Heat-shock protein 70 Cloning in *Tetrahymena thermophila*
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Abstract:

Heat shock response occurs in quite a few species. Surprisingly there is not much known about the gene responsible for this response (*HSP70*). One widely accepted function is that the “proteins of [HSP] families function by binding to unfolded regions of polypeptide chains” (Cooper, 2000). Researchers have hypothesized that there is a role between the decline of this protein due to stress and, because of this, plays a role in aging. This means that understanding the *HSP70* gene could possibly lead to prolonging the aging process. Because the heat shock response in zebrafish (*Danio rerio*) utilizes a similar signaling pathway to that of mammals, zebrafish are a good model for comparative studies of heat shock response (Keller et al, 2009). In this experiment we extracted the DNA from *Tetrahymena thermophila* and amplified the gene using PCR. The new DNA was introduced into a chemically competent *Escherichia coli* vector and was tested to confirm the presence of *HSP70*. This process produced a very good sample of the newly cloned *HSP70* gene in *Tetrahymena* that has been preserved and can be studied to possibly understand the process of aging.

Introduction:

The heat shock proteins (HSPs) are ubiquitous, highly conserved proteins that have been found in every organism, including plants, bacteria, yeast, flies, and vertebrates. “Both the MAPK and HSP families of proteins function in stress-related pathophysiological states of the cell and play critical roles in maintaining normal cellular homeostasis. It is well documented that
expression of HSPs are induced in response to various types of metabolic or environmental stresses such as heat, heavy metal ions, ethanol, nicotine, viral agents, surgical stress, and reactive oxygen species” (Keller et al, 1999). “Members of the HSP70 family stabilize unfolded polypeptide chains during translation as well as during the transport of polypeptides into a variety of subcellular compartments, such as mitochondria and the endoplasmic reticulum. These proteins bind to short segments (seven or eight amino acid residues) of unfolded polypeptides, maintaining the polypeptide chain in an unfolded configuration and preventing aggregation” (Cooper, 2000). Researchers have discovered that spontaneous high expression of heat shock protein 70 (HSP70) was detected in zebrafish (Danio rerio) at early larval stage, but the HSP70 level was either low or barely detectable four hours after fertilization and continued to decrease. The extracts of zebrafish days after fertilization formed a “clear protein-DNA complex with a probe containing heat shock elements (HSEs), suggesting that this spontaneous expression of HSP70 may be turned on via the binding of stage-specific HSE-binding factors to HSP 70 gene promoter” (Yeh & Hsu, 1999). This shows that heat shock response similarly declines with age in zebrafish (danio rerio). However, signaling pathways that regulate the heat shock response in zebrafish are unknown. This pattern, however, is continually being studied. Scientists do know that "HSP70 is induced in response to both intrinsic and extrinsic stress. Because aging theory postulates that life span is causally linked to the ability of a host to resist these stresses, the ability of HSP70 to respond to and resist these stresses may lead to increased life span” (McConnell et al. 2011). If the decline shown in zebrafish can be understood, it could provide great insight regarding the aging process. By cloning the HSP70 gene in Tetrahymena Thermophilia we may come to fully understand its role in aging and potentially expand the lifespan of many different organisms.
Methods/Procedure:

**Bioinformatics Report:**

The NCBI website (http://www.ncbi.nlm.nih.gov/) was searched for the amino acid sequence of HSP70 in *Danio Rerio*. The homolog was found using the Tetrahymena Genome Database Wiki (http://www.ciliate.org/). The protein sequence, nucleotide sequence, coding sequence, and expressed sequencing tags were also found on this website. The MGAlignIt site (http://proline.bic.nus.edu.sg/ mgalign/mgalignit.html) was used to align the homolog coding sequence with the genomic sequence. We compared the predicted homolog of our protein with the original coding sequence once again using the NCBI homepage which confirmed that we had the correct coding sequence for our *Tetrahymena* homolog. See BMS110 handout entitled Laboratory 3: Bioinformatics and Molecular Computational Tools for full protocol (Smith 2011).

**Tetrahymena DNA Isolation**

The DNA was collected from a prepared culture of *Tetrahymena*. 1.4 µL was obtained of *Tetrahymena* culture. 700 µL of Urea Lysis Buffer was added to break open these cells and release the DNA. The proteins were removed from the sample using 600 µL of phenol:chloroform:isoamyl alcohol mixture. 150µL of 5M NaCl was added to the solution in order to lower the carbohydrate content in the precipitate. An equal volume (700 µL) of isopropyl alcohol was added to the solution to precipitate the DNA. The solution was added to 50 µL of Tris-EDTA (TE) buffer for storage and 1 µL of RNase A which broke apart the RNA components left of the original culture.
The genomic DNA was later quantified using a nanodrop. The A260 reading was 58.075 nm and the A280 reading was 28.746 nm. The concentration of the DNA was found using the standard A260/A280 ratio which resulted in a very pure reading of 2.02. See Lab 4: Genomic DNA Isolation (Smith 2011) for full protocol.

**Polymerase Chain Reaction**

Primers were designed for this lab using the predicted oglionucleotides from the homolog. They were resuspended in ddH2O to a final concentration of 200 µM in both the stock and the working stock. The working stock contained 20 µL of either the forward or reverse primer and 180 µL of water (obtained by using the dilution formula C1V1=C2V2). The forward and reverse primers were made and brought in from Integrated DNA Technologies. 5’-CAC CCT CGA GGC TGA CAA GAA AGC TGA AGG TG-3’ was the sequence used to make the forward primer that was .23 mg and 23.7 nMoles. 5’- AGA GCC TAG GTC AAT CGA CTT CAT CGA CGT TG-3’ was the sequence used to make the reverse primer which was .31 mg and 31.5 nMoles. For the forward primer 118.5 µL of sterile ddH2O was added and for the reverse primer 157.5 µL of sterile ddH2O was added (both quantities obtained by simple dilution formula).

Both the Genomic DNA and the cDNA underwent Polymerase Chain Reaction. The master mix for the genomic DNA consisted of 3 µL of genomic DNA, 1.5 µL forward primer, 1.5 µL reverse primer, 1.5 µL *Phusion* polymerase, 30 µL of GC buffer, 30 µL Betaine, 3 µL dNTPs, and 79.5 µL of distilled water making the solution run with a total volume of 150 µL. The master mix for the cDNA consisted of 3 µL of cDNA, 1.5 µL of forward primer, 1.5 µL of reverse primers, 1.5 µL *Phusion* polymerase, 30 µL GC buffer, 30 µL Betaine, 3 µL of dNTPs,
and 79.5 µL of sterile distilled water also making the solution run with a final volume of 150 µL. Three tubes were filled with 50 µL of the genomic DNA master mix and three were filled with 50 µL of cDNA and placed in a thermocycler. The thermocycler heated reactions for 1 minute at 98°C to denature the genomic DNA. It then ran 34 cycles of the following times and temperatures: 20 seconds at 98°C, 25 seconds at primer annealing temperature, 1.5 minute at 72°C. Finally it ran for 10 minutes and 72°C then dropped down to 4°C for storage. The primer annealing temperatures were 52.5°C, 54.4°C, 56.8°C constituting that the first samples of both genomic DNA and cDNA ran at 52.5°C, the second samples of both ran at 54.4°C, and the third samples of both ran at 56.8°C. These temperatures were obtained from the number of Adenine, Cytosine, Guanine, and Thymine bases in the forward and reverse primers. The number of Adenine and Thymine bases were multiplied by 2 and the Cytosine and Guanine bases were multiplied by three. These two numbers were 55 and 54 which were averaged to reach the median temperature of 54.5°C. The other temperatures were either 2°C higher or lower (the predicted numbers aren’t the exact used numbers based only on the settings of the thermocycler).

See Lab 5: Polymerase Chain Reaction (Smith 2011) for protocol, Pre-Lab 5: Polymerase Chain Reaction (Smith 2011) for master mix calculations and instructions, and primer handouts in lab notebooks for primer information.

**Agarose Gel Electrophoresis**

.5 ml (1% of 50 mL) agarose was added to 50 mL of 1X TAE in making our agarose gel. This was placed in the microwave for 1 minute and 20 seconds. .5 µL Ethidium Bromide was added to solution when it cooled and then poured into casting tray. The gel solidified for 35 minutes. The electrophoresis chamber was filled with 1X TAE to cover the gel. 1 µL of 10X sample dye (containing both xylene cyanol-blue and brophenol blue-purple) was mixed with
each of the six tubes of both genomic and cDNA. Each well was filled with 10 µL of the mixed sample and dye in the following order: well 2, Genomic DNA sample 1; well 3, cDNA sample 1; well 4, genomic DNA sample 2; well 5, cDNA sample 2; well 6, genomic DNA sample 3; well 7, cDNA sample 3; well 8, kb ladder; well 9, kb ladder. The agarose gel ran at constant voltage (90-120 volts) for 45 minutes. See Lab 6: Agarose Gel Electrophoresis (Smith 2011) handout for full lab protocol.

Cloning PCR Product

To set up the TOPO cloning reactions the reaction components are .5 µL PCR product, 1 µL salt solution, 3.5 µL sterile water, 1 µL TOPO vector for a total volume of 6 µL. This incubated for 30 minutes at 22-23° C. For the transformation of E.coli, chemically competent E. coli cells were thawed on ice for about five minutes. 2 µL of the TOPO cloning reaction was added to a vial of chemically competent E. coli and then incubated on ice for 25 minutes. The cells were heat shocked at 42°C for 30 seconds and then transferred to ice for 1.5 minutes. 250 µL of room temperature SOC Medium was added and then placed in a shaking incubator set at 37°C for 40 minutes. 200 µL of TOPO cloning reaction is spread on one pre-warmed LB plate with 50 µg/mL kanamycin and 50 µL on another pre-warmed LB plate containing 50 µg/mL and mixed using glass beads. The plate was incubated overnight in 37°C incubator. See Lab 7 titled Cloning PCR Reaction (Smith 2011) for full lab protocol.

Construction of Plasmid Map & Restriction Enzyme Digestion Design

Gene construction kit 3.0 was the program used to create the pENTR/TOPO-D plasmid map. Our gene was inserted into the plasmid map using the sequence 5’-CACCCTCGAGGCTGACAAAGAAAGCTGGTG-3’.
GACAAGAAAGCTGAA GGTG. The restriction enzymes present were PvuII, NheI, HindII,
XhoI, EcoRV, NcoI, EcoRI, Sall, AvrII, Asc I, NrvI, NsiI, PvuI. The restriction enzyme digest EcoRV allows us to confirm the gene made it into the plasmid. See Lab 8: Plasmid Map Construction & Restriction Enzyme Design (Smith 2011) for full protocol.

**Plasmid Purification and Restriction Enzyme Digest**

The day before the actual lab, we inoculated six 2 mL cultures of LB liquid media tubes (containing 50 µg/ml Kanamycin) with six transformant colonies from the pENTR/TOPO cloning (Lab 7). On lab day, the culture was prepared for digest by adding Sucrose Lysis Buffer, to provide a hypotonic environment, along with lysozyme solution. It was then heat shocked at 99°C for one minute and then centrifuged. The DNA was then precipitated with 3M NaOAc and isopropanol. After centrifuging the plasmid, it was washed in ethanol and then centrifuged again. It was then resuspended in 50µL of Tris-EDTA (TE) Buffer.

The second part of the lab involved the confirmation of plasmid by restriction enzyme digestion. EcoRV and AvorII/XhoI were the restriction enzyme used because they were restriction enzymes present in our gene shown on the plasmid map. The cocktail used for the EcoRV digest consist of the following: 14µL of NE Buffer 3, 3.5µL of EcoRV, 1.4µL of 100X BSA (100mM of NaCl, 50 mM of Tris HCl, 10 mM of MgCl, 1 mM of diothitheritol), and 100.1µL of water. The cocktail used for the AvorII/XhoI consist of the following: 14µL of NE Buffer 3, 3.5µL of XhoI, 3.5 µL of AvrII, 1.4µL of 100X BSA (100mM of NaCl, 50 mM of Tris HCl, 10 mM of MgCl, 1 mM of diothitheritol), and 96.6µL of water. Each cocktail was put into tubes and 3µL of each plasmid was added to the appropriate tube. These were both incubated at 37°C for 45 minutes. A 10X sample dye was then added and then they were run on a gel using the same procedure as the earlier gel electrophoresis lab (Lab 6). The only variations from the
original lab were that a 75 mL gel was used and it was shared with another research group. Each well was filled with 20 mL of each sample. The gel ran at 120 voltz for 30 minutes then 140 voltz for 10 minutes. See Lab 10: Plasmid Isolation & Digest (Smith 2011) and Lab 6: Agarose Gel Electrophoresis (Smith 2011) handout for full lab protocol.

**Cryopreservation of *E.Coli* Containing Plasmid with *HSP70***

The day prior to the lab, we inoculated a 2-mL culture of LB liquid media tubes with their positive clone containing the *HSP70* gene. We used samples one and three. The samples were then placed in a 37°C shaking incubator for the evening. For the actual lab, 700 µL of Glycerol and 700µL of bacterial cells were added to the solution. They were placed in powdered dry ice until its solid. It was then transferred to the -80°C in the freezer where it will be stored for future use. See Lab 14: Cryopreservation of Bacteria Containing Plasmid (Smith 2011) for full lab protocol.

**Results:**

The bioinformatics lab revealed a very possible homolog for *HSP70* in *Tetrahymena Thermophilia* with an expected value of 2.5e-20. This gave us the amino acid, coding, and genomic sequence (Figure 1). After extracting the DNA from the cells we quantified the results using the nanodrop to find a purification of 2.02 which is much greater than the pure value of 1.8. Using the information previously gathered, we determined proper annealing temperatures for the sample and ran the samples through the thermocycler. We then tested the results using Agarose Gel Electrophoresis (Figure 2). The gel is set up according to the annealing temperatures used. Genomic DNA and cDNA sample 1 used 52.5°C for their annealing
temperature, Genomic DNA and cDNA sample 2 used 54.4°C for their annealing temperature, and Genomic DNA and cDNA 3 used 56.8 for their annealing temperature. Using this information we used the genomic DNA that was incubated at 54.4°C for the rest of the experiments. This information was also used to compare the predicted size (1959 kb) of the DNA to the actual size.

Together all of this proved that the genomic sequence seemed to have been predicted correctly. No introns were ever discovered in our DNA. To finally clone the PCR product and the \textit{HSP70} gene we used the pENTR/D-TOPO vector. The sample was cloned using \textit{E. coli} as the host organism on LB kanamycin plates. After a few days of allowing the colonies to grow, we found too many colonies to count growing on our plate. The Invitrogen cloning kit provided the vector used in the cloning. In order to test the success of the cloning we used the plasmid map (Figure 3). The plasmid map allowed us to select restriction enzymes to test to confirm \textit{HSP70} was still in the sample after the cloning process. This was shown when we ran the restriction enzyme digest gel electrophoresis. For the digest containing the EcoRV restriction enzyme, sample one showed bright, correctly sized bands without any primer dimers. Sample three didn’t seem to show up. For the digest containing the XhoI/AvrII restriction enzyme, sample one and three both showed the bright, correctly sized bands without any primer dimers. These were the two samples saved via cryopreservation.

\textbf{Conclusions:}

We seemed to have successfully cloned the \textit{HSP70} into \textit{Tetrahymena}. In the original bioinformatics report we found a very good homolog which has been verified throughout the
experiment and especially in the agarose gel electrophoresis. The gel electrophoresis produced the expected number and sized bands that the homolog gave, which suggests that TThERM_00105110 was the appropriate homolog for our gene. The *E. coli* colonies grew in great abundance, which is a very good sign that our DNA was present and active in these vectors. The restriction enzyme digest showed one sample that was exactly as predicted and another that has a strong possibility of containing the DNA as well. Samples one and three very likely contain the *HSP70* gene and have been saved for future use. We didn’t come across any major problems in this process, so the protocol is very accurate and successful. We have however had a few slight problems. One error is shown in our original gel electrophoresis in lane one where the DNA didn’t show up, most likely due to error loading the gel (this also could have been caused by an error in isolating the gel or simply by the fact that we are trying to clone a heat-shock protein and it doesn’t work right at that low of temperature). Another error was shown when we tested the second sample of DNA on the Nanodrop and came up with a negative result (this sample was discarded). Also lane three in the EcoRV restriction enzyme digest didn’t produce any real bands. Since lane three in the Xhol/AvrII restriction enzyme digest did show up exactly as predicted, it’s most likely that the EcoRV lane three was loaded incorrectly. This lab taught me many new procedures and more about how cloning works. This lab has provided great insight to how cells work and how DNA can be taken from one organism and grown in another. It’s probably the first lab that I’ve done that could actually have some meaningful impact. Now that I know we’ve created two viable samples, I hope they can be used to help aid researchers into understanding what exactly the *HSP70* gene is responsible and hopefully lead to more knowledge of its effect on aging
Figure 1: Genomic Sequence of HSP70. Nucleotide sequence of the *Tetrahymena HSP70* (TTherm_001015110). BOLD GREEN represents the start codon for the gene and the Stop codon is in BOLD RED. Exons that are used to make the protein are in RED. Underlined sequence represents the ESTs for the gene. Highlighted regions represent the area that the PCR primers were constructed from (YELLOW: Forward Primer; BLUE: Reverse Primer).
Figure 3: pENTR; HSP70 Plasmid Map. The pink section is the kanamycin resistance cassette. The yellow segment is the origin of replication (ori). The brown striped regions are the LR recombinase sites. The green region is the gene sequence (entry vector). The red sections are the TF primer and TR complementary primer.
Figure 4. Restriction Enzyme Digest Agarose Gel Electrophoresis. Digest 1 Contains the EcoRV restriction enzyme and digest 2 contains the XhoI/AvrII restriction enzymes. The figure on the left shows the predicted band size for all the restriction enzymes. Samples one and three were selected to continue with.

References:


<http://www.jimmunol.org/content/186/6/3718.long>.

Smith, J.J. Lab 3-8,10, 14 Protocols. (2011). BMS110 Section 999