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Modeling the Adaptive Immune Response to Mutation-Generated Antigens

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**Modeling the Adaptive Immune Response to
Mutation-Generated Antigens**

Rory Geyer

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ABSTRACT

Somatic mutations may drive tumorigenesis or lead to new, immunogenic epitopes (neoantigens). The immune system is thought to represses neoplastic growths through the recognition of neoantigens presented only by tumor cells. To study mutations as well as the immune response to mutation-generated antigens, we have created a conditional knockin mouse line with a gene encoding, 5' to 3', yellow fluorescent protein (YFP), ovalbumin (which is processed to the immunologically recognizable peptide, SIINFEKL), and cyan fluorescent protein (CFP), or, YFP-ovalbumin-CFP. A frame shift mutation has been created at the 5' end of the ovalbumin gene, hence YFP should always be expressed, while ovalbumin and CFP should only be expressed after a single base pair deletion that can restore the frame, has occurred. Experiments *in vitro* have been conducted to test whether the transgene behaves they way we expect. Transfection of EL4 cells with the transgene caused cells to express YFP. After growing the transfected cells (thus allowing time for spontaneous mutations), populations of YFP+CFP⁻ and YFP+CFP⁺ cells were sorted and grown. The YFP+CFP⁻ cells did not present SIINFEKL on H2-K^b on their surfaces, while YFP+CFP⁺ cells did; this was tested by incubating the cells with B3Z cells, which respond to the SIINFEKL/H2-K^b complex. The mouse model will be used to measure the rate of mutation in the transgene *in vivo*, investigate the immune response to mutation-generated antigens in a growing tumor in a live host, and test the hypothesis that an immune response to neoantigens on non-transformed somatic cells contributes to aging.

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INTRODUCTION

RANDOM SOMATIC MUTATIONS ACCUMULATE WITH AGE AND IN TUMORS

Random somatic mutations occur in all mitotic cells during the DNA synthesis phase of each passage through the cell cycle. Although there are DNA repair mechanisms in place to ensure fidelity in the synthesis of new DNA strands, these mechanisms do not work with complete efficiency and some errors persist in the new strands (Echols and Goodman, 1991). Studies performed in cell lines have reported that random mutations occur at a rate between 10^{-9} and 10^{-5} mutations per base pair per cell division (Beckman and Loeb, 2005; Simpson, 1997).

There are several types of mutations that occur during DNA synthesis. Among the most common are the insertion of an incorrect base leading to improper basepairing, the insertion of an entirely new base that was not previously present, and the deletion of a base (Zhang and Gerstein, 2003). Assuming they occur in a protein-coding gene, these different mutations can have vastly different effects on the structure (and thus the function) of the protein. Base pair substitutions may either be silent (not change the amino acid produced from the altered codon) or may cause a missense mutation (the amino acid produced from the altered codon is a different one than is produced from the unaltered codon). Silent mutations have no effect on the translated protein, but missense mutations may. Depending on how the new amino acid in the protein interacts with the others, the overall protein structure may or may not be changed. Insertion and deletions of bases tend to have a greater effect on the structure

of the mutated protein because they cause the nucleotides in the open reading frame to shift (hence, these are called frameshift mutations). This completely changes the codons that are read and, thus, the amino acids that are transcribed. Frameshift mutations tend to either lead to an entirely new protein structure or a truncated protein product from the generation of an early stop codon (Ogura et al., 2001).

A particular type of insertion and deletion mutation is more likely to occur in mononucleotide repeat tracts, which are 8 or more of the same nucleotide repeated consecutively (Stringer et al., 2004; see Levinson and Gutman, 1987). As DNA polymerase reads a mononucleotide repeat tract, a nucleotide in either the new strand or the template strand may “slip” backward (the template strand would move 3’ to 5’ and the new strand would move 5’ to 3’). Either strand may form a basepair with a nucleotide on the opposite strand that is upstream of the nucleotide it is supposed to pair with. If the template strand slips back, this will lead to a deletion in the new strand, whereas if the strand being synthesized slips back, it will have an insertion. Several trinucleotide repeat disorders are caused by slipped strand mispairing mutations (Axford et al., 2013), including Huntington’s disease, in which CAG trinucleotide repeat tracts are expanded by insertions made in the newly synthesized strand (however, there is no frameshift with these diseases because three nucleotides “slip” at a time).

Mutations (especially frameshift mutations) will often cause proteins to misfold and/or prevent them from functioning properly, which may lead to apoptosis of the cells harboring these mutations. However, in addition to causing various forms of cellular dysfunction, certain mutations drive tumorigenesis (Greenman et al., 2007; Stratton et al., 2009) and have been shown to accumulate in cancers (Greenman et al.,

2007; Stratton et al., 2009). Driver mutations enhance the survival and proliferative ability of tumor cells. They may allow a tumor cell to evade apoptosis (Hanahan and Weinberg, 2000) or cell cycle checkpoints (Hanahan and Weinberg, 2000). The mutations that do not drive a cell into malignancy, and do not promote or inhibit cell division or survival, and that occur randomly as a necessary part of cell division, are called “passenger” mutations. Passenger mutations also occur in genes of tumor cells that are not essential to the cell’s transformation or maintaining its transformed phenotype (Greenman et al., 2007). These mutations can generate new antigens (neoantigens) that allow the immune system to distinguish cancer cells from non-transformed cells (Matsushita et al., 2012). It is possible that driver mutations can generate neoantigens as well, although driver mutations are far fewer in number than passenger mutations, and so may account for a very small minority of tumor neoantigens at best. An immune response to cancer is discussed in the next section.

The rate of spontaneous somatic mutations has not been rigorously measured *in vivo* and only two studies have approached the accumulation of mutations (but not the rate at which they occur) in a living organism (Stringer et al. 2004; Fischer and Stringer 2008). These studies showed that mutations accumulate in growing tumors as well as in whole, aging organisms. Stringer et al. (2004) and Fischer and Stringer (2008) used a transgenic mouse model, in which a particular mutation in the transgene led to the expression of the enzyme, human placental alkaline phosphatase (hPLAP). An insertion of 7 guanines was made into an early part of the transgene in which 4 guanines were already present. This insertion has two effects on the transgene. First, this will create a frameshift mutation in the transgene. Second, these 11 guanines make up a

mononucleotide tract, which are prone to slipped strand mispairing, leading to either an insertion or deletion in the newly synthesized strand. When a deletion of one of these guanines occurs, the frame of the transgene will be restored and the full hPLAP will be transcribed and translated. 5-bromo-4-chloro-3-indolyl phosphate and nitro blue tetrazolium chloride were then used to stain for hPLAP activity, allowing for the visualization of cells that had mutated.

In both tumors and non-transformed aging tissues, multiple factors led to the accumulation of mutant cells, including genomic instability (single mutant cells were observed) and proliferation of mutant cells (detected by clusters of mutant cells). In the study of aging tissues, they also found that mutant cells accumulated in tissues with age. Both findings in aged tissues were true for both proliferative and non-proliferative tissues, although the rate at which mutant cells accumulate in tissues was not measured.

ANTI-CANCER IMMUNITY

Cytotoxic CD8⁺ T cells are the primary direct effectors of an anti-cancer immune response; they are required for the immune system to reject a tumor (DuPage et al. 2012; Matsushita et al., 2012). Antigens recognized by CD8⁺ T cells are complexes of an 8-11 amino acid peptide and an MHC class I molecule. These peptide-MHC (pMHC) complexes are recognized via a T-cell receptor (TCR) that is specific to one cognate pMHC complex (although, some TCRs do cross-react with more than one pMHC complex). TCRs can interact with a peptide only if it is presented on a MHC molecule

because the TCR interacts with amino acids on both the peptide and the MHC molecule; free peptides are not recognized.

Peptides presented on MHC class I molecules are generated through the processing of either endogenously synthesized or exogenously acquired proteins (Neefjes et al., 2011); the presentation of the former is termed direct presentation, while the latter can only be presented on MHC class I by cross-presentation.

Endogenous proteins are processed and presented by all nucleated somatic cells in the body, while the latter can only be presented by dendritic cells (DCs), though it is not clear specifically which subsets of DCs are capable of this (Joffre et al., 2012). Direct presentation is essential for immune surveillance by T cells, while cross-presentation is necessary for the priming of naïve T cells.

In the case of cancer, both mutated and non-mutated peptides will be presented on MHC class I (Englehorn et al., 2006). The immune system distinguishes between “self” (non-mutated) and “nonself” (mutated) by being taught not to respond to a particular set of “self” epitopes. In T cells, this occurs in the thymus. After being produced from the lymphoid lineage of hematopoietic cells, immature CD4-CD8- T cells migrate to the thymus where they receive a signal to rearrange the genes encoding each chain of the T-cell receptor (TCR) and express CD4 and CD8. These CD4+CD8+ cells then interact with thymic epithelial cells that present “self” peptide antigens on MHC class I and MHC class II molecules. T cells that recognize a peptide-MHC class I complex will receive a survival signal and a signal to downregulate expression of CD4, but will continue to express CD8. T cells that recognize a peptide-MHC class II complex will also receive a survival signal, but will receive a signal to downregulate CD8, and instead will

continue to express CD4. The survival signals are only received provided that the T cell does not interact with the thymic epithelial cells with too high avidity or too low an avidity; if the avidity of the interaction does not fall within a very narrow range, the immature T cell will not receive a survival signal and will apoptose. About 2% of immature T cells survive this selection process in the thymus. Thus, only cells that may recognize self MHC molecules, but cannot recognize self peptides presented on MHC survive selection and exit the thymus to begin circulating the periphery. For a more detailed discussion of T-cell selection, see reviews by Klein et al. (2009) and Klein et al. (2014).

It has been hypothesized that through cancer immunosurveillance, (Burnet, 1970; Burnet, 1971, Dunn et al., 2002) circulating cytotoxic CD8+ T cells can recognize neoantigens presented on MHC class I by neoplastic cells and repress the growth of neoplasia. However, direct evidence for an immune against mutation-generated neoantigens presented on a tumor growing in a live host is lacking. This has been approached by studies using genetic engineering models to simultaneously remove tumor suppressor genes and induce the expression of oncogenes and exogenous antigens (DuPage et al., 2011; DuPage et al. 2012). These studies do show that tumor-specific antigens are required for tumor rejection by the immune system, but they have relied on using viral vectors to introduce exogenous antigens into tumor, not mutation-generated antigens. While this is how some tumor antigens are created in virus-induced tumors (which means these antigens are really viral antigens), the vast majority of tumor-specific antigens do not arise in this fashion and instead are thought to be created through random somatic mutations in tumor cells.

Other than studies that genetically engineer tumors to express exogenous tumor-specific antigens, one other study has approached the issue of demonstrating an immune response against mutation-generated antigens (Matsushita et al., 2012). This study sequenced the exome of a tumor cell line and the mouse strain from which it is derived and found genetic differences between the two. It was found that T cells responded to antigens derived from the protein products of some of the genetic differences in the tumor cell line, which presumably exist because of random mutations in the tumor cells. However, the antigens had already been established in the cell line and were not generated after being injected into mice. More mutations may have occurred in the tumor cells after the tumor challenge and some of these mutations may have led to neoantigens. However, the immune response was found to occur against the neoantigens that had been established prior to the challenge. Thus, an immune response to neoantigens generated in a tumor growing in a live host was not tested here.

OUTLINE OF THESIS

- 1. Creation of transgene encoding YFP-ovalbumin-CFP with a frameshift-creating polyA tract between YFP and ovalbumin.** To study mutation and antigens generated from mutation, a transgenic mouse line will be created with a gene encoding, 5' to 3', yellow fluorescent protein (YFP), ovalbumin (which is processed to the immunologically recognizable peptide, SIINFEKL), and cyan fluorescent protein (CFP), or, YFP-ovalbumin-CFP. A frame shift mutation has

been created at the 5' end of the ovalbumin gene, hence YFP should always be expressed, while ovalbumin and CFP should only be expressed after a single base pair deletion that can restore the frame, has occurred. The expression of CFP can be used as a marker for mutated cells. Thus SIINFEKL (within ovalbumin) can only be generated from mutation and is a neoantigen in this model and an immune response toward it can be monitored.

2. ***In vitro* testing of the CAG-YFP-m7aOVA-CFP transgene.** Before injecting the transgene into a mouse embryo to create a transgenic line, it was transfected into cultured cells and tested to determine if it mutated to express CFP and present the SIINFEKL/H2-K^b complex as it was designed to do.

MATERIALS AND METHODS

MICE AND CELL LINES

C57BL/6 mice were obtained from the Jackson Laboratory. EL4 cells are a T cell lymphoma derived from C57BL/6 mice; these were obtained from a frozen stock in the lab. B3Z cells are a T cell hybridoma with a TCR specific to the SIINFEKL/H2-K^b complex that produce β -galactosidase in response to recognition of this antigen. β -galactosidase will convert the substrate chlorophenol red- β -D-galactopyranoside to chlorophenol red, which can be detected by measuring the absorbance of the solution the

cells are in at 570 nm. B3Z cells were obtained from a frozen stock in the lab and have been described by Kanaseki and Shastri (2013).

CREATION OF THE *CAG-YFP-m7a-OVA-CFP* TRANSGENE

An ovalbumin (OVA) plasmid was taken and a citrine yellow fluorescent protein (YFP) plasmid was placed upstream of OVA and a cerulean cyan fluorescent protein (CFP) plasmid was placed downstream of OVA. At the beginning of the OVA gene, 7 adenines have been inserted (YFP-7AOVA-CFP; Fig. 1), causing a frameshift and nonsense mutation, allowing only for the translation of YFP. This insertion also creates a mutation-prone mononucleotide repeat tract. The deletion of a single base pair in this tract will restore the frame, making translation of the full YFP-OVA-CFP possible (Fig. 2); this is similar to the model used by Stringer et al. described above. In mutated cells, CFP will be expressed and processing of this full protein product will generate the H2-K^b-restricted epitope, SIINFEKL. Two transgenes were created, one with 7 inserted adenines and another with only 6 inserted adenines; the two transgenes are referred to as the 7A transgene and 6A transgene, respectively. The 6A transgene mimics the sequence we expect the 7A transgene to have after a deletion occurs in the region of 11 repeated adenines. The transgene was created by the Gene Targeting and Transgenic Facility at the University of Connecticut Health Center.

TRANSFECTION

Electroporation was performed using the Amaxa® Nucleofector®. Nucleofection kits containing Nucleofection solution and cuvettes were purchased from the Lonza Group. Two million EL4 cells were centrifuged at 1500 rpm for 5 min to remove medium, washed in phosphate-buffered saline (PBS), and resuspended in 100 µL of Nucleofection solution in a cuvette.

FACS ANALYSIS

Cells were analyzed by flow cytometry using the FACSCalibur and FACS Aria II (Becton Dickinson; University of Connecticut Health Center FACS facility). To check for expression of YFP 8 hours after transfection, medium was removed and the cells were washed in PBS. After washing, they were resuspended in PBS and run on the FACSCalibur. To sort using the FACS Aria II, cells were put 2% FBS in PBS at a concentration of 20×10^6 cells/mL. Sorted cells were collected in 10% FBS in PBS. After sorting, the sorting medium was washed off and the cells were either immediately used in a B3Z assay or were put back in culture.

B3Z ASSAY

B3Z cells are used to detect the presentation of the SIINFEKL/H2-K^b complex. A mixed culture of APCs and B3Z cells is incubated for 16 hours. The plate is centrifuged at 2000

rpm for 2 min and the medium is removed. The cells are then washed twice with PBS. After washing, the β -galactosidase substrate CPRG is added to the cells and they are incubated for 2 hours in a 37°C (5% CO₂) incubator. The contents of the plate are sensitive to light, so the plate must be kept in the dark (cover with aluminum foil). If the reaction mixture has changed from yellow to red/purple in the positive control wells, the plate can be measured for the absorbance of 570 nm light. If the reaction has not led to a color change after 2 hours, the incubation is continued at room temperature until a color change is observed, at which point the absorbance of 570 nm light is measured.

MICROINJECTION

A transgenic mouse line was created by microinjection of the CAG-YFP-m7aOVA-CFP transgene into a C57BL/6 embryo. A superovulated female C57BL/6 mouse is mated with a male C57BL/6 mouse and the fertilized eggs are harvested. The transgene is then microinjected into the sperm pronucleus and the microinjected single-cell embryos are put into a surrogate mother. The microinjection was performed by the Gene Targeting and Transgenic Facility at the University of Connecticut Health Center.

FLUORESCENCE MICROSCOPY

Tissues were harvested from C57BL/6 mice, fixed in formalin, frozen in optimum freezing temperature (OCT) medium, and cut into thin sections with a cryostat. The

thin sections were placed on microscope slides, dehydrated , and then stained with hemotoxylin and eosin. The stained slides were imaged using a fluorescence microscope. Dr. Kevin Claffey helped with tissue sectioning and imaging and provided the fluorescence microscope.

RESULTS

CREATION OF A CELL LINE THAT EXPRESSES THE TRANSGENE

To test whether the transgene could be expressed by cells growing in culture, EL4 cells were electroporated with the 7A transgene. If the transgene is expressed by the transfected cells, a signal should be detected when they are analyzed by the FACSCalibur in the channel FL1, which detects light in the wavelength range that is emitted by fluorescein isothiocyanate (FITC). Following transfection, the cells were put back in culture and after 8 hours in culture, a small portion were taken and analyzed by flow cytometry (Fig. 3). The emission spectra of FITC and YFP overlap so YFP can also be detected by the FL1 channel. Compared to untransfected cells, a greater signal was detected in FL1 from the transfected cells, indicating greater expression of YFP. Thus, the transgene is expressed by the *in vitro* culture of EL4 cells.

The portion of transfected EL4 cells that were not analyzed by FACS was kept in culture for two more weeks. At this time, they were sorted into YFP+ cells and YFP- cells (all cells were CFP-). The YFP- cells were discarded and the YFP+ cells were grown

for two more weeks before being analyzed by FACS again (Fig. 4). This analysis showed three populations of cells:

1. YFP-CFP-

These cells are probably cells that were untransfected or had lost the transgene (e.g. because it did not integrate into every cell's genome) before the sort.

2. YFP+CFP-

This population consists of cells that have been transfected (and retained the transgene) and express the transgene; these cells have not mutated to also express CFP.

3. YFP+CFP+

This population expresses YFP, which means that these cells were successfully transfected with the transgene. This cell population also expresses CFP, suggesting that these cells underwent the expected deletion mutation to express CFP. If this is the case, SIINFEKL/H2-K^b complexes should be presented on the surfaces of these cells. However, it is also possible that a random deletion mutation occurred at such a location in the transgene that only restored the frame of the CFP-encoding portion of the gene.

ANTIGEN PRESENTING ABILITY OF THE TRANSGENE-EXPRESSING CELL LINE

To test whether cells transfected with the 7A transgene that express CFP were also capable of presenting SIINFEKL/H2-K^b complexes on their surfaces, transfected cells were incubated with B3Z cells. In this assay, EL4 cells served as the antigen presenting cells (APCs) to the B3Z cells. After this incubation, the β -galactosidase substrate CPRG was added to the cells and the absorbance of the solution was measured at 570 nm (Fig. 5). Four different APCs were used to stimulate B3Z cells: EL4 cells pulsed with free SIINFEKL, YFP+CFP- EL4 cells that were transfected with the 7A transgene, YFP+CFP+ EL4 cells transfected with the 7A transgene, and EL4 cells transfected with the 6A transgene. The YFP+CFP- EL4 cells and the YFP+CFP+ EL4 cells were used in the B3Z assay immediately after being sorted. Four different ratios of APC number to B3Z cell number were used in each condition: 2×10^4 , 4×10^4 , 8×10^4 , or 10×10^4 APCs to 10×10^4 B3Z cells. EL4 cells pulsed with SIINFEKL gave a strong response that was not titratable over the different APC:B3Z ratios because the solution became saturated with chlorophenol red, even when a small number of APCs was used. YFP+CFP- EL4 cells did not stimulate a B3Z response. YFP+CFP+ cells gave a positive titratable response. EL4 cells transfected with the 6A transgene, like the untransfected cells pulsed with SIINFEKL, gave a strong response. Theoretically, the YFP+CFP+ EL4 cells and the EL4 cells transfected with the 6A transgene should be expressing the same transgene, however, the cells transfected with the 6A transgene gave a much greater response than the YFP+CFP+ cells. This is probably because the cells transfected with the 6A transgene had been transfected the day before, fewer cells had lost the

transgene, and the cells may have had multiple copies of the transgene. The YFP+CFP+ cells had been transfected 4 weeks earlier and while they were probably stably transfected, the transgene was probably present in these cells at a much lower copy number and was expressed at a lower level compared to the 6A-transfected cells.

TISSUES OF THE TRANSGENIC MOUSE EXPRESS THE TRANSGENE

The data described above show that *in vitro* the transgene mutation-dependent expression of CFP and presentation of the SIINFEKL/H2-K^b complex. To create a transgenic mouse line, the 7A transgene was microinjected into a C57BL/6 embryo. At 6 weeks of age, pups that developed from these embryos were sacrificed and their tissues were harvested to check for expression of YFP. After harvesting, the tissues were fixed overnight in formalin and then put into optimum cutting temperature medium and frozen. The frozen tissues were cut using a cryostat and the thin sections from the cutting were placed on slides. The slides were stained with hematoxylin and eosin and were imaged using a fluorescence microscope. Lung and colorectal tissue of the transgenic mice were found to be noticeably more fluorescent than the same tissues from a wild type mouse (Fig. 6). Because the transgene is expressed under the CAG promoter (chicken β -actin promoter with CMV enhancer), it should have strong, global expression. This is not the case here as the lung and colon of the transgenic mouse were the only two tissues to express the transgene at a noticeably greater level than their wild type counterparts. This is probably because of the location in the genome where the transgene inserted. The microinjection method of creating a transgenic

mouse line does not allow for directed insertion of the transgene into the genome. Instead, insertion occurs randomly (if it occurs at all) and this affects the level of expression of the transgene and may lead to different expression levels in different tissues, which was observed here.

DISCUSSION

The data shown here demonstrate that the CAG-YFP-m7aOVA-CFP transgene encodes mutation-dependent expression of CFP and presentation of the SIINFEKL/H2-K^b complex on the surface of cells. *In vitro*, it can be used as another tool for measuring the mutation rate of cultured cells. *In vivo*, the transgenic mouse strain carrying the transgene can be used to test the following:

- 1. Measure the rates of spontaneous mutations in various somatic tissues.**

The rate of somatic mutation will be measured *in vivo* in various tissues by using confocal microscopy to measure CFP fluorescence at different time points in the mouse's life; older mice (6 months old) should have higher levels of CFP fluorescence than younger mice (3 weeks old). Both slow- (e.g. brain and liver) and fast-dividing tissues (e.g. colon) will be examined, to determine if there are differences in mutation rate related to proliferative rate.

- 2. Measure the rates of spontaneous mutations in a tumor and the host**

immune response to the generated antigens. In addition to non-transformed

tissues, the rate of mutation in a methylcholanthrene-induced fibrosarcoma in the transgenic mice will be measured as described above. As a mutation in the CAG-YFP-m7aOVA-CFP transgene should lead to MHC class I presentation of SIINFEKL, we expect an immune response to be elicited against cells that develop a mutation in the transgene. MHC class I tetramers will be used to determine the presence of SIINFEKL-specific T cells in the tumor and the degree to which infiltration of the tumor by these T cells occurs. If T cells are not found this may suggest that the tumor is capable of some form of immunosuppression.

As somatic mutations make cancer cells antigenically distinct from non-transformed cells of the same tissue (Matsushita et al., 2012), this study has significant bearing on our understanding of the immune response to cancer.

Non-transformed cells will accumulate somatic mutations as well, albeit likely fewer than cancer cells, and these mutations may still generate new epitopes. Thus, in the future, the second question stated above can be probed in non-transformed tissues. If evidence of an immune response is found, it would likely be in an aged mouse (an older mouse will have more mutations and, therefore more mutation-generated epitopes than a younger mouse). We would then ask, “Does an immune response to mutation-generated epitopes contribute to the damage and dysfunction undergone by tissues with age?” (Geyer et al., 2013). Applying these present studies in the context of aging mice may go toward giving us a greater understanding of a mechanism behind aging.

FIGURES

mOVA7A double fluorescence construct

mOVA6A double fluorescence construct

Citrine YFP->
 ATGGTGAGCAAGGGCGAGGAGCTGTTACCGGGGGTGGCCATCTGCTCGAGCTGGACGGCGACGTAAACGGCCACAA
 ▶ M V S K G E E L F T G V V P I L V E L D G D V N G H K
 GTTCACGCTGTCGGCGAGGGCGAGGGCGATGCCACCTACGGCAAGCTGACCTGAAGTTTCATCTGCACCAACGGCAAGC
 ▶ F S V S G E G E G D A T Y G K L T L K F I C T T G K
 C->T in OVA7
 TGCCCGTGCCTGGCCACCTCGTGACCACCTTCGGCTACGCGCTGATGTGCTTGCCTGCTACCCGACCATGAAG
 ▶ L P V P W P T L V T T F G Y G L M C F A R Y P D H M K
 CAGCAGACTTCTTCAAGTCGCGCATGCCGAAGGCTACGTCCAGGAGCGACCATCTTCTCAAGGACGACGGCACTA
 ▶ Q H D F F K S A M P E G Y V Q E R T I F F K D D G N Y
 CAAGACCCGCGCGAGGTGAAGTTTCAGGGCGACACCTGGTGAACCGCATCGAGCTGAAGGGCATGACTTCAAGGAGG
 ▶ K T R A E V K F E G D T L V N R I E L K G I D F K E
 ACGGCAACATCTGGGGCACAAGCTGGAGTACAACACAGCCACACGCTTATATCATGGCCGACAGCAGAAGAAC
 ▶ D G N I L G H K L E Y N Y N S H N V Y I M A D K Q K N
 GGCATCAAGGTGAACCTCAAGATCCGCCACAACATCGAGGACGGCAGCTGCGGACCACTACGAGCAGAACAC
 ▶ G I K V N F K I R H N I E D G S V Q L A D H Y Q Q N T
 CCCCACGGCGACGGCCGCTGCTGCTGCCGACAACCACTACCTGAGCTACCACTCCGCTGAGCAAGACCCCA
 ▶ P I G D G P V L L P D N H Y L S Y Q S A L S K D P
 ACGAGAAGCGGATCACATGGTCTGCTGAGTTCGTGACCGCGCGGGATCACTCTCGCATGGACGAGCTGTAC
 ▶ N E K R D H M V L L E F V T A A G I T L G M D E L Y
 OVA-7A->
 AAGATGGCTTCTGAGAA^{AAAAAA}AATGAAGATCCTGGAGCTTCATTGCGAGTGGGACAATGAGCATG
 ▶ K M A S E K K K N E D P G A S I C Q W D N E H
 Stul
 TTGGTGTCTTGCCTGATGAAGTCTCAGGCTTGAAGCTGAGAGTAAATCACTTTGAAAACTGACTGAATGGAC
 ▶ V G A V A • • • S L R P • A A • E Y N Q L • K T D • M D
 CAGTTCTAATGTTTGAAGAGAGGAAGATCAAGGTGACTTACCTCGCATGAAGATGGAGAAAAATACAACCTCACAT
 ▶ Q F • C Y G R E E D Q S V L T S H E D G G K I Q P H I
 AflIII
 CTGTCTTAATGGCTATGGCATTACTGACGTGTTTAGCTCTTCAGCCAACTGTCTGGCATCTCTCAGCAGAGAGCCTG
 ▶ C L N G Y G H Y • R V • L F S Q S V W H L L S R E P
 EcoRV
 AAGATATCTCAAGCTGCTCATGACGACATCGAGAAATCAATGAAGCAGGACAGAGAGGTGGTAGGGTCAGCAGAGGCTGG
 ▶ E D I S S C P C S T C R N • • S R Q R G R V S R G W
 OVA seqF->
 AGTGGATGCTCAAGCGTCTCTGAAGAATTTAGGGCTGACCATTCCTCTCTGTATCAAGCAGCATCGCAACC
 ▶ S G C K R L • R I • G • P S I P L L Y Q A H R N
 CFP->
 AAGCGGTTCTCTCTTTGGCAGATGTGTTTCCCTTATGGTGAAGGGCGAGGAGCTGTTACCGGGGGT
 ▶ Q R R S L L W Q M C F P Y G E Q G R G A V H R G
 GTGCCATCTGCTGAGCTGGACGGCGACGTAAACGGCCACAACTGACGCTGTCCGGCGAGGGCGAGGGCGATGCCAC
 ▶ G A H P G R A G R R R K P Q V Q R V R R G R G R C H
 <-CFPr
 CTACGGCAAGCTGACCTGAAGTTTCATCTGCACACCGGCAAGCTGCCGTGCCGTGCCACCTCGTGACCACTG
 ▶ L R Q A D P E V H L H H R Q A A R A L A H P R D H P
 ACCTGGGGCTGAGTCTTCCGCGCTACCCGACCATGAAGCAGCAGCTTCTTCAAGTCGCGCATGCCGA
 ▶ D L G R A V L R P L P R P H E A A R L L Q V R H A R
 AGGCTACGTCAGGAGCGACCATCTTCTCAAGGACGACGGCACTACAAGACCCGCGCAGGTGAAGTTCAAGGGCG
 ▶ R L R P G A H H L L Q G R R Q L Q D P R R G E V R G R
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 ▶ H P G E P H R A E G H R L Q G G R Q H P G A Q A G V
 AACGCCATCAGCGACAAGCTTATATCACCGCGCAGCAGAAGCAAGCATCAAGGCCAATCTCAAGATCCGCGACAA
 ▶ Q R H Q R Q R L Y H R R Q A E E R H Q G Q L Q D P P Q
 CATCGAGGACGGCAGCTGCGGACCACTACGACGAGAACCCCCATCGGCGACGGCCGCTGCTGCTGCTGCGCG
 ▶ H R G R Q R A A R R P L P A E H P H R R R P R A A A R
 ACAACCACTACCTGAGCACCAGTCCAAGCTGAGCAAGACCCCAACGAGAAGCGGATCACATGGTCTGCTGAGATTG
 ▶ Q P L P E H P V Q A E Q R P Q R E A R S H G P A G V

Citrine YFP->
 ATGGTGAGCAAGGGCGAGGAGCTGTTACCGGGGGTGGCCATCTGCTCGAGCTGGACGGCGACGTAAACGGCCACAA
 ▶ M V S K G E E L F T G V V P I L V E L D G D V N G H K
 GTTCACGCTGTCGGCGAGGGCGAGGGCGATGCCACCTACGGCAAGCTGACCTGAAGTTTCATCTGCACCAACGGCAAGC
 ▶ F S V S G E G E G D A T Y G K L T L K F I C T T G K
 C->T in OVA7
 TGCCCGTGCCTGGCCACCTCGTGACCACCTTCGGCTACGCGCTGATGTGCTTGCCTGCTACCCGACCATGAAG
 ▶ L P V P W P T L V T T F G Y G L M C F A R Y P D H M K
 CAGCAGACTTCTTCAAGTCGCGCATGCCGAAGGCTACGTCCAGGAGCGACCATCTTCTTCAAGGACGACGGCACTA
 ▶ Q H D F F K S A M P E G Y V Q E R T I F F K D D G N Y
 CAAGACCCGCGCGAGGTGAAGTTTCAGGGCGACACCTGGTGAACCGCATCGAGCTGAAGGGCATGACTTCAAGGAGG
 ▶ K T R A E V K F E G D T L V N R I E L K G I D F K E
 ACGGCAACATCTGGGGCACAAGCTGGAGTACAACACAGCCACACGCTTATATCATGGCCGACAGCAGAAGAAC
 ▶ D G N I L G H K L E Y N Y N S H N V Y I M A D K Q K N
 GGCATCAAGGTGAACCTCAAGATCCGCCACAACATCGAGGACGGCAGCTGCGGACCACTACGAGCAGAACAC
 ▶ G I K V N F K I R H N I E D G S V Q L A D H Y Q Q N T
 CCCCACGGCGACGGCCGCTGCTGCTGCCGACAACCACTACCTGAGCTACCACTCCGCTGAGCAAGACCCCA
 ▶ P I G D G P V L L P D N H Y L S Y Q S A L S K D P
 ACGAGAAGCGGATCACATGGTCTGCTGAGTTCGTGACCGCGCGGGATCACTCTCGCATGGACGAGCTGTAC
 ▶ N E K R D H M V L L E F V T A A G I T L G M D E L Y
 OVA-7A->
 AAGATGGCTTCTGAGAA^{AAAAAA}AATGAAGATCCTGGAGCTTCATTGCGAGTGGGACAATGAGCATGTT
 ▶ K M A S E K K K M K I L E L P F A S G T M S M L
 Stul
 GGTGCTGTTGCTGATGAAGTCTCAGGCTTGAAGCTGAGAGTAAATCACTTTGAAAACTGACTGAATGGACCA
 ▶ V L L P D E V S G L E Q L E S I I N F F K L D T E W
 GTTCTAATGTTTGAAGAGAGGAAGATCAAGGTGACTTACCTCGCATGAAGATGGAGAAAAATACAACCTCACATCT
 ▶ S S N V M E E R K I K V Y L P R M K M E E K Y N L T S
 AflIII
 GTCTTAATGGCTATGGCATTACTGACGTGTTTAGCTCTTCAGCCAACTGTCTGGCATCTCTCAGCAGAGAGCCTGAA
 ▶ V L M A M G I T D V F S S S A N L S G I S S A E S L K
 EcoRV
 GATATCTCAAGCTGCTCATGACGACATCGAGAAATCAATGAAGCAGGACAGAGAGGTGGTAGGGTCAGCAGAGGCTGGAG
 ▶ I S Q A V H A A H A E A I N E A G R E V V G S A E A G
 OVA seqF->
 TGGATGCTCGAAGCGTCTCTGAAGAATTTAGGGCTGACCATTCCTCTCTGTATCAAGCAGCATCGCAACC
 ▶ V D A A S V S E E F R A D H P F L F C I K H I A T N
 CFP->
 CGCGGTTCTCTCTTTGGCAGATGTGTTTCCCTTATGGTGAAGGGCGAGGAGCTGTTACCGGGGGTGGT
 ▶ A V L L F F G R C V S P M V S K G E E L F T G V V
 CCCCATCTGCTGAGCTGGACGGCGACGTAAACGGCCACAACTGACGCTGTCCGGCGAGGGCGAGGGCGATGCCAC
 ▶ P I L V L D G D V N G H K F S V S G E G E G D A T Y
 <-CFPr
 CGGCAAGCTGACCTGAAGTTTCATCTGCACACCGGCAAGCTGCCGTGCCGTGCCACCTCGTGACCACTGAGC
 ▶ G K L T L K F I C T T G K L P V P W P T L V T T L T
 TGGGGCTGAGTCTTCCGCGCTACCCGACCATGAAGCAGCAGCTTCTTCAAGTCGCGCATGCCGAAGGCG
 ▶ W G V Q C F A R Y P D H M K Q H D F F K S A M P E G
 TACGTCAGGAGCGACCATCTTCTTCAAGGACGACGGCACTACAAGACCCGCGCAGGTGAAGTTCAAGGGCGACAC
 ▶ Y V Q E R T I F F K D D G N Y K T R A E V K F E G D T
 CTTGGTGAACCGCATCGAGCTGAAGGGCATGACTTCAAGGAGGACGGCAACATCTGGGGCACAAGCTGGAGTACAACG
 ▶ L V N R I E L K G I D F K E D G N I L G H K L E Y N
 CCATCAGCGACAAGCTTATATCACCGCGCAGCAGAAGCAAGCATCAAGGCCAATCTCAAGATCCGCGACAACATC
 ▶ A I S D N V Y I T A D K Q K N G I K A N F K I R H N I
 GAGGACGGCAGCTGCGGACCACTACGACGAGAACCCCCATCGGCGACGGCCGCTGCTGCTGCTGCTGCTGCTGCTG
 ▶ E D G S V Q L A D H Y Q Q N T P I G D G P V L L P D N
 CCATCTGAGCAGGACGCTCAAGCTGAGCAAGACCCCAACGAGAAGCGGATCACATGGTCTGCTGAGATTGCTGA
 ▶ H Y L S T Q S K L S K D P N E K R D H M V L L E F V

Citrine YFP Fluorescence only

Citrine YFP and CFP Fluorescence

Figure 1. Construct for two versions of YFP-ovalbumin-CFP. The left panel shows the construct when a seven adenine tract is inserted between YFP and ovalbumin-CFP, leading to a frameshift, such that YFP, but not ovalbumin-CFP, will be translated. The right panel shows the construct with a six adenine tract inserted in-frame, such that all three proteins (YFP-ovalbumin-CFP) will be translated.

A

GAG	AAA				ATG	AAG	...	GAG	AGT	ATA	ATC	AAC	TTT	GAA	AAA	CTG
Glu	Lys				Met	Lys	...	Glu	Ser	Ile	Ile	Asn	Phe	Glu	Lys	Leu
E	K				M	K	...	E	S	I	I	N	F	E	K	L

B

GAG	AAA	AAA	AAA	AAT	GAA	GAT	...	TGA	GAG	TAT	AAT	CAA	CTT	TGA	AAA	ACT
Glu	Lys	Lys	Lys	Asn	Glu	Asp	...	STOP	Glu	Tyr	Asn	Gln	Leu	STOP	Lys	Thr
E	K	K	K	N	E	D	...	STOP	E	Y	N	Q	L	STOP	K	T

C

GAG	AAA	AAA	AAA		ATG	AAG	...	GAG	AGT	ATA	ATC	AAC	TTT	GAA	AAA	CTG
Glu	Lys	Lys	Lys		Met	Lys	...	Glu	Ser	Ile	Ile	Asn	Phe	Glu	Lys	Leu
E	K	K	K		M	K	...	E	S	I	I	N	F	E	K	L

Figure 2. The 7A insertion into the transgene. (A) Shows the region near the N-terminus of ovalbumin where the insertion of the 7A tract (B) creates a frameshift or the 6A tract (C) is inserted in-frame.

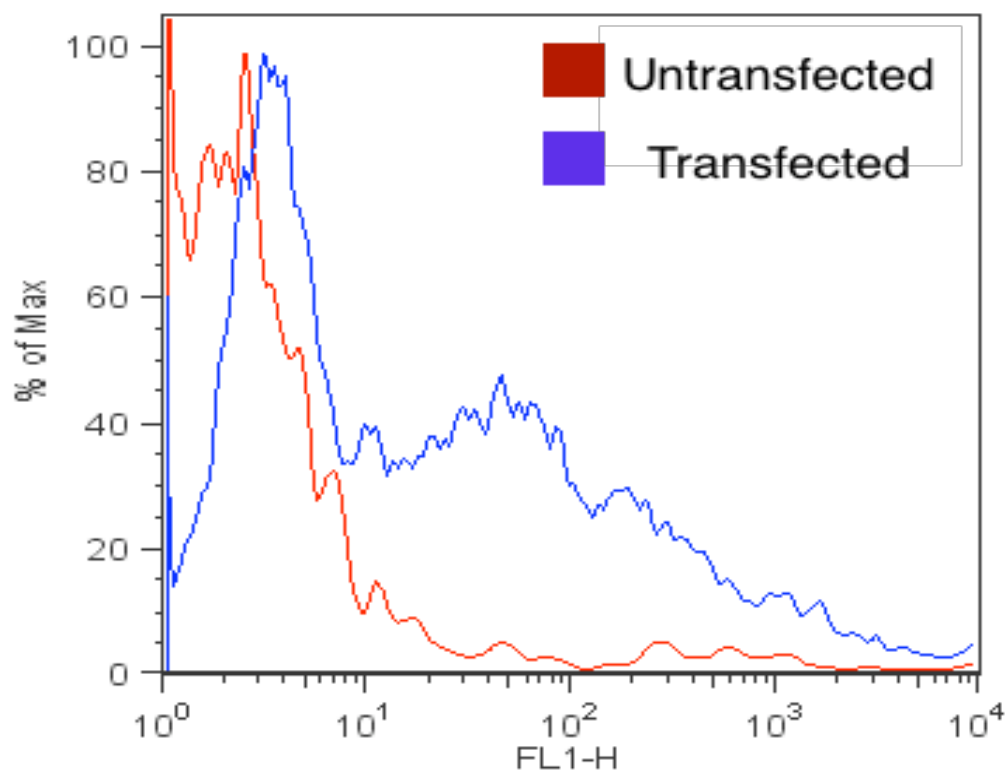


Figure 3. FACS analysis of transfected cells. Unttransfected EL4 cells or EL4 cells transfected with the 7A transgene are analyzed by FACS for the expression of YFP.

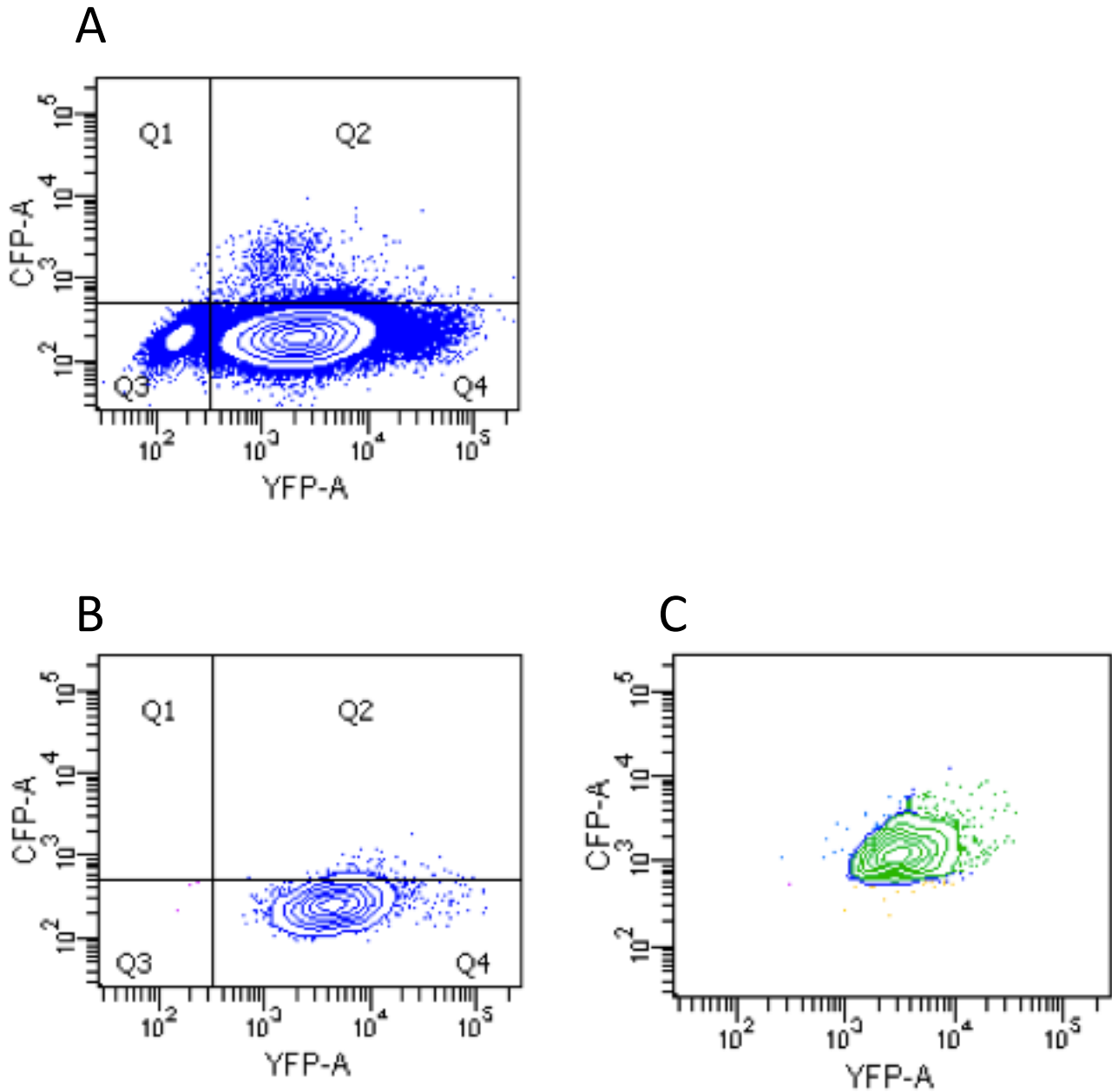


Figure 4. FACS analysis of transfected cells for YFP and CFP expression. EL4 cells were transfected with the 7A transgene, grown in culture for 2 weeks, at which time they were analyzed by FACS (A) and sorted into YFP+CFP⁻ and YFP+CFP⁺ populations. These two populations were then cultured for 2 more weeks, after which they were sorted to remove contaminating cells and analyzed by FACS. The YFP+CFP⁻ (B) and YFP+CFP⁺ (C) populations were then re-analyzed by FACS to confirm YFP and CFP expression.

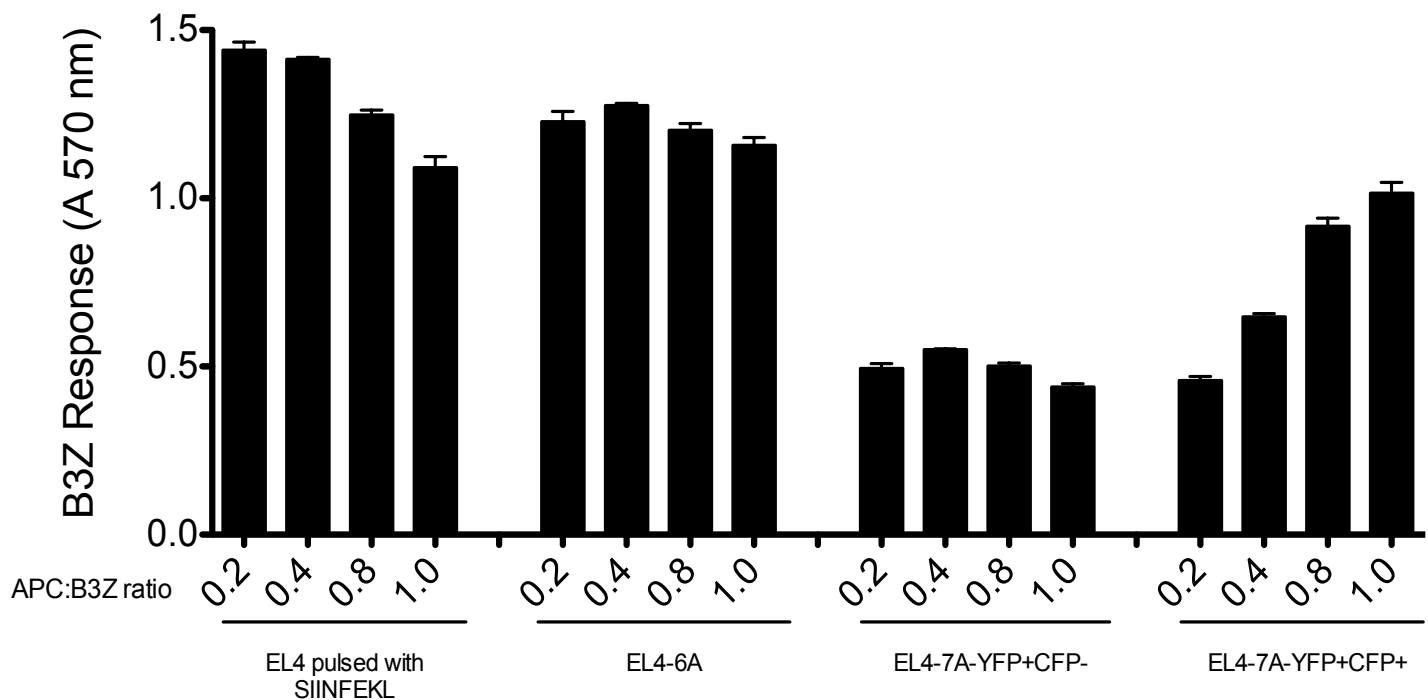


Figure 5. Presentation of SIINFEKL/H2-K^b by EL4-7A cells after a spontaneous mutation. EL4 cells were used as APCs to stimulate B3Z cells. Titrations are shown for four different ratios of APCs:B3Z cells that were used for stimulation; these ratios increase from left to right for each APC. Starting from the left, EL4 cells pulsed with SIINFEKL were used as a control to show that B3Z cells could be stimulated by SIINFEKL/H2-K^b. Second, EL4 cells transfected with the 6A transgene (EL4-6A) were used as a control to show that the product of the in-frame gene is processed to SIINFEKL and presented on H2-K^b. Third, YFP+CFP- EL4 cells that had been transfected with the 7A construct were used to show that the out-of-frame construct does not present the SIINFEKL/H2-K^b complex (The level of response seen here represents the background response). Fourth, YFP+CFP+ EL4 cells that had been transfected with the 7A construct (so they were initially YFP+CFP-) and cultured for 4 weeks were used to show that dividing cells mutate to present the SIINFEKL/H2-K^b complex.

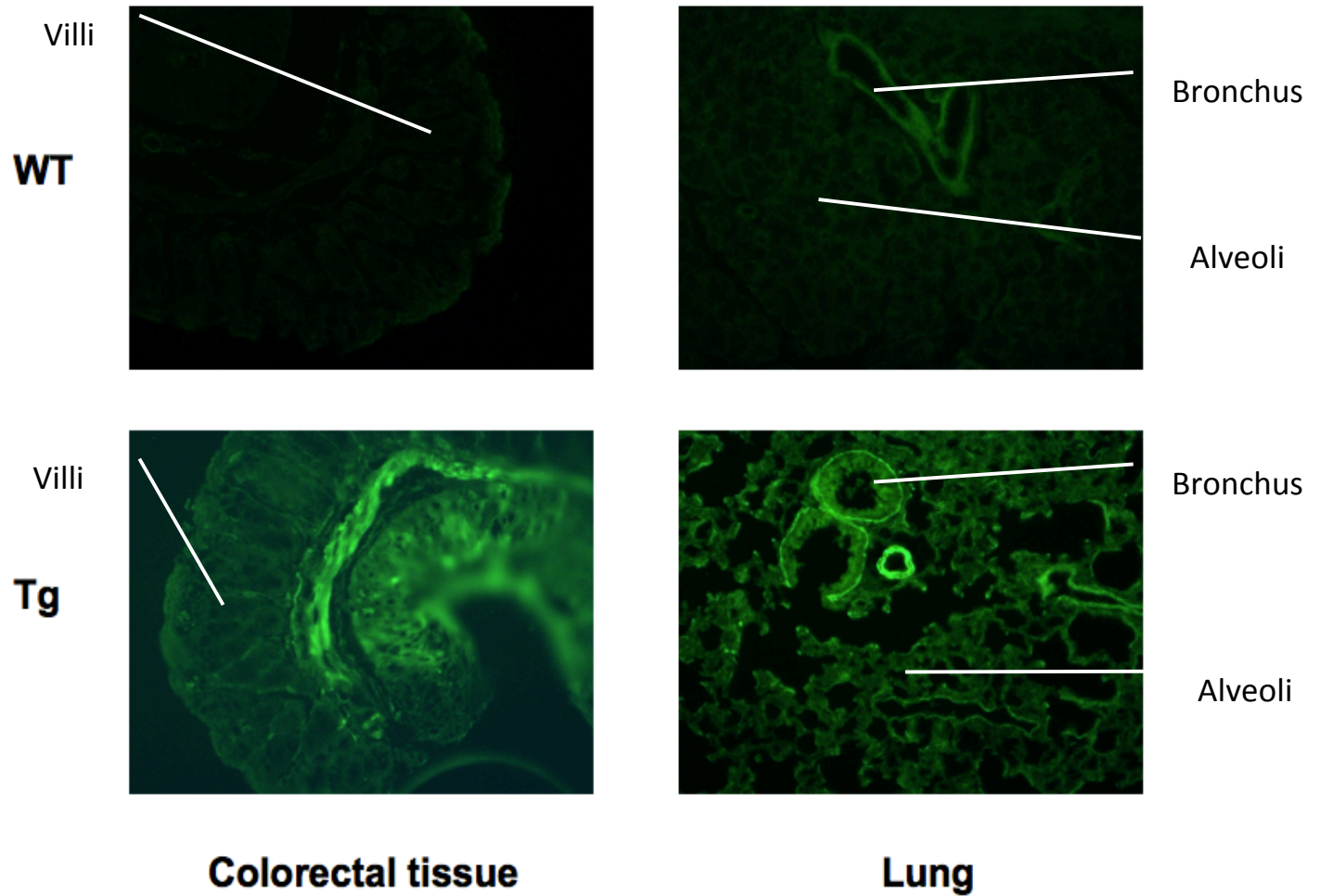


Figure 6. Expression of the 7A transgene *in vivo*. Colon and lung tissues from wild type and transgenic C57BL/6 mice are imaged with a fluorescence microscope. Wild type tissues are on top and transgenic tissues are on the bottom; colorectal tissue images are on the left and images of the lung are on the right. Structures in each tissue are indicated.

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