MQP-BIO-DSA-2511
MQP-BIO-DSA-8072
MQP-BC-DSA-3826

ORAL AVIAN INFLUENZA VACCINE

A Major Qualifying Project Report

Submitted to the Faculty of the

WORCESTER POLYTECHNIC INSTITUTE

in partial fulfillment of the requirements for the

Degree of Bachelor of Science

in

Biology and Biotechnology

and Biochemistry (Webber)

by

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April 30, 2009

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ABSTRACT

Avian influenza is a highly mutable RNA virus that is readily passed between bird species, and from birds to humans. It is possibly the greatest airborne pandemic threat to the human population. An inexpensive vaccine that is easy to produce, store, and administer is necessary for the prevention of this disease. We purified a novel protein construct (U65-mHAt) which includes the mature transcript of Influenza A subtype H5N1 virus and loaded it into particles made of beta-glucan, a known adjuvant that has been shown to improve immune system response to the influenza vaccine. This system is cost effective, can be rapidly amplified, and is easily administered making it a viable option for preventing a worldwide H5N1 pandemic.

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ACKNOWLEDGEMENTS

First and foremost we would like to thank Professor Emeritus Donald J. Tipper PhD for his wisdom, guidance, and willingness to mentor us through our project. We are especially grateful for his day-to-day support and inspiration, as well as for the use of his laboratory, reagents, and constructs. We would also like to thank Gary Ostroff PhD at Umass Medical School for arranging this opportunity and networking us with Donald. Additionally we would like to acknowledge the scientists of the Leong and Wong labs for their assistance with microscopy and reagents that we needed, and Tammy Brailey on the 6th floor of the Medical School for her support. Finally we would like to extend a big 'thank you' to Dave Adams for his support and wisdom throughout our project, for editing this MQP report, and for being able to provide insight into the multitude of questions that we had. This project would not have been possible without the aforementioned individuals.

BACKGROUND

Written accounts of influenza outbreaks date back as far as 212 BC among the Roman army and their opponents during the besieging of Achradina. There are also claims that Hippocrates documented an influenza epidemic in 412 BC but there is no conclusive evidence to verify this (Livius, 1905). Influenza is an *Orthomyxoviridae* virus, which are enveloped virus' containing a single-stranded RNA genome. One deadly feature of this particular virus is its high rate of mutation (Barry, 2005). Influenza is so mutable that 99% of all virions don't even end up as live virus. Influenza pandemics have been a reoccurring trend throughout human history, and have had some of the largest mortality rates of any pandemics the world has seen. The fear of a worldwide outbreak is a constant threat to humanity.

Influenza Types

Three types of influenza are able to infect humans; type A, which is the only type responsible for causing pandemics, type B, and type C. Influenza binds to the cell sialic acid receptors of a host cell using the hemagglutinin and neuraminidase proteins expressed on the surface of the virus (**Figure-1**). These two chemicals are responsible for enabling the virus to enter the host cell nucleus to allow replication of the viral genome. After many cycles of replication, up to 1 million times greater than cellular DNA replication, the virus destroys the host cell. The newly replicated viruses are released into the organism. There are 15 different characterized subsets for hemagglutinin (which bind to sialic acid receptors to begin infection of the host cell), and nine of neuraminidase (which cleaves sialic acid receptors in order to release

the progeny to attack other cells) that we use to classify current known influenza viruses (Wagner et al., 2002).

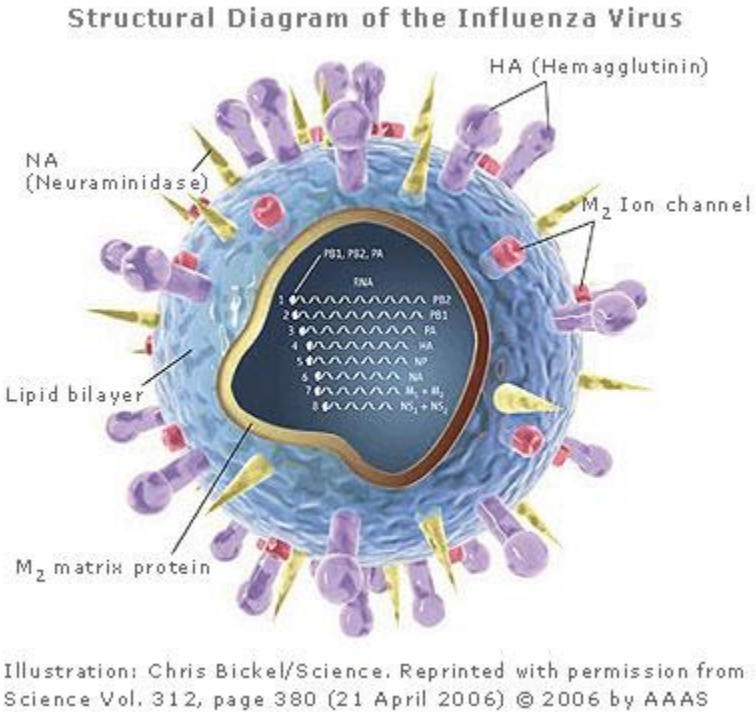

Figure 1: Diagram of an Influenza Virus *(Bickel, 2006).*

H1N1 influenza contains the type 1 of both hemagglutinin and neuraminidase. It was in fact, H1N1 influenza that caused the pandemic of 1918, killing up to 100 million people worldwide. H5N1 influenza, also known as the bird flu, is a variant strain that originated in Hong Kong (Lederberg, 2001). This virus was first discovered in 1997. Novel mutations have allowed other strains of the virus to infect humans throughout history, and have resulted in other influenza pandemics in the past, including the Asian flu of 1956, and the Hong Kong flu of 1968, both which contain RNA found in avian influenza. "While the pandemic human influenza viruses of 1957 (H2N2) and 1968 (H3N2) clearly arose through reassortment between human

and avian viruses, the influenza virus that caused the 'Spanish flu' in 1918 appears to be entirely derived from an avian source" (Belshe, 2005).

Currently, there is a worldwide outbreak of swine influenza (MacKenzie, 2009), that according to early characterization, is an H1N1 strain. This swine strain seems to been able to pass from human to human, previously been seen only in the H1N1 'Spanish flu' in 1918. Swine influenzas have been found in multiple forms in the past, including H5N1. The last scare of a swine influenza pandemic was in 1976 when an army recruit died from H1N1, but it did not appear to spread. Lack of preparation in terms of vaccine production and supply is very evident with this most recent H1N1 scare, and shows just how crucial influenza vaccines are for protecting our society. While H1N1's potential for a pandemic is more immediate, H5N1 has a greater potential for a more deadly pandemic.

Avian Influenza

Many wild birds that become infected with avian influenza do not display any symptoms. However, their saliva and feces are capable of transferring the virus to other birds and mammals. Humans on the other hand, are capable infection by avian influenza, and will initially display symptoms similar to the common cold. Symptoms can vary depending on the individual, and can range from mild effects of coughing, headache, sore throat and sneezing, to more serious effects such as pneumonia. However there is a more pathogenic form of the virus that "advances at a rapid speed and may stimulate illnesses on visceral organs which could lead to 90-100% mortality rate within 48 hours" (CDC, 2009). Most mortality from influenza is due to pneumonia that develops after infection. Currently avian influenza cannot easily be passed from human to human, unlike the current H1N1 outbreak, which is why a pandemic outbreak has

not occurred. Unfortunately avian influenza's mutable nature could lead to a mutation that allows for an easier human to human infection which, combined with the lack of a potent vaccine, creates the worry of a pandemic outbreak.

Most seasonal strains of the influenza virus are highly contagious and attack the upper respiratory track. But a recent paper has helped explain why H5N1 is normally less contagious. According to a paper published in *Nature* the expression of a specific surface molecule, SA α2,6Gal of respiratory cells is largely responsible for the ability of H5N1 to attach to human cells and replicate (Shinya, 2006). This surface molecule is found only in the lower regions of the human lung, which significantly reduces H5N1 human infection. Since influenza infection generally occurs in upper respiratory cells, replication of the H5N1 virus in humans becomes difficult which helps to explain its low incidence from bird to human, and the virtual lack of incidence from human to human (Shinya, 2006).

H5N1 influenza is not easy to diagnose because early symptoms are nearly identical to the common flu, and therefore early diagnosis of a mounting H5N1 pandemic is unlikely. There are only a few labs in the world that are capable of identifying the virus. If a human was infected and went to his primary care physician, they would probably dismiss it as the common cold. But even if the person were lucky enough to know they were infected with H5N1 influenza (such as during an outbreak), the >90% mortality rate combined with a rapid onset of the disease leaves little chance of survival.

The only current treatment for H5N1 influenza is an antiviral drug called Oseltamivir. ”Oseltamivir is a neuraminidase inhibitor which acts as a transition-state analogue inhibitor of influenza neuraminidase, preventing progeny virons from emerging from infected cells” (Moscona, 2005). This treatment was invented by United States Gilead Sciences, which is a

pharmaceutical company focused on developing therapeutics to prevent deadly diseases, and was trademarked under the name "Tamiflu." The problem with this treatment though is it was not created specifically to fight H5N1. The following is an excerpt from a Daily Net News article after Tamiflu's initial testing in December of 2005:

"Dr. Nguyen Tuong Van, who runs the intensive care unit of the Center for Tropical Diseases in Hanoi, followed World Health Organization guidelines in her treatment of patients but concluded it had no effect on the disease. "We place no importance on using this drug on our patients," she said. "Tamiflu is really only meant for treating ordinary type A flu. It was not designed to combat H5N1 ... [Tamiflu] is useless." Van said bird flu is far worse than SARS, an avian-linked respiratory illness, which she has also treated. Caring for H5N1 victims requires intensive patient "support" with modern technology, like ventilators and dialysis machines, if patients are to be kept alive. Even Western countries with wide access to technology would see their medical infrastructure strained to the limit if the dreaded pandemic comes." (World Net Daily)

Therefore if a severe avian influenza epidemic were to occur in any nation, there would be a serious problem, as there are currently no effective treatments, making worldwide spread of the disease possible. An oral vaccine designed to stop the replication of this virus is important for being proactive towards fighting this disease.

Influenza Vaccines

Prevention of a disease with a vaccine is better than treating the symptoms of a disease after infection. However, the traditional needle injection by healthcare professionals is not the most cost or time efficient option. The United States contains approximately 300 million people. If an avian influenza pandemic were to break out, an intravenous version of this vaccine would require 300 million sterilized needles to be used and disposed of properly, and would result in massive lines at health care facilities waiting for limited licensed personnel to administer the vaccine.

Oral vaccines are not only easily stored and distributed, but also have other inherent characteristics that make them superior to injected vaccine. The largest benefit is that "oral vaccines stimulate both systemic and mucosal immune responses, while injected vaccines only lead to serum antibody production" (Chen et al., 2001). The initial stimulation of mucosal antibodies close to the virus's point of entry has been known to elicit a quicker immune response, sometimes preventing the pathogen from ever entering lymphatic tissue. It has been demonstrated in viruses that an efficient immune response is not elicited through traditional oral vaccines, as compared to traditional intramuscular vaccines (Lin, 2007). This decrease in oral vaccine effectiveness is usually attributed to loss of antigen due to natural degradation of the delivered antigenic proteins in the digestive tract. Thus, a great deal of research has focused on a way to protect an oral vaccine as it travels from the mouth to the gut-associated lymphatic tissue, and any strategy to increase the potency is encouraged.

Avian influenza's high virulence is due to high rates of mutation in the hemagglutinin (HA) gene, non structural genes (NS), and the PB2 gene. As previously mentioned, HA is responsible for the binding of the virus to the host cell, and "alterations of HA occur during adaptation of influenza viruses to a new host species, as in the 1957 and 1968 influenza pandemics" (Hughes et al., 2001). NS genes are viral regulatory genes that help cause viral infections and incapacitate interferon that initiate signaling to the immune system. The PB2 gene "encodes an internal polymerase that influences the outcome of infection" and contains mutations that are underlying mechanisms for species to species evolution (Webby and Webster, 2003).

The adaptive molecular evolution of these three genes were studied for avian influenza and simulated to determine an average rate of mutation for each component (Webby and

Webster, 2003). The simulation was based on the Markov model of codon substitution for detecting significant rate shifts using comparisons of synonymous and non-synonymous nucleotide substitutions. The results concluded "that NS genes have the fastest rate of evolution and seem to be most significant for molecular adaptation of the parasite. This was also confirmed by determining the trees generated for these genes by a maximum likelihood algorithm". **Figure-2** shows avian influenza phylogenetic trees that illustrate the variance in mutagenicity of each genetic component.

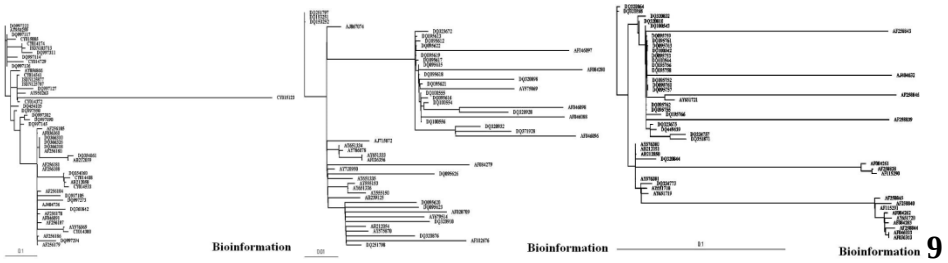

Figure 2: Phylogenetic Tree of Avian Influenza. *(Webby and Webster, 2003)*

In contrast to the NS gene conclusion of Webby and Webster (2003), Hughes et al (2001) noted, "when serially passaged in this cell line, human H3N2 viruses lost sialidase activity due to a large internal deletion in the NA gene, without alteration of the HA gene. So these 2001 findings indicate that NA mutations can contribute to the adaptation of influenza A virus to new host environments and hence may play a role in the transmission of virus across species (Hughes et al., 2001). Thus neuraminidase should not be discredited as a factor of a strain's virulence.

Hemagglutinin is the second most mutable gene in avian influenza and is responsible for binding to the receptors of host cells. The mature hemagglutinin sequence was chosen as the antigen of interest for this project.

Hepatitis B Surface Antigen (HBsAg)

Blumberg and Alter first isolated the Hepatitis-B surface antigen (HBsAg) in 1965, and the first Hepatitis-B vaccine became available in 1981. Today, many children are vaccinated with HBsAg protein, and this antigen has become the basis of a very effective Hepatitis-B vaccine. The current wealth of knowledge on the structure of HBsAg (**Figure-3**) allows the use of recombinant constructs with added domains on the N-terminus end. For example, adding Green Fluorescent Protein (GFP) to the N-terminus of HBsAg allows the presence of the recombinant protein to be visualized (Huang and Mason, 2004).

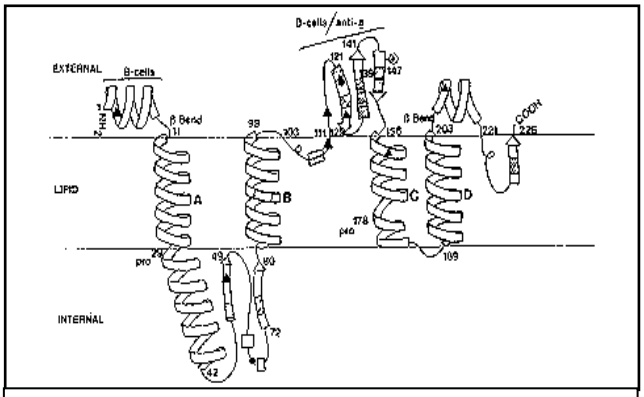

Figure 3: Structure of the Hepatitis-B Surface Antigen (HbsAg) Secondary Structure. *(Image: Mahoney and Kane, 1999)*

Hepatitis B Surface Antigen forms structures referred to as Virus-Like Particles (VLP). These VLP structures resemble the assembled virus (without the genome) and are generally a mixture of both protein and lipid. HBsAg has three characterized forms. The most antigenic form is the similar to what is found naturally in the human body. With domains added to the N-terminus, HBsAg should still form VLP's and have the added domains decorating the outside of the particles.

1→3 Linked β-D-Glucan Particles

Yeast cell walls are multilayered (**Figure-4**), composed of an outer fibrillar layer, and β-glucan layers sandwiched between layers of mannoprotein. β-Glucan is a polymer of glucose residues that is the main cell wall structural component in fungi, plants and some bacteria. The glucose residues are joined in a backbone of (1→3)-linked β-D glucopyranosyl units, with 1→6 side chains of different lengths. The β-glucan particles used in this project were derived from yeast (*Saccharomyces cerevisiae*) by removing all the mannoproteins found on the outer layer, as well as the soluble proteins that are exposed within the yeast cell. This leaves only β-glucan particles (diagram right side), which can act as a vessel for proteins, DNA, or any macromolecule of interest. Their shape is reminiscent of a yeast cell, as would be expected, and reasonably sized proteins can easily diffuse in or out of the particle due to the porous nature of β-glucan. This diffusion property was important to overcome in our project, since the protein should not diffuse out of the particle prior to uptake by the macrophage or monocyte cells. We attempted to trap our protein of interest (U65-mHAt) in these particles, as well as other proteins we were investigating.

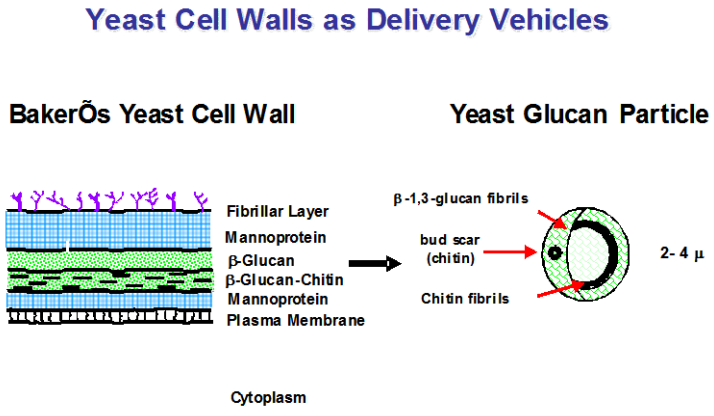

Figure 4: Derivation of Yeast Glucan Particles (YGP) from Yeast Cell Membranes.
The diagram shows what is left of the yeast membrane after GP transformation.

An important characteristic of β-glucan is that it is a proven adjuvant that safely and effectively boosts the immune system (Chihara, 1992). A variety of cell surface receptors bind β-glucan, including lectins, scavenger receptors, integrins on monocytes and macrophages, neutrophils, natural killer (NK) cells, and various lymphocyte subpopulations (Brown and Gordon, 2003). β-Glucan particles stimulate an immune reaction, but they also have a characteristic that is unique to most adjuvants. "Agents that stimulate the immune system can push the system to over-stimulation, and hence are contraindicated in individuals with autoimmune diseases, allergies, or yeast infections." (Chihara, 1992). 1,3 β-D-glucans seem to make the immune system work better without becoming overactive. They accomplish this by activating phagocytes, whose function is to trap and destroy foreign substances in our bodies such as bacteria, viruses, fungi, and parasites. 1,3 β-D-glucan has also been noted to "enhance anti-tumor and anti-infection functions in animals" (Xiao et al., 2004) making it ideal for cancer prevention and a means to prevent infection after high risk surgeries. Therefore the glucan particles not only are used as a cage to protect the loaded protein until it transported into the gut associated lymphatic tissue, but also to boost the immune system reaction to produce more antibodies to the enclosed antigen (Hong, 2004).

β-Glucan's unique properties as an adjuvant make it an ideal oral vaccine delivery system for avian influenza HA (**Figure-5**). The vaccine protein could be packaged into yeast glucan particles, ingested, and would be taken up by glucan receptors on monocytes and macrophage cells to stimulate an immune response against the packaged protein.

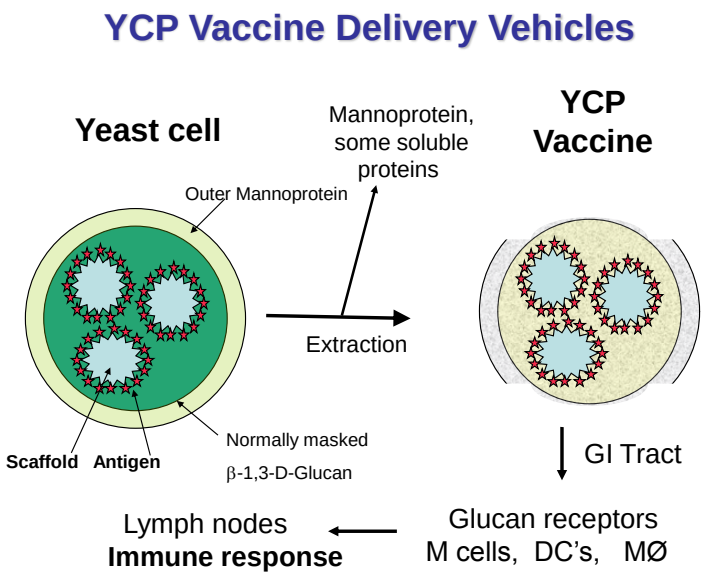

Figure 5: Yeast Glucan Particles as Vaccine Delivery Vehicles. *(Tipper, unpublished 2009)*

Although using β-glucan particles as an oral delivery mechanism seems like a potent, effective way to deliver a vaccine, the method of association between the antigen and the glucan particle could be a large factor for vaccine efficacy. Since the glucan protects the antigen through the digestive track to the gut associated lymphatic tissue, keeping the antigen as far from the surrounding proteins would be ideal. The Ostroff lab developed a charged core approach to achieve this purpose. By incubating the glucan particles with negatively charged RNA followed by positively charged PEI the antigens will aggregate inside the glucan particles to form clusters too large to escape through the porous membrane. It was hypothesized that due to the nature of the charged core, using oppositely charged trapping polymers to surround the particle would assist in binding or "trapping" the protein of interest to the newly created core of the particle, preventing escape. This trapping can later be visualized by microscopy of the glucan particles at

100x magnification after tagging the isolated protein of interest with fluorescein isothiocyanate (FITC) or GFP.

U65 Fibrillar Scaffold

The construct used for the oral avian influenza vaccine in this project was composed of a yeast U65 fibrillar scaffold (**Figure-6**) attached to the mature form of H5 hemagglutinin found in avian influenza. "U74 consists of U65, the N-terminal prion-forming 65 residues of the yeast URE2 gene product, Ure2p, followed by a 9 residue linker. Ure2p and U65 self-assemble into polymeric amyloid fibrils when over-expressed in yeast. Any coupled protein will decorate the surface of these fibrils, often in natively folded form, as demonstrated by GFP fluorescence" (Tipper, unpublished 2009).

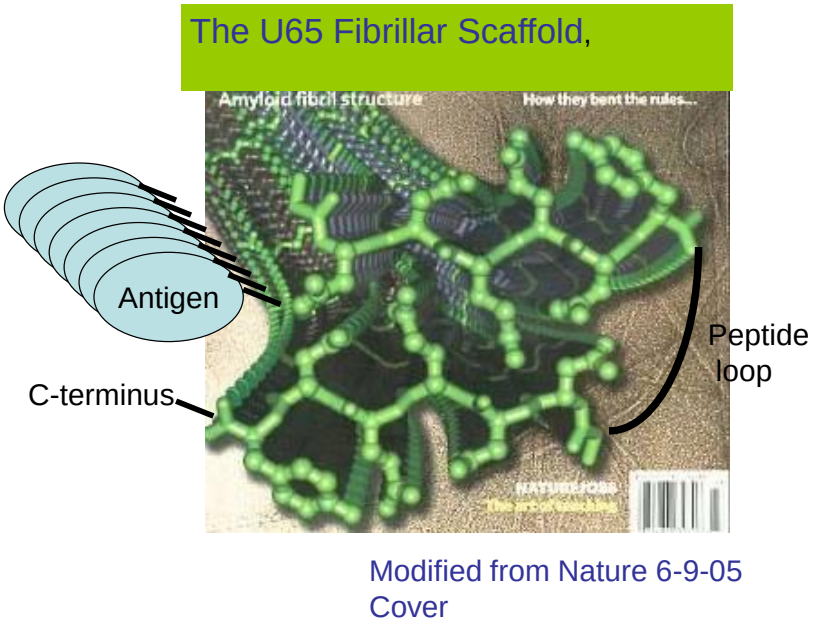

Figure 6: Structure of the U65 Yeast Fibrillar Scaffold.

In *Saccharomyces cerevisiae*, the protein Ure2p is a regulator of nitrogen catabolism. The prion form of Ure2p, is referred to as [URE3]. There are two regions on Ure2p that allow binding of Ure2p to itself. The first is contained in the primary 65 residues, and was the U65 domain used in this project. The U65 constructs were designed by Donald Tipper PhD, and contain those first 65 residues, followed by a nine amino acid linker sequence, and then the antigenic domain(s) of interest. As shown in Figure-6, U65 can create a large molecule if many of the proteins aggregate, especially into polymeric amyloid fibrils. U65 also expresses any domain attached on the C-terminus end of the protein outside of the aggregate, decorating it with the desired domain, assuming the protein was stable after translation.

Multiple U65 constructs were studied during the development of this avian influenza vaccine, and are named U65-GFP, U65-mHAt, and U65-mHAt-GFP. The mHAt domain is the H5 antigenic sequence for H5N1, and thus is the sequence necessary to elicit proper immune system response to avian influenza. Green Fluorescent Protein (GFP) is a very stable protein that folds easily and can be expressed in many different organisms. It is commonly used to track a protein of interest visually since it can usually be detected either by eye or more accurately by fluorescence microscopy.

Delivery and Immune Response to an Oral Vaccine

An oral vaccine must successfully deliver an antigen to lymphatic tissue without protease or other degradation of the antigen occurring. Donald Tipper PhD and Gary Ostroff PhD have successfully shown in mice that an antigen loaded in a glucan particle can be orally administered and achieve the appropriate immune response thereafter (Tipper, unpublished 2009). The yeast

β-glucan shell cannot easily be digested by gastrointestinal enzymes. After successful loading of a payload, the β-glucan has been shown to adequately protect its cargo until it reaches the targeted lymphatic tissue (**Figure-7**). It is hypothesized that phagocytosis by a macrophage occurs at this point (diagram upper right), degrading the β-glucan particle and subsequently presenting the antigen on its surface and initiating the desired immune response. At this point the β-glucan has stimulated the release of lymphokines (diagram right side), causing inflammation and a boost to antibody production to the presented antigen.

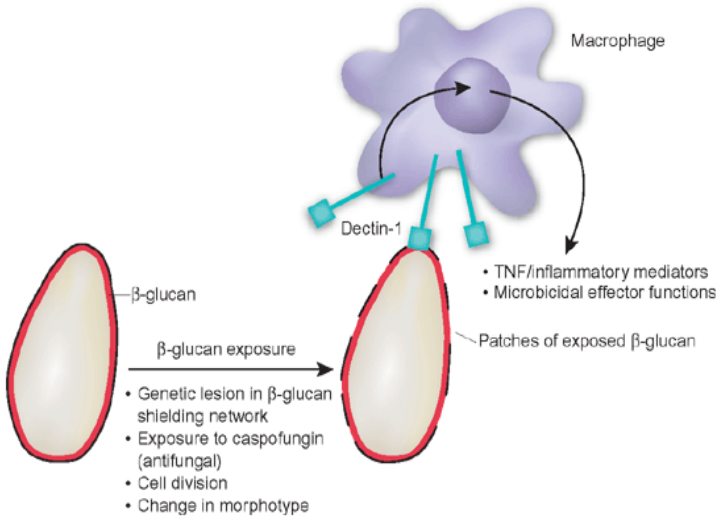

Figure 7: Example of β-Glucan's Interaction with Macrophages and Their Effect in the Immune System. *(Hohl and Pamer, 2006).*

The route of vaccine administration is the path the vaccine takes to come into contact with the body's immune system. There are two main routes that vaccines are usually distributed, intravenously and mucosal, and each has a major effect on how the drug is taken up by the body and distributed. Intravenous injections of drugs have many advantages including that they are fast, effective, and one injection is usually good for months. The disadvantages to injections are

they require sterile equipment, patients should not inject themselves, and intravenous injections are more dangerous because they bypass natural defenses.

Mucosal (oral) administered vaccinations do not come with any of the negative side effects that intravenous injections do. Oral vaccinations are taken up by the gut and interact with macrophages. Unlike intravenous vaccines, oral vaccines are usually taken in multiple doses for a few days in order to achieve maximal effect. Additionally, oral vaccines do not require a specialist to administer the injection, nor does it require a high degree of sterility that if compromised could cause infection and even death in serious cases. With intravenous injections, inflammation of a vein, known as phlebitis, is possible and can allow bacteria to enter can cause infection. However, if an oral vaccine is taken, the skin is never punctured, allowing no chance of infection. "With the exception of critically ill patients and those unable to absorb oral drugs, clinicians should consider oral therapy before resorting too quickly to *i.v.* antimicrobial agents" (Cunha, 2009). Additionally the β-glucan particles used in this project act as adjuvants, in addition to carrying the antigen to macrophage cells. The benefits of oral vaccines include being safer, cheaper, and easier to administer than current vaccination methods.

Testing has been done concerning the way β-glucan particles interact with the body, specifically dendritic cells. Dendritic cells form in the immune system of mammals and serve as antigen processing cells. The interaction of dendritic cells with β-glucan is an important interaction to understand. It was found that, "Dectin-1, the major receptor for β-glucan, is a C-type lectin that is highly expressed on dendritic cells (DCs). Its expression can also be detected in macrophages, monocytes, and neutrophils. Although the exact signal transduction pathways have not been elucidated, these events can lead to activation of nuclear factor of activated T cells (NFAT), nitrogen-activated protein kinases, and nuclear factor kappa B (NF-κB), leading to

cytokine production" (Ostroff et al., 2009). Therefore, β-glucan particles loaded with an antigen of interest will cause a boost in the immune system via activation of T cells, which cause cytokine production.

PROJECT PURPOSE

One of the largest possible pandemic threats to our society is avian influenza, as discussed in the Background. Gary Ostroff PhD previously used β-glucan particles in an oral vaccine for anthrax and found "these results demonstrate the potential for β-1,3-glucan immune modulators to provide a significant degree of protection against anthrax, a potential biological warfare (BW) agent in a mouse model of anthrax infection" (Kournikakis et al., 2003). Since previous experiments demonstrated great success with β-glucan, it was hypothesized that this approach could be used to create a cheap and efficient oral vaccine against avian influenza if the proper antigen were attached.

The purpose of this project was to create a viable, oral avian influenza vaccine by encapsulating a known H5 antigen sequence, specifically the mature hemagglutinin in the specific H5N1 variant, into glucan particles. Reaching that goal required that we determine ideal growth conditions, extraction and isolation processes, and glucan particle trapping conditions for proteins derived from yeast transformants expressing different proteins of interest. These trapped proteins would then be inspected within their glucan shells using microscopy and SDS gel analysis to determine whether the protein was packaged within the shell. If successful, the goal would then be to analyze the oral vaccine efficacy in producing an immune response in mice given gastrointestinally or subcutaneously.

MATERIALS AND METHODS

MATERIALS

Buffer A (modified)
50 mM Tris-HCl pH 7.6
150 mM NaCl
10 mM NaN3
10 mM KF
2.5 mM EDTA

BufferA1= buffer A + 1X protease inhibitors

100X protease inhibitors

100 mM PMSF. 17.4 mg/ml in 100% ETOH.
100X Pepstatin A 0.2 mg/ml in same solution,
Store @ -20 ^{o}C

XP Buffer: 10 ml Buffer pH 9.5 + 20 µl NBT stock and 20 µl BCIP stock
pH 9.5 Buffer: 12g Tris base, 0.8 g Tris/HCl, 5.8g NaCl, and 5 ml of 1M MgCl2
Coomassie IPA (IPA). Per liter, 500 mg Coomassie R250, 250 ml IPA, 70 ml glacial acetic acid

Stain 20-60 min. Destain same without dye.
YPG: 20g yeast extract, 40g peptone, and 54 ml 75% glycerol per two liters.
YEPD made of 5.1 grams yeast nitrogen base, 15 grams of ammonium sulfate, 6 grams of Leu/Trp D/O powder, 18 grams of sucrose, 81 ml of 75% glycerol, and 3 ml of 1000x vitamins (1x) per three liters of media.

METHODS

Media and Growing Conditions

Several different yeast media compositions were used throughout these experiments for growing yeast transformants. Selection for plasmid maintenance at normal copy number in pG4 transformants of strain PAP1502 required growth at 30°C in Ura drop out (D/O) media, which is made of 1.7g yeast nitrogen base, 5 grams of ammonium sulfate, 2 grams of Ura D/O powder, and 20 grams of glucose per liter. Transformants are re-grown in non-selective YEPD medium for storage at 4°C, since cells grown only in D/O media die rapidly when stored.

The YEPD-grown cells were later used to inoculate 2.5 liter Fernbach flasks containing 450 mL Leu/Trp drop out media, which is made of 5.1 grams yeast nitrogen base, 15 grams of ammonium sulfate, 6 grams of Leu/Trp D/O powder, 18 grams of sucrose, 81 ml of 75%

glycerol, and 3 ml of 1000x vitamins (1x) per three liters of media. All of the media used above were filter sterilized before use or storage. Growth in Leu/Trp drop out media requires a much higher plasmid copy number, and the shift to the glycerol carbon source relieves all catabolite repression of Gal promoter (**See Figure-8**) expression so that after 24 to 30 hours, when a spectrophotometric absorbance reading at 600nm should be approximately 5.0, expression is induced - the cells should be removed from the shaker, spun down at 3,000 RPM, and have YPG added to half the original Leu/Trp volume. After being suspended in YPG at room temperature, the cells were shaken in a two-liter flask without foaming for 10 minutes. Then 10% of the total volume of 20% galactose was added to induce expression, and the cultures were grown for an additional 18-24 hours.

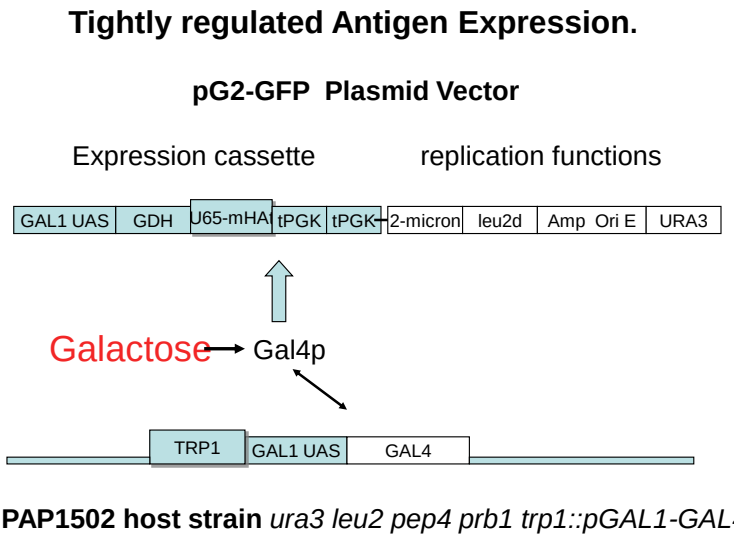

Figure 8: Expression Cassette and Regulation of Induction.

Harvesting After Inducing and Breaking Procedure to obtain 16kg Sup and Pel

After approximately 24 hours of inducing the cells, the cultures were removed and transferred to a plastic J10 rotor bottle. A spectrophotometric absorbance reading at 600nm was taken of all samples and they were spun at 4,000 RPM for 5 minutes. If the samples contained GFP, a quantitative assay was carried out before centrifugation. The supernatant was then removed, and the pellet was suspended in less than 20 mL of 0.15 M NaCl. The sample was then spun at 4,000 RPM for another 5 minutes, and then the pellet was suspended in approximately 10 mL of buffer A1.

In order to break the cells, the samples were filled with glass beads to half of their total volume. The samples were cooled on ice for 90 seconds, and then vortexed on the maximum setting for 90 seconds. This process was repeated approximately 8 times, and the samples were viewed under a microscope to check for breakage. If the cells were not entirely broken, continue cooling and vortexing.

Next the bottom of the plastic containers holding the samples were punctured multiple times, then placed within identical tubes bonded together. This construct was centrifuged at less than 500 RPM until the entire sample was passed to the punctured tube to the lower tube, leaving the glass beads behind. The glass free, broken sample was spun for an hour to separate out the 16kg supernatant and pellets, both of which were stored for SDS-PAGE gel analysis.

SDS-Page Gels

The protein samples were run on a 12.5% bis-acrylamide denaturing gel after being heated in the presence of sodium dodecyl sulfate (SDS) so that proteins would not fold and affect gel migration. Samples were consistently made up of approximately 15X concentration.

Additionally β-mercaptoethanol (BME) was added at a 1% concentration to the samples after incubation in order to disrupt disulfide bonds and ensure proper mobility through the gel. All gels were run at 100 volts, and included a protein ladder for size determinations, and were stained for 20 minutes using Coomassie blue, then destained.

Semi Dry Electroblotting to Immobilon

Three pieces of 3MM paper and one sheet of immobilon membrane were cut to the size of the gel to be electroblotted. A corner of the immobilon membrane was cut in order to recognize Lane 1. Two pieces of 3MM paper were soaked in transfer buffer and then placed on the bottom electrode. The membrane was then soaked initially in methanol (MeOH) and then in transfer buffer, and placed on top of the first two pieces of 3MM paper. The SDS-PAGE gel was placed on top of the membrane after a wash with transfer buffer, and then the third piece of 3MM paper was added to cover the gel. A glass rod was gently rolled back and forth over the final piece of 3MM paper to remove any air bubbles, and then the electroblot was run at a constant current at 0.2 A for 4 minigels (0.15 if only 1-2 gels) for one hour. Once complete, the membrane was removed and stored in a plastic dish, and shaken in 5% fat-free dried milk in 1X TTBS for one hour. The membrane should now have all protein that was in the gel; a quick indicator is being able to see the pre-stained ladder.

Western Blot Procedure

5% fat-free dried milk in 1X TTBS was poured off of the membrane, and the primary antibody of interest was used at approximately 1:5,000 dilution overnight at 4°C on an oscillating table. The next day the primary antibody was recovered and stored for future use, and

the membrane was washed in 1X TBST and placed on a oscillating table a series of times first for 1 minute, then 5 minutes, and finally 10 minutes, using new TBST in each wash. The membrane was then probed using 1:5,000 Goat anti-mouse-AP for two hours at room temperature. The 1, 5, and 10-minute TBST washes were repeated, and the membrane was washed in pH 9.5 buffer for ten minutes. The membrane was then developed in XP at room temperature. Color development was stopped at any time by washing with water. The duration of time before color development ceases was recorded and a picture was taken of the resulting blot.

Ultracentrifugation for VLP Isolation

This technique was performed by Lori in the Morrison lab. After selective solubilization of U65-mHAt into a minimal volume, the sample was loaded onto a sucrose gradient. A sucrose gradient contained in dialysis tubing was constructed to have 3 concentration differentials of 30%, 60% and 80% and volumes of 1ml, 2ml, and 4ml, respectively. Dialysis tubes were used with an 11 ml capacity, so the maximum amount of loadable sample per centrifugation tube is 4 ml. The tube was spun in an ultracentrifuge at 35,000 RPM for approximately 18 hours at 4°C, then the fractions were recovered separately in 1mL tubes. U65-mHAt was consistently in the bottom four fractions after ultracentrifugation.

Sizing Column

The column was 90 ml of Sephadex and was run in 0.1M Methylamine buffer + 0.1% Sarkosyl. We collected 25 samples of 3 ml each after letting the first 10 ml through. We had previously determined that nothing eluted in that first 10 ml.

β-Glucan Trapping

β-Glucan particles with charged cores (yeast cell walls stripped of all proteins, leaving solely the shell) were added in solution with our protein of interest. A trapping polymer was then added with the intent of our protein being suspended between the core and trapping polymer. See Appendix for details.

RESULTS

Protein Constructs

The proteins referred to in this project are novel constructs created by Donald Tipper PhD using avian influenza H5 HA and GAG genes, provided by Antigen Express. All proteins were expressed in *Saccharomyces cerevisiae* (**Tables I and II**). Although our MQP team aided in making several new constructs, the ones referred to in this paper were previously transfected yeast strains. U65-mHAt was the antigenic protein chosen for the avian influenza vaccine. Other proteins of interest were also cultured simultaneously for the purposes of either visualization by GFP, or the development of a different vaccine.

Table I: Potential Vaccine Proteins

U65-mHAt	Novel protein consisting of the first 65 residues of the Ure2p gene followed by a 9 amino acid linker and finally by the mature hemagglutinin sequence from H5N1, provided by Antigen Express, with the final transmembrane domain not present
HBs	This is Hepatitis B Surface Antigen would make a potential oral Hepatitis B vaccine and would allow antibody comparisons to be made to the injectable form
GAG-HBs	This novel construct consists of GAG sequence provided by Antigen Express fused to HBsAg, GAG is the second closest protein to the exterior of HIV and is not nearly as mutable as the membrane protein, which is closest to the outside – would make a potential oral HIV vaccine as well as Hepatitis B vaccine
VP1	The Mouse Polyoma Virus capsid protein, cloned from intact viral DNA by DJT makes Virus Like Particles similar to HBsAg – would make a potential Mouse Polyoma Virus vaccine

Table II: Proteins Expressing GFP

U2N-GFP/U74-GFP/U65-GFP	Novel protein containing the first 65 residues of the Ure2p gene followed by a 9 amino acid linker sequence followed by GFP – used to track U65 constructs visually to evaluate expression and glucan particle loading
U65-mHAt-GFP	Same as previous but with the mHAt sequence between the linker and the GFP – used same as previous but could elicit an antibody response to HA if used as a vaccine
GFP-HBs	Hepatitis B Surface Antigen with GFP on the N terminus end – used same as previous but was expected to behave more like HBs
VP1-GFP6	VP1 with GFP on the C terminus end – same as previous but for VP1

Protein Purification

16,000x g Centrifugations

The first step in producing this vaccine was purifying the proteins of interest. For every protein, the first enrichment step was a 16,000 x g centrifugal spin after the grown yeast cells were harvested and broken to separate cell wall debris and membrane proteins from soluble proteins. This centrifugation step pellets the U65-mHAt proteins, leaving the smaller soluble proteins in the supernatant, and allows the pellet to be resuspended in a different buffer. Almost all of the proteins mentioned in Tables I and II were found in the 16,000 x g pellet. However, GAG-HBs remained in the supernatant. **Figure-9** shows some supernatants and pellets collected after the 16kg centrifugation as analyzed on an SDS-PAGE gel. In the figure, a solid circle surrounds the U65-mHAt band (at 66.2 kDa) found in the pellet fraction, and the dotted circle surrounds the same band (far less abundant) in the supernatant. Even though most of the proteins ended up in the pellet with the protein of interest, the supernatant not only gets rid of the soluble protein, but also allows for selective solublization of the target. There is a band at approximately 66.2 kDa in protein that remained suspended in the supernatant. As this band appears to be

slightly shifted up from the comparable band in the pellet fraction, it remains unclear as to whether this is the protein of interest.

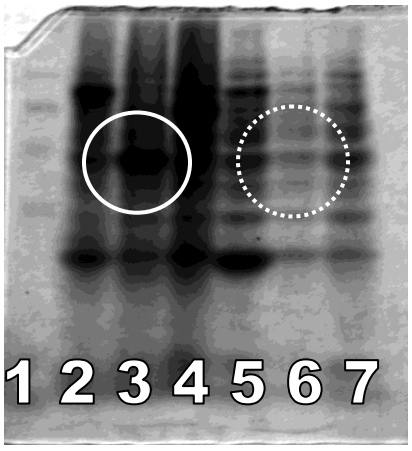

Figure 9: SDS PAGE Analysis of Supernatants and Pellets from 16kg Centrifugation Step. *The lanes are as follows: (from left to right) Ladder, GAG-HBs 16kg pellet, U65-mHAt 16kg pellet, GFP-HBs 16kg pellet, GAG-HBs 16kg supernatant, U65-mHAt 16kg supernatant, GFP-HBs 16kg supernatant. The solid circle indicates U65-mHAt in the 16kg pellets, and the dotted circle indicates U65-mHAt in the 16kg supernatants.*

Selective Solubilization

Through numerous centrifugation trials, U65-mHAt and GFP-HBs were both found initially in the 16,000 x g pellet, separating them from all soluble yeast proteins. Both pellets were soluble in a solution of 0.1M Methylamine buffer at pH 10.7 with 0.1% Sarkosyl, when incubated at 37°C for 30 minutes. This was verified by SDS-page gel for U65-mHAt and by GFP assay for GFP-HBs (data not shown).

Ultracentrifugation on a Discontinuous Sucrose Gradient

The solubilized proteins were run in the SW41 rotor on the ultracentrifuge at 35,000 x g (35kg) for 18-24 hours, using a step gradient from 0 to 80% sucrose in MN buffer. This process separates proteins by density, with the antigenic proteins expected to run between the 60% and 80% sucrose concentrations. The 0.1% sarkosyl buffer solubilizes an unidentified protein

polymer that forms a gel like polymer layer after 18hr centrifugation in sucrose. These polymers, which are believed to be mannoproteins, were not readily suspended, and became a problem when more than a minimal amount was present. The polymer formed throughout the region with the protein of interest, retaining it inside the gel, significantly decreasing the yield of U65-mHAt. Fortunately, this polymer was largely eliminated by repeated 16 kg centrifugation before use of sucrose gradients. The separation of U65-mHAt is shown in **Figure 10**. Lanes 9, 11, and 13, contained the antigenic protein in high levels showing how this step provided strong U65-mHAt enrichment. Lane 15 is not shown, but it was subsequently reanalyzed and was verified to contain U65-mHAt. Since the lanes were relatively similar, they were pooled, and used for the next step, thus allowing little to no loss of the antigenic protein.

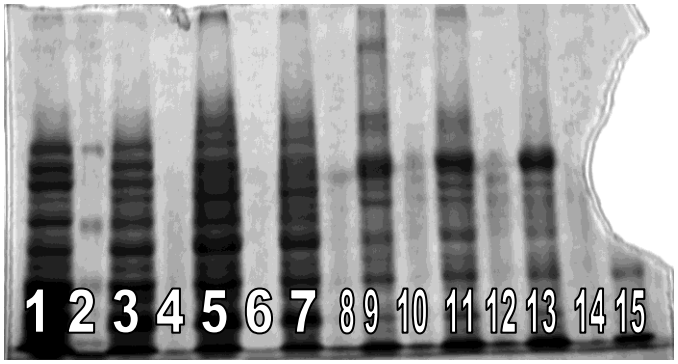

Figure10: SW41 Fractions of mHAt*. The sample on the left was the top fraction and the one ripped off on the right was the bottom one, with the intermediate ones in proper order from top to bottom. The ripped sample that was the bottom of the gradient looked similar to the fractions beside it, but with less overall protein. The four bottom most fractions were pooled for the next purification step.*

Acid Precipitation of U65-mHAt from a SW41 Gradient Spin

Proteins from pooled sucrose gradient fractions in MN buffer were quantitatively precipitated by the addition of acetic acid, reducing the pH to about 5 (**Figure-11**). The total protein of the starting material is represented in lane 2. Even though this step provides limited enrichment, the ability to suspend the precipitate in a minimal volume is essential for the next enrichment step.

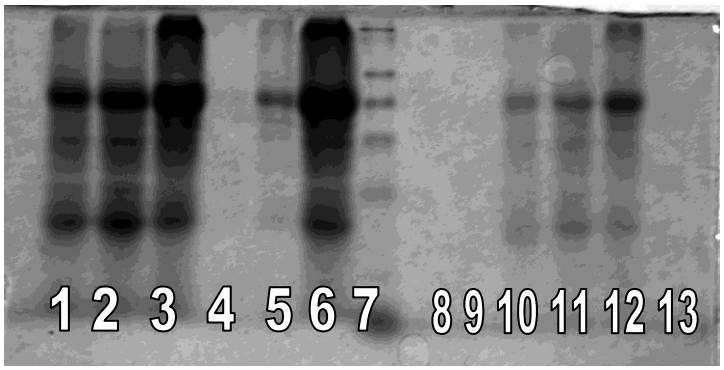

Figure 11: Acid Precipitation of the Sucrose Gradient Proteins*. Lane 5 shows the precipitated fraction used for the next step.*

Isolation of U65-mHAt Samples Using a Size Exclusion Column

After isolation of the sucrose gradient fraction-5 protein, it was loaded onto a size exclusion column. The fractions were acid precipitated to reduce the volume and allow a buffer change, then analyzed by PAGE (**Figure-12**). This column allowed almost complete separation of U-65-mHAt from any impurities. Note that the large molecular weight material at the top of the column is what we later found to be an aggregate of some sort that either disaggregated before loading or failed to load properly in the next step. Access to a longer column would be ideal since an increase in resolution would completely get rid of the small impurity we saw at this point.

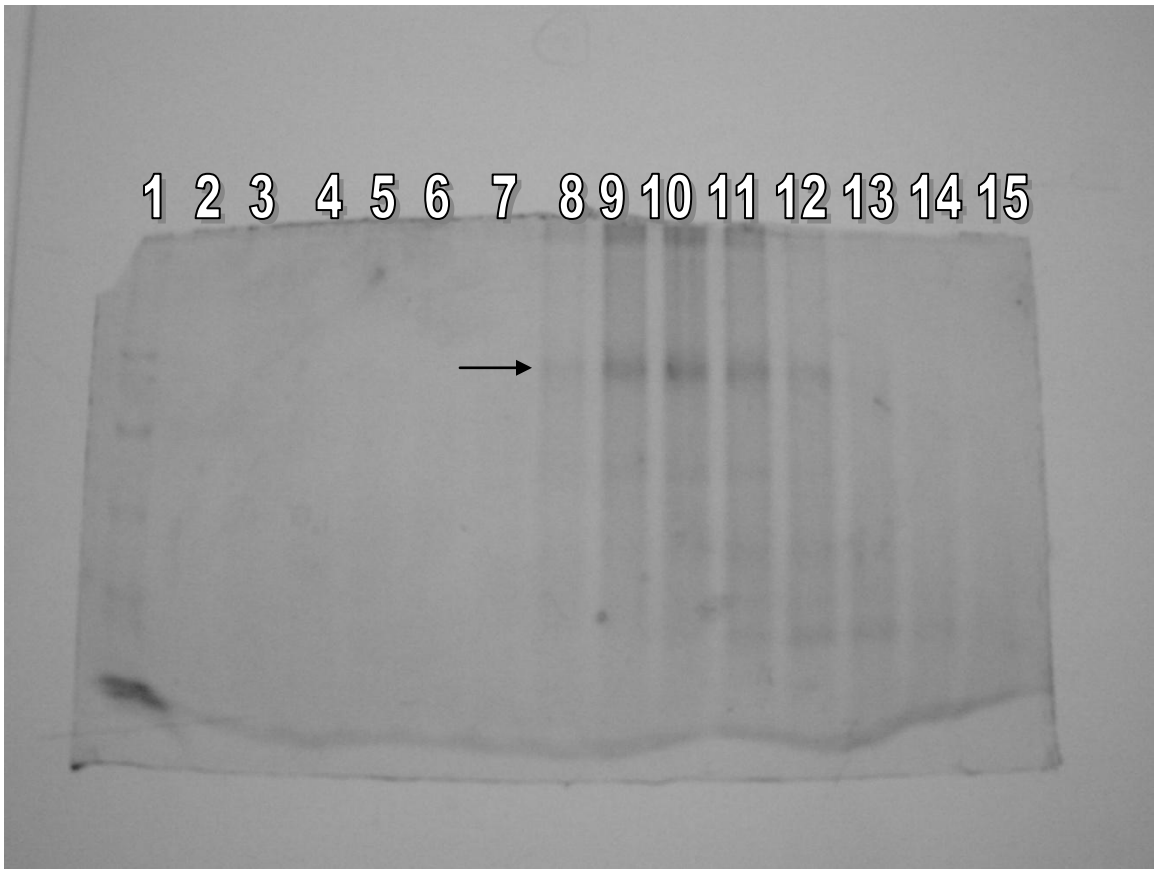

Figure 12: PAGE Analysis of the Column Fractions*. Proteins were separated on a size exclusion column, the fractions were precipitated, the analyzed by PAGE. Lane 1 - Ladder, Lanes 8-12 contain the U-65-mHAt (see arrow)*

Trapping U-65-mHAt in Glucan Particles

Using 8 potential trapping polymers, glucan particles were loaded with two purified proteins, U-65-mHAt and GFP-HBs. Using a variety of trapping polymers is necessary since some are bound to trap more efficiently than others for different proteins. The loading of the glucan particles was performed separately for U-65-mHAt and GFP-HBs. First, trapping of GFP-HBs in glucan particles was attempted since it would allow GFP visual confirmation and analysis of loading. U65-mHAt was the other protein of interest for use as an oral vaccine. Subsequently, FITC-labeled U65-mHAt also was used for trapping in glucan particles.

Verifying and Characterizing Loaded Glucan Particles

Finally, the loaded glucan particles were lysed and analyzed by PAGE to verify correct loading. **Figure-13** shows an SDS page gel of four loaded glucan particles next to an overloaded control lane. The right lane shows a ladder and an arrow at 66.2 kDa indicating where U65-mHAt should migrate. The protein migrated to the correct size, indicating no degradation had occurred in the packaging. The lack of other proteins shows efficient isolation, and the relatively similar band intensity of the four shows similar trapping success for the four different polymers used. These particular U65-mHAt samples were not labeled with FITC, so microscopy of the loaded particle is not a viable option for verification of loading.

The loaded GFP-HBs however is clearly seen under 1000x magnification in **Figure-14**. Using a blue filter to easily see the GFP and phase contrast to see the glucan particle, the image of a loaded glucan particle shows properly loaded glucan particle. **Figure-15** shows multiple forms of loading. Some are ideal, and some were not properly loaded.

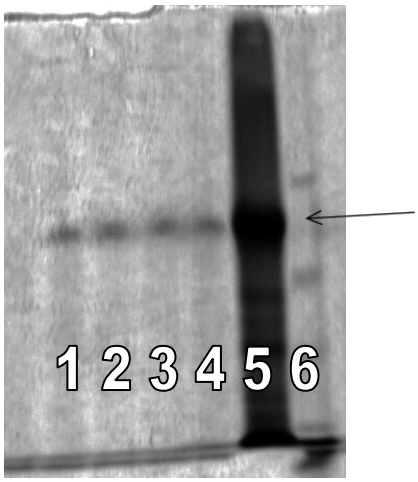

Figure 13: PAGE Analysis of U65-mHAt From Loaded Glucan Particles*. Lanes 1-4 are loaded glucan particles ran to analyze loaded protein content. Lane 5 is overloaded with our sample with U65-mHAt as a positive control and lane 6 is a ladder.*

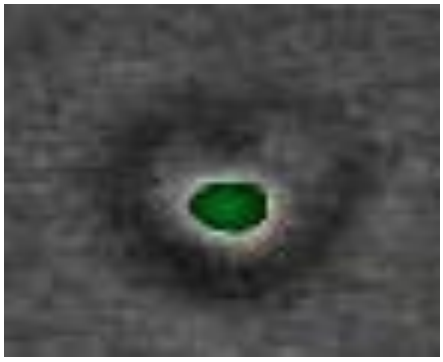

Figure 14: GFP Microscopy of a Properly Loaded Glucan Particle. *Note, the green is in the middle. The black outline around it (the heart shaped one) is the glucan particle*

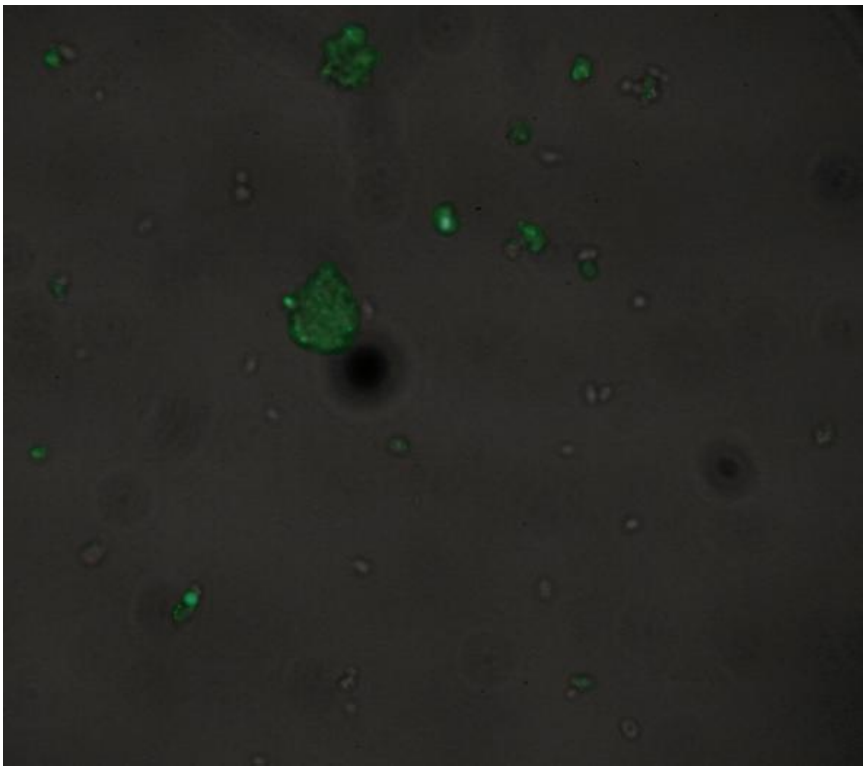

Figure 15: GFP Microscopy of a Mixture of Properly and Improperly Loaded Glucan Particles.

DISCUSSION

The rapid rate of influenza viral RNA mutation, lower than ideal levels of antibody production in patients, high mortality rates, and short time between infection and death, all indicate that effective protection against an avian influenza pandemic is imperative. Creating a potent vaccine against influenza is not only a convoluted, difficult task by itself, but the rapid rate of mutation of the virus makes complete vaccination against all forms of influenza even more improbable. U65-mHAt loaded in glucan particles delivered orally will theoretically provide excellent protection against the H5N1 variant of avian influenza. A dehydrated oral vaccine would also allow for cheap and easy storage, transport, and distribution.

We ran into many unforeseen obstacles on the path to developing this vaccine. We started this project in September of 2008. Our first goal was to get proficient at producing large amounts of target proteins and getting those proteins into solution. Quick proficiency at properly growing and inducing cells was obtained and very few cultures were lost to contamination. Working with anywhere from three to seven different proteins at a time, we attempted to successfully affect solubility of target proteins by altering urea concentrations, presence of different detergents in varying amounts, buffers, temperature, protein concentration, salt concentration, and pH. The goal was to manipulate the proteins of interest's solubility easily, to allow a subsequent 16,000 x g centrifugation (or a series of them) until the protein of interest was pelleted. It was not until February 2009 that we successfully made significant enrichment on our main proteins of interest (U65-mHAt and GFP-HBs). Another complication we ran into right around February was the discovery that the HBsAg in our yeast strain expressing HBsAg was not actually present. We spent many hours attempting to isolate HBsAg and verify its presence by Westerns. Due to the

use of old antibodies that were not giving consistent results, we were unable to determine the lack of HBsAg presence until we ran two GAG-HBs samples concurrently with HBsAg samples. We then saw clear HBsAg presence in the GAG-HBs samples and none in any of the supposed HBsAg samples. We had, up until that point, spent a significant fraction of our time specifically on HBsAg isolation. We also did not have an avian H5 HA antibody, so HA confirmation by Western blots of HA was not available.

The original concept for trapping the proteins of interest in glucan particles was to have them assemble inside the glucan particles into complexes too large to diffuse out. For all of the HBsAg and VP1 containing vaccines, attempted assembly into large virus-like particles after diffusion into glucan particles was the concept for trapping without using trapping polymers. Similarly, U65's ability to self assemble was hypothesized (as with the virus-like particles) to catalyze into the larger aggregates under specific conditions. The original concept of the project was based on the previous successes. Unfortunately control of assembly was not found so we went with the backup plan of trapping polymers. Fortunately, the polymers worked fine for trapping and did not end up being a huge problem.

There are many future experiments to this project that should be explored. One is the obvious continuation of the project to test whether the U65-mHAt loaded glucan particles when fed to mice create an antibody response to mHAt. Experiments on the ability of U65 to polymerize, and HBsAg and VP1 to assemble into virus-like particles, would be interesting and perhaps a necessary next step if loading with trapping polymers does not provide the desired antibody response for any reason. Finally, the loading of glucan particles with other antigens for vaccine production is the next logical step. While swine influenza (H1N1) seems like an obvious candidate for consideration, we have already started working on a couple variants of GAG, as an

antigen for HIV, which are to be loaded in glucan particles and eventually tested for antibody response. Using this method for producing oral vaccines could potentially be very valuable to our society and will hopefully find all the funding it needs to be explored in the future.

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APPENDIX A

BUFFERS

		pKa (25º)	Other	
FORMIC ACID-NA FORMATE		3.7		
LACTIC ACID-NA LACTATE		3.86		
ACETIC ACID-NA ACETATE		4.68		
NaH_2 CITRATE-Na_2H CITRATE		4.68	3.12	6.4
SUCCINIC ACID-NA SUCCINATE		5.4	4.2	
KH_2PO_4-Na_2HPO_4	Na_3PO_4	6.86		12.0
TRIS-HCL		8.1 at 25 C		
BORIC ACID-NA BORATE		9.18		
PIPES		6.8		

Grams per 400 ml 1M	Tris base	TrisHCl
pH 7.5 at 25 C (7.2 at 37)	9.44	50.8
pH 8.0 at 25 C (7.7 at 37)	21.2	35.5

pH STANDARDS

	0º	**25º**	**37º**
0.02M KH_2 CITRATE	3.93	3.84	3.82
0.05M ACETIC/ 0.05M NA ACETATE	4.71	4.68	4.69
25mM NaH Succinate/ 25mM Na_2 Succinate	5.46	5.40	5.41
50mM KH_2 PO_4 / 50mM Na_2H PO_4	6.98	6.86	6.84
10 mM Na BORATE (CO2-FREE H2O)	9.46	9.18	9.07
50 mM Na_3 PO_4	12.75	12.04	11.74

WORKING SOLUTIONS

0.5 M EDTA pH 8.0 94 g Na_2 EDTA.2 H_20 + ~ 10 g NaOH pellets, stir in 400 ml water, adjust to pH 8.0, dilute to 500 ml

Ethidium bromide. Dissolve in water at 2 mg/ml and store in the dark, use 1-2µl /10 ml of agarose. Use gloves.

Sodium chloride, 4M Dissolve 117g in 400 ml water, dilute to 500 ml
Sodium hydroxide, 4M Dissolve 32g in 150 ml water, dilute to 200 ml
50X TAE for Agarose gels 121 g Tris base, 28.5 ml glacial acetic, 50 ml 0.5 M EDTA to 500 ml

Buffer A (modified)
50 mM Tris-HCl pH 7.6
150 mM NaCl
10 mM NaN3
10 mM KF
2.5 mM EDTA

BufferA1= buffer A + 1X protease inhibitors
100X protease inhibitors
100 mM PMSF. 17.4 mg/ml in 100% ETOH.
100X Pepstatin A 0.2 mg/ml in same solution,
Store @ -20 ^{o}C

Coomassie IPA (IPA). Per liter, 500 mg Coomassie R250, 250 ml IPA, 70 ml glacial acetic acid. Stain 20-60 min. Destain same without dye.

Media

LB per liter: 10g tryptone
5g yeast extract
10g NaCl

Ampicillin, 100 mg/ml, store at -20°C

YEPD per liter: 10g yeast extract
20g peptone
20g glucose

YM1 Mix equal volumes of
2X YEPD and
2X YNB

YPG per 2 liters: 20g yeast extract
40g peptone
54ml 75% glycerol
8 ml Pen/Strep mix, as used for TC (to reduce bacterial contamination)
NB, it is more important to seal the 2L flasks used for 36-48 hr Gal induction with both plugs and foil to exclude dust from the shaker

Ura D/O per liter Filter-sterilize
1.7g YNB (no AS or amino acids)
5g AS (ammonium sulfate)
2g Ura D/O powder
20g glucose
For plates Autoclave above + 20g Agar in 900 ml water, then add 100 ml 20% glucose

Leu/Trp D/O Suc/Gly as used routinely since 10/06. Filter-sterilize
Increased sucrose (9g/liter) improves cell yield.

Per 3 Liters
5.1g YNB (no AS or amino acids)
15g AS (ammonium sulfate)

6g	Leu-Trp D/O powder
18g	sucrose (0.6%)
81ml	75% glycerol (2 %)
3 ml	1000x vitamins (1x). Frozen stock
(12 ml	Pen/Strep mix . Frozen stock- NOT essential)

GFP assay; Gal-induced expression of GUL2-U2N-GFP -UNL-GFP, etc.

Normal growth and induction procedure (at 30C):

Inocula. Grow pGUL transformants of strain PAP1502 in Ura/ D/O 2% glucose to saturation, then re-grow in YEPD. Store at 4C, can pour off 50% of settled sup to give 2X cells.

Use 1-2 ml 2x or 2-4 ml 1x YEPD-grown cells to inoculate 450 ml Leu/Trp D/O with 0.9% sucrose and 2% glycerol (+/- additional vitamin supplement) in fluted Fernbachs, 200 rpm. Small scale: 25-50μl inoculum in 5-10 ml Leu/Trp D/O in max tilted 25 ml tubes.

After 24-30 hr, A600 should reach about 0.5-6, indicating significant growth on glycerol, resulting in de-repression of the GAL pathway. Harvest cells (450 ml fills a J10 bottle, 10 ml in a 12 ml culture tube), suspend in 50% original volume of RT YPG, pour into 2L flask and grow with lower aeration (125 rpm; AVOID ANY FOAMING) for 10-20 min and then add 10% volume of 20% galactose. Harvest induced cells after 18-24 hr.

1. Measure A600 of the 2X culture (eg, if use 1/40 dilution, multiply result by 40).
2. Measure GFP fluorescence of n μl + 150 μl PBS, in duplicate, in microtiter plate reader, using 488 excite and 520 emission = F520

THEN, since A600 of 1 = 10^7 cells/ml

F520 per 10^6 cells = 100/n X F520/A600

For well-induced U2N-GFP, this should be 2500.
For UNL-GFP, about 1500
For VP1-GFP, about 1000
For GFP-HB, about 900
For U74-GAG-HBs about 100

Assume GFPbex has 6x fluorescence of GFP

Prep of Electroporation-Competent E. coli.

NB, it is convenient to do two cultures at once to balance centrifuge.

Day 1; PM Inoculate 20 ml LB in 125 ml flask with DH5α, or XL1Blue.
Autoclave 1 L LB in 2 liter flask (best, 2.7L Fernbach) and leave at 37 ON.
Autoclave 1 L water and 600 ml 10% glycerol and store at 4 ºC.
Make sure to have 6 sterile 450 ml bottles, 10 ml pipettes and 50 ml dispo tubes.

Day 2; AM

1. Inoc the 1.2 L LB in Fernbach with 2-10 ml ON culture. Shake till A600 is 0.4 to 0.6 (2-3 hr) then immediately cool in ice. Pour into 3 ice-cold 450 ml bottles and store on ice 15 min. Pre-cool J10 rotor to 4 ºC, spin bottles 10 min 7.5k.

2. Pour off sup, remove residual sup w pipette, suspend pellets in 5 ml ice-cold water using a 10 ml pipette.
Combine in one bottle, dilute to 450 ml total (35% original vol), mix.
Spin 10 min 8k at 4 ºC. CARE! Pellets are loose in water.

3. Repeat wash in 450 ml water.

4. Suspend each pellet in 10 ml ice cold 10% glycerol and combine in 50 ml tube. Dilute to 20 ml in ice cold 10% glycerol (2% orig vol), spin 10 min 10k. Pour off sups.

5. Suspend in 10ml (1% orig vol) ice cold 10% glycerol and spin 10 min 10k.

6. Suspend in 2.5 ml (0.25% orig vol) ice cold 10% glycerol. Aliquot 50 X 50 µl in Epps, freeze in dry ice and store at -70 ºC.

ELECTROPORATION of *E. coli*

Ligations; Dialyse 15-30 min on 0.05-0.2 micron filters floating on water to remove salts.

NEED; sterile 1 mm cuvettes. SOC or LB or TB (50 ml tube). Sterile 18 mm tubes. Amp plates.

1. Remove frozen EP competent cells from -75 and melt in ice (20 min).

2. To 50 µl cells in Epp tube in ice, add 1- 5 µl of DNA in water or 1: 1 TE.

Transfer to sterile 1 mm cuvettes in ice. Include a No DNA control.

3. Pulse in Goguen EP apparatus: 2.2 Kv; depress to charge (1 sec), release to pulse. OR use commercial apparatus at 1.25 Kv, depress both buttons to pulse, time constant should be >4

4. Immediately add 0.6 ml RT ºC SOC (or LB) and transfer to suitable tube

5. Rotate at 37 ºC 60 min

7. Pour total on LB-Amp plate, sprea and incubate lid-up at 37 ON.

DAY2. set up miniprep cultures. DAY3. Prepare minipreps and analyze.

NB: Test EP cells with 1 ng control plasmid and plate 100 µl of 1 ml. 10^6/µg = 100 cfu's.

OR test colonies by PCR on day 2 (saves a day if probes good)

YEAST LiOAc TRANSFORMATION.

One Step procedure (best, simplest).

1. **One step buffer, 0.2 m LiOAc pH 5.0, 30% PEG 3350 100 mM DTT, kept at -20C.**
2. 1 ml of freshly grown ON cells, A600 about 5, wash in 1 ml water, suspend in 100 µl One step.

2A. OR grow cells as for older procedure to get fresh culture at A600 1-2, wash in water and suspend in 1/40 original volume of 0.1M LiOAc. Spin 0.1 ml, spin and suspend in 100 µl One step buffer.

3. Add 5 µl denatured 10 mg/ml HS DNA and 1-2 µl plasmid DNA

Incubate 42 C 30 min, spin 5 sec, remove sup (use fresh sterile tips) suspend in 200 µl water and plate. Use Ura D/O plates for GUL vectors. Incubate 2-3 days at 30C

OLDER Cells; should be growing healthily in early post-exponential phase at A600 of 1.5-2.5 in rich broth such as YEPD or YM1. Dilute a fresh ON in YM1 or YEPD 1/20 and grow ~4-5 hr. For transformants, etc requiring growth ON in D/O medium, dilute in YM1 for second growth.

1. Day 1: Fresh ON culture in 2.5 ml YEPD or DO inoc in late afternoon with 2 loops full (20µl) of refrigerated saturated broth culture (grown in YM1).

2. **On day2,** pre-warm YEPD 20-50 ml to 30 ºC in 250 ml flask in WB shaker.
   Inoc with 1/20 vol of ON shake t**o A600~ 1.5 to 2** (~ 8.30AM-12.30PM)
   Cells must be in exp phase when washed, Gietz recommends 10^7 cells/ml.
   Higher OD seems optimal for SEY6210, must be optimized for each strain.
3. **Harvest cells** in 50 ml dispo tubes**,** 5 min at 2700 RPM (top speed) in GLC2B, RT ºC.
4. **Pour off sup**, suspend (vortex) in original vol sterile water.
5. **Spin again**, suspend in 1 ml 100 mM LiOAc (not buffered), transfer to Epp tube.
6. **Spin 5 sec,** pour off, suspend by vortex in 1 ml LiOAc again.
7. **Spin 5 sec,** pour off, suspend by pipeting in 1/100 orig vol LiOAc.

eg, 50 ml cells, add 0.5 ml, final vol about 0.7 ml. **Store on ice. 15 min to 24 hr 0°C.**

Procedure.

1. **Epp tube + 50-120 µl cells**
   + 1/10 vol Herring sperm DNA (heated BWB 2 min, cooled in ice, or quick frozen)
   + 1-10 µl DNA in TE or water
   + **3.2 vol sterile 45% PEG** 4000 in 100 mM LiOAc (9 vol 50% PEG + 1 vol 1M LiOAc).
2. **Mix, incubate 30 min at 30 ºC on rotator, then 20 min 42 ºC**.

3. Spin 5 sec, remove all sup with 200 µl tip (PEG inhibits growth)

suspend in 1X TE or water, store in ice, plate at appropriate dilution.

STOCKS: filter sterilised.

1. **10X TE**, 100 mM Tris/HCl pH 7.5, 10 mM EDTA.
2. **10X LiOAc**, 1M, pH NOT adjusted. Making 1X fresh recommended, don' t know why.
3. **45% PEG. 9** vol 50% PEG, 1 vol 10X LiOAc. Filters very slowly!

Protein Methods.

Solutions for Western Blot, IP, EndoH, Protease K, Zymolyase etc.

AntiBodies. Store in 25 µl aliquots at –75^{o}C. Unfreeze an aliquot only once and then keep at 4 ^{o}C.

Primary AB's are diluted in 5% DM for Western detection and stored at4 ^{o}C. These can be used at least 10 times, although the DM tends to decay. To prevent microbial contamination, after first use, add Na Azide to 1 mM, but this must be completely washed out before secondary, since azide inhibits lumifos, etc

Examples:

Mouse monoclonal anti-HA (F-7) Santa Cruz biotech Cat # sc-7392 200 µg/ml, $275 per ml
Dilute this primary AB 1/2000-4000 (10-20 µl/40 ml) in 5% DM.

Mouse 3F4 anti-PrP ascites fluid (Lingappa,). Dilute 1/10,000 (4 µl/40 ml) in 5% DM.

Mouse anti-HBs clone NE3

Secondary AB. Santa Cruz Cat #Sc-2031 HR peroxidase-labeled goat anti-mouse $60 per 200 µg/0.5 ml
Use 2 µl / 15 ml 5% DM, do not re-use, cannot add azide.
OR Santa Cruz Cat #Sc-2066 AP (Alkaline phosphatase)-labeled goat anti-mouse. Can use azide.

100X Protease inhibitors (PI's)

To be added to solutions containing at least 1 mM EDTA. This inhibits metallo-proteases

100 mM PMSF. 17.4 mg/ml in 100% ETOH.
100X Pepstatin A 0.2 mg/ml in same solution, Store @ -20 ^{o}C.
100X TPCK an optional additional inhibitor; 7 mg/ml in 100% anhydrous ETOH
Keep separate, Store @ -20 ^{o}C

Buffer A (modified)
50 mM Tris-HCl pH 7.6
150 mM NaCl
10 mM NaN3
10 mM KF
2.5 mM EDTA
BufferA1= buffer A + 1X protease inhibitors
BufferA2= buffer A + 1X pepstatin (no PMSF). Use with protease K (see below)
Buffer A3= buffer A/4 = 1X PI's. use to disperse HSP's prior to adding EndoH buffer.

10 X TTBS: per liter

24.2 grams Tris base
Store 4 ^{o}C
292.2 grams NaCl
pH solution to 7.5 by adding ~ 14.3ml of conc HCl.
Add 5 ml Tween 20 (0.5% Final), and dH2O to 1L

5% DM (Non-Fat Dried Milk) in TTBS, Store 4 ^{o}C

Antisera; dilute in 5% DM in TTBS.

RIPA Buffer for Immunoprecipitation

50 mM Tris-HCl pH 8, (5 ml 1M/100 ml)
150 mM NaCl, (3.75 ml 4M/100 ml)
1 mM EDTA (0.2 ml 0.5M/100 ml)
1% NP40, 0.5% DOCholate, **0.1% SDS**

Protease K Stock: 1 mg/ml in 50 mM Tris
pH7.5, 20% glycerol, Store @ -20 ^{o}C.
Stability has not been tested.
is a potential problem, so keep only two weeks.
Also made w/o SDS and + PI's

Coomassie stain after SDS-PAGE.
Cut off stacking gel and transfer gel to small plastic tray or Tupperware box.

Coomassie IPA. 0.05% in 25% IPA, 7% acetic acid. Stain 20-60 min.
Per liter, 500 mg Coomassie R250, 250 ml IPA, 70 ml glacial acetic acid
Destain same w/o dye + kimwipes, ON. Can re-stain if desired

Older less useful:Coomassie stain (Maniatis) 0.25% in 50% methanol + 10% acetic acid. Hard to dissolve stain. Stain for not more than 20 min, or background is too high. Can only re-use a few times
De-stain in MeOH-acetic acid –water 30:7:63.-first one hour in used destain + kimwipes to adsorb stain
-then ON in fresh destain + kimwipes in sealed bag/box.

To store gels, rinse water then soak in 20% glycerol 1-18 hr and place between sheets of cellophane soaked in 20% glycerol and dry on clamped plastic frame ON.

Quantitation of stained bands after SDS-PAGE.

A. Stain and de-stain

B. To image on Kodak 200 Imager before glycerol step.

NEED: Gels and data sheet to identify by #

Tongue depressors, paper towels
Memory stick, Pen, White sheet

Insert memory stick in USB drive (front bottom right). Start → My computer → Drive Icon, Choose show files → See new window of files on the stick. Can now drag image **.tif** files to this from DJT folder.
Using tongue depressors, transfer de-stained gel to clear sheet-holder containing letter-quality white paper.
Epi-White. Center, using preview to observe image. Set diaphragm (top ring) to max. Exposure 0.1 sec.
Adjust exposure time to just over-expose, then use diaphragm to get band contrast best emulating the gel.
Adjust magnification to get largest image.
Capture. Select **Crop** tool, remove unwanted areas (Edit →crop). **Print 2 copies** (contrast is > than image)

C. File → export data→ image
Name the file (e. g., 4-10-07 gel #). **File type**, choose **8 bit ….min/max .tif** (default).
Save in DJT folder. Drag Gel Image .tif files to memory stick window.
Open dragged icon to check transfer. Close gel images (Save changes? NO).
Repeat with other gels. Eject flash drive by proper procedure (2nd icon bottom right).

D. Quantitation on own computer

Open Photoshop CS version 9

1.Open the image file. Should be grey scale, 8 bit, 256 levels

2. Adjust brightness/contrast so that background is white and bands have a relative density similar to the original gel. Save.

3. Window→ Histogram → Expanded view → show statistics

4. Select lasso tool. Drag around a white area to establish maximal mean, should be 255. Drag around a band. Observe Histogram. Record Band mean and pixels.

5. Drag the lasso to the same area of an adjacent "blank" lane (same protein loading but lacking the same expressed protein). Record Blank mean and pixels.

6. Drag around entire lane for both the band and blank lanes. Record Mean and pixels.

Then 1.Band density = 255-Band mean X pixels.
2.Blank density = 255-Blank mean- X pixels.
3. Lane density = 255- Lane mean X pixels. **Ratio 1/3 or 2/3 x100 = % of total stain in the band.**
Then true % in band = % in band - % in blank.
If the protein loadings are similar, so that total lane stain density appears consistent, then easier to calculate **true band density = [255-(Band density-Blank density)] X pixels**

SDS-PAGE. BioRad Miniprotean III.

Stocks. 40% Acrylamide/bis 37.5/1 mixture, Bio-Rad, stored at 4 ^{o}C, brown bottle lab refrigerator.

Temed from Pharmacia (17-1312-01), on bench shelf.

10% Amonium persulfate, store at –20 ^{o}C. for 1 month maximum, then re-make.

4X Separating gel buffer = 1.5 M Tris pH 8.8 + 0.4% SDS

4X Stacking gel buffer = 0.5 M Tris pH 6.8 + 0.4% SDS

Volumes/gel

The miniprotean gels have about 8X5 cm area of separating gel and 8X2 cm of stacking gel. **Volumes/gel for 1 mm and 1.5 mm gels are 4 and 6 ml (separating) and 1.6 and 3.2 ml stacking**.

To allow excess gel as a check on polymerization, make following volumes (ml)

	SEPARATING			STACKING		
# Gels	2	4	6	2	4	6
1mm gels	10	20	30	4	8	12
1.5 mm gels	15	30	45	6	12	18

10% Resolving Gel composition. **Stacking gel composition**

Volume, ml	**10**	**20**	**30**	**0.5M Tris pH 6.8→**	**2**	**3**	**4**	**6**	**8**
Water	4.8	9.6	14.4		1.5	2.3	3	4.5	6
40% mix	2.5	5	7.5		0.25	0.38	0.5	0.75	1
1.5 M Tris 8.8	2.5	5	7.5		0.25	0.38	0.5	0.75	1
10% SDS	0.1	0.2	0.3		20 □l	30 □l	40 □l	60 □l	80 □l
10% APS	0.1	0.2	0.3		20 □l	30 □l	40 □l	60 □l	80 □l
TEMED	4 □l	8 □l	12 □l		2 □l	3 □l	4 □l	6 □l	8 □l

For 10 ml of 8.5, 12.5 or 15% gels use 2.12, 3.1 or 3.75 ml 40% mix and reduce water so total is 7.3 ml

5X Running Buffer: per liter

15.1 g Tris Base

94 g Glycine Add 50 ml of 10% SDS (final 0.5%) and dH_2O to 1L.

pH should be around 8.3, check at 1X, must not be >8.7.

2X sample buffer 20% glycerol, 4% SDS 0.04% Bromophenol blue, 125 mM Tris-HCl pH 6.8.

3X sample buffer 30% glycerol, 6% SDS 0.06% Bromophenol blue 125 mM Tris-HCl pH 6.8

per 100 ml: 30 ml glycerol, 6 g SDS, 60 mg BrP blue, 12.5 ml 1M Tris pH 6.8, water to 100 ml (56ml)

PROCEDURE.

1. Clean plates thoroughly with water, finally with ethanol and kimwipes. Normally use 1 mM plates/combs. Both 0.75 and 1.5 mm plates are available and both 10 and 15 place combs. 10 work better.

2. Rubber mats of pouring apparatus should be clean.
Align bottom edges on flat (bench) surface in green frame, clamp frame (turn arms out) then clamp top of large plate onto gasket in holder.

3. Make running gel mix in a 50 ml tube and stacking in a 15 ml tube eg, for four 10% 1 mm gels:

10.7 ml water, 5 ml 40% acrylamide, 5 ml 5X buffer pH 8.8,
0.2 ml 10% APS, 15 µl TEMED.
Mix stacking gel at same time, but do not add TEMED.

Pour gel from tube directly between plates (fill about 75%) and fill to top with water from squeeze bottle to exclude bubbles and O_2 which inhibits polymerization (can also use 70% ethanol).
Observe rest of mix in tube for gelling. When set,
Can now remove from frame and store in frig covered with Saran wrap, in sealed box to prevent evaporation.

Addition of stacking gel and run.
pour out the water, remove rest with paper towel, add TEMED to Stacking gel and pour in till full. Insert the comb (use Kimwipe to divert splash at point of final insertion). Wait until excess in tube is set before removing comb (~20 min).

• Wash sides of the gels to remove any excess acrylamide. Place two gels in Miniprotean III apparatus, short plates inwards to create upper well. Push both ends down as clamps are applied. Remove combs and fill wells with running buffer. Fill internal space with buffer, check for leaks. Add outer buffer, load samples and run.

Protein Samples; typically from 100 µl saturated culture ~ 5.10^6 cells, or about 20 µg total protein.

Mix protein samples with 50% volume of 3X buffer . Heat 2-3 min at 100 ^oC (10 min at 37 for membranes)

Add β-ME to 2 to 5% just before use
Load with Hamilton syringe or gel loading 200 µl tips (SuperMkt # 287040).

Run. 1- 2 hr at 100 volts (or ON 10 volts) till bands focused then 150 volts till dye and 20 kDa pre-stained marker near the bottom. Should be about **mAmps.**

Markers = Invitrogen Benchmak pre-stained (5 µl). Visible transfer to membrane should occur.

2. **Semi-Dry Electroblotting to immobilon**. BIO_RAD TRANS BLOT

Western semi-dry electro-blot transfer

10X Transfer Buffer: per liter		**1X Transfer Buffer**
30g	Tris-base	100 ml 10X Transfer Buffer
145.8g	Glycine	300 ml Methanol (30%)
pH should be around 8.9, check at 1X.		Bring volume to 1L with dH2O

Need three pieces of 3MM and one of immobilon per gel. Size not larger than 8.5 X 6 cm (slightly >gel). Use guillotine to cut.

Cut off one corner of membrane, to correspond to nick at the top of Lane 1 (markers).
With soft pencil, Label gel number at nick. Mark as UP the side in contact with the gel during transfer.

Need small dish with ~15 ml transfer buffer and dish with MeOH.

Soak two pieces of 3MM in transfer buffer, place on bottom electrode.
Soak membrane in MeOH then transfer buffer. Place membrane Up side Up on top of papers.
Separate gel plates with razor blade and use it to remove stacking gel and any other unwanted sections. Cut off corner at top of lane 1 (markers), this marks the top of lane 1.
Transfer to transfer buffer, wash 30 sec then place on membrane so that nicks correspond.
Place third soaked paper sheet on top. Roll gently in two directions with glass rod to remove bubbles.
Run at constant current at 0.2 A for 4 minigels (0.15 if have only 1-2 gels, 0.3 if have 6) **1 hour**.

Western Blot Procedure.

Blocking. Remove lid and upper plate; remove top paper sheet and gel. Should see pre-stained markers transferred to membrane. Helps confirm orientation . Move membrane to 5X10 plastic dish.
Shake membranes in fresh 5% fat-free dried milk in 1X TTBS 1 hr

DETECT Primary Antibody (overnight)

A. In closed plastic boxes (works more consistently) with primary AB ~1/5,000 (2 to 10 µl + 40 ml 5% dry milk), overnight 4 C on oscillating table. Add azide to 5 mM and add an extra washing step before the second wash to ensure complete azide removal. Azide inhibits HRP detection, NOT AP detection. Store the primary AB/5% DM mix at 4C for re-use.
OR
B. Seal in bags with primary AB 1/4000 (1 µl + 4 ml 5% dry milk), overnight 4 C on tilt table.
8X 20 cm Bags, cut in half, open all but one long side. Insert membrane, seal two more sides.
Sealer set at 2, about 1/2 sec per seal
Add AB in 1 ml 5% dry milk slowly from base of bag on UP side, to leave as few bubbles as possible. Remove any bubbles on UP side, seal 4th side, Place on rocker at 4C ON.

Next day, Secondary Antibody: HRP (peroxidase) Goat anti-mouse.

1. Transfer the 5%DM/primary AB to storage tube, or pour from the plastic box.
2. WASH: Immerse membrane in 1X TTBS (15-20ml) place on rotating table, setting 6, for 1 min then replace buffer, wash 5 min then replace and wash 10 min in 1X TTBS
3. Shake in 15 ml 5% dry milk in 1X TTBA containing 2-4 µl HRP-labeled Goat-anti mouse (1/5000 final). Rock 1-2 hr RT C.
4. Wash 1 min in TTBS then 5 min then 10 min in 1X TTBS as in step 2

Development fro HRP-labelled secondary.

After wash, place membranes UP side (face in contact with gel during transfer) UP in flat-bottomed dish.

SuperSignal reagents Mix 0.5 ml of A and 0.5 ml of B for each blot (do NOT cross-contaminate bottles)

Add 1 ml mix to each membrane blot, incubate 5' RT C.

Remove blots, drain on Kimwipe, place UP FACE DOWN on cling film, wrap and place UP FACE UP in cassette. Fix with tape,

Place two fluorescent tag labels between blots for localization of bands

IN dark room, add film, close cassette.

Develop after 10" to 30 '

Alternate Secondary Antibody: Goat anti-mouse-AP (alkaline phosphatase) conjugate.

Promega S372B AP conjugate. 100 µl at 1mg/ml. Dilute 1/5,000 (2 µl/10ml) in 5% DM in PBST or TBST plus 4 mM azide (no interference). Can store at least 1 week at 4C.

1. Wash off primary in TBST 1' 5' 10'
2. Probe with 1/5,000 Goat anti-mouse-AP (10 ml) 2hr RT
3. Pour off and store IIy, wash in TBST 1' 5' 10'
4. Wash in pH 9.5 buffer (10 ml 10 min)
5. Develop in XP at RT, stop color development by washing with water. Note relative times of band appearance. Can wash briefly in MeOH BRIEFLY to remove background

pH 9.5 Buffer (1X):	12 g	Tris base	
Per liter	0.8 g	Tris/HCl	**NBT stock** 2.5% in 100% DMF
	5.8g	NaCl	**BCIP stock** 5% in 70% DMF
	5 ml	1M MgCl2	**Store** Al foil-wrapped at -20C
XP Reagent:	10 ml Buffer pH 9.5 + 20 µl NBT stock and 20 µl BCIP stock		

Washing glass beads. (0.5 mm-yeast cells). BioSpec Products, Inc. (800) 617-3363 or (918) 336-3363

In most cases, cleaning new glass or ceramic bead media is unnecessary. The only contaminate - carbon black - is so inert that its presence in your prep has no effect. Do not acid wash beads. It is a waste of time.

Clean used beads by soaking over night in a solution of laboratory detergent. Then rinse away all detergent with several changes of tap water and then with distilled water. Dry the beads in an open stainless steel or glass tray at 40 to 70ºC. If the dried beads do not pour freely (i.e., they are caked together), then they were not cleaned or rinsed well enough. Repeat the cleaning protocol.

If you are isolating nucleic acids from disrupted cells, beads can be soaked in a 1:10 dilution of ordinary household bleach (Clorox or equivalent) for 5 minutes. This not only cleans and sterilizes the beads, but completely destroys contaminating nucleic acids. See Biotechniques, Vol 12, 358-360 (1992).

You can reuse beads about ten times before they wear down to too small a size.
Autoclave only after cleaning.

Ultracentrifuge runs for VLP isolation

1. SW41 tubes, 11 ml capacity, can run at 35k. Overnight, VLP's and membrane fragments will pellet through a 20% sucrose layer, separating them from soluble proteins.

To each tube add 5-6 ml of 20% sucrose in PBS (or other buffer)
Layer on 6-5 ml of the solubilized VLP's (eg, in buffer A1 +/- detergent, pH 10.7 carbonate +/- urea)
Run at 35k ON 4C
There will probably be a visible pellet of membrane.
Remove all of the sup except for the last 0.5-1 ml
Finally remove the last 0.5-1 ml
Disperse the pellet in PBS or pH 10.7 carbonate +/- urea , etc. This should be the VLP fraction

2. SW41 tubes or SW50.1 tubes, 5.5 ml capacity, can run both at 35k. This provedure can b eused once the VLP's are separated from most of the soluble proteins, e.g by:

- Pelleting through 20% sucrose as above Sample = dissolved pellet
- For HBsAg by binding to and elutinig from silica Sample = silica eluate
- For U65-fusions and for VP1-GFP and GFP-HBs by extensive washing of the 16 kg pellet from yeast cell breakage Sample = pH 10.7 carbonate +/- urea extract of the washed 16kg pellet

Dissolve sucrose in the sample to make 60% final

SW50.1 volumes	**SW 41 volumes**
To each tube add 0.5-1 ml of 80% sucrose in PBS (or other buffer)	1 ml
Layer on the sample in 60% sucrose (not more than 3 ml)	Up to 6 ml
Layer on 1-1.5 ml 50% sucrose	2-3 ml
Layer on 05-1 ml 10% sucrose	1 ml

Protein extracts from yeast for Western by Bead Breakage

1. Stocks, storage of Yeast transformants;
(eg, PrP YEp352 URA3 derivatives) grow in 5 ml Ura D/O ON to saturation.
Spin cells, remove sup, suspended cells in 5 ml YEPD, grown again 24 hrs, then store at 4 ^{o}C.

2. Cultures: fresh 3 ml ON cultures in UraD/O; inoc with 3 to 30 µl stocks.
Generation time in D/O is ~ 2 hr so 10 gen (2^{10} = 1024) = 20 hr, allowing 3µl in 3 ml to reach saturation.
Max A600 in D/O = 5 or about 5.10^{7} cells/ml. **Then 3 ml = 1.5×10^{8} cells = ~ 600µg total protein**

Grow cells to early stationary phase unless protein half lives are short or spheroplasts are wanted,
in which case grow to late log, A600 = 1.5 to 2.5.
Harvest cells by 2X 4 sec full speed spin in 1.5 ml in Epp tubes,
Wash cells with 0.5 ml buffer A, spin 4 sec
Suspend in 200 µl buffer A1 (A + protease inhibitors).

4. BREAK: Add 0.5 mm beads to just below meniscus (use standard scoop), cool in ice 5 min.
Vortex 8 X 45 sec using multi-tube holder and newer mixer at max with 1 min cooling in wet ice between.

5. Isolation of LSS ; Open tubes, pierce bases with a hot needle, place in a labeled second set of tubes.
Spin 3 min ~3000g spin (min speed setting on microfuge).
Pellet = **LSP** = walls and unbroken cells + trapped membranes + any large aggregates
Sup = **LSS.**

To analyze protein in the LSP for solubility in sarkosyl and sensitivity to protease K.
PrP's in unbroken cells will be insoluble in 1% sarkosyl and completely resistant to protease K.
TM species trapped in ruptured cells will be soluble in 1% sarkosyl and sensitive to protease K + sark.
Aggregates of PrP^{Res} will be insoluble in 1% sarkosyl but may converted by protease K + sarkosyl to a highly resistant C-terminal fragment (residues ~111-231 = 13 kDa + any N-CHO).
LSP, removal of walls and unbroken cells. This may be necessary to allow identification of true aggregates.
Suspend LSP in 0.2 ml buffer A1, spin 3 sec (or allow to settle 10 min) and recover the sup.
Pellet residual LSP from sup by 60 sec spin and wash 2X in buffer A2 with 60 sec spin (to remove PMSF).
Add sarkosyl to 1%, after 10 min RT ^{o}C
a: remove 50% as control, add 2% by vol 100X PI's..
b: other 50%. Add protease K to 50 µg/ml, incubate 30 min RT or 37 ^{o}C, then add 2% by vol 100X PI's.

separate in airfuge as fro LSS below. Run all 4 samples on gel

6. Airfuge to separate HSP and HSS. Transfer LSS (200 µl) to airfuge tube, spin 20 min 26-28 psi

Pellet = HSP, membranes and ribosomes Sup = HSS, cytosol

Pellet should be clearly visible as a translucent orangish button.

7. HSP pellet; add 30-50 µl 1% SDS in buffer A1, incubate 10' at 37 ^{o}C to dissolve. run gels later.

SDS-PAGE adjust sample to represent about 0.1 ml of a saturated yeast culture (~1/30 of total). Eg, 5-15 µl HSP solution, etc, + buffer A1 to 15 µl and 15 µl 2X sample buffer.
5 µl of the 200 µl LSS = membranes from ~ 10^8 cells or ~ 15µg total protein
Heat again 3' BWB (for membranes may be better to use 10' at 37 ^{o}C). Load 20-30 µl/well.

Western blot Tests for integral transmembrane (TM) insertion.

In TM form of PrP fusions should behave as i**ntegral membrane proteins**.
These should be in the LSS from broken cells and:

- In the HSP isolated by airfuge from the LSS.
  Cytoplasmic and secreted proteins are in the HSS.
- Remain in the HSP in the presence of 0.1 M Na carbonate buffer (pH 11.5).
  peripheral membrane proteins are solubilized
- Move to the HSS (dissolve) in the presence of non-ionic detergents such as 1% triton X100 or NP40
  or 1% of the stronger zwitterionic detergent sarkosyl (or 1% triton + 0.1% sarkosyl).
  Anything remaining in the HSP in 1% sarkosyl may be an aggregate (see LSP below).

Procedure

1. Grow 3 ml of transformed cells in Ura D/O to early stationary phase, as usual.
   Prepare LSS by bead breakage of 15 OD of cells (eg, 3 ml of cells at A600 = 5) in 200 µl buffer A1.
   Split LSS into 3 X 60 µl in airfuge tubes.
   a → ice (control).
   b + 7 µl 10% sarkosyl (or triton) mix gently, → ice 20 min,
   c + 7 µl 1 M $Na_2 CO_3$ mix gently, → ice 20 min.
2. Spin all tubes in airfuge 10 min 28 psi.
   Remove HSS, disperse HSP's in buffer A1 + 1% sarkosyl by pipetting
3. Dissolve all 6 samples in equal vol SDS sample buffer, 10", 37" or 3 min BWB.
   Load 10% → SDS-PAGE. Detect as usual, 6 lanes per transformant.

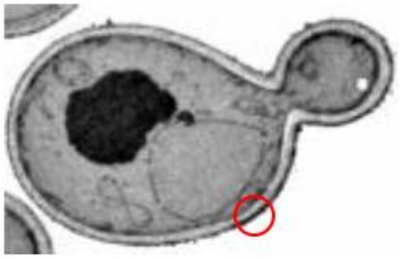
APPENDIX B

Architecture of a Yeast Cell

Scanning Electron Micrograph

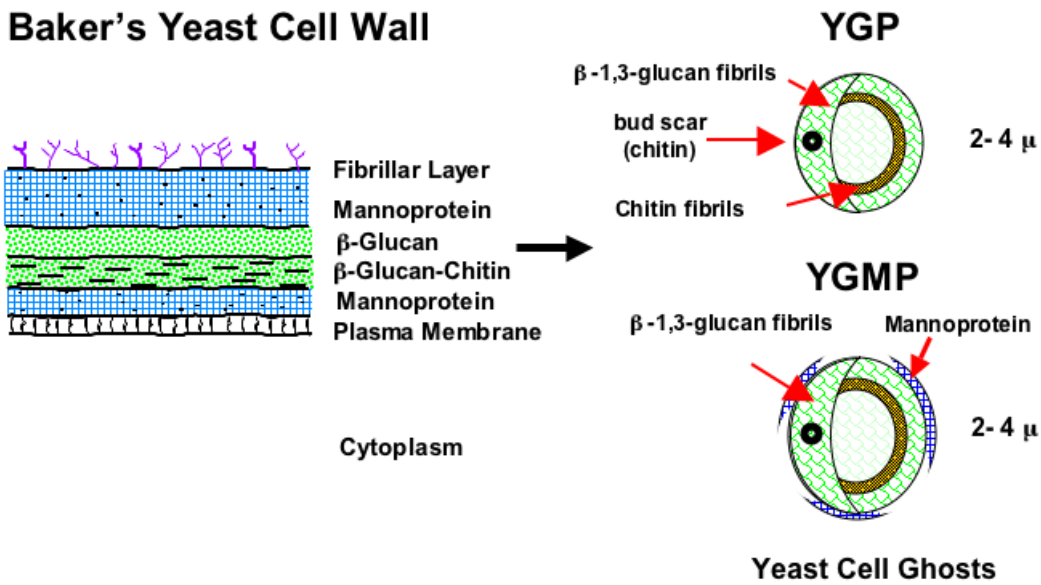
Surface

Transmission Electron Micrograph

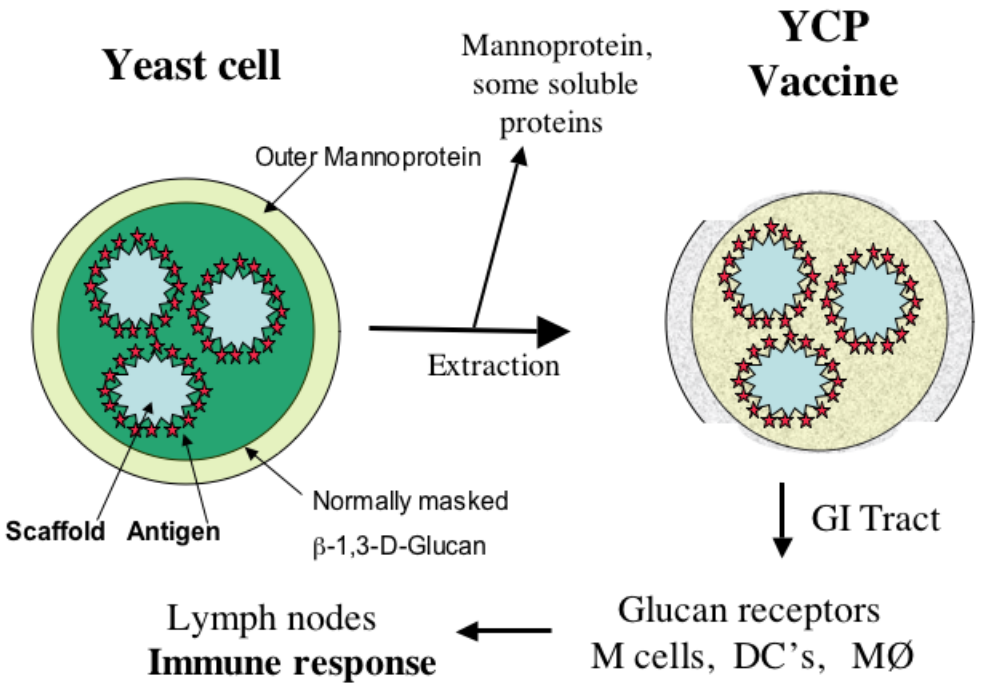


Cross Section

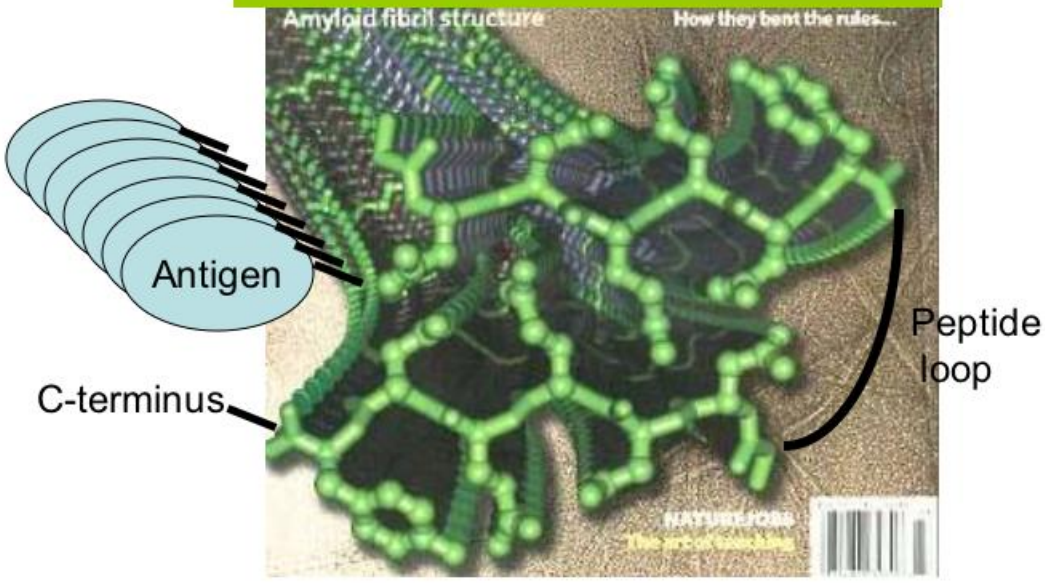
Yeast Cell Walls as Delivery Vehicles



YCP Vaccine Delivery Vehicles



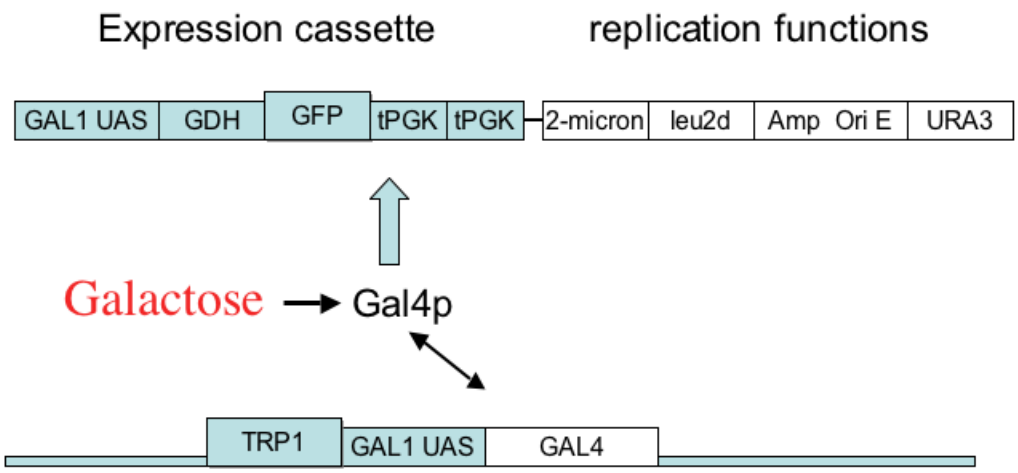
The U65 Fibrillar Scaffold,



Modified from Nature 6-9-05 Cover

Tightly regulated Antigen Expression.

pG2-GFP Plasmid Vector



PAP1502 host strain *ura3 leu2 pep4 prb1 trp1::pGAL1-GAL4*

Expression in strain PAP1502
Ura3 leu2 trp1::pGal1-GAL4;

1. Growth on Ura D/O + glucose selects for normal plasmid CN and tightly suppresses expression

2. Growth on Leu D/O + sucrose and glycerol greatly increases CN and de-represses pGal.

3. Addition of galactose stops growth and induces expression

GFP is used as a convenient model antigen.