicillin in methanol stock solution was made.

3-Series phage-based mutagenesis using Myli 534 and Myli 535

In order to obtain successfully mutagenized pSel-GFP, yet another modification to the phage-based mutagenesis procedure was attempted. This time, only JM109 E. coli transformed with candidate plasmid 7 were used. JM109 E. coli containing either pSelect or the pSelect variant pSel-ESV from a previous study were thawed from the -80°C freezer for use as positive controls. R408 helper phage was added to saturated cultures as described in the methods, but at a multiplicity of infection of ~7000. In addition, the infection incubation period was extended from 6 hours to 9 hours. When ssDNA from these reactions was assayed on a gel, the signal in all three lanes was much stronger than in the 2-series ssDNA (figure 11B), thus indicating a sufficiently high yield of ssDNA for second strand synthesis. Second strand synthesis was performed on 3-series pSel-GFP candidate 7 ssDNA. Three second strand reactions were created: reaction 1, which had no mutagenic oligonucleotides, reaction 2, which had the AmpR oligo, and reaction 3, which had the AmpR oligo, the main mutagenic oligo Myli 534, and the hybrid restriction site repair oligo Myli 535. When these were transformed into mutS BMH71-18
*E. coli* and plated on ampicillin-supplemented media, colony counts were 0 for reaction 1, 483 for reaction 2, and 1860 for reaction 3. Thus, the system for selection against unmutagenized phagemid seems to have worked as desired – no resistant colonies were contained when the AmpR oligo was not used, but many were present in second strand synthesis reactions with the oligo. The increase in colonies from reaction 2 to 3 was most likely due to increased second strand synthesis efficiency resulting from the presence of the additional primers in reaction 3. After demonstrating the success of the *in vitro* synthesis, DNA was isolated from reaction 3 *E. coli* and transformed into DH5α *E. coli*. These were used to create a patch plate of 9 mutagenesis candidates, from which DNA was isolated for restriction analysis.

**Restriction analysis of mutagenized pSel-GFP candidates**

As a preliminary check for successful mutagenesis, plasmid from the candidate *E. coli* clones was digested with several restriction enzyme combinations. These tests took advantage of the new sites introduced by the main mutagenic and EcoRV site repair oligos (figure 7B). Because the plasmid already contained two *Hind*III sites and an *Eco*RV site (figure 7A), digesting with these enzymes should produce an additional, low molecular weight band in successfully mutagenized plasmids. Such a band can be seen in most of the clones tested (figure 12A, orange arrows), suggesting that both a successful main insertion and a successful hybrid site conversion had occurred. As expected, this band is not present in unmutagenized control digested with the same enzyme. A clone displaying the desired behavior in both digestions is denoted with an asterisk. In order to further test the integrity of the insertion, a promising subset of these clones were selected for digestion with *Spe*I (New England BioLabs) and *Not*I-HF (New England BioLabs). These sites are present in the desired insertion, but are found in neither pGLO nor pSelect. Therefore, digestion with either one should linearize mutagenized plasmids, while having no effect on unaltered plasmids. As shown in figure 12B, all candidates were linearized (further run distance than unmutagenized control) by *Not*I but not *Spe*I. Upon examining the primer design notes, it was discovered that the sequence of the *Spe*I site had been incorrectly transcribed during the ordering process (figure 12C). To remedy this, a correct version of the main mutagenic oligo, Myli 536, was ordered (table 2). When mutagenesis was repeated as previously described with the corrected oligo, many of plasmids isolated from the new DH5α *E. coli* candidates once again displayed the correct banding pattern with both the *Hind*III/*Eco*RI-RV
digestion set (figure 13A; a candidate with the correct band pattern in both lanes is denoted by an asterisk). Furthermore, digesting a subset of the candidate plasmids with \textit{SpeI} and \textit{NotI} resulted in the linearization of all samples for both enzymes. Thus, all the desired restrictions sites were successfully inserted into these candidates.

\textbf{Preparation of mutagenized candidates for sequencing}

Plasmid DNA from two candidates displaying the desired banding pattern in the previously described restriction analysis, candidates 1 and 2, were prepared for sequencing by Roche prep, as described in the methods. Both prep samples were electrophoresed on an agarose gel to assess purity. Both samples had no visible low molecular weight contamination and a small amount of genomic DNA (data not shown). DNA yields were then were assessed via nanodrop 2000. The prep from candidate 1 was 258.1 ng/µl, while the prep from candidate 2 was 179.3 ng/µl. The samples were diluted to 100 ng/µl for sequencing by the Penn State Hershey Macromolecular Core Facility.
Discussion

This study has demonstrated the construction of an altered GFP gene, which according to preliminary restriction analysis was successfully mutagenized to introduce two unique restriction sites just downstream of the start codon. Once complete, this system will be very useful in the fine-mapping of CD4+ T cell epitopes in the SV40 Tag protein by allowing the production of any length of Tag desired in a fusion protein with GFP. In addition Tag is maintained in a pSelect derivative, so specific mutations can be induced before amplification and insertion into the modified pGLO. Once the Tag gene sequence has been PCR amplified with primers introducing the NotI and SpeI, the PCR product and the pGLO epitope test vector can be digested and ligated together. Provided that the PCR primers are designed such that the Tag sequence inserts in the correct orientation and in frame, expression of the fusion protein can be induced in *E. coli*, which can then be fed to cultured dendritic cells and screened by CD4+ hybridoma clones specific for the epitope being characterized (figure 2).

The process of introducing the restriction sites was not without difficulty, however. We initially elected to use a PCR-based mutagenesis site-directed mutagenesis technique, as it could be performed without moving the GFP gene to another plasmid and thus represented the simplest method for introducing the sites. Unfortunately, even after several attempts using different GFP concentrations and competent cells, we were unable to obtain mutagenized plasmid. When the products of the mutagenesis reaction were analyzed by gel electrophoresis, no band was ever observed in the lane containing DNA taken from the reaction following digestion of the input plasmid by *DpnI*, which suggested that no product was being produced (figure 5). In addition, no truly ampicillin-resistant colonies resulted when this DNA was transformed into *E. coli*. Taken together and with the successful mutagenesis of the pWhiteScript control plasmid, these suggest a problem with the mutagenic primers used. The primers, which are self-complimentary, contained a relatively large 21 bp insertion and at 69 bp total length were at the upper limits of the primer size recommended by the Agilent literature (table 1). One possible explanation for the failure of these primers is that the large insertion means that it is significantly more favorable for the primers to self-anneal than to anneal to the pGLO template. Furthermore, the length of the primers results in a melting temperature of 65.3°C for the 5’ end of the sense primer. This may also have been problematic in and of itself, as the relatively low melting temperatures of each side may have led to poor annealing to the template.
When PCR-based mutagenesis did not appear to be working, we changed strategies and attempted phage-based altered-sites mutagenesis instead. This technique was less convenient because it required the transfer of the GFP gene to the pSelect plasmid containing the broken ampicillin resistance gene, which is needed for selection of mutagenized plasmid. However, phage-based mutagenesis has the advantage of only necessitating mutagenic oligos that anneal to one strand and not involving PCR. This circumvents both the problems that we believe may have contributed to the failure of the PCR method. Unfortunately, difficulties were encountered in this procedure as well, primarily in the form of the incorrectly ordered main mutagenic oligo. However, the construct resulting from mutagenesis with this oligo may prove to be a useful control for fusion protein translation. Although the oligo still contains the GFP start codon, it is shifted in position by one nucleotide. Translation beginning from that start codon will therefore produce a frame-shifted protein, which will not fluoresce. However, if translation begins from an internal ATG instead, some fluorescence may still be observed. This would be an important result because it would mean that fluorescent *E. coli* clones harboring correctly mutagenized GFP with a Tag insert might not actually be producing the fusion protein, but could instead be starting translation from the alternate ATG.

When a new main oligo was ordered, we were able to perform another round of mutagenesis, which according to the results of our restriction analysis successfully introduced the *SpeI*, *HindIII*, and *NotI* sites. However, it is important to make sure that the sites are correctly oriented and in frame with the rest of the GFP gene. It is also important to check the integrity of the GFP gene, as it is always possible that undesired mutations may have occurred during second strand synthesis. Once it has been confirmed that the mutagenized GFP gene has the correct sequence, the *EcoRI*-*EcoRV* fragment must be excised from pSelect and returned to pGLO so that the gene can be expressed. Finally, the DC presentation system must be tested before the vector can be used for epitope fine mapping. This will be performed by inserting a portion of Tag known to contain the 529 epitope into the vector, expressing this in *E. coli*, and then probing DCs which have been fed these bacteria with CD4+ hybridomas specific for the 529 epitope. If a hybridoma response is observed, it indicates that everything is working as planned, and the system can then be put to use.
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References


Figure 1. T cell response to SV40 Large T antigen-driven tumors
A.) MHC molecules bind peptides via specific residues known as anchor residues (1). These residues interact with binding pockets in the floor of the MHC molecules. The TCR can then interact with this complex in a highly specific manner by binding outward facing residues in the peptide as well as polymorphic amino acid sequences on the MHC. B.) In order to be activated, T cells must encounter their cognate epitope presented by a specialized antigen presenting cell such as a dendritic cell. CD8+ killer T cells recognize epitopes presented by MHC class I molecules, while CD4+ helper T cells recognize epitopes presented by MHC class II molecules. Studies have demonstrated that successful tumor control is correlated with the simultaneous activity of both types of T cells. CD8+ T cells eliminate compromised host cells, while CD4+ T cells enhance CD8+ proliferation and presence in the tumor microenvironment and delay the onset of CD8+ tolerance. C.) T cell epitopes in the SV40 Tag sequence for C57BL/6 (H-2b) mice. Exact amino acid locations within the Tag protein are given for each previously-characterized CD8+ epitope (red). The approximate amino acid location of each newly-discovered putative CD4+ epitope (purple) is also given. We have generated T cell hybridoma clones that respond to each of these epitopes.

Figure 2. A fusion protein-based system for CD4+ epitope characterization
In order to better characterize the precise locations and important residues of the putative CD4+ epitopes, a system will be generated in which pieces of the SV40 Tag gene sequence can be inserted at the 5’ end of a GFP gene under the control of an arabinose-inducible promoter (A). The fusion protein can then be expressed in E. coli transformed with the vector by growing them on arabinose-supplemented media (B). The bacteria will be fed to cultured dendritic cells (C), which will phagocytose the E. coli and break down the fusion protein in phagocytic vesicles. These vesicles will fuse with MHC-II-bearing vesicles, where the epitope or epitope variant from the fusion protein can bind to MHC-II molecules and displayed on the cell surface (D) (1). Finally, the response to the peptide by CD4+ hybridoma clones, which express a LacZ gene under the control of the NFAT promoter system, can be quantified in a colorimetric assay (D inset). In order to create a system in which such a fusion protein can be generated for any portion of Tag desired, two unique restriction sites must be introduced to the 5’ end of the GFP gene in the pGLO plasmid via a site-directed mutagenesis method. Then, epitopes can be amplified by PCR primers that introduce the same restriction sites, and the epitope can be ligated into the GFP vector in a direction-specific manner.

Figure 3. Target vector construct
A.) A restriction site map of the unaltered pGLO plasmid (8). The plasmid includes an E. coli origin of replication, a beta-lactamase ampicillin resistance gene, and a GFP gene under the control of the arabinose/arabinose repressor system. B.) The sequence of the target construct at the 5’ end of the GFP gene. The red ATG represents the GFP start codon. The next three colored sequences are the SpeI site, the EcoRI site, and the NotI site, respectively. Finally, the A offset by asterisks was included to make it easier to introduce in-frame insertions (the NotI site consists of 8 base pairs).
**Figure 4. Restriction analysis of pGLO**

In order to ensure that pGLO did not contain the *Spe*I and *Not*I sites, the plasmid was digested with these enzymes. The single pGLO *Eco*RI site was also tested. The band pattern from the *Spe*I and *Not*I digestions was indistinguishable from the undigested control, indicating that they did not cut the plasmid. In contrast, the apparent size of pGLO digested with *Eco*RI increased dramatically, indicating that *Eco*RI linearized the plasmid. pGEM T-easy, which contains a *Spe*I site, was digested as a positive control. As expected, the plasmid was linearized by the enzyme, as evidenced by the significantly shorter run distance of the main band.

**Figure 5. Mutagenic PCR of the pGLO plasmid**

In order to introduce the desired restriction sites into pGLO, mutagenesis was carried out using the QuickChange Lightning Site-Directed Mutagenesis Kit (Agilent Technologies). PCR reactions were initially carried out using two concentrations of pGLO plasmid (100 and 33 ng of DNA in the reaction mixture) with the Myli 531 and 532 mutagenic oligos, alongside the pWhiteScript mutagenesis control included in the kit. Small samples were removed before and after digestion of input plasmid with *Dpn*I, which cuts methylated and hemimethylated sites, and analyzed by gel electrophoresis (A). All reactions except the for the pWS control reaction with no primers (lane 2) had a visible band before digestion, but only the pWS control with primers (lane 3) had a band afterwards. When the resulting DNA was transformed into DH5α *E. coli*, no ampicillin resistant colonies were obtained. Given the post-mutagenesis band for the pWS control, the lack of colonies for this sample was surprising. Mutagenesis was repeated using only the pWS control, and samples were removed for electrophoresis as before (B). This time two of the three concentrations of pWS mutagenized had a post-digestion band, and transformation yielded between 500 and 1500 colonies for each sample. The control reaction with no primers also produced about 100 colonies. When pGLO mutagenesis was repeated (C), no band was observed in the post-digestion lane, and no ampicillin-resistant colonies were obtained, as before.

**Figure 6. Sequence map of the pSelect phagemid**

The 5.7 kb pSelect vector contains all the important components of a plasmid, including a tetracycline resistance gene for selection following transformation. It also has the frame-shifted ampicillin gene and f1 viral origin of replication required for altered-sites mutagenesis (11). The *Sma*I and *Eco*RI sites used to insert the GFP fragment are found in the MCS.
Figure 7. Selected restriction sites in the pSel-GFP phagemid

HindIII and EcoRI/EcoRV digestions were performed as a preliminary screen of phage mutagenesis candidates for the desired restriction sites. Because both the pSelect and the GFP fragments contain 1 HindIII site, cutting with enzyme excises a 1112 bp fragment from the remaining 6241 bp of the plasmid. The main mutagenic oligo is designed to introduce another HindIII site, so successfully mutagenized candidate plasmids should have three fragments: a large 6241 fragment, a smaller 1003 bp fragment, and a very small 130 bp fragment. Likewise, an EcoRI/EcoRV double digestion of pSel-GFP should cut at the EcoRI site used to insert the GFP fragment as well as an EcoRV site that is present in pSelect, producing a 5057 bp fragment and a 2296 bp fragment. It will not cut at the EcoRV/SmaI hybrid site produced by blunt-blunt ligation. In contrast, EcoRV will cut at this site once it has been repaired by the hybrid site repair oligo. Therefore, double digestion of a mutagenized plasmid should excise the 1698 bp GFP fragment as well as generating two other bands running at 619 and 5057 bp.

Figure 8. Double digestion of pGLO and pSelect

pGLO was double digested with EcoRI and EcoRV to excise the GFP gene (A). The apparent size of the lower molecular weight band fits very well with the expected 1698 bp size of the fragment. Likewise, pSelect was digested with EcoRI and SmaI (B). In contrast to the pGLO digestion, these sites are almost adjacent, and the second band produced is so small that it is undetectable. As expected, the main pSelect band ran differently after digestion, indicating successful linearization.

Figure 9. GFP fragment and pSelect linear vector isolation

pSelect vector double digested with EcoRI and SmaI was purified by extraction, and a sample was analyzed by gel electrophoresis (A). A single band was observed, thus indicating that the DNA had been successfully purified. During large-scale preparation of the GFP fragment, samples were removed before (B lane 1) and after (B lanes 2 and 3) gel isolation of the GFP band. The band was removed from the prep gel (C) such that the sides of the band in each lane remained. The significant fluorescence remaining after isolation of the DNA in the band (C vs B lanes 2 and 3) indicates a good yield.

Figure 10. Restriction analysis of pSel-GFP candidates

As a screen for successful fragment insertion, pSel-GFP candidates were analyzed by electrophoresis (A). Since the fragment inserted was 1698 bp, candidates running above the 5000 bp marker band (e.g. candidate 4) probably contain the insert, while bands running below it (e.g. candidate 1) do not. The candidate phagemids were also digested with HindIII and EcoRI. HindIII digestion (B) should produce two bands of sizes 6241 and 1112 bp in candidates with the insert (e.g. candidate 4), but will only linearize candidates without it (e.g. candidate 1). Finally, EcoRI digestion (C) should simply linearize both types of plasmids, so the interpretation of the results is identical to the undigested gel (plasmids with the insert will run slower).
Figure 11. Single stranded template preparation for pSel-GFP
In order to perform altered-sites mutagenesis, cultures of JM109 *E. coli* bearing two of the pSel-GFP candidates, as well as a control with the unaltered pSelect phagemid, were infected with the R408 helper phage. The second attempt at this procedure yielded some single stranded product, as seen in the lower band in all lanes of (A), but not enough to generate ampicillin-resistant colonies when transformed into BMH71-18 *E. coli*. Some genomic DNA (the band running above the 12 kb ladder band) can also be seen. The third attempt, performed with a very high multiplicity of infection and a long infection time, yielded significantly more product for all three samples (B). The R408 genetic material can also be seen as the lower band in the candidate 8 lane, although for reasons that are unclear it is not present in visible amounts in the other lanes.

Figure 12. Preliminary restriction analysis results for phage mutagenesis candidates
Single stranded template from pSel-GFP candidate 7 were mutagenized using the Myli 534, Myli 535, and AmpR oligos. In order to determine whether the desired sites had been successfully introduced, the resulting plasmids were first digested with *Hind*III and *Eco*RI/*Eco*RV (A). For the *Hind*III digest, 2 bands with sizes 6241 and 1112 bp are expected in the unmutagenized plasmid, while 3 bands with sizes 6241, 1003, and 130 bp should result from an appropriately mutagenized candidate. Likewise, the *Eco*RI/RV double digest should yield two bands with sizes 5057 and 2296 bp from unmutagenized plasmid and three bands with sizes 5057, 1698, and 619 bp from mutagenized plasmids. Most candidates, such as the one with the asterisk, displayed banding patterns like those expected for mutagenized plasmids in both digestions, including the telltale low molecular weight bands (orange arrows). However, when a subset of the samples was digested with *Spe*I and *Not*I, *Spe*I failed to linearize any of the candidates, suggesting a problem with the main mutagenic oligo (B). It was discovered that the Myli 524 oligo sequence had been incorrectly inverted just prior to ordering (C).

Figure 13. Preliminary restriction analysis results for phage mutagenesis with the corrected main oligo
Phage-based mutagenesis was repeated using the corrected Myli 536 oligo instead of the faulty Myli 524. In the *Hind*III and *Eco*RI/*Eco*RV digestions, many of the candidates, including the one with the asterisk, displayed the desired band pattern. In addition, when the candidates were digested with either *Spe*I or *Not*I, the samples were linearized, as evidenced by the difference in run distance from the unmutagenized control (the second control band contains an erroneously included, irrelevant sample). Thus, the candidates appear to have been successfully mutagenized.
Table 1. Mutagenic oligonucleotides used for Quick Change Strategy

<table>
<thead>
<tr>
<th>Name</th>
<th>Sequence (5' to 3')</th>
</tr>
</thead>
</table>
| MYLI 531 | CTTTAAGAAGGAGATATACATATGACTAGTGAATTTC  
                      CGGCCGCAGCTAGCAAAGGAGAAGAACTTTTC 
                      GAAAAGTTCTTCTCCTTTGCTAGCTGCGGCCGCGAAT  
                      TCACTAGTCTATATGTATATCTCCTTCTTTAAAG |
| MYLI 532 | 

Table 2. Oligonucleotides used for phage-based mutagenesis

<table>
<thead>
<tr>
<th>Name</th>
<th>Purpose</th>
<th>Sequence (5' to 3')</th>
</tr>
</thead>
</table>
| MYLI 534 | Main mutagenic oligo (error in SpeI site) | GAAAAGTTCTTCTCCTTTTGTCT  
                      AGCTGCGGCCGCGAAGCTTTCT  
                      AGTCATATGTATATCTCCTTCT  
                      TAAAG |
| MYLI 536 | Main mutagenic oligo (corrected) | GAAAAGTTCTTCTCCTTTTGTCT  
                      AGCTGCGGCCGCAAGCTTACT  
                      AGTCATATGTATATCTCCTTCT  
                      TAAAG |
| MYLI 535 | Repair the SmaI/EcoRV hybrid site to an EcoRV site  
                      Activate the ampicillin resistance gene | CTGGCAGACAGCAATTGATTGTCA  
                      ATCGATCCTCTAGAGTCCACC  
                      GTTGCCATTGCTGAGGCATC  
                      GTGGTG |
Fig. 1
Fig. 2
Fig. 3
Fig. 5

A. pWS no primers  pWS  pGLO no primers  pGLO 100 ng  pGLO 33 ng

DpnI Digested?  N  Y  N  Y  N  Y  N  Y  N  Y  N  Y
Amp-resistant colonies?  0  0  0  0  0  0  0  0
+ ctrl: 279  - ctrl: 0

B. pWS no primers  pWS 25 ng  pWS 5 ng  pWS 1 ng

DpnI Digested?  N  Y  N  Y  N  Y  N  Y  N  Y
Amp-resistant colonies?  116  495  450 >1000
- ctrl: 0

C. pGLO 33.3 ng  pGLO 16.7 ng  pGLO 6.7 ng

DpnI Digested?  N  Y  N  Y  N  Y  N  Y
Amp-resistant colonies?  0  0  0  0

3000 bp
Fig. 6
Fig. 7

A. pSelect-GFP (unmutagenized) 7353 bp

B. pSelect-GFP (mutagenized) 7374 bp
Fig. 9
Fig. 12

A. HindIII Unmutagenized
   HindIII Mutagenized
   EcoRI/EcoRV Unmutagenized
   EcoRI/EcoRV Mutagenized

HindIII Digestion
EcoRI/EcoRV Double Digestion

B. Spel Digestion
   NotI Digestion

Spel Mutagenized
Unmutagenized Control
NotI Mutagenized

C. PRIMARY OLIGO
   HindIII - within 581 epitope
   BamHI - ~106 bp fragment produced
   CURRENTLY HindIII!!!

Coding (In ss strand):
5′:CTTTAGAAGGAGAATACATATGACTAGCTAGCTGCGCCGGAGCGTAGCAAGGAGAGAAACTTTTCG:3
3′:GAAACTCTTCTCTATTGATACATATCAAGCCGGCGCCTGATCGTTTCCCTTGTTTTAGG:5
Noncoding (ORDER):

ORDER THIS ONE
5′-GAAAGATTCTTCTCTTTGCTAGC|GGGGGGGCGFAGCTTCTAGTCAATUTATATATCTCTTTTAAG-3′
Fig. 13