Production of murine BCR-ABL fusion protein

B- acute lymphoblastic leukemia (B-ALL) affects adults and children. Treatments with tyrosine kinase inhibitors such as Imatinib and Nilotinib are effective at treating patients with chronic myeloid leukemia (CML), but are much less effective in patients with B-ALL (Mishra 2006). About 25% of adult B-ALL express the BCR-ABL fusion protein due to a chromosomal translocation involving human chromosomes 9 and 22 (Rowley 1973). Our preliminary data show that numbers of CD4+ T cells specific for BCR-ABL in a naïve mouse are far lower than naïve precursor frequencies for other antigen-specific CD4+ T cell populations. Immunizing mice with the BCR-ABL antigen and standard adjuvants results in a fifty-fold expansion of BCR-ABL specific T cells. However, an effective immune response against BCR-ABL+ acute lymphoblastic leukemia will probably require much more dramatic expansion of these antigen-specific T cells. My project was to amplify the BCR and ABL genes from *Mus musculus* and synthesize a BCR-ABL fusion protein using those mouse genes.

We use human BCR-ABL in our mouse experiments and there may be areas of the human BCR and ABL fusion protein that are immunogenic to mice but not to humans. Thus, in mice, introduction of human BCR-ABL protein potentially elicits an immune response not solely against the region spanning the fusion between the two proteins, which is what we are interested in. Therefore, I searched the NCBI database for mRNA sequences of BCR (GenBank: BC060270.1) and of ABL (NCBI Reference Sequence: NM_001112703.2) in *Mus musculus*. One result appeared for BCR, but there were several mRNA alternative splice variants of c-ABL.

With this information, I designed primers to amplify mouse BCR and ABL. I also designed primers to amplify the truncated proteins to produce the p190 fusion that is associated with B-ALL. Literature searches indicated that the first 426 amino acids of BCR are present in P190. The Abl portion of P190 is comprised of the kinase, SH2, and SH3 domains of c-ABL (Ren 2005, Ghaffari et al 1999). The website Uniprot provided valuable information about the location of the domains; this tool helped me to uncover the exact amino acid composition of the P190 fusion. The expected lengths of the full-length and truncated forms of BCR and ABL are in the table below:

Table 1: Length of *M. musculus* BCR and ABL cDNA

Full length BCR	2952 nucleotides
Full length ABL	3429 nucleotides
Truncated BCR	1279 nucleotides
Truncated ABL	3249 nucleotides

I included a three-nucleotide long DNA tail at the 5’ end of the primers to act as a landing pad for DNA restriction enzymes. I also included NotI and EcoRI restriction enzyme cut sites and a Kozak consensus sequence (ACC) directly upstream of the annealing sequence. To select the restriction enzymes, I ensured that there were no restriction enzyme recognition sites for NotI or EcoRI within the BCR or ABL sequences, nor in the pMIGR vector, in which BCR-ABL would be inserted, other than in the cloning polylinker.

To obtain template DNA to amplify BCR and ABL, we collected blood from a wild type mouse and isolated RNA. Messenger RNA from the total RNA was copied into cDNA, which served as template in PCR. During PCR, I encountered problems amplifying the DNA. After running 25 cycles of PCR in the thermocycler, there were no distinguishable bands of DNA after the gel was visualized, with the exception of primer dimers at the bottom. Even a control reaction with a completely different set of primers and template (to rule out that the primers for BCR and ABL were just incorrect) resulted in little to no amplification, indicating that the cDNA library was at fault. To troubleshoot this, I increased the number of cycles to 35 and used two times the volume of template cDNA. This seemed to help: with this approach, I saw bands at around 3kb for

truncated ABL and a band below 1500 nt, which matched well with the expected band size of 1279 nt. For full-length ABL, there was a band at 900 nt - this band was excised from the gel for further investigation.

Bands in the gel were cut out and purified by gel extraction. The next step was to add adenines onto the blunt ends of the PCR amplicons to facilitate insertion of the amplicons inside a pGEM-T-easy plasmid. The TA cloning procedure that we used did not lead to successful amplification of pGEM-tr-ABL because we lacked dNTPs. dATPs are required so that Taq polymerase can add adenines to the ends of blunt DNA. The lack of this reagent resulted in lower transformation efficiencies and could explain why we only obtained empty pGEM-TA vectors from analytical restriction digests and sequencing.

After completing the TA cloning and PCR cleanup, the pGEM-BCR, pGEM-ABL, pGEM-trBCR (truncated BCR), and pGEM-trABL (truncated ABL) were introduced into DH10B *Escherichia coli* via transformation and the bacteria were plated on LB + carbenicillin (an ampicillin analog) + Xgal media; if the gene of interest was inserted into the pGEM vector, it would disrupt the lacZ gene and thus colonies would appear white in the presence of the substrate Xgal. I picked white colonies and isolated the plasmid DNA via a miniprep procedure. The DNA isolated from the bacteria was then digested using restriction enzymes PvuII and HindIII. If the truncated ABL DNA had been inserted into the pGEM vector and was digested with PvuII, we would expect to see five different bands on a gel. For pGEM-trBCR digested by PvuII, we would expect a total of four bands. Because there are no HindIII cut sites in the pGEM vector, nor in the sequence of the inserts, we would expect no distinct bands. Results were mixed: for several pGEM-trABL samples, I observed bands just below the 3 kb level and at 1.5 kb, which coincide with the expected results, but I did not observe any bands at 279 nt or 723 nt. It could be likely that the gel was running for too long and that the DNA ran off of the gel; in future experiments, I would let the gel run for a shorter period of time, or I would visualize the gel once after ten minutes, and once again after 20 minutes have passed to ensure that smaller bands are visualized before they disappear from the gel. As for the pGEM-trBCR samples, I observed bands corresponding to 2.5 kb and 1 kb. As a summary, the expected DNA band sizes for the plasmids when cut with HindIII and PvuII are below:

Table 2: Expected band sizes of pGEM-trBCR, pGEM-trABL incubated with PvuII

pGEM-trBCR digested with PvuII	pGEM-trABL digested with PvuII
2.5 kb	2.5 kb
1 kb	1.6 kb
361 nt	957 nt
279 nt	723 nt
	279 nt

To increase efficiency of primer binding, I can design a primer with a higher GC percentage or use a primer of shorter length (hence a lower Tm). Additionally, having guanine or cytosine at the end of the primer will also enhance binding of the primer to the template strand due to those bases having three hydrogen bonds as opposed to two.

We sent samples of pGEM-trBCR and pGEM-trABL to the University's Biomedical Genomics Center (BMGC) for sequencing to confirm the presence of BCR and ABL in the pGEM vector. The sequencing results were not conclusive and did not match the sequence of BCR or ABL. I entered some of the sequence into a BLAST search: one sample aligned to c-Abl in *M. musculus* and *Rattus norvegicus*. For this reason, we continued to work with these samples. I changed the TA cloning procedure to increase the efficiency of insertion of our genes of interest

into the vectors and resubmitted for sequencing in hopes of better results. This is where the experiment currently stands at the end of this UROP. We are still attempting to clone BCR and ABL and insert the DNA into a plasmid.

Once the cloning issues are resolved and we have confirmed that BCR and ABL indeed have been inserted into pGEM, we can begin to produce the BCR-ABL fusion peptide. I already designed primers for the construction of trBCR-e1a2-trABL and trBCR-2W-trABL. The BCR and ABL would be derived from mouse, and DNA encoding an e1a2 peptide from human leukemia patients or the 2W1S peptide, which would serve as a control experiment. These amplicons would then also be inserted into pGEM, and eventually into a pMIGR retroviral vector containing two long terminal repeats (LTRs) that are usually involved in insertion of viral DNA into the host genome. The pMIGR vector is used for introducing and expressing target genes into mammalian cells such as human or murine hematopoietic cells. Our goal would be to transduce mouse bone marrow cells in culture with the pMIGR-trBCR-e1a2-trABL or pMIGR-trBCR-2W1S-trABL. We would then examine these cells under a microscope to check for transformation; that is, we would want to know if cells expressing murine BCR-ABL have the ability to grow uncontrollably (e.g lack of contact inhibition, development of anchorage independence, etc). These cancerous cells would then be injected into mice in order to induce leukemia. Our lab is interested in antigen-specific CD4+ T cells that can orchestrate an adaptive immune response against these BCR-ABL+ leukemic cells. Using flow cytometry, we would quantify and characterize these pivotal cells.

This type of work was oftentimes tedious and repetitive, but it made me appreciate the patience that scientists and researchers must have. It took over three months to insert the genes of interest into a bacterial vector because of various problems. With this UROP, I have realized that science moves at a glacial pace. However, I am grateful to have had this research experience because it challenged me intellectually and tested my ability to problem solve and troubleshoot when things did not go as planned. I learned to communicate better and more effectively as well as finding the right balance between asking for help when needed and figuring something out on my own. I have more trust in my ability to think like a scientist, thanks to UROP, my sponsor Dr. Michael Farrar, and my mentor Luke Manlove.

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