DIMERIZATION SPECIFICITIES OF LEUCINE ZIPPER MUTANTS

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Abstract

Dimerization Specificities of Leucine Zipper Mutants

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The dimerization specificity of a leucine zipper is partially determined by the interactions of charged amino acids on the surfaces of dimer interfaces (*e* and *g* positions). A series of *e* and *g* position GCN4 mutants has been described that do not dimerize with the wild-type GCN4 but are able to dimerize with each other. Two hydrophobic leucine residues present in the wild-type GCN4 leucine zipper are suspected to be causing this difference in dimerization specificity. To determine the importance of these leucine residues in dimerization specificity, I constructed a series of mutants in which the leucine residues were substituted with alanine. The dimerization specificity of these mutants was determined based on the phenotypes of cells expressing wild-type and dominant negative λ repressor-leucine zipper fusion proteins. Cells that were immune to infection by phage λ contain homodimeric fusion proteins. Cells that were sensitive to infection by phage λ are forming heterodimeric fusion proteins. The two leucine residues in the wild-type GCN4 leucine zipper are not sufficient to explain the difference in dimerization specificity.

The amino acid sequence of a protein determines whether or not it will interact with another protein. The model used in our lab for studying protein-protein interactions is the leucine zipper. The leucine zipper is a structural motif consisting of two α-helices coiled together. It allows two proteins to dimerize when the α-helix of one protein monomer coils together with the α-helix of another protein monomer. The leucine zipper is required for dimerization in a class of DNA binding transcription factors (bZIP proteins). Landschulz *et. al.* [1988] showed that these proteins consist of two structural domains, one for DNA binding and the other for dimerization. The dimerization of bZIP proteins is important for their activities as activators or repressors of transcription. The natural proteins containing leucine zippers are very specific, which means they will only dimerize with a few other proteins. Since the leucine zippers are ubiquitous in nature, the specificity of dimerization is important to our understanding of the regulation of transcription.

The structure of the leucine zipper was first described by Landschulz [1988], but the α-helices he suggested were antiparallel. X-ray crystallography has shown that the α-helices are parallel [1991]. The α-helix of each monomer is only 30 to 40 amino acids long. It contains a leucine residue every seventh position (***d***) over four to five heptad repeats (Fig. 1). Each heptad forms two turns of the α-helix. The residues of one monomer are labeled ***abcdefg*** and the corresponding residues of the other monomer are labeled ***a'b'c'd'e'f'g'***. The ***a***, ***d***, ***e***, and ***g*** positions form the interface where the two α-

helices come together. The *a* position contains mostly hydrophobic residues, while the *d* position contains the leucine residues. Two monomers form a dimer by joining on the hydrophobic surface, with the leucines forming a zipper. The *e* and *g* positions contain mostly charged residues which can stabilize the leucine zipper by forming salt bridges between the *e* position of one heptad and the *g'* position of the next heptad.

The leucine zipper allows the protein to form homodimers and heterodimers. Homodimers contain two subunits of the same protein. Heterodimers contain two subunits from different proteins. The formation of heterodimers allows nature to make a large number of protein dimers by mixing and matching just a small number of monomers. Vinson *et. al.* (1993A) studied the formation of heterodimers by looking at the *e* and *g* positions of 20 bZIP proteins. He suggested that homodimers would form if the residues at the *e* and *g* positions in one heptad were of opposite charges. If the residues were of the same charge, then the formation of a heterodimer with a protein that has residues of opposite charge would be favored. Using this hypothesis, Vinson was able to predict which monomers would dimerize.

Zeng applied Vinson's hypothesis to a series of mutants at the *e* and *g* positions of the GCN4 leucine zipper [1997A]. The mutants contain different combinations of four charged or neutral amino acids, threonine, lysine, glutamic acid and alanine, at the *e* and *g* positions (Table 1). They differ from the wild-type GCN4 protein by 2 to 8 amino acids. The mutants were tested to see if they would form heterodimers or homodimers with each other and with the wild-type. Zeng was not able to predict the dimerization specificity of the mutants based on Vinson's hypothesis. He found that predicting the dimerization

specificity of mutants was more complicated than looking just at the charge interactions of amino acids at the ***e*** and ***g*** positions. His results did show that the mutants were promiscuous, meaning they dimerized with each other. The wild-type was more specific, meaning it would only dimerize with itself and not with the mutants. These results show that there is a difference in dimerization specificity between the wild-type and the mutants. The differences in the amino acid sequence of the mutant and wild-type leucine zippers must account for this difference in specificity. The most noticeable difference in the sequences is that the wild-type zipper contains two leucines at positions ***e13*** and ***g29***. This is unusual because leucine is a hydrophobic residue. Usually the ***e*** and ***g*** positions contain charged residues.

I constructed two series of mutants to test the effect of these leucines on the dimerization specificity. The first series of mutants, the alanine mutants, was constructed by changing the wild-type zipper so it contains alanine instead of leucine at either position ***e13***, ***g29***, or both (Table 2). This mutation is referred to as a molecular haircut because it removes the long hydrophobic side chain of leucine and replaces it with the methyl group of alanine (Fig. 2). Alanine was also chosen because it was one of the amino acids used to construct the original series of mutants. The specificity of each of these mutants was tested with the wild-type GCN4, each of the other mutants, and each of the original mutants from Zeng's work. The working hypothesis suggests that if the leucines are causing the difference in dimerization specificity, then the alanine mutants will be able to dimerize with the original series of mutants and will not be able to dimerize with wild-type GCN4. The results of the specificity assay showed that the mutants were able to dimerize

with the wild-type but not with the original set of mutants. These results indicate that the leucine residues at ***e13*** and ***g29*** do not have an affect on the dimerization specificity.

The second series of mutants, the leucine mutants, was constructed by introducing leucines into three of the original mutants at positions which contain alanine residues (Table 3). Two of the original mutants, s26 and s67, contain 2 alanine residues, and one, s20, contains 3 alanine residues. The working hypothesis suggests that if the leucines are responsible for the difference in dimerization specificity, then the leucine mutants will be able to dimerize with the wild-type and will not be able to dimerize with the original series of mutants. The specificity assays on these mutants have not been done yet.

Materials and methods

Construction of zipper sequences

The series of alanine mutants was made by substituting alanine for leucine at positions ***e13*** (AL), ***g29*** (LA) or both (AA) in the wild-type GCN4 zipper. Four oligos coding for the mutant and wild-type leucine zippers were ordered from Genosys (Table 4). The first oligo, L13A N-terminal, codes for the N-terminal half of the leucine zipper and contains a *Sal*I site and the L13A mutation. The second oligo, L29A C-terminal, codes for the C-terminal half of the zipper and contains a *Bam*HI site and the L29A mutation. The third oligo, WT N-terminal, codes for the N-terminal of the wild-type GCN4 zipper and contains a *Sal*I site. The fourth oligo, WT C-terminal, codes for the C-terminal of the wild-type GCN4 zipper and contains a *Bam*HI site. The oligos were resuspended in 300μL of TE (10mM Tris HCl pH 7.5, 1mM EDTA) and then purified on 10% acrylamide gel (50% urea, 1X TBE). 150μL of DNA was mixed with 50μL Sequenase Stop Solution

(95% formamide, 20mM EDTA, bromphenol blue and xylene cyanol). The DNA was loaded onto the gel and run at 25W for 3 hours. The bands were visualized using UV shadowing. The desired band was cut from the gel and the DNA was eluted in 0.5mL of 0.3M NaOAc, 5μM EDTA overnight at 37°C. The pieces of the gel were removed by filtering the solution through empty Wizard miniprep columns. The pieces were washed with 0.5mL of 0.3M NaOAc and filtered again. The overall eluate was ~1mL of 0.3M NaOAc containing the DNA. The DNA was concentrated by ethanol precipitation and resuspended in 100μL TE. The concentration of the DNA was determined by measuring the OD260 and OD280. The DNA was then diluted with TE to 10μM stocks to be used in the annealing and extending reactions. The N-terminal and C-terminal oligos overlap by 9 nucleotides so they can be used as mutual primers. Four annealing reactions were set up to make the three mutants and the wild-type. Mixing L13A N-terminal and WT C-terminal made the L13A mutant (AL) with a *Sal*I site on the 5' end and a *Bam*HI site on the 3' end. Similarly, mixing WT N-terminal and L29A C-terminal makes the L29A mutant (LA) with a *Sal*I site on the 5' end and a *Bam*HI site on the 3' end. The double mutant (AA) was made by mixing L13A N-terminal and L29A C-terminal. The wild-type zipper (LL) is made by mixing WT N-terminal and WT C-terminal. Each of the annealing reactions contained 1μM of each oligo, 1X Sequenase buffer, 0.25μM dNTPs, and 10mM DTT in a 100μL reaction. The annealing reactions were done by heating to 70°C for one minute and cooling by 1°C per 15 seconds to 0°C. After annealing the oligos, they were extended by the addition of 13 units of Sequenase T7 DNA polymerase v2.0 to each annealing reaction to give double stranded DNA coding for all three mutant and the wild-

type GCN4 zippers. The double stranded DNA was then digested with *Sal*I and *Bam*HI. Each digestion reaction contained approximately 10ng of extended DNA, 1X Restriction buffer (100mM Tris HCl pH 7.5, 100mM EDTA), 1X BSA, 150mM NaCl, 1mM DTT, and 10 units each of *Sal*I and *Bam*HI in a 100μL reaction. The digestions were carried out at 37°C for two hours. The DNA was concentrated by ethanol precipitation. The DNA was resuspended in 20μL of TE. A sample of the digested DNA was run on a 12% acrylamide gel to check the purity of the digest and to estimate the concentration of DNA.

The series of leucine mutants was made by substituting leucine for alanine in three mutants from the original series (Table 3). The three mutants selected were s20, s26, and s67. These three mutants are referred to as s20L, s26L and s67L, respectively. Six oligos were ordered from Genosys to code for the three new mutants (Table 4). The first oligo, s20L N-terminal, codes for the N-terminal half of s20L and contains a *Sal*I site. The second oligo, s20L C-terminal, codes for the C-terminal half of s20L and contains the A20L, A22L, and A29L mutations and a *Bam*HI site. The third oligo, s26 N-terminal, codes for the N-terminal half of s26L and contains the A6L mutation and a *Sal*I site. The fourth oligo, s26L C-terminal, codes for the C-terminal half of s26L and contains the A20L mutation and a *Bam*HI site. The fifth oligo, s67 N-terminal, codes for the N-terminal half of s67L and contains a *Sal*I site. The sixth oligo, s67L C-terminal, codes for the C-terminal half of s67L and contains the A27L and A29L mutations and a *Bam*HI site. The N-terminal and C-terminal oligos of each mutant overlap by nine nucleotides. The oligos were purified using the same methods as the alanine mutants. The DNA

concentration was measured and the oligos were annealed, extended and digested following the same protocol described for the alanine mutants.

Construction of the repressor fusion proteins

In order to use the heterodimer specificity assay, these leucine zipper sequences were cloned into plasmids with the cI^+ and dominant negative fusion proteins. For the cI^+ fusion protein constructs of the alanine mutants, the double stranded DNA fragments containing the leucine zipper sequences were cloned between the *Sal*I and *Bam*HI sites of pXZ240. pXZ240 is identical to pJH391 [1993B], except it expresses cI^+ fusions from the p7107 promoter, a constitutive mutant of the *lac*UV5 promoter. It also has the ampicillin resistance gene. pXZ240 was digested at 37°C for two hours with *Sal*I and *Bam*HI in a reaction containing 100ng of pXZ240, 1X Restriction buffer, 1X BSA, 150mM NaCl, 1mM DTT, and 10 units of *Sal*I and *Bam*HI in a 100μL reaction. pXZ240 was then gel purified on a 1% TPE agarose gel. A 100μL digestion reaction was mixed with 10μL of tracking dye (0.6g Ficol 400, 75mg bromphenol blue, 75mg xylene green, 1% SDS, and 0.1M EDTA) and loaded on the gel. The gel ran for 2 hours at 100V. The DNA was visualized with ethidium bromide. The desired band was cut from the gel and the DNA was eluted using the Gene Clean protocol. The band of DNA was weighed and three times the volume of NaI was added. The mixture was incubated at 55°C until the agarose melted. 5μL of glass milk was added and the solution was incubated on ice for 10 minutes. The solution was spun in a microfuge for 10 seconds and the supernatant was removed. The pellet was resuspended in 500μL of NEW solution. The solution was spun 10 seconds, the supernatant removed and the pellet was resuspended in 500μL NEW

solution. The solution was spun 10 seconds and the pellet was resuspended in 25μL of TE. The solution was incubated 5 minutes at 55°C and then spun 30 seconds. The supernatant was saved in a new tube and the pellet was resuspended in 25μL TE. The incubation and spinning were repeated and the supernatant saved. The total volume of DNA in the end was 50μL. A sample of the purified DNA was run on a 1% TBE agarose gel and the concentration of DNA was estimated visually by comparing the intensity of the ethidium bromide stained band to the marker bands. 100ng of pXZ240 and 50ng of the zipper sequence were ligated together using 0.1 units of T4 ligase in a reaction containing 1X ligase buffer(0.1M Tris HCl pH 7.5, 10mM $MgCl_2$, 1mM ATP, and 25μg/mL BSA) and 20mM DTT in a 100μL reaction. The ligation reactions were carried out at room temperature overnight. The ligated plasmids were then transformed into *E. coli* strain AG1688 using electroporation (AG1688 is MC1061 F'128 *lacI*^{q} *lacZ::Tn5*) [1993B]. To select for the desired plasmid, the transformed cells were plated on LB plates containing 200μg/mL of ampicillin. Colonies that grew on these plates were tested for immunity to phage λKH54 by cross-streaking. A line of phage was plated down the center of an LB ampicillin plate. Transformed colonies were streaked across the line of phage. Streaks that were immune to the phage were selected as the desired candidates. The sequence of the candidates was confirmed by dideoxy sequencing. One correct candidate of each mutant and the wild-type was used to make the dominant negative fusions and in the specificity assay.

For the dominant negative fusion protein constructs, the mutant and wild-type zipper fragments were subcloned from the cI^+ constructs into a *SalI-Bam*HI cut backbone of pXZ610. pXZ610 expresses the dominant negative fusion proteins from the *tac* promoter. The DNA binding domain of λ cI^+ repressor has a glutamine to leucine mutation at position 44 and an isoleucine to serine mutation at position 84. The linker region contains a Flag-M2 epitope tag (IBI). pXZ610 also has the tetracycline resistance gene. The ligated plasmids were transformed into *E. coli* strain AG1688 using electroporation. To select for the desired plasmid, the transformed cells were plated on LB plates containing 20μg/mL of tetracycline. Colonies that grew on these plates were selected as candidates. The sequence of the candidates was confirmed by dideoxy sequencing. One correct candidate of each mutant and the wild-type was used in the specificity assay.

For the leucine mutants, only the cI^+ fusion protein of the s20 mutant has been made. The mutated zipper sequence was cloned between the *SalI* and *Bam*HI sites of a purified digest of pXZ240. Transformed colonies were selected by plating on LB ampicillin plates. The colonies that grew were tested for immunity to λKH54 by cross-streaking. Immune colonies were selected as candidates and sequenced by the dideoxy method. One correct candidate has been identified and will be used to construct the dominant negative fusion and in the specificity assay.

Several control plasmids were also used in the specificity assays. The ampicillin resistant controls are pJH706, pXZ1130, and pAH4083. pJH706 contains the DNA binding domain of the 434 repressor fused to the GCN4 leucine zipper so it cannot confer immunity to phage λ. Any tetracycline resistant plasmid paired with pJH706 will be

sensitive to phage λ. pXZ1130 and pAH4083 have the N-terminal domain of cI repressor fused to an *a* position mutant of the GCN4 leucine zipper [1997B]. The *a* position sequence of pXZ1130 is IINI, and of pAH4083 is INNI. These mutants are known to be immune to infection when paired with the wild-type. They are also known to be able to titrate themselves so they will be sensitive when the same zippers are expressed on both fusion proteins. Their interaction with the mutants is unknown. The tetracycline resistant controls are pJH541, pXZ1090, and pAH4099. pJH541 is the tetracycline resistant vector. It is used to make sure that the tetracycline resistant plasmids do not affect the expression of the ampicillin resistant plasmids. Any ampicillin resistant plasmid, except pJH706, paired with pJH541 should be immune to phage λ. pXZ1090 and pAH4099 contain the dominant negative N-terminal domain of cI repressor fused to an *a* position mutant of the GCN4 leucine zipper. pXZ1090 contains the same zipper as pXZ1130 and pAH4099 contains the same zipper as pAH4083. These mutants are immune to infection when expressed with wild-type GCN4. Cells containing the wild-type ampicillin resistant plasmid and one of these two plasmids will be immune to infection. When they are expressed with the ampicillin resistant plasmid with the same zipper they will be sensitive. Their interaction with the mutants is unknown.

Plasmids with cI^+ fusions were introduced into cells containing the dominant negative fusions by M13-mediated transduction. A single colony of cells containing each cI^- fusion plasmid was used to inoculate overnight cultures in LB ampicillin. 20μL of the overnight culture was mixed with 0.1mL of a 1:10 dilution of phage M13 rv-1(original titer $2x10^{11}$pfu/mL). This mixture was incubated for 10 minutes at 37°C. 2mL of 2xYT

broth (16g Tryptone, 10g yeast extract, 10g NaCl per liter) was added to the mixture and the cells were grown for 8 hours with aeration. The cells were spun down in a microfuge for 10 minutes. The supernatant containing the M13 transducing phage was removed to a new tube and incubated for 15 minutes at 60°C to kill any remaining cells. Recipients were prepared by using a single colony of cells containing each dominant negative fusion plasmid to inoculate overnight cultures in LB tetracycline. 50μL of each overnight was mixed with 5μL of each cI^+ fusion M13 transducing stock, to make all possible combinations of cI^+ and dominant negative fusions. The mixtures were incubated at 37°C for 30 minutes. 5μL of each transduction was spotted on an LB ampicillin/tetracycline plate. After the spot dried, it was struck out for single colonies. The single colonies that grew were struck for singles again the next day. These single colonies were used in the specificity assays.

Specificity Assays

The specificity of the mutants was tested using λ repressor gene fusions. The gene fusion technique modifies the properties of proteins that do not give detectable phenotypes so that the protein expresses a detectable phenotype. The λ repressor is a homodimer with two domains. The N-domain is for DNA binding and the C-domain is for dimerization. When the C-domains dimerize, they form the functional λ repressor by bringing the N-domains together in the correct arrangement to allow DNA binding. When λ repressor binds to DNA, it prevents transcription of the λ DNA which prevents expression of genes in the lytic pathway. These cells that contain dimeric λ repressor cannot be infected by

phage λ. To test mutants that only form homodimers, the C-domain of λ repressor was replaced with the leucine zipper domain of each mutant. The cells with functional leucine zippers being made are able to bind DNA and repress the lytic cycle so they are immune to infection by phage λ. To test mutants that can form either heterodimers or homodimers, this process must be modified slightly. The homodimeric λ repressor was expressed in a cell along with the mutant leucine zipper attached to a dominant negative repressor mutant (Fig. 3). The dominant negative repressor mutant has a dominant mutation in λ repressor that makes it unable to bind to DNA. In order for a cell to be immune to phage λ, it must produce homodimeric wild-type λ repressor. If heterodimers form, they will not be able to bind to the DNA, so the cell will be sensitive to infection by phage λ [1995].

The plasmid containing cells were tested for immunity λKH54. A culture of the double plasmid cells was inoculated from a single colony and was grown in LB ampicillin/tetracycline at 37°C. When the first specificity assays were done, overnight cultures were used. A large number of the plaques were turbid, meaning not all of the cells in the plaque were being killed. I began using eight hour cultures because this eliminated the presence of most turbid plaques in the immunity tests. Eight hour cultures were used in the specificity assays except where specifically noted. 50μL of the culture was mixed with 2mL of tryptone top agar (10g tryptone, 5g NaCl, 13g Bacto-Agar per liter). The agar was poured onto an LB ampicillin plate. Tetracycline was omitted from the plates because it was affecting the ability of the cells to be immune to phage λ. Dilutions of λKH54 were spotted on the lawn. Strains that did not show any plaques

were considered immune; they form homodimers. Strains with clear or turbid plaques were considered sensitive; they form heterodimers at a high enough level to reduce the intracellular concentration of homodimers below the level required for immunity.

Results

Specificity assays with the original series of mutants

The specificity assays done by Zeng on the original series of mutants were repeated to make sure the results were valid [1997A]. The specificity of interactions between the original mutants and the wild-type was tested using λ repressor gene fusions. The gene fusion technique modifies the properties of proteins that do not give detectable phenotypes so that the protein expresses a detectable phenotype. This assay is fairly quick to carry out so very large numbers of mutants can be tested at one time. To test if the mutants are forming either heterodimers or homodimers, the homodimeric λ repressor is expressed in a cell along with the mutant leucine zipper attached to a dominant negative repressor mutant (Fig. 3). In order for a cell to be immune to phage λ, it must produce homodimeric wild-type λ repressor. If heterodimers form, they will not be able to bind to the DNA, so the cell will be sensitive to infection by phage λ [1995]. The original tests were done by cross-streaking with λKH54. The tests were redone using the more sensitive plating method described in materials and methods. The tests pairing the mutants with the wild-type fusions and with the controls were done using 8 hour cultures, while the tests pairing the mutants with each other were done using overnight cultures.

Figure 4 shows the results of the specificity assays. Each row shows the results for one cI^+ fusion protein expressed with one of the dominant negative fusion proteins in each

column. The results of the assays I did are shown in the big boxes. The results of the assays done by Zeng are shown in small boxes inside the big boxes. Zeng's results are only shown if his result differed from the result I obtained.

The control plasmids all gave the expected results. Each of the dominant negative fusions paired with pJH706 was sensitive to infection by phage λ. This result is correct because pJH706 contains the DNA binding domain of the 434 repressor fused to the GCN4 leucine zipper so it cannot confer immunity to phage λ. All of the cI^+ fusions, except pJH706, paired with pJH541 were immune to infection by phage λ. This result is correct because it shows that the tetracycline resistant vector, pJH541, does not affect the expression of the cI^+ fusions and it does not affect the ability of the cell to be immune to phage λ. The ***a*** position mutants were immune to infection when paired with wild-type GCN4 and sensitive to infection when paired with themselves (i.e. XZ1130 with XZ1090 was sensitive and AH4083 with AH4099 was sensitive). The ***a*** position mutants were immune when paired with the mutants, indicating the ***a*** position mutants could not interact with the mutants.

Zeng found that all of the mutants were immune when paired with the wild-type fusions, meaning the mutants and the wild-type leucine zippers were unable to interact with each other. My results show that the mutants paired with the wild-type are immune to infection, except for n34. The cI^+ fusion of n34 was sensitive when paired with the dominant negative fusion of the wild-type, but the n34 dominant negative fusion was unable to inhibit the cI^+ fusion of the wild-type. My results confirm that overall the mutants are unable to interact with the wild-type.

The boxes along the diagonal show combinations where the same leucine zipper is present in both fusion proteins. Zeng found that the wild-type and all of the mutants were sensitive to infection when the same zipper was expressed on both fusion proteins. My results confirmed that the wild-type, n34, s20, s24, and s67 were sensitive to infection. My results for s03, s26, s56 and s64 show that the cells are immune to infection when the same zipper is expressed on both fusion proteins.

Zeng's results and my results differ greatly when the mutants are paired with each other. Of the 81 possible combinations, there were 30 discrepancies. The nature and possible origin of these discrepancies are described in the discussion.

Construction of the alanine mutants

To test the importance of the ***e13*** and ***g29*** leucine residues in wild-type GCN4 on the dimerization specificity, a series of mutants containing alanine instead of leucine at these positions was constructed. The mutant and wild-type zippers were fused to the DNA binding domain of λ cI repressor (cI^+ fusions). These fusion proteins are expressed in *E. coli* from a weak constitutive promoter, P7107. Cells that express dimeric cI^+ fusions are immune to infection by phage λ. The leucine zippers will form dimers that bind to the λ DNA, preventing it from being transcribed. Cells that express monomeric cI^+ fusions are sensitive to infection by phage λ. Each of the mutants and wild-type cI^+ fusion proteins were tested by cross-streaking with λKH54 to make sure they were forming stable dimers.

The mutant and wild-type zippers were subcloned into a plasmid containing the DNA binding domain of λ cI repressor with two mutations that make it unable to bind to DNA (dominant negative fusions). The cI^+ fusions and the dominant negative fusions

were expressed in the same cell using M13-mediated transduction as described in materials and methods.

Construction of the leucine mutants

A second series of mutants was constructed by adding leucine residues to three of the mutants in the original series. The three mutants chosen, s20, s26, and s67, contained alanine at two or three positions. The cI^+ fusion of the s20 leucine mutant has been constructed the same way the alanine mutants were made. The other mutants are in the process of being made and then their specificity will be tested to each other, the wild-type GCN4 and the original series of mutants.

Specificity assay results of the alanine mutants with each other

The results of phage immunity tests on the alanine mutants expressing pairwise combinations of wild-type and mutant leucine zippers are shown in Figure 5. Each row shows the results for one cI^+ fusion protein expressed with one of the dominant negative fusion proteins in each column.

The control plasmids all gave the expected results. Each of the dominant negative fusions paired with pJH706 was sensitive to infection by phage λ. All of the cI^+ fusions, except pJH706, paired with pJH541 were immune to infection by phage λ. The ***a*** position mutants were immune to infection when paired with wild-type GCN4 and sensitive to infection when paired with themselves. The ***a*** position mutants were immune when paired with the mutants, indicating the ***a*** position mutants could not interact with the alanine mutants.

The cI^+ fusion of the wild-type GCN4 zipper was sensitive when paired with its dominant negative fusion, indicating that heterodimers are forming. The wild-type is able to interact with itself. The wild-type paired with each of the mutants was sensitive to infection, indicating that the mutants and the wild-type are forming heterodimers.

The cI^+ fusion of AL was immune to infection when paired with the dominant negative fusion of the wild-type. This result suggests that AL is not able to interact with the wild-type by forming heterodimers. The cI^+ fusion of AL was immune when paired with its own dominant negative fusion. Since AL must be able to interact with itself, this suggests something else is happening (see discussion). The cI^+ fusion of AL was immune when paired with the dominant negative fusion of LA and is sensitive when paired with the dominant negative fusion of AA. The dominant negative fusion of AL inhibits repression by the cI^+ fusions of wild-type and LA.

The cI^+ fusion of LA is sensitive when paired with the dominant negative fusions of the wild-type, AL, and AA, indicating that it can form heterodimers with each one. The cI^+ fusion of LA was sensitive when paired with its own dominant negative fusion, indicating that it is able to interact with itself. The dominant negative fusion to LA inhibits repression by cI^+ fusions to wild-type but not the AL or AA mutants.

Like the LA mutant, the cI^+ fusion of AA is sensitive when paired with the dominant negative fusion of the wild-type, suggesting that it is able to interact with the wild-type. The cI^+ fusion of AA was immune when paired with the dominant negative fusion of AL and LA, suggesting that it is also unable to interact with them. The cI^+ fusion of AA was sensitive when paired with its dominant negative fusion, indicating that it is

able to interact with itself. However, the dominant negative fusion of AA inhibited repression of wild-type, AL, LA and itself.

Specificity assay results of the alanine mutants with the original mutants

The specificity of each alanine mutant was also tested with the original series of mutants. The results of the specificity assay using the cI^+ fusion of the alanine mutants and the dominant negative fusion of the original series of mutants is shown in Figure 6. The control plasmids all gave the expected results. Each of the dominant negative fusions paired with pJH706 was sensitive to infection by phage λ. The ***a*** position mutants, XZ1130 and AH4083, were immune when paired with the mutants, indicating that they could not interact with the mutants. All combinations of the wild-type and alanine mutants paired with the original mutants were immune to infection by phage λ. These results indicate that the alanine mutants cannot form heterodimers with the original series of mutants.

The results of the specificity assay using the cI^+ fusions of the original mutants and the dominant negative fusions of the alanine mutants is shown in Figure 7. The control plasmids all gave the expected results. Each of the dominant negative fusions paired with pJH706 was sensitive to infection by phage λ. The ***a*** position mutants, XZ1130 and AH4083, were immune when paired with the mutants, indicating that they could not interact with the mutants. All of the combinations were immune to infection by phage λ, except n34 with wild-type, s56 with AL and s67 with AL. These results indicate that overall the alanine mutants do not interact with the original series of mutants.

Discussion

The sequence of the 67 naturally occurring leucine zippers identified as of 1994 [1994] was analyzed to determine the number of leucines occurring in the *e* and *g* positions. Only three zippers were found that contain two leucines at the *e* and *g* positions. One zipper was found that contains three leucines. This shows that the leucines present in GCN4 are not usually found in the naturally occurring leucine zippers.

Despite the observed discrepancies, the specificity assays done with the original series of mutants show the same general trend as the assays done by Zeng. The wild-type is specific; it will only dimerize with itself. The mutants are promiscuous; most of the mutants will dimerize with each other. There were many discrepancies in the assays testing the mutants with each other. Some reasons for these discrepancies are that the original tests done by Zeng were done by cross-streaking and the tests I conducted were done by spotting dilutions of phage on a lawn of cells. Also, the tests pairing the mutants with the wild-type fusions and with the controls were done using 8 hour cultures, while the tests pairing the mutants with each other were done using overnight cultures. I will redo the assays of the mutants with each other using 8 hour cultures to determine if this changes the results. Even though these discrepancies exist, the results of my assays and of Zeng's assays show that the mutants are promiscuous, meaning they dimerize with each other and the wild-type is very specific, meaning it will only dimerize with itself and not the mutants. The results of my specificity assays show that there is a difference in the dimerization specificity of the wild-type and the mutants.

The specificity assays done with the alanine mutants show several interesting results. The AL mutant was immune when it was expressed in the same cell on both fusion proteins. Similar results were seen with other mutants in our lab when tetramers were being formed instead of dimers [1997C]. The tetramer can still bind to the phage λ DNA and confer immunity as long as it contains two wild-type copies of the DNA binding domain. The AL mutant will be tested for tetramer formation using the assay in Figure 8. Two reporter strains are used. The strain on the left has two operators. The weak operator overlaps the promoter for *lacZ*, which codes for β-galactosidase and *cat*, which confers chloramphenicol resistance. The strong operator site is upstream of the weak operator and does not affect transcription by the promoter. Cooperative binding to both of these promoters increases the efficiency of repression. The second reporter strain, shown in the middle of the picture, contains one weak promoter that overlaps the promoter for *lacZ* and *cat*. Repression of the *lacZ* gene is measured using a β-galactosidase assay. The top of Figure 8 gives an example of a dimeric fusion protein. On the right, the dimeric protein does not have a high enough affinity to bind to the weak operator. The graph shows the level of β-galactosidase activity for the wild-type dimer is repressed to 60% for one operator. On the left, the dimeric fusion will bind to the strong operator. The dimeric protein does not have a high enough affinity to bind to the weak operator. The level of β-galactosidase activity for the wild-type dimer is repressed to 40% for two operators. The bottom of the figure gives an example of a tetrameric fusion protein. On the right, the tetrameric protein does not have a high enough affinity to bind to the weak operator. The graph shows the level of β-galactosidase activity for the *aLdl*

tetramer is repressed to 40% for one operator. On the left, the binding of two DNA binding domains to the strong operator brings the other two DNA binding domains into a configuration that allows them to bind to the weak operator. The cooperative binding of the tetrameric fusion protein to both operators represses the level of β-galactosidase activity to less than 10%. This assay allows tetrameric and dimeric fusion proteins to distinguished phenotypically based on the ability of tetrameric fusions to bind cooperatively to adjacent operator sites.

The dominant negative fusion of AA was the only combination that was sensitive to infection when paired with the cI^+ fusion of AL. This result can also be explained by formation of tetramers. Consider a situation where the dominant negative fusion of AA forms dimers and the cI^+ fusion of AL forms tetramers. When the two fusion proteins are expressed in the same cell, they are able to interact. The resulting fusion protein could be a dimer, tetramer or higher order oligomer. As long as the dimer contains one dominant negative DNA binding domain the cell will be sensitive to infection. As long as a tetramer or higher order oligomer contains enough copies of the dominant negative DNA binding domain to make it inactive, the cell will be sensitive to infection.

The working hypothesis predicted that the leucine residues at positions ***e13*** and ***g29*** were causing the wild-type GCN4 to be more specific. By replacing the leucine residues with alanine, the new series of mutants would be more similar to the original series of mutants. If the leucines are causing the difference in specificity, then the new mutants should be able to dimerize with the original series of mutants and should not be able to dimerize with the wild-type GCN4. Instead, removing those leucine residues and

replacing them with alanine allowed the mutants to dimerize with the wild-type but not with the original set of mutants. This result indicates that the leucines are not sufficient to explain the differences in dimerization specificity. Thus, our working hypothesis was shown to be incorrect based on the results of the specificity assays.

The properties of the AL and AA mutants suggest that leucine at position ***e13*** may be involved in causing the wild-type leucine zipper to prefer dimers over other oligmerization states. We cannot rule out the possibility that the leucines do have some effect on the dimerization specificity due to the limitations of the specificity assay. The new series of alanine mutants may be less promiscuous than the original series of mutants and still have a different specificity than the wild-type. These degrees of interaction cannot be determined by the assay used here; we plan to do a quantitative measurement of heterodimer formation *in vitro*.

Nevertheless, my results suggest that the difference in dimerization specificity between the mutants and the wild-type is due to the pattern of amino acids in the ***e*** and ***g*** positions. A specific pattern of positively charged, negatively charged and neutral amino acids may be required in order to see the specificity exhibited by the wild-type GCN4 leucine zipper and the promiscuity exhibited by the mutants.

References

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Table 1. ***e*** and ***g*** sequence of mutants in the original series

mutant	*e*/*g* sequence
n34	KKTK/AAEA
s03	KEKT/KEAE
s20	EEAE/KKAA
s24	AKKK/TETA
s26	AEAE/KKKE
s56	EKTA/EKTK
s64	ATKK/AEET
s67	KKEA/EKEA

Table 2. The alanine mutants

Mutant name	Mutated positions	*e/g* sequence
LL	wild-type	ELEK/KKEL
AL	***e13***	EAEK/KKEL
LA	***g29***	ELEK/KKEA
AA	***e13*** and ***g29***	EAEK/KKEA

Table 3. The leucine mutants

Mutant name	Mutated positions	original *e/g* sequence	mutated *e/g* sequence
s20L	***e20***, ***g22***, and ***g29***	EEAE/KKAA	EELE/KKLL
s26L	***e6*** and ***e20***	AEAE/KKKE	LELE/KKKE
s67L	***e27*** and ***g29***	KKEA/EKEA	KKEL/EKEL

Table 4. Oligos used to make alanine and leucine mutants

Oligo name	Description	Sequence[1]
WT N-terminal	N-terminal half containing the wild-type zipper and a *Sal*I site	GAGAGATGGGTGTCGACACATATGAAAC AGCTGGAAGACAAAGTTGAAGACTGCGC TTCTAAAAAC
WT C-terminal	C-terminal half containing the wild-type zipper and a *Bam*HI site	AGCAGCGCCGGATCCTCAACGTTCACCAA CTAGTTTTTTCAGGCGCGCAACTTCGTTCT CGAGGTGGTAGTTTTTAGA
L13A N-terminal	N-terminal half containing L13A mutation and a *Sal*I site	GAGAGATGGGTGTCGACACATATGAAAC AGCTGGAAGACAAAGTTGAAGAGCTCGC TTCTAAAAAC
L29A C-terminal	C-terminal half containing L29A mutation and a *Bam*HI site	AGCAGCGCCGGATCCTCAACGTTCACCAA CAGCTTTTTTCAGGCGCGCAACTTCGTTC TCGAGGTGGTAGTTTTTAGA
s20L N-terminal	N-terminal half of s20 mutant containing a *Sal*I site	GAGAGATGGGTGTCGACACATATGAAAC AGCTGGAAGACAAAGTTGAAGAGCTCGA ATCTAAAAAC
s20L C-terminal	C-terminal half of s20 mutant containing the A20L, A22L, and A29L mutations and a *Bam*HI site	AGCAGCGCCGGATCCTCAACGTTCACCAA CCAGTTTTTCCAGGCGCGCAACCAGGTTC AGGAGGTGGTAGTTTTTAGA
s26L N-terminal	N-terminal half of s26 mutant containing A6L mutation and a *Sal*I site	GAGAGATGGGTGTCGACACATATGAAAC AGCTGCTGGACAAAGTTGAAGAGCTCGA ATCTAAAAAC
s26L C-terminal	C-terminal half of s26 mutant containing A20L mutation and a *Bam*HI site	AGCAGCGCCGGATCCTCAACTTTCACCAA CTTCTTTTTCCAGGCGCGCAACTTTGTTCA GGAGGTGGTAGTTTTTAGA
s67L N-terminal	N-terminal half of s67 mutant containing a *Sal*I site	GAGAGATGGGTGTCGACACATATGAAAC AGCTGAAAGACGAAGTTGAAGAGCTCAA ATCTAAAAAC
s67L C-terminal	C-terminal half of s67 mutant containing A27L and A29L mutations and a *Bam*HI site	AGCAGCGCCGGATCCTCAACGTTCACCAA CCAGTTTCAGCAGGCGCGCAACTTCGTTT TCGAGGTGGTAGTTTTTAGA

[1]The underlined nucleotides indicate bases that were changed to make the mutations.

Figure Legends

Figure 1. The leucine zipper of GCN4.

A. The sequence of the wild-type GCN4 leucine zipper. The lower case letters indicate the positions in the heptad repeat. The ***e*** and ***g*** positions are indicated by outline font.

B. Each α-helix is represented by a cylinder with a dotted line showing the path of the polypeptide chain. The side chains of the amino acids are represented by circles. The ***e'*** and ***g*** positions are highlighted to show how salt bridges can form between charged amino acids at the ***e'*** position of one heptad and the ***g*** position of the next heptad.

C. An end view of the dimer. The residues of one monomer are labeled ***abcdefg*** and the corresponding residues of the other monomer are labeled ***a'b'c'd'e'f'g'***. The ***a*** and ***d*** residues form the hydrophobic surface where the two α-helices come together. The ***e*** and ***g*** positions are on the side of the dimer, so they can interact by forming salt bridges.

Figure 2. The molecular haircut

Leucine has a long hydrophobic side chain. The interactions of this side chain at ***e13*** and ***g29*** in the wild-type GCN4 can be eliminated by substituting alanine in these positions in the mutants.

Figure 3. The specificity assay for testing heterodimer formation.

The homodimeric λ repressor (white circle with striped zipper) is expressed with the mutant leucine zipper attached to a dominant negative repressor mutant (poison circle

with white zipper). If the cell only produces homodimers, then the cell will be immune to phage λ infection. If heterodimers form, the cell will be sensitive to infection by phage λ.

Figure 4. Specificity assay results for the original series of mutants
Each row shows the results for one cI^+ fusion protein expressed with one of the dominant negative fusion proteins in each column. The results of the assays I did are shown in the big boxes. The results of the assays done by Zeng are shown in small boxes inside the big boxes. Zeng's results are only shown if the result differed from the result I obtained. The boxes along the diagonal show combinations where the same leucine zipper is present in both fusion proteins. Boxes on the diagonal that were sensitive to infection by phage λ are black. Gray boxes indicate combinations that were sensitive to infection. White boxes indicate combinations that were immune to infection.

Figure 5. Specificity assay results for the alanine mutants tested with each other
The wild-type and mutants are designated by their ***e13/g29*** sequence (L = leucine, A = alanine). The shading codes are the same as Figure 4.

Figure 6. Specificity assay results for the cI^+ fusion of the alanine mutants with the dominant negative fusion of the original series of mutants
The shading codes are the same as Figure 4.

Figure 7. Specificity assay results for the cI$^+$ fusion of the original series of mutants with the dominant negative fusion of the alanine mutants
The shading codes are the same as Figure 4.

Figure 8. Tetramer assay
This assay allows tetrameric and dimeric fusion proteins to distinguished phenotypically based on the ability of tetrameric fusions to bind cooperatively to adjacent operator sites. Two reporter strains are shown, one on the left and one on the right. The dimeric protein in the strain on the right does not have a high enough affinity to bind to the weak operator. The graph shows that the level of β-galactosidase activity for the wild-type dimer is repressed to 60% for one operator. The dimeric fusion in the strain on the left will bind to the strong operator. The dimeric fusion protein does not have a high enough affinity to bind to the weak operator. The level of β-galactosidase activity for the wild-type dimer is repressed to 40% for two operators. The tetrameric protein in the strain on the right does not have a high enough affinity to bind to the weak operator. The graph shows the level of β-galactosidase activity for the *aLdI* tetramer is repressed to 40% for one operator. In the strain on the left, the binding of two DNA binding domains to the strong operator brings the other two DNA binding domains into a configuration that allows them to bind to the weak operator. The cooperative binding of the tetrameric fusion protein to both operators represses the level of β-galactosidase activity to less than 10%.

Figure 1. The leucine zipper of GCN4

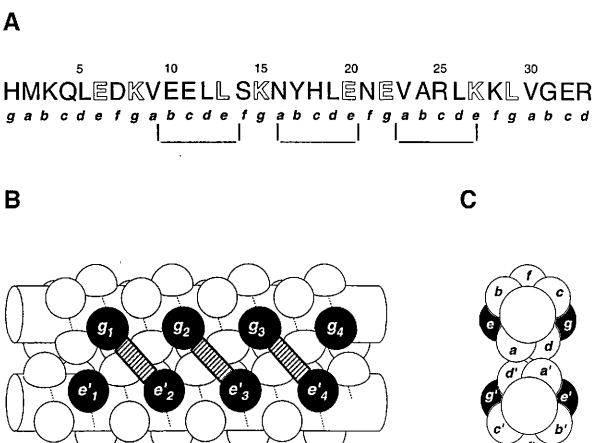


Figure 2. The molecular haircut

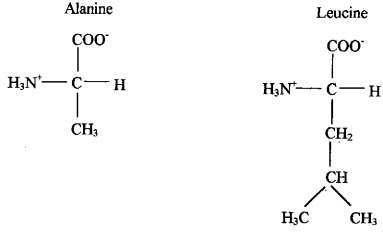
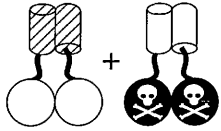
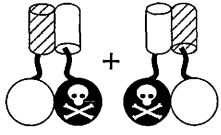


Figure 3. The specificity assay



Immune

or



Sensitive

Figure 4. Specificity assays using the original series of mutants

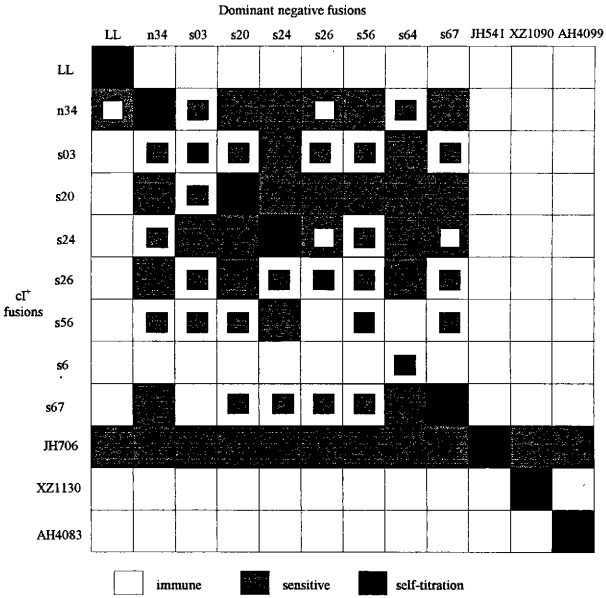


Figure 5. Specificity Assays using alanine mutants with each other

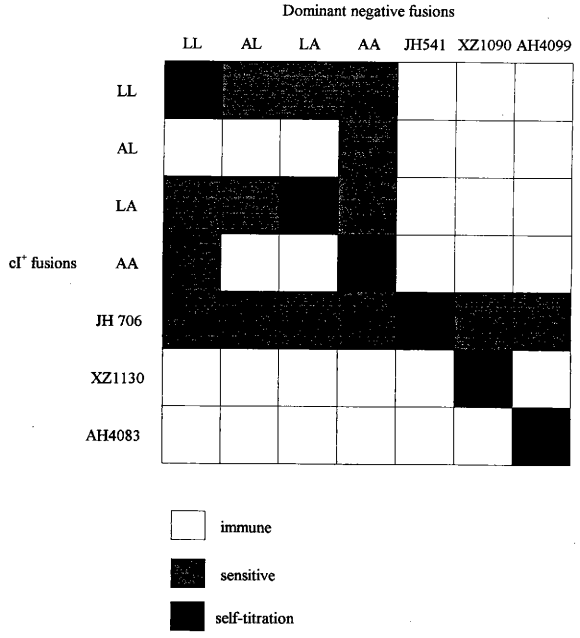


Figure 6. Specificity Assays using cI^+ fusion of alanine mutants with the dominant negative fusion of the original series of mutants

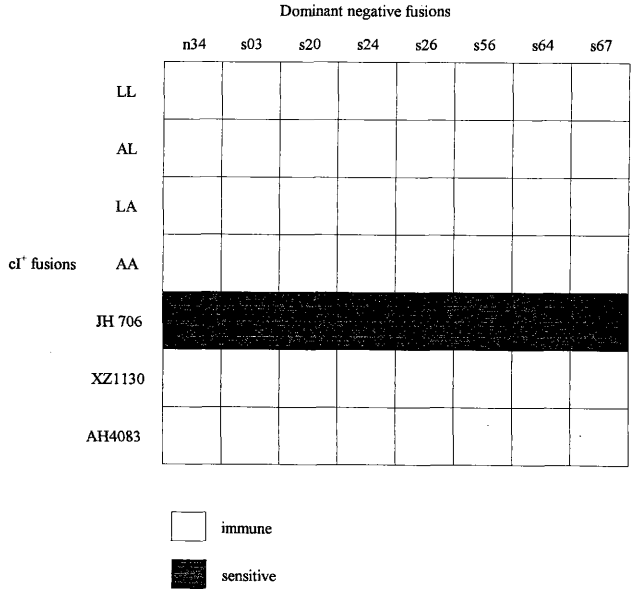


Figure 7. Specificity Assays using cI^{+} fusion of the original series of mutants with the dominant negative fusion of the alanine mutants

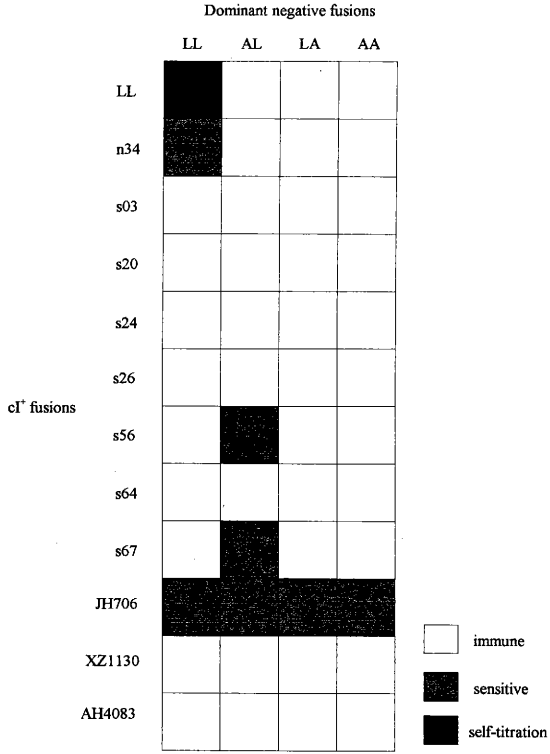


Figure 8. Tetramer assay