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. Results indicate that Ats1p can stimulate nucleotide exchange on Ran but it does so much less efficiently than Prp20p. This finding suggests that Ats1p may be a nucleotide exchange factor for a G-protein but perhaps the G-protein is not Ran. Thus, some other cellular GTP-binding protein may be the *in vivo* target for Ats1p.

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 which 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.

We were able to express and purify a 6-His-tagged version of the Ats1 protein in *E. coli* using an established protocol. This protein was found to modestly stimulate guanine nucleotide exchange on Ran, approximately 100-fold as compared to 10^5-fold stimulation for Prp20p.

Methods and Results

The Ats1 protein was expressed with an N-terminal 6-His tag. 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 expression plasmid for Ats1p was generated by amplifying the *ATS1* coding region by PCR with Vent polymerase (New England Biolabs) using oligos AC135 and 136 engineered with a 5'-*BamH*I site and a 3'-*Hind*III site, respectively. The template used for the PCR was the plasmid pAC214 which contains the genomic *ATS1* clone. The resulting PCR product, which was the expected 1.1 kb in length, was digested with *BamH*I and *Hind*III and cloned into the expression plasmid pET15b to generate pAC341 (Figure 1). Cloning was confirmed initially by miniprepping and finally by sequencing the entire open-reading frame of the *ATS1* gene including the N-terminal 6-His tag. For control exchange experiments the Prp20p protein was expressed from the existing expression construct pAC215. Purified Ran protein was available in the laboratory.

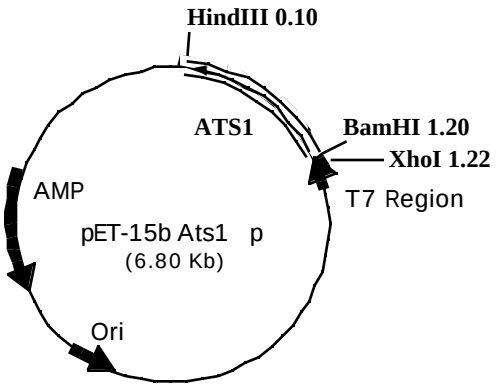

Figure 1. Schematic of Ats1p expression plasmid.

Both the pAC341 (Ats1p) and the pAC215 (Prp20p) expression plasmids were transformed into the BL21 (DE3) expression strain of *E. coli*. Two transformants were chosen for each protein. These transformants were inoculated into 2 ml overnight cultures of Luria Broth containing 100 µg/ml ampicillin (LB-AMP) and grown to saturation. In the morning, the overnight cultures (2 ml) were inoculated into separate 500 ml cultures of LB-AMP. These cultures were grown at 30°C with shaking until they reached an OD600 of 0.375. Cultures were then induced with 0.5 mM isopropylthio-β-D-galactoside (IPTG). One ml samples of the cultures were collected just prior to the addition of IPTG and then at one hour intervals until a three hour induction was completed. Following the three hour induction, the *E. coli* were pelleted by centrifugation at 6000Xg for 15 min at 4°C. Pellets were then stored at -20°C until needed.

To assay protein expression in the induced *E. coli*, the one ml samples were analyzed by SDS-PAGE (12) to determine whether a band of the appropriate size was induced. Each of the one ml samples was pelleted in a microcentrifuge and then resuspended in 50 µl (no induction), 100 µl (1h induction), 150 µl (2h induction), and 200 µl (3h induction) of protein sample buffer (50 mM Tris-HCl, 100 mM dithiothreitol, 2% SDS, 0.1% bromophenol blue, 10% glycerol, pH 6.8) to compensate for the increase in cell number during the logarithmic growth of the culture. Cell pellets were lysed by boiling at

100°C for 10 min in sample buffer. Samples (10µl/lane) were then loaded onto a 10% polyacrylamide gel. Following electrophoresis, proteins were visualized by staining with coomassie brilliant blue. As shown in Figure 2 both of the Prp20p samples induced a band corresponding to the expected size of 6-His-tagged Prp20p (~47 kDa) following a three hour induction. In the case of the Ats1p samples only one culture induced a band of ~38 kDa that corresponds to the expected size of 6-His-tagged Ats1p.

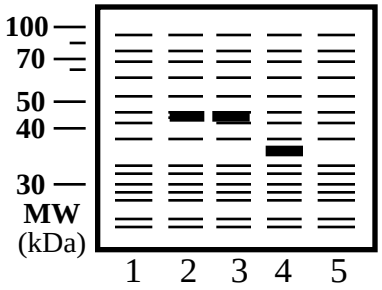

Figure 2. Induction of Prp20p and Ats1p. Lane 1, no induction; Lanes 2 & 3, Prp20p 3h induction; Lanes 4 & 5, Ats1p 3 h induction.

Immunoblotting was used to confirm that the bands observed correspond to the Prp20 and Ats1 proteins. A commercially available anti-His antibody (Qiagen) was used to blot samples that were resolved by SDS-PAGE and transferred to nitrocellulose by standard methods (13). Bands corresponding to the predicted size for both 6 His-Prp20p and 6-His-Ats1p are recognized by the anti-His antibody (Figure 3).

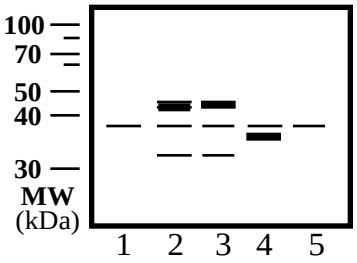

Figure 3. Detection of induced proteins by anti-His Antibodies. Lane 1, no induction; Lanes 2 and 3, Prp20p 3h induction; Lanes 4 and 5, Ats1p 3 h induction.

The protocol for purification of the 6-His tagged proteins was essentially as described in for the Invitrogen X-press system protein purification protocol. This protocol has been used successfully by the Corbett laboratory in the past to express functional 6-His-tagged Prp20p. Pellets corresponding to the expressed Prp20p and Ats1p were resuspended in 10 ml native binding buffer (20 mM $NaPO_4$. pH 7.8, 500 mM NaCl). Cells pellets were lysed by five 30 s pulses with a Biosystems microprobe sonicator. Soluble and insoluble fractions were separated by centrifugation at 6000Xg (5000 RPM in the GSA rotor) for 15 min at 4°C. The supernatant was then poured into a new bottle and recentrifuged to remove any particulate matter. This soluble fraction was then analyzed as described above for the presence of the recombinant Prp20p and Ats1p. Previous experiments in the laboratory suggested that both proteins were soluble under these conditions and this was confirmed (See Figure 4).

In order to purify the tagged proteins, the soluble fractions from both proteins were bound in batch to 1 ml Ni-NTA agarose (Qiagen) that had been pre-equilibrated in native binding buffer. Binding was carried out in a 15 ml conical tube at 4°C for one hour. The matrix was then pelleted by a 5 min spin at 1000Xg. Non-specific proteins bound were removed by three 10 ml washes with wash buffer (20 mM $NaPO_4$, pH 6.0, 500 mM NaCl). These washes were followed by a single wash with stringent wash buffer (20 mM $NaPO_4$, pH 5.5, 500 mM NaCl). Samples were then eluted by incubation with 2 times 2 ml of elution buffer for 10 min each (20 mM $NaPO_4$, pH 4.0, 500 mM NaCl). These samples were pooled and concentrated with Centriprep-30 (Amicon, Inc.). Final protein concentrations were determined using the Bio-Rad protein assay using purified BSA as a standard. Purity was also assessed by analyzing the final concentrated samples by SDS-PAGE followed by silver staining (Figure 4).

With the purified proteins in hand, the goal was to test whether Ats1p acts as a guanine nucleotide exchange factor for Ran.

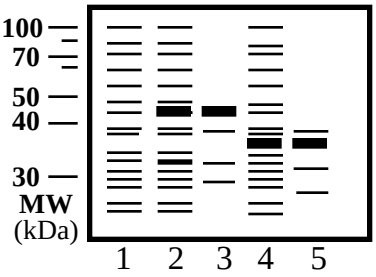

Figure 4. Silver stained gel of purified proteins. Lane 1, uninduced lysate; Lane 2, induced Prp20p lysate soluble fraction; Lane 3, purified 6-His-Prp20p; Lane 4, induced Ats1p lysate soluble fraction; Lane 5, purified 6-His-Ats1p

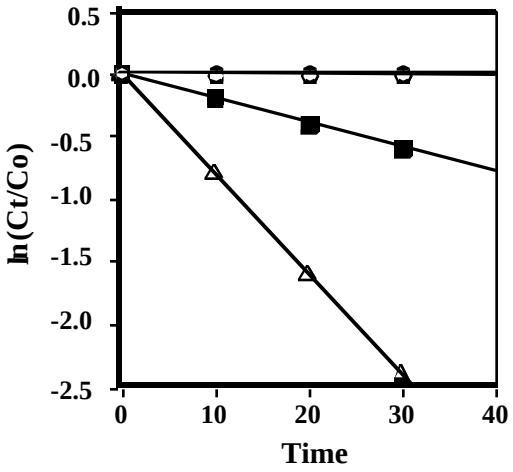

Figure 5. Activity assay for exchange activity of Ats1p. Assays were carried out as described in the text. Results are plotted as rates of exchange of GTP for [^{3}H]GDP on Ran in the presence of buffer (O) or 50 fmol 6-His-Prp20p (Δ) or 6-His-Ats1p (■).

Exchange assays were carried out as described by Bischoff and Ponstingl (6). Purified recombinant yeast Ran protein (~150 pmol) was charged with 200 pmol radiolabeled [^{3}H]GDP in 200 µl of binding buffer (200 mM $NaPO_4$, pH 7.0, 1 mM DTT) for 30 min at 30°C. The charged Ran was then purified and washed into assay buffer (20 mM Tris-HCl, pH 7.5, 100 mM NaCl, 5 mM $MgCl_2$, 1 mM DTT) on a NAP-5 gel filtration column (Pharmacia). The flow through from the column was brought to a total volume of 1.5 ml with assay buffer. To 350 µl samples was added 5 µl of 1 mM unlabeled GTP and either After incubation at 30°C for the time indicated , the ratio of nucleotide bound to Ran at the time t to the amount bound at t_0 (C_t/C_0) was determined by vacuum filtering 50 µl samples through nitrocellulose (Schleicher & Schuell, 0.45 µM), washing with assay buffer, and measuring the protein-bound radioactivity retained by the filter. The data was plotted as the $\ln(C_t/C_0)$ vs time (Figure 5) to indicate the rates of exchange.

Discussion

Our results indicate that the rate of nucleotide exchange induced on Ran by purified Ats1p is not comparable to those observed for the known Ran exchange factor Prp20p. In our control experiments Prp20p stimulated the rate of nucleotide exchange on Ran by approximately 10^5, a fold-stimulation which is consistent with that observed by previous researchers (4). Ats1p, however, only stimulated the exchange activity by approximately 100-fold as compared to buffer. These results suggest that perhaps Ats1p does indeed act as an exchange factor for a G-protein but perhaps its true *in vivo* substrate is not Ran but some other cellular G-protein.

There are several considerations with regard to the exchange activity observed for Ats1p. The protein used for these experiments is a recombinant tagged protein so it may not retain its full activity. As of yet, the Corbett lab has not identified an *in vivo* phenotype for strains lacking the *ATS1* gene so it is difficult to test whether the N-terminal tag might interfere with protein function *in vivo*. In addition, these experiments employed the human Ran protein as the substrate. Human Ran and *S. cerevisiae* Ran

(encoded by *GSP1*) are 85% identical at the amino acid level so this is unlikely to influence the outcome of the experiments, but they should be repeated with yeast Ran that is now available in the lab.

The finding that the *in vivo* substrate for Ats1p may not be Ran is consistent with preliminary localization data obtained for Ats1p. The Ran protein is primarily nuclear although a fraction is also found in the cytoplasm (8). When the Ats1p protein is tagged at the C-terminus with the naturally fluorescent green fluorescent protein (GFP), it appears to localize to mitochondria (Ahktar and Corbett, unpublished results). Thus it seems likely that the Ats1 protein may play a role in regulating the nucleotide-bound state of a mitochondrial G-protein.

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. In addition, given the recent information regarding the localization of Ats1p, the strain lacking *ATS1* will be tested for mitochondrial phenotypes such as the inability to grow on non-fermentable carbon sources (i.e. glycerol). The combination of these genetic and biochemical approaches should ultimately identify an *in vivo* role for Ats1p.

References

1. M. P. Rout, S. R. Wente, *Trends Cell Biol.* **4**, 357-365 (1994).
2. A. H. Corbett, P. A. Silver, *Microbiol. Mol. Biol. Revs.* **61**, 193-211 (1997).
3. M. S. Moore, G. Blobel, *Trends Biol. Sci.* **19**, 211-216 (1994).
4. C. Klebe, T. Nishimoto, F. Wittinghofer, *Biochemistry* **32**, 11923-11928 (1993).
5. A. H. Corbett, et al., *J. Cell Biol.* **130**, 1017-1026 (1995).
6. F. R. Bischoff, H. Ponstingl, *Nature* **354**, 80-82 (1991).
7. D. M. Koepp, P. A. Silver, *Cell* **87**, 1-4 (1996).
8. P. Belhumeur, et al., *Mol. Cell. Biol.* **13**, 2152-2161 (1993).
9. A. K. Hopper, H. M. Traglia, R. W. Dunst, *J. Cell Biol.* **111**, 309-321 (1990).
10. M. Fleischmann, et al., *Mol. Gen. Genet.* **227**, 417-423 (1991).
11. D. Kirkpatrick, F. Solomon, *Genetics* **137**, 381-392 (1994).
12. U. K. Laemmli, *Nature* **227**, 680-685 (1970).
13. H. Towbin, T. Staehelin, J. Gordon, *Proc. Natl. Acad. Sci. USA* **76**, 4350-4354 (1979).