Rotation Report Sample
Version 3

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Analysis of the Guanine Nucleotide Exchange Activity of the *S. cerevisiae* Ats1 Protein

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Abstract: The goal of my rotation project was to express, purify, and examine the exchange activity of a putative guanine nucleotide exchange factor, Ats1p. The *S. cerevisiae ATS1* gene encodes a protein with homology to the guanine nucleotide exchange factor for Ran, Prp20p. My approach was to use an established purification protocol and exchange assay to determine whether Ats1p, like Prp20p, acts as a nucleotide exchange factor for Ran. Due to technical difficulties, the expression clone was constructed but no protein expression was carried out.

All macromolecules that move into and out of the nucleus transit the nuclear envelope via large proteinaceous complexes termed nuclear pores (1). These nuclear pore complexes essentially serve as channels that perforate the double membrane that surrounds the nucleus. Transport substrates do not, however, flow freely back and forth through the nuclear pores. The traffic through these channels is meticulously regulated by a number of soluble factors that are required for both import and export of substrates from the nucleus (2). The most central of these soluble factors is the small GTP-binding protein Ran that is required for virtually all substrates to traverse the nuclear pore (3).

As with other cellular G-proteins, Ran cycles between a GTP-bound and a GDP-bound state. The rate of GTP hydrolysis by isolated Ran protein is extremely slow (4). In the cell, several accessory factors enhance the rate at which Ran cycles between the two nucleotide-bound states. The GTPase activating protein (GAP) for Ran is the Rna1 protein, which stimulates the rate at which Ran hydrolyzes GTP to GDP (5). The guanine nucleotide exchange factor (EF) for Ran is the Prp20 protein, which stimulates the release of GDP from Ran (6). The concerted action of these two Ran regulatory proteins allows Ran to cycle rapidly between the GTP- and the GDP-bound states.

Many current models for how directional transport of substrates into and out of the nucleus is achieved rely heavily on the absolute cellular compartmentalization of the Ran GAP and EF (7). While Ran is located throughout the cell (8), the GAP is primarily confined to the cytoplasm (9) and the EF is confined to the nucleus (10). The localization of these proteins suggests that Ran is mostly in the GDP-bound state when it is in the cytoplasm due to the action of the GAP. In contrast, logic would dictate that Ran should be mostly in the GTP-bound state within the nucleus due to the activity of the EF. This distribution of Ran-GTP and Ran-GDP melds nicely with a directional model in which Ran-GDP initiates import of proteins into the nucleus and Ran-GTP causes the release of these proteins to the nuclear interior. This model has never been directly tested because it is difficult to determine what the nucleotide-bound state of Ran is *in vivo* in any specific cellular compartment.

Obviously the model described above is dependent on the presence of only the defined Ran regulators, Rna1p and Prp20p. However, the complete sequencing of the *S. cerevisiae* genome has revealed that there are at least two yeast genes that encode proteins with significant homology to Prp20p. One of these genes, *ATS1*, was identified several years ago as an alpha-tubulin mutant suppressor (11) although subsequent experiments have not revealed any direct connection to alpha-tubulin. The goal of

this work was to use a biochemical approach to determine whether the Ats1 protein might serve as a guanine nucleotide exchange factor for Ran.

Methods and Results

The Ats1 protein will be expressed in a construct with an N-terminal 6-His tag (Figure 1). In the past, the Corbett Laboratory has used a 6-His tagged version of the Prp20 protein and found that the tag does not interfere with the exchange activity. The strategy for constructing the Ats1p expression plasmid was to amplify the *ATS1* coding region by PCR using oligos AC135 and 136 engineered with a 5'-*BamH*I site and a 3'-*Hind*III site, respectively. The template for the PCR was the plasmid pAC214, which contains the genomic *ATS1* clone.

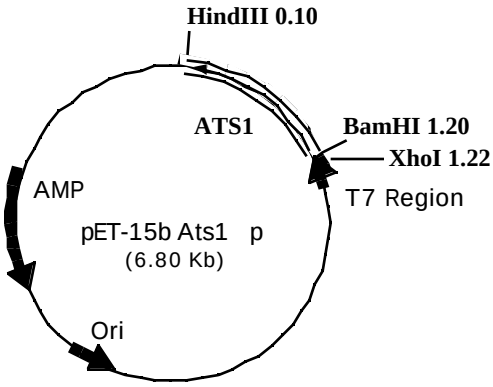

Figure 1. Schematic of Ats1p expression plasmid.

The first attempt to PCR amplify the *ATS1* gene employed Vent DNA polymerase (New England Biolabs). No PCR product was obtained under standard reaction conditions using the manufacturer supplied Thermopol buffer and 2.0 mM $MgCl_2$. Several attempts were made to alter either the annealing temperature of the reaction (45°C, 48°C, 52°C, 55°C, 60°C) or the concentration of MgCl2 in the reaction (1.5 mM, 2.0 mM, 2,5 mM, and 3.0 mM). A control reaction using oligonucleotides AC21 and AC22 that amplify the *S. cerevisiae NTF2* gene from pAC217 was carried out in parallel with the *ATS1* reaction. The *NTF2* PCR reaction yielded a band of the expected size (450 bp) under many conditions where no significant product was obtained for the *ATS1* reaction.

The original plan was to use Vent to amplify the *ATS1* gene because Vent DNA polymerase has an inherent 3'-5' proofreading exonuclease activity that makes its ability to faithfully replicate DNA significantly greater than *Taq* polymerase which lacks the 3'-5' exonuclease activity. Given, however, the difficulty in amplifying the *ATS1* gene with Vent, we decided to try using *Taq* polymerase (Fisher Scientific) as a next approach. *Taq* polymerase introduces approximately one error/kb of DNA amplified so it will obviously be critically important to fully sequence our PCR amplified *ATS1* gene before using it in any further experiments. It would have been necessary to do this with the Vent amplified gene as well but there would have been a lower probability that we would identify polymerase induced mutations. Since there is a significant possibility that *Taq* will introduce mutations into the 1.1 kb *ATS1* gene, three independent PCR reactions were carried out. This should yield three independent pools of the amplified DNA and it is extremely unlikely that each one will contain errors. All subsequent methods described were carried out in triplicate with the three independent PCR products obtained.

Initial PCR reactions with *Taq* were carried out under the manufacturer recommended conditions in Fisher Buffer B supplemented with 1.5 mM $MgCl_2$. For all reactions amplification of the *NTF2* gene was employed as a positive control to assure that the reactions were set up correctly. In the initial reactions the *NTF2* gene was efficiently amplified and a faint band was observed at the predicted size for the *ATS1* gene. In order to enhance the yield from the *ATS1* PCR reaction, the $MgCl_2$ was varied. Reactions were carried out with 1.5 mM, 2.0 mM, and 2.5 mM MgCl2. At 2.0 mM $MgCl_2$ the yield of the 1.1 kb *ATS1* PCR product was greatly enhanced (Figure 2).

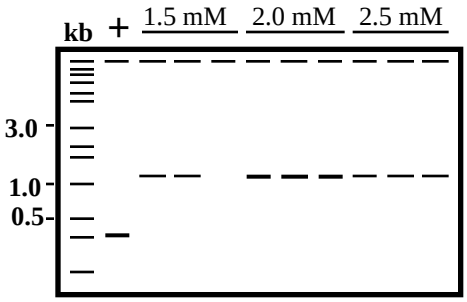

Figure 2. PCR of *ATS1* with *Taq* polymerase. The *ATS1* gene was amplified as described in Methods. Lane 1, 1 kb ladder; Lane 2 (+), *NTF2* control. In each of the subsequent lanes three independent reactions were carried out at the indicated concentrations of $MgCl_2$.

The *ATS1* PCR product was purified by passing over a Qiagen PCR spin column. A 15 µl sample of the purified DNA (total volume following purification 50 µl) was digested with *BamH*I and *Hind*III in NEB *BamH*I buffer. In parallel, the pET15b vector was digested with *BamH*I and *Hind*III. Following a two hour incubation, the cut vector was treated with 1 µl of alkaline phosphatase. Each of the samples was loaded on a 1% agarose gel and the gel was run for 2 h at 100 mA. Bands of the appropriate size were excised and purified by gene cleaning (Bio 101). Each purified fragment was run on a gel to assure recovery of the purified fragment. These purified fragments were then ligated using either a 1 µl:5 µl or 1 µl:10 µl ratio of vector to insert in a total reaction volume of 20 µl. Control ligations were vector alone (1 µl) or insert alone (5 µl). Ligations were carried out overnight at 14°C. The following day 10 µl of each reaction mix was transformed into a 100 µl aliquot of competent XL-1 Blue *E coli* (Stratagene). Following an overnight incubation at 37°C, no colonies were obtained.

Since this cloning attempt was apparently unsuccessful, it was suggested that perhaps a two hour digestion was not sufficient to digest the ends of the PCR product efficiently. Thus, the same exact procedure was followed except that the PCR product was digested for 12 h (overnight) at 37°C. Transformation of the ligation from the second cloning attempt yielded no colonies on either the vector or insert alone plates, ~25 colonies on the vector + 5 µl of insert, and ~80 colonies on the vector + 10 µl insert. Four colonies were chosen from each of the vector + insert plates. These colonies were incubated overnight in LB-AMP and then miniprepped. The resulting minipreps were cut with *BamH*I and *Hind*III to determine whether the appropriate clones were obtained (Figure 3). As controls, the vector alone was cut with *BamH*I and *Hind*III and included on the diagnostic gel along with some of the

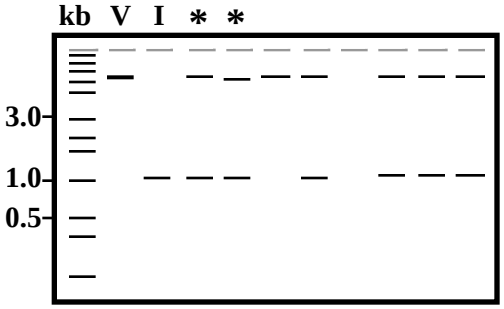

Figure 3. Diagnostic digests of Ats1p expression clones. Minipreps 1-8 were digested with BamHI and HindIII. Controls, vector (**V**) and insert (**I**) were also loaded. Minipreps chosen for sequencing are indicated by the *****.

original PCR product to demarcate the positions expected for both the vector and the insert. Out of the 8 minipreps performed 6 of the clones obtained appear to be correct.

To ensure the absence of PCR-induced errors, two of the clones were chosen for further analysis. The entire clone was sequenced by the Emory Sequencing facility using the T7 promoter and terminator oligonucleotides present in the pET15b plasmid. Since sequencing of two independent minipreps revealed no errors in the DNA sequence as compared to the Saccharomyces Genome Database and also demonstrated an in frame fusion with the N-terminal 6-His tag, this Ats1p expression clone which is designated pAC341 will be used in the future to express and purify 6-His-tagged Ats1p.

Discussion

Due to difficulties in obtaining the Ats1p expression clone, we were not able to test the ability of Ats1p to enhance the nucleotide exchange activity of Ran. It is not clear why it was not possible to amplify the *ATS1* gene using Vent polymerase. A single attempt was made to amplify the *ATS1* gene with *Pfu* polymerase, which is another polymerase that has an inherent 3'-5' proofreading exonuclease activity. As with the Vent polymerase, this was unsuccessful. The use of *Taq* polymerase to amplify the gene simply meant that sequencing was critical before moving forward with any experiments, but this would have ultimately been the case regardless of which polymerase was used. The clone, however, is now available for these experiments and has been checked by DNA sequencing to assure that there are no mutations in the PCR-amplified *ATS1* gene.

The next step is to transform the expression clones for both Ats1p (pAC341) and the expression clone for Prp20p (pAC215) into the *E. coli* expression strain BL21(DE3). Individual transformants will be tested for the expression of the appropriate protein initially by simply comparing the protein patterns from induced and uninduced cultures on SDS-PAGE gels. Expression of the 6-His-tagged proteins will then be confirmed by immunoblotting with a commercially available anti-His antibody. Previous experiments in the Corbett lab suggest that 6-His-Prp20p should be found in the soluble fraction when the *E. coli* are lysed. This will be tested for Ats1p as well. If both proteins are soluble, it is a straightforward matter to purify the 6-His tagged proteins using a Ni-NTA matrix (Qiagen). These purified proteins will then be used to carry out nucleotide exchange activity assays using purified Ran that is already available in the laboratory.

The nucleotide exchange experiments will be conducted in the following manner, which is essentially as described by Bischoff and Ponstingl (6). Purified Ran protein will be incubated with [^{3}H]GDP. This charged Ran will then be incubated with various amounts of either buffer, Prp20p, or Ats1p over a time course in the presence of excess unlabeled GTP. The amount of exchange that occurs will be quantitated by a filter binding assay. At each time point a portion of the reaction will be vacuum filtered through a 0.45 µm nitrocellulose filter. Protein will bind to the filter while free nucleotide will pass through. Thus, any protein associated radioactivity will remain bound to the filter and can be

quantitated by scintillation counting. Rates of exchange will be determined by plotting time *vs* ln (C_t/C_0) where C_t= number of counts at any particular time point and C_0= number of counts at time zero.

Three outcomes are possible for the exchange assay: 1) Ats1p may not stimulate nucleotide exchange on Ran; 2) it may stimulate as well or better than Prp20p (~10^5-fold); or 3) it may stimulate the exchange activity but not as well as Prp20p. If Ats1p does not stimulate guanine nucleotide exchange on Ran this might suggest either that Ats1p is not an exchange factor or that is specific for some G-protein other than Ran. If this is the outcome of the assay, it will also be necessary to consider the possibility that the recombinant epitope-tagged Ats1 protein is not functional. This is currently extremely difficult to test as there are no activities yet known for Ats1p. If Ats1p is comparable to Prp20p at stimulating exchange on Ran, it will be necessary to carry out *in vivo* experiments to determine whether Ats1p does act on Ran *in vivo*. Finally, if Ats1p has an intermediate ability to enhance the exchange rate on Ran, this could suggest that Ats1p is indeed an exchange factor for a G-protein but perhaps Ran is not its *in vivo* target. Alternatively, it would again be necessary to consider the fact that the assays are being carried out with a recombinant protein that may not retain its full functionality. Regardless of the results, these experiments should lend insight into the function of Ats1p.

As a complement to this biochemical approach, future experiments in the Corbett laboratory will use a genetic approach to characterize the *in vivo* role of Ats1p. The laboratory has generated a deletion strain that lacks the *ATS1* gene and this strain will be used as the basis for a synthetic lethal screen. The combination of these genetic and biochemical approaches should ultimately identify an *in vivo* role for Ats1p.

References

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