CASE WESTERN RESERVE UNIVERSITY

Discussions

Volume 6 | Issue 2 Article 2

2024

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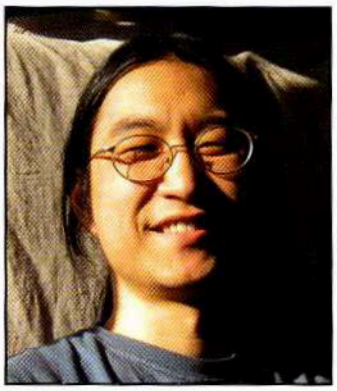
Recommended Citation

Ishida, Kenji (2024) "Identification of a Heat Shock Transcription Factor in Schistosoma Mansoni," *Discussions*: Vol. 6: Iss. 2, Article 2.
DOI: https://doi.org/10.28953/2997-2582.1112
Available at: https://commons.case.edu/discussions/vol6/iss2/2

Identification of a Heat Shock Transcription Factor in *Schistosoma Mansoni*



Kenji Ishida

Kenji Ishida is a senior at Case Western Reserve University, where he is pursuing a BS degree in Biology. His interests include crew, ballroom dancing, and taking power naps. For the next year, he plans to continue working in his current lab.

ABSTRACT

Schistosomiasis, a disease caused by the parasite worm, *Schistosoma mansoni*, affects over 200 million people and ranks second only to malaria in terms of its impact on quality of life. Little is known about the specific regulators of gene transcription in *S. mansoni* or about many of the biological pathways in which they participate. In this study, we identified a schistosome gene that encodes for a transcriptional activator. We designed fusion proteins consisting of the yeast Gal4p DNA binding domain (DBD) and a putative schistosome transcriptional activator and asked whether these fusion proteins could drive expression of several reporter genes in a modified yeast one-hybrid system. The genes coding for the putative transcriptional activators were found through predicted protein homology BLAST comparison between confirmed activator-coding genes in yeast and uncharacterized genes in *S. mansoni*. We describe in this paper the cloning and analysis of a schistosome homolog to the yeast gene HSF1, a gene involved in activating heat shock response genes. The identification of this schistosome transcriptional activator will provide a strong foundation from which we can build a better understanding of the biological pathways involved in gene activation, expression, and development in response to stress.

INTRODUCTION

Schistosomiasis is a debilitating disease affecting over 200 million people worldwide. However, as a neglected tropical disease, it has received little attention from the global scientific community (Chitsulo et al., 2004). *Schistosoma mansoni* is a species of parasitic worms responsible for schistosomiasis and is endemic in many developing countries with poor water treatment systems (Steinmann, et al., 2006). The parasitic schistosome has a complex life cycle with many developmental stages, two of which involve infection of a host and all of which are distinctly different from one another (see Figure 1). For the mammalian hosts, cercariae are the infective agents. Once a swimming cercaria contacts a mammalian host, it passes through the skin, losing its tail, while its head transforms into a schistosomulum. The schistosomula develop into adult worms, which mate and produce eggs in the liver. These eggs are then excreted from the host into freshwater, where they hatch, yielding miracidiae, which then find and infect a molluscan host,

-Acknowledgments-

My thanks go to Dr. Emmitt Jolly for his wonderful guidance and support and for offering me the opportunity to do great research. I also thank all of the members of the Jolly Lab for providing assistance, as well as the Howard Hughes Medical Institute for funding much of this ongoing project.

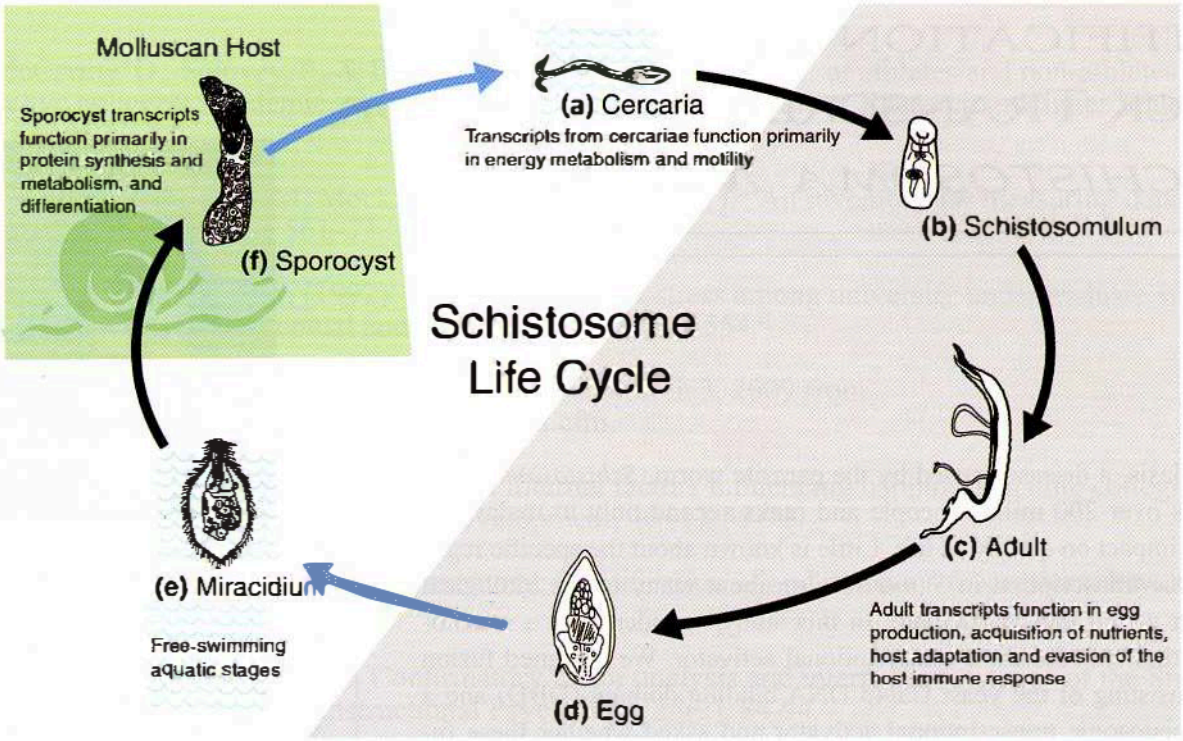


Figure 1: Life cycle of the Schistosome parasite, which includes six different stages adapted to different environments. Free-swimming cercariae (a) infect mammalian hosts, after which the tail is lost and the head transforms into the schistosomulum (b). When further adaptation to the host is complete, the schistosomula become adult worms (c), which mate in the liver to produce eggs (d). After shedding through the feces into freshwater, the eggs hatch and release miracidia (e), which infect molluscan hosts and transform into sprocysts (f), mass producers of cercariae (taken from Jolly, et al., 2007).

transforming into sporocysts, mass producers of cercariae (Jolly et al., 2007). The focus of this study is to attempt to uncover information about the genetics of the complex parasite *S. mansoni* to expand the possibility of drug development. This is an opportune time for such an investigation, as there is concern for schistosome worms resistant to the one current drug, praziquantel, (Doenhoff, et al., 2007).

To begin the process of uncovering biological pathways associated with the stress response in the schistosome, this study attempts to identify genes that code for transcriptional activators in schistosomes. Transcriptional activators belong to a class of proteins that cause transcription of specific genes under a specific set of conditions. In order to drive gene expression, the activator must be present in the nucleus and able to access the genome. If post-translational modification or protein-protein interaction is required to satisfy this condition, the activator cannot function. To address this problem, the sequence of the gene coding for the potential schistosome activator can be cloned with a well-characterized DNA binding domain (DBD), such as that encoded by the separable DNA binding domain of the yeast gene GAL4, which will produce a fusion protein consisting of the Gal4p-DBD and putative transcriptional activator protein (Titz et al., 2006). The yeast Gal4p-DBD will bind to known promoter sites that regulate transcription of reporter genes. The ability of the putative activator/Gal4p-DBD complex to induce reporter gene expression will permit us to determine whether the putative transcription factor is a positive regulator of gene expression.

MATERIALS AND METHODS

Candidate Gene Selection

Genes were first selected from a Saccharomyces cerevisiae transcriptional activator screen by Uetz et al. and Ito

et al. and used sequence information in a protein BLAST query hosted by GeneDB against the *Schistosoma mansoni* genome. After choosing a schistosome gene that had significant homology with a yeast transcriptional activator, we proceeded to design oligonucleotides to amplify the gene from schistosome RNA.

Oligonucleotide Design

Two sets of oligonucleotides were designed in this study. One of these sets was for use with standard TA vector cloning and the other was for use with a newer InFusion cloning technique. Each set of these oligonucleotides consisted of forward and reverse primers, which contained 30 base pairs of homology with the respective ends of the schistosome gene (the forward primer contained the sequence of the first 30 base pairs and the reverse primer contained the reverse complement of the last 30 base pairs).

The first set contained the addition of two different restriction endonuclease cut sites for Nde I and EcoR I, respectively, for the forward and reverse primers in order to facilitate cloning into the final plasmid vector, pGBKT7 (Clontech). These two restriction endonuclease cut sites were chosen by comparing available cut sites in the pGBKT7 multiple cloning site (MCS), which is directly downstream of the GAL4 DBD gene, with the gene sequence and using a restriction endonuclease cut site search tool (New England Biolabs). Both Nde I and EcoR I had potential cut sites in the pGBKT7 MCS and did not exist in the gene.

While the other set of oligonucleotide primers also contained the first and last 30 base pair homology to the schistosome gene, they did not contain the same cut sites. Instead, they contained a 15 base pair sequence homologous to the respective side of the Sma I cut site of the pGBKT7 MCS at the 5' end, as well as a Sma I cut site after the 15 base pair homologous region to allow the subsequent isolation of the gene and the verification of successful cloning into pGBKT7.

RT-PCR

cDNA of the schistosome gene was generated using SuperScript One-Step RT-PCR (Invitrogen) with both sets of forward and reverse oligonucleotide primers and 14 ng of schistosome RNA as the template strand in a MultiGene Gradient Thermal Cycler (Labnet). For reverse transcription, the samples were placed in the thermal cycler at 45°C for 30 minutes, followed by a 95°C denaturing period of 2 minutes and polymerase chain reaction (PCR) amplification of the cDNA, which consisted of 25 cycles of denaturing at 94°C for 30 seconds, annealing at 50.4°C for 30 seconds, and extension at 72°C for 2½ minutes. A final extension at 72°C was programmed for 10 minutes after PCR.

To verify the presence of the schistosome gene, the RT-PCR product was run on a 0.8% agarose gel and visualized with a Fotodyne FOTO/Eclipse and FOTO/Analyst transilluminator system.

Cloning

Using the InFusion Liquid PCR Cloning Kit (Clontech), the PCR product amplified by the InFusion-specific primers was cloned into the prepared pGBKT7 at the Sma I cut site. The pGBKT7 vector was prepared using a standard digest protocol from New England Biolabs with the Sma I restriction endonuclease, followed by gel purification with Nucleospin Extract II (Macherey-Nagel).

Bacteria Transformation

To complete the InFusion cloning, the InFusion product was immediately transformed into FusionBlue competent E. coli bacteria (Clontech) according to the standard Clontech bacteria transformation protocol. After the final 37°C shaker incubation period, 30μL of cells were diluted with super optimal with catabolite repression medium (SOC), and the remaining cells were centrifuged and resuspended in a smaller volume of SOC (70μL less) to achieve an approximate total volume of 200μL. Both the diluted and normal cell concentration semples were plated on 37°C Luria-Bertani (LB)/kana-mycin plates to select only the cells that were transformed with pGBKT7. The cells were allowed to grow overnight in a 37°C incubator, after which eight colonies were picked and grown in liquid LB/kana-mycin medium in the 37°C shaker overnight.

Using the PureYield Miniprep System (Promega), plasmid DNA was extracted from the cells. A portion of these extracts were digested with Sma I and run on a gel to verify the presence of the gene in the extracted plasmid. The samples that yielded a band at the location corresponding to the size of the gene were subsequently used to transform yeast.

Yeast Transformation

First, four 10mL samples of yeast strain AH109 (Clontech) were grown from a frozen stock in yeast extract peptone dextrose (YEPD) medium in a 30°C roller overnight, after which the optical absorbance (at 600nm) of the samples were verified to be between 0.6 and 1.0 (OD600 = 0.6-1.0).

In order to transform the yeast cells, a variation of the protocol presented in Guthrie and Fink (1991) was used with the positive control, negative control, and experimental plasmids, which are, respectively, pGBKT7 cloned with a complete GAL4 gene downstream of the constitutive promoter PADH1, pGBKT7 alone, and 3μL (600ng DNA) from a sample of extracted pGBKT7 (cloned with the schistosome gene) that showed a band of the gene size in the previous gel verification.

After the transformation, the three yeast samples were first plated on synthetic dextrose complete medium (SDC) lacking the essential amino acid tryptophan (SDC-Trp) to screen for those cells carried each plasmid. Single colonies were chosen from each of the samples and streaked for single colonies on a separate SDC-Trp plate to further verify transformation. For each sample, three single colonies were chosen from the new streak plate to make an SDC-Trp master patch plate, from which all subsequent samples would be obtained.

Gene Expression Assay and Screening

LacZ, HIS3, ADE2 in the yeast were the three main genes targeted for activation in this experiment. Either an assay or simple screening was performed to verify the successful expression of these genes.

For the LacZ gene expression assay, a new SDC-Trp plate was supplemented with 100μL alpha-galactosidase, and samples from each of the patches on the SDC-Trp master patch plates were transferred to the alpha-galactosidase plate to verify LacZ expression. Blue color indicates successful LacZ expression, while white color indicates no LacZ expression.

Verification of HIS3 and ADE2 expression involved a simple streaking from the master patch plates to SDC plates missing histidine (SDC-His) or adenine (SDC-Ade), respectively. In order to observe relative activation strength, these plates were separated into three compartments, with each compartment containing singles streaks for the positive control, negative control, or experimental sample.

RESULTS

Selection and Amplification

The schistosome gene, Smp_068270.5 was chosen as the focus of this study because of its high degree of homology to a known yeast transcriptional activator. Comparing the protein sequence produced by the HSF1 yeast gene with that produced by the Smp_068270.5 schistosome gene using BLAST yielded a probability of 5.8x10-18 of dissimilarity between the two genes, meaning the two genes are likely to be homologous.

Oligonucleotide primers designed to amplify the Smp_068270.5 gene with the addition of a 15 base pair region of homology to the respective sides of the Sma I cut site in the pGBKT7 vector, as well as the addition of a Sma I cut site, were used for the RT-PCR reaction, the product of which was run on a gel and found to contain DNA fragments of 2000 base pairs as can be seen in Figure 2. Lanes 3 and 4 show the 2000 base pair fragment with darker bands further along the gel, which are the excess primers used in the RT-PCR reaction. No DNA fragment product was amplified in lanes 1 and 2, in which different oligonucleotide primers were used to prepare the gene for standard TA vector cloning.

Restriction Endonuclease Digest and Cloning

In order to clone the schistosome gene (amplified by the InFusion-specific primers) into the pGBKT7 vector, a restriction endonuclease digest with Sma I as well as a verification gel of the digest was necessary. Figure 3 shows that pGBKT7 digested with Sma I (rightmost lane) ran further on the gel compared to the uncut pGBKT7 (middle lane) by a difference of between 1000 and 2000 base pairs. The brighter band of the digested pGBKT7 resulted from a greater sample size of 0.75μg of vector compared to the 0.1μg of uncut pGBKT7.

Combining the RT-PCR product prepared for the InFusion reaction, the gel purified pGBKT7 vector, and InFusion enzyme and buffer yielded a partially cloned

Figure 2: 0.8% agarose gel of the RT-PCR product. Lanes 1 and 2 are duplicate samples of schis-tosome RNA amplified with primers for TA cloning and lanes 3 and 4 are duplicate samples of the RNA amplified with primers for InFusion cloning. Lane 9 is the 1 kb ladder molecular marker.

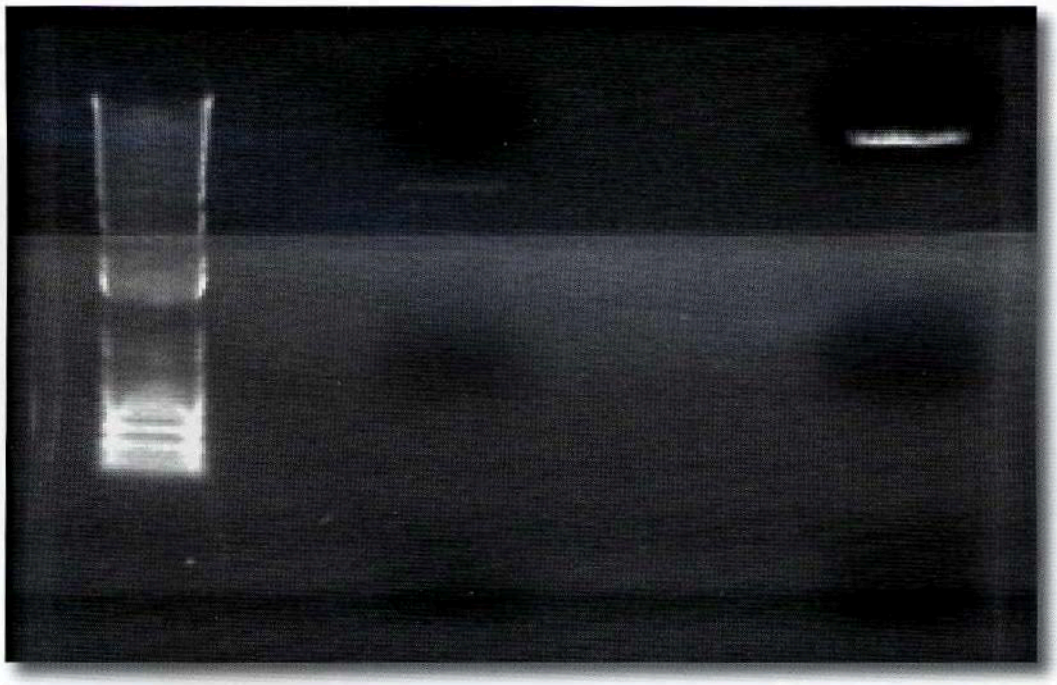


Figure 3: 0.8% agarose gel of the pGBKT7 plasmid. The middle lane is uncut pGBKT7 and rightmost lane is pGBKT7 cut with Sma I. The leftmost lane is 1 kb ladder molecular marker.

gene to be completely cloned via a bacteria transformation. After transforming, growing, and picking bacteria colonies, a miniprep was performed, the product of which was digested with Sma I and run on a gel along with undigested vector extract (Figure 4). Out of the eight different samples of extracted and digested pGBKT7 cloned with Smp_068270.5, samples 1, 4, 6, and 8 show a DNA fragment at about 2000 base pairs and 7000 base pairs, while the other samples show a DNA fragment only at 7000 base pairs. Again, the undigested samples of pGBKT7 alone as well as the undigested samples 6, 7, and 8 traveled less on the gel than the digested samples. Between the undigested samples 6, 7, and 8, sample 7 traveled less than samples 6, and 8, which both traveled about the same distance of 8000 base pairs.

Yeast cells of strain AH109 were transformed with sample 6 from the bacteria miniprep with other samples transformed with positive control vector pGBKT7 cloned with the complete GAL4 gene and negative control vector pGBKT7 alone and plated on SDC-Trp, SDC-Trp+x-alpha-galactosidase, SDC-His, and SDC-Ade (Figure 5, 6, 7, pages 18, 19). As expected, the positive control transformant grew on all plates and exhibited blue color in the LacZ assay, while the negative control transformant grew only on SDC-Trp and remained a white color in the LacZ assay. The experimental transformant showed growth in all plates and exhibited blue color in the LacZ assay.

DISCUSSION

Smp_068270.5 was chosen as the gene of interest in this study by looking at its homology to a yeast gene confirmed to function as a heat shock transcriptional activator. Yeast was used as a starting point for the reason that many yeast genes have been thoroughly investigated and characterized. If one such gene has homology with a completely different species such as *S. mansoni*, there may be significance in the conservation of the sequence and, therefore, a possibility that the functions of the two genes may be similar. Yeast HSF1 is an example of such a case, especially considering that heat shock factor not only acts when an organism encounters a heat shock but also acts under other types of stress, during which cellular proteins fold incorrectly and the integrity of the cytoskeleton degrades (Shamovsky et al., 2008).

For designing the two sets of oligonucleotide primers to amplify Smp_068270.5, in addition to verifying that the correct sequences were used in the correct orientation for the homologous regions, one more consideration was

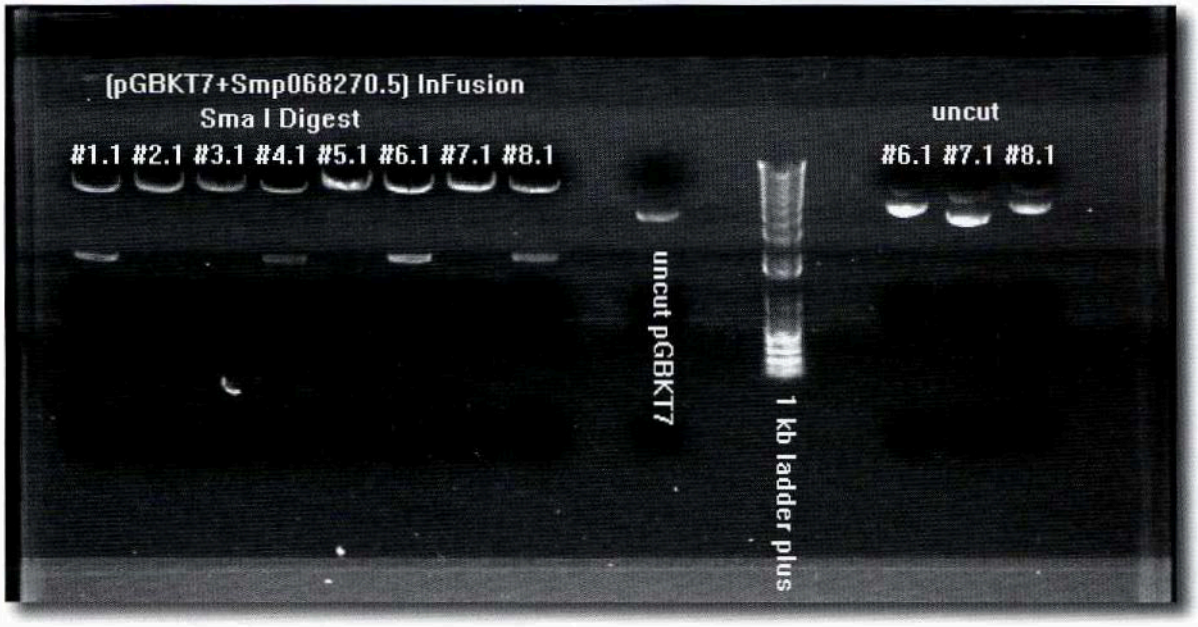


Figure 4: 0.8% agarose gel of the Sma I restriction endonuclease digest of vector pGBKT7 containing the heat shock gene Smp_068270.5. Lanes 1-8 represent digest samples of vector (with gene) extracted from eight different bacterial colonies. Gene product was detected in samples 1, 4, 6, and 8. Lane 10 is uncut vector alone, lane 12 is a 1 kb ladder molecular marker, and lanes 13-16 are uncut vector (with gene) samples 6-8.

required when inserting the restriction endonuclease cut sites into the primers. After the cloning of the gene into pGBKT7, the reading frame of the gene must match the reading frame of the vector in order to obtain a working protein from the gene sequence. One or two arbitrary nucleotide bases were added immediately following the restriction cut site sequence, if adjustment was necessary, to allow the start codon (ATG) to be read in the correct frame.

Out of the two sets of oligonucleotide primers used for RT-PCR, only the second set designed for the InFusion reaction yielded a gel that showed a DNA fragment at about the size of 2000 base pairs. The first set only showed light bands toward the bottom of the gel, indicating that the target PCR product could be formed. Since all samples were amplified in the same machine under the same conditions, certain temperatures and cycle lengths may have been incompatible between the two different primers in terms of obtaining a PCR product. As both samples for the InFusion cloning yielded clean PCR products, the samples could be immediately used without purification to perform the InFusion cloning.

In order for the InFusion cloning reaction to work correctly, the pGBKT7 vector needed to be digested by Sma I, and the RT-PCR product needed to be amplified by oligonucleotide primers containing the cut site for the same restriction endonuclease and homologous sequences to the regions immediately surrounding the Sma I cut site in the pGBKT7 MCS. All of these conditions must be met in order for the InFusion 3' to 5' exonuclease to be able to create sticky ends on the linearized (non-circular) vector and the gene insert.

Following the InFusion cloning, bacteria transformation, and plasmid extraction, a digest with the appropriate restriction endonuclease, Sma I, was necessary to verify that the gene was successfully cloned into the Sma I site of the pGBKT7 MCS. Since the MCS is immediately downstream of the GAL4 gene, given that the inserted gene is in the correct orientation and correct reading frame, a fusion protein of the Gal4p DBD and putative transcriptional activator will be produced when this plasmid is transformed into the AH109 yeast strain.

AH109 is specifically designed with many Gal4p binding elements (genomic promoters) that are upstream of the reporter genes LacZ, HIS3, and ADE2, and it is also deficient in that without either the presence of histidine and adenine in the medium or a transcriptional activator to drive expression of the HIS3 and ADE2 genes, it cannot survive. When the Gal4p DBD portion of a fusion protein consisting of the DBD and a transcriptional activator binds to its binding element, expression of the genes downstream of the promoters will occur.

Plating the transformed yeast on specific media missing tryptophan, histidine, or adenine ensures that only those cells that received the expressing the correct genes can survive. A successful transformation with the pGBKT7 vector should allow the cells to grow in SDC-Trp plates, since pGBKT7 contains a TRP synthesis gene with its own promoter.

In the assay and screens, growth for all three transformants was observed on SDC-Trp plates, indicating the transformation was successful. In the LacZ assay, both the positive control and experimental exhibited blue color while the negative control did not (Figure 5a, page 18), indicating activation of the LacZ reporter gene. In the histidine and adenine screen, both the positive control and experimental produced viable single colonies while the negative control did not, indicating the successful activation of both HIS3 and ADE2 reporter genes by the positive control and experimental. There was also comparable growth between the two transformants in both the histidine and adenine screen. Comparable growth of the experimental to the positive control in the adenine screen indicates that the schistosome codes for a strong activator, as the ADE2 reporter gene is the most stringent in terms of the strength of activation required for adequate growth.

All gene expression testing showed a positive result for the experimental transformant, especially for the stringent ADE2 reporter gene, indicating that the schistosome gene Smp_068270.5 has strong transcriptional activator activity. Now that the activator activity has been confirmed, the activator must be further characterized by identification of its DNA binding requirements and genomic DNA binding targets in schistosome, as well as verification of its protein sequence by mass spectroscopy.

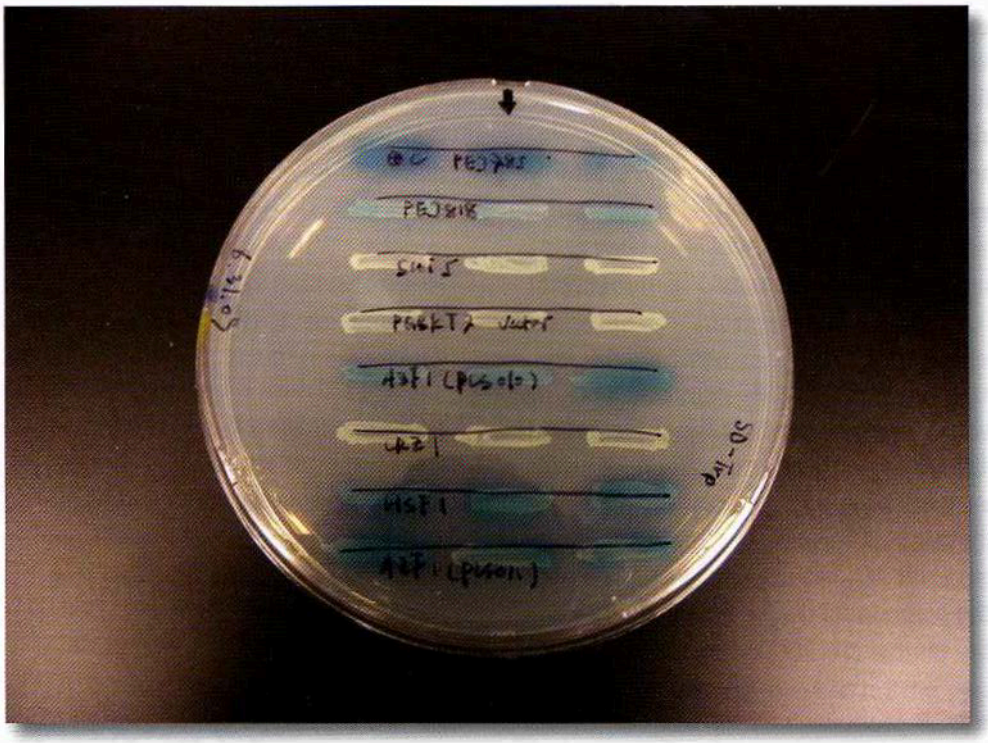


Figure 5: LacZ assay with alpha-galactosidase on SDC-Trp media. The positive control is pEJ785 (complete yeast GAL4) in the first row and the negative control is pGBKT7 (DBD) alone in the fourth row. Smp_068270.5 shows a positive result and is labeled HSF1 in the seventh row.

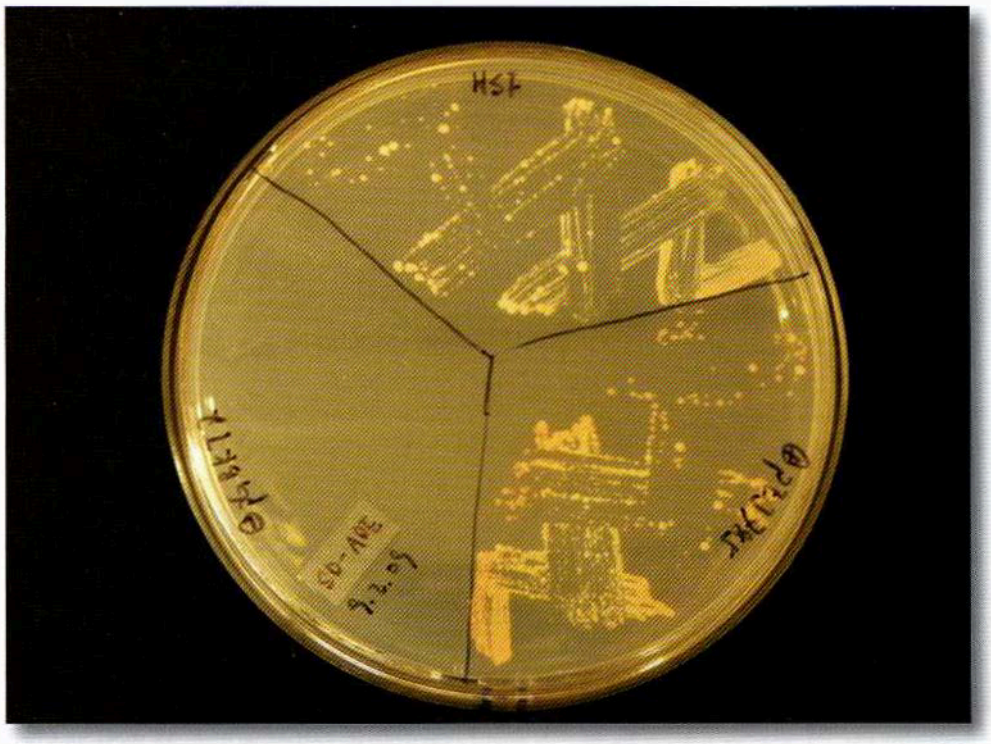


Figure 6: Growth of transformed yeast on SDC-Ade media. Clockwise from top, Smp_068270.5 (HSF1) followed by the positive and negative controls. Again, the schistosome gene shows comparable growth with the positive control.

CONCLUSIONS

The successful expression of LacZ, histidine, and adenine reporters by the schistosome homolog to yeast HSF1 shows exciting possibilities for the future of schistosome genetics: first, a previously unknown schistosome transcriptional activator was identified; and second, this activator showed functionality in a heterologous yeast system. In other words, not only can this activator function in schistosomes but also in yeast, given the correct conditions. Therefore, such expression in the yeast system is not only limited to this specific activator but includes other schistosome activators as well. The techniques used in this study can be applied to other schistosome genes, from identification of a putative transcriptional activator candidate, to the determination of whether expression can be driven. Once such an activator is determined, the sites in the schistosome genome to which it binds could be identified, uncovering pathway information, which can be used in drug development for schistosomiasis.

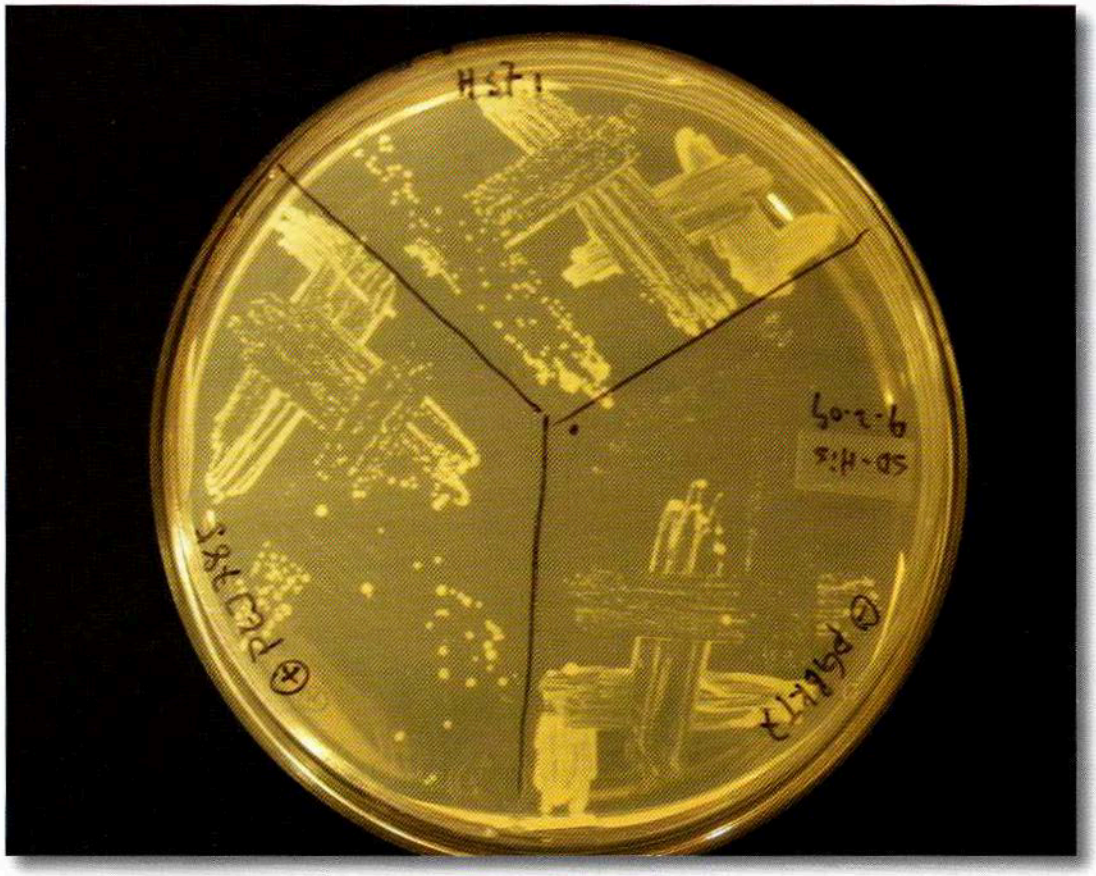


Figure 7: Growth of transformed yeast on SDC-His media. Clockwise from top, Smp_068270.5 (HSF1), followed by the negative and positive controls. The schistosome gene shows comparable growth with the positive control.

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