Characterization of the RAD6 homologue in *Tetrahymena thermophila*

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Abstract:

RAD6 is ubiquitin conjugating enzyme known to play a role in post replication repair in yeast. Post replication repair allows a means for cells to bypass DNA damage during replication in what is known as a “stop gap” measure. RAD6 uses its ubiquitin conjugating capability to promote post replication repair. It was the goal of this project to create two tagging constructs for the *T. thermophila* homologue of RAD6 and to determine expression of RAD6 using real-time PCR. The tagging constructs included a Flag-HisX6 tag that will be used for immunoprecipitation and western blotting and red fluorescent pigment (RFP) that will be used for determining the localization of RAD6 under various conditions. Western blotting confirmed the transformation of the FLAG-HisX6 construct into *T. thermophila*. qPCR revealed that RAD6 may be highly expressed following UV damage. This may indicate that RAD6 in *T. thermophila* is involved in post replication repair.
Introduction:

RAD6 was the first gene found to have a role in post replication repair. The term postreplication repair (PRR) is sort of a misnomer. The process does not actually repair damage and occurs during repair. This process is used by the cell to bypass damage during replication. This allows a cell to continue replication even in the presence of DNA lesions like pyrimidine dimers. By bypassing the damage the cell is capable of continuing replication without the replication machinery separating from the DNA. RAD6 uses its ubiquitin conjugating capabilities to promote PRR (Anderson et al., 2008).

Ubiquitin is a small peptide that can be covalently attached to proteins via lysine residues. The process of ubiquitinating a protein requires several steps and is ATP dependent, Figure 1. The first step in the process uses ATP to conjugate a single ubiquitin at its carboxy terminal glycine to a cysteine within the active site of the E1 enzyme, also known as an ubiquitin-activating enzyme. The next step involves a transfer of the ubiquitin form the E1 enzyme to an active site cysteine on an E2 enzyme, also known as an ubiquitin-conjugating enzyme. Finally an E3 enzyme forms a complex with the substrate protein and the ubiquitinated E2. This allows the transfer of the ubiquitin from the E2 to the substrate protein, forming a linkage between a lysine on the substrate and the carboxy terminal glycine of ubiquitin. This process can be repeated to yield a polyubiquitinated substrate (Nobelprize.org)
RAD6 has been shown to form a complex with RAD18 an E3 ubiquitin ligase (Bailly et al., 1994). This complex is capable of ubiquitinating PCNA (Hoege et al., 2002). PCNA plays an important role in replication. It acts as a clamp that holds the polymerase tightly on the DNA strand. Monoubiquitination of PCNA by RAD6/18 allows the replication machinery to bypass damage through PRR. This process has been well defined in lower eukaryotes like budding yeast but has not been shown in higher eukaryotes (Anderson et al., 2008).

The goal of this project is to characterize the closest RAD6 homologue in *Tetrahymena thermophila*. This organism was chosen because it is more closely related to higher eukaryotes than budding yeast. Bioinformatics has shown that THERM_00550720 is the closest homologue of RAD6 in *Tetrahymena*. The gene for RAD6 was previously cloned into a pENTR plasmid by BMS 110 honors students; Kelsey Marquardt and Kelsey Harner. This plasmid was sequenced to confirm that it contains RAD6 in an un-mutated form. The pENTR-RAD6 was used to produce Flag-HisX6 (FH6) and red fluorescent pigment (RFP) tagging constructs of RAD6.
Both of these constructs produce N-terminally tagged RAD6 and were transformed into \textit{T.t.} using biolistic transformation. Real-time PCR primers were constructed to determine the expression of RAD6 under various conditions. These primers were used in Real-time PCR (qPCR) to determine expression in \textit{T.t.}

**Materials and Methods:**

**Plasmid isolation and digest:** The 5 Prime FastPlasmid mini kit was used to isolate pENTR-RAD6. As described in BMS 558 Lab Protocols, boil preps were used to isolate pBMFH6-RAD6 and pBMRFP-RAD6 plasmids. The procedure for this is as follows: a liquid culture containing the plasmid of interest was grown overnight culture. The culture was centrifuged at maximum speed for 2 minutes. The supernatant was then decanted and resuspended in sucrose lysis buffer. Lysozyme was then added and allowed to incubate at room temperature for 5 minutes. The sample was then heated to 99 °C for 1 minute followed by centrifugation for 15 minutes at maximum speed. The pellet of cellular debris was removed with a toothpick. The DNA was precipitated for 5 minutes at room temperature using NaOAc and isopropanol. The sample was centrifuged for 10 minutes. The supernatant was discarded and the pelleted DNA was washed with 70% ethanol. The pellet was resuspended in Tris-EDTA.

The isolated plasmid DNA was digested with various restriction endonucleases to confirm its identity using the protocol described in BMS 558 Lab Protocols. The digest reactions consisted of 1X buffer, 1X BSA, 0.5 μL of each enzyme (New England Biolabs) and 3 μL of plasmid. Gene Construction Kit was used to predict the digestion product sizes. To confirm the pENTR-RAD6 plasmids, the plasmids were digested with Nhe1 and Xba1. To confirm the RAD6-
FH6 construct, the plasmid was digested with NsiI and PvuI. The RAD6-RFP construct was confirmed by digesting the plasmid with NsiI.

**LR Clonase™ reaction:** This procedure was performed as described in BMS 558 Lab Protocols. 150 ng of pENTR-RAD6 plasmid and 200 ng pMTFH6-GTW or 400 ng of pbs2_MCherry (pBMRFP-GTW) were added to 4 μL of H2O and 1 μL 5x LR Clonase™II enzyme. The reaction was incubated at room temperature overnight and then stored at -20 °C. Proteinase K solution was added and incubated at 37 °C for 10 minutes.

**Production of electrocompetent cells:** This procedure was performed as described in BMS 558 Lab Protocols. 10mL culture of DH10B was grown in SOB + MgCl₂. This was used to inoculate a 1 L culture of SOB. This culture was grown to an O.D. of 0.8-1.0. Cells were pelleted at 3000 g at 4 °C for 10 minutes. The cells were resuspended in cold ddH2O and pelleted this process was repeated once more. Cells were then resuspended in 20 mL of cold 10% glycerol. The cells were pelleted once more and aliquotted into 1.5 mL microcentrifuge tubes. The cells were frozen using dry ice in ethanol and then stored at -80 °C.

**Electroporation of E. coli:** This procedure was performed as described in BMS 558 Lab Protocols. DH10B cells and plasmid DNA were thawed on ice. 50 μL of DH10B cells and 1 μL of DNA were added to a chilled 1 mm electroporation cuvette. Cells were electroporated at the following conditions 2.5 kV, 200 ohms and 25 μF for 3-4 ms. 0.5 mL of SOC media was used to remove the cells from the cuvette. The cells were incubated for 1 hour at 37 °C. Cells were plated on LB plates with ampicillin or kanomycin.

**Bioinformatics:** Analysis of RAD6 bioinformatics was carried out using the protocol described in BMS 558 Lab Protocols. The amino acid sequence of *T.t.* RAD6 was analyzed using
BLASTp to determine its closest homologues. The amino acid sequences of *T. t.* RAD6, *D. m.* RAD6a, *H. s.* RAD6b, *S. c.* RAD6, *D. m.* RAD6b, *S. p.* E2Rhp6 (RAD6), *A. t.* E2(RAD6), TTHERM_00450870, TTHERM_00547960, TTHERM_00659090, TTHERM_00659080, and TTHERM_01053020 were aligned using Tree-based Consistency Objective Function For alignment Evaluation (TCOFFEE). The molecular phylogeny of the sequences was then evaluated using Multiple Sequence Alignment-CLUSTALW. The CLUSTAL protein alignment was performed using a gap open penalty of 10, a gap extension penalty of 0.05, a hydrophobic gap, no weight transition, and a BLOSUM weight matrix. Distances were computed using the Poisson Correction Distance method in Molecular Evolutionary Genetic Analysis (MEGA) software version 5.0. The unweighted-pair group method using average linkages tree was constructed with 500 bootstrap replicates.

**Protein Isolation:** Cells were grown to a density of 1-3x10^5 cells/ml. For immunoprecipitation experiments 200 mL of cells were grown for confirmation of 2-HA transformants smaller cultures of 20-50 mL were grown. Cells were treated with CdCl₂ at 1.0 µg/mL to induce production of 2-HA tagged Thd14. Cells were centrifuged at 3000 RPM for 3 minutes, the supernatant was removed and cells were resuspended in 30 ml of 10 mM Tris (pH7.5). The cells were centrifuged/decanted a second time and resuspended in 1 mL of Tris. Cells were transferred to a 1.5 mL centrifuge and spun at 5000 RPM in microfuge for 2 minutes. The supernatant was removed and 600 µl of Breaking Buffer (350 mM NaCl, 40 mM HEPES, 1% TritonX-100, 10% glycerol, 1 mM DTT and water) with Protease inhibitors was added. The cells were vortexed for 1 minute in cold-room (4 °C). Cellular lysate and debris was spun for 5 minutes at max speed at 4 °C and supernatant was transferred to a new 1.5 ml tube. Cellular
lysate and debris was spun again at max speed for 15 minutes; the supernatant was added to the previous. Total protein was quantitated using Bradford protein assay reagent and a standard curve made from BSA. Isolated proteins were stored at -80 °C. The isolated proteins were used for Western blotting.

**SDS-PAGE:** 12% polyacrylamide gel was used to analyze the proteins isolated during the previous procedure. Samples were loaded to maintain a set concentration for all samples. Proteins were separated at 225 v through the stacking gel and 250 v through the resolving gel.

**Western Transfer:** Gel, nitrocellulose membrane and two pieces of extra thick blotting paper were soaked in transfer buffer for 10 minutes. After soaking one piece of blotting paper was placed on the positive plate of a semi-dry gel transfer rig. On top of the paper was added the membrane, followed by the gel and finally the second piece of blotting paper. A roller was used to eliminate any bubbles that might have formed during the stacking process. The gel was transferred at 25 volts for 1 hour. After the gel had been transferred the membrane was stained briefly with Ponceau. This was done to confirm the transfer process was successful. Excess stain was washed away and the membrane was used for Western blotting.

**Western Blot:** The membrane was first blocked in 5% non-fat milk (in TBS) overnight at 4 °C. The next day the milk was poured off and 1:5000 dilution of monoclonal anti-Flag antibody in 5% milk in TBST was added. The membrane was allowed to incubate while shaking overnight at 4°C. The primary antibody was removed and the membrane was washed 4 times with TBS, each wash was five minutes. After the final wash, 5% milk with 1:5,000 anti-mouse antibody was added and allowed to incubate for 1 hour at room temperature. The secondary antibody was poured off and the membrane was washed three times with TBS. A working solution of 300
µL SuperSignal West Dura Luminol/Enhancer Solution + 300 µL of SuperSignal West Dura Stable Peroxide Solution was added to the transfer. The membrane was covered with plastic wrap, and exposed to X-Ray film in a dark room. The film was developed.

**Biolistic Transformation:** Prior to transformation a plasmid DNA was isolated from one RAD6-FH6 clone and one RAD6-RFP clone. The plasmid DNA was digested with EarI/BplI in the case of the RFP construct and Sacl/KpnI for the FH6 construct. The linear plasmid was then transformed into the background strains Cu522 (RAD6-FH6) and Cu428 (RAD6-RFP), using biolistic transformation as follows: Cultures were grown to 1-3 x 10^5 cells/mL. The media was removed and replaced by 10 mM Tris-HCl pH 7.5. The cells were allowed to starve in this solution at 30 °C for 18 hours. Following starvation the cells were counted and concentrated to 1 x 10^7 cells/mL. DNA to be transformed was coated onto gold beads using the following steps: To a tube containing 25 µl of AU Beads, 5 µl of the DNA (1.0 µg/µl) was added. Then 25 µl of 2.5 M CaCl_2 and 10 µl 100 mM spermidine was added. The mixture was then vortexed for 30 minutes. Samples were then centrifuged 2-3 seconds and the supernatant removed and 100 µl of 70% ETOH was added. Samples were then centrifuged 2-3 seconds and the supernatant removed and 100 µl of 100% ETOH was added. The sample was then centrifuged 2-3 seconds and the supernatant removed and 25 µl of 100% ETOH was added. To transform the cells the following steps were employed: 1 mL of cells was placed onto a filter paper that had been soaked with 2 mL of 10 mM HEPES and placed in an upside down Petri dish. This was inserted into the gene gun along with a macrocarrier coated with 12.5 µL of DNA on gold beads (from above). The DNA was shot into the cells using a gene gun. The chamber of the gene gun was placed under a vacuum at 27 mmHg. The gun shot at a pressure of 900 mmHg provided by a
helium tank. The cells were then transferred along with the filter paper into 50 mL of pre-warmed 2% PPYS + PSF. The cells were allowed to recover at 30 °C for 3 hours in the case of paclitaxel treated cells and 4 hours for cyclohexamide treated cells. The cells were then drugged and plated on 96 well plates, 100 μL/well. Paclitaxel was administered at 20 μM while cyclohexamide was used at 125 μg/mL. Surviving cells were further selected with drug before confirming transformation success. Transformation success was confirmed via Western blot for the FH6 construct and fluorescence microscopy for the RFP construct.

**Primer Dilution and Annealing Temperature Determination:** Stocks were resuspended in water to a final concentration of 200 pmol/μL. A working solution of primers was made at a concentration of 20 pmol/μL. A PCR “master mix” was made containing 1X GoTaq Green Master Mix, 0.4 pmol/μL forward and reverse primers, 12.8 ng/μL gDNA (Cu428) and water for dilution. The master mix had a final volume of 100 μL it was aliquotted in to 4 PCR tubes containing 25 μL. Each PCR tube was loaded in to a thermocycler (Bio-Rad, Mj mini Personal Thermocycler) set to the following parameters: Step 1. 95 °C for 2 min., Step 2. 95 °C for 45 s., Step 3. annealing temperature gradient (example 52.0, 53.7, 57.0 or 59.5 °C), Step 4. 72 °C for 1.75 min., Step 5. repeat steps 2-4 32 times, Step 6. 72 °C for 5 min., Step 7. hold at 4 °C. All 25 μL of the PCR reaction was loaded onto a 2% agarose gel containing ethidium bromide and electrophoresed. The gel was then imaged. The annealing temperature that provided the best amplification was used for further experiments.

**qPCR:** This technique was used several times during this research the following parameters were used for most experiments. The protocol provided with the SsoFast EvaGreen supermix from BioRad was used for this experiment. A PCR “master mix” was made containing
1X SsoFast EvaGreen supermix with low ROX, 0.5 pmol/µL forward and reverse primers, and water for dilution. The master mix was aliquotted into PCR tubes. 1.0 µL of gDNA or cDNA was loaded into each tube. Each PCR tube was loaded into a thermocycler (Bio-Rad, Mj mini Personal Thermocycler with Mini Opticon RT-PCR system) set to the following parameters: Step 1. 98.0 °C for 2 min., Step 2. 98.0 °C for 0:05 min., Step 3. Annealing temperature 54.0 °C for 0:20 min, Step 4. repeat steps 2 and 3, 39 times, Step 5. 54.0 °C for 0:10 min., Step 6. 95.0 °C until products melt completely. Starting quantity of DNA was used to determine normalized and relative expression.

**Sequencing of pENTR-RAD6:** DNA sequencing was carried out at the MU DNA Core Facility. Reactions containing either M13F or M13R primers and plasmid DNA were sent to the facility for sequencing.

**Cryopreservation of E. coli containing pBMTFH6-RAD6 and pBMTTCherry-RAD6:** Liquid cultures were started from colonies streaked during previous experiments. The cultures were grown in 2 mL of LB media with ampicillin. The cultures were allowed to grow overnight at 37 °C overnight. The next day 700 µL of cells was mixed with 700 µL of 50% glycerol in a cryotube. The cells were frozen at -80 °C.

**Results:**

**Bioinformatics:** The complete amino acid sequence of the *T.t.* RAD6 protein was obtained from Tetrahymena Genome Database (TGD) and compared to amino acid sequence of RAD6 from other organisms using MEGA 5.0 and T-COFFEE. The T-COFFEE results (data not shown) showed that TTHERM_00550720 (*T.t.* RAD6) is more closely related to RAD6 in other organisms than the other *T.t.* RAD6 homologues. A phylogenetic tree was constructed using
MEGA 5, Figure 2. The tree was constructed using the unweighted pair group method with arithmetic mean (UPGMA) with bootstrapping. The tree confirms the T-COFFEE results and indicated that *T.t.* RAD6 is most closely related to RAD6 in *Arabidopsis thaliana*. The tree also shows that the other RAD6 homologues in *T.t.* do not branch with the RAD6 homologues from other species. This indicates they are not as closely related.

![Phylogenetic tree](image)

Figure 2: The evolutionary history was inferred using the UPGMA method. The optimal tree with the sum of branch length = 3.99933560 is shown. The percentage of replicate trees in which the associated taxa clustered together in the bootstrap test (500 replicates) are shown next to the branches. The tree is drawn to scale, with branch lengths in the same units as those of the evolutionary distances used to infer the phylogenetic tree. The evolutionary distances were computed using the Poisson correction method and are in the units of the number of amino acid substitutions per site. The analysis involved 13 amino acid sequences. All positions containing gaps and missing data were eliminated. There were a total of 116 positions in the final dataset. Evolutionary analyses were conducted in MEGA5

ExPASy PROSITE was used to determine the structural motifs/domains within each RAD6 homologue described in the phylogenetic tree. The results are shown in Table 5. The analysis shows that all of the proteins studied possessed the same ubiquitin conjugating domain. This indicates that all of the proteins are likely E2 enzymes.
<table>
<thead>
<tr>
<th>Species</th>
<th>Protein Name</th>
<th>Structural Diagram</th>
<th>Domain Location (a.a.)</th>
</tr>
</thead>
<tbody>
<tr>
<td>S. pombe</td>
<td>E2 Rhp6</td>
<td><img src="image" alt="Diagram" /></td>
<td>7-139</td>
</tr>
<tr>
<td><em>Saccharomyces cerevisiae</em></td>
<td>RAD6</td>
<td><img src="image" alt="Diagram" /></td>
<td>7-139</td>
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<tr>
<td>Homo sapiens</td>
<td>RAD6a</td>
<td><img src="image" alt="Diagram" /></td>
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<td>Homo sapiens</td>
<td>RAD6b</td>
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<tr>
<td><em>Drosophila melanogaster</em></td>
<td>RAD6a</td>
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<tr>
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<td><img src="image" alt="Diagram" /></td>
<td>1-106</td>
</tr>
<tr>
<td>Arabidopsis thaliana</td>
<td>UCE E2</td>
<td><img src="image" alt="Diagram" /></td>
<td>7-139</td>
</tr>
<tr>
<td><em>Tetrahymena thermophila</em></td>
<td>TTERM 00550720 (RAD6)</td>
<td><img src="image" alt="Diagram" /></td>
<td>7-139</td>
</tr>
<tr>
<td><em>Tetrahymena thermophila</em></td>
<td>TTERM 00659080</td>
<td><img src="image" alt="Diagram" /></td>
<td>10-148</td>
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<tr>
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<td><img src="image" alt="Diagram" /></td>
<td>7-150</td>
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The coding sequence of *T.t.* RAD6 was obtained from the Tetrahymena Genome Database genome browser and used to construct real-time PCR primers. These will be used to determine the expression of the gene at the mRNA level. The coding sequence and primers are shown in Table 2.

|| Coding Sequence of RAD6 (Red = exonic information, underlined = EST compliment) ||
|---|---|
| ATGACAACAGCTGCTAAGAGAGAATTTTAGATTTCCACCAATATGGATTAATCTCTCCTATGGAAGATAATATAATGAACTGGGATGCAAGTAATTTTTGTCAGAAAGACACACTCTGG | GAAAGGTGCAACATTCAGATTAAC |
| GCAACATTACAGATTAACATTAGAGTTCTGAGGATTTATCCCAATAAGCCACCTACTTGTTAGATTAAAAACTACCATTCCACCTAATGTATACACGATGGATCTATATGTTTAGACATTCTTTAAAACTAATGGAGTCCTATTACGATGT | GAAGTGAACGTATAGATGTGAAG |
| TTGGGCGCATTCGATATCAGTCATACCTTTGTGATCCAAATCCAAATAGTCCTGGCAATAGCGAAGCCAAATTGTATTAAGAAGATCGAATATATTCGTAGAGTAAGAGAAATATGAGATGCTGGAATTATCTGA | GAAGTGAACGTATAGATGTGAAG |

Table 2: Real-time PCR Primers for RAD6

<table>
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<th>Forward Primer</th>
<th>Reverse Primer</th>
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<tr>
<td>Sequence (5’-3’)</td>
<td>GAAGTGAACGTATAGATGTGAAG</td>
</tr>
<tr>
<td>Annealing Temp. (°C)</td>
<td>55</td>
</tr>
<tr>
<td>GC Content</td>
<td>39%</td>
</tr>
</tbody>
</table>

Product size = 193 bp
Construction of Flag-His6 and RFP tagged RAD6: Previously T.t. Rad6 was cloned into a pENTR plasmid, the plasmid map of pENTR-RAD6 is shown in Figure 3. This plasmid was stored in E. coli. Plasmid DNA was isolated from the bacteria and digested to confirm that the pENTR-RAD6 plasmid was present and that the gene for RAD6 was in the correct orientation. Gene Construction Kit (GCK) was used to determine the band sizes produced by the digestion of pENTR-RAD6 by Nhe1 and Xba1. The prediction showed bands produced with a size of 2299, 720 and 260 bp. The digestion products were resolved via agarose gel.
electrophoresis. An image of the gel can be seen in Figure 4. The gel shows band sizes of roughly 3000, 700 and 300 bp. The size of the smaller bands is consistent with the GCK predictions. However, the 3000 bp band should be closer to 2300 bp. This indicates that there may be something wrong with the plasmid. However, later digest showed correct plasmid structure. Also the presence of three bands confirms that RAD6 is in the plasmid because it harbored one of the cut sites. This confirms that both pENTR-RAD6 strains from which plasmid DNA was isolated possess the RAD6 plasmid and that RAD6 is in the correct orientation.

Using this information an LR Clonase reaction was setup to create two RAD6 tagging constructs; Flag-HisX6 and Cherry (RFP). Figures 5 and 6 show the predicted maps of the tagging plasmids. The products of the LR Clonase reactions were transformed into the E. coli strain DH10B. The transformed bacteria were allowed to replicate the plasmids before the plasmids were isolated and digested. The digests were setup to confirm that the RAD6 tagging plasmids were present. GCK was again used to predict the sizes of the digestion products. Figure 7 shows the gel that was loaded with digestion products of pBMFH6-RAD6.
Figure 5: pBMFH6-RAD6 plasmid map constructed using Gene Construction Kit. Green = RAD6 gene, Gray = beta-tubulin locus sequence, Pink = MTT1 promoter, Blue = BTU2 Polyadenylation signal

Figure 6: pBSMTmCherry-RAD6 plasmid map constructed using Gene Construction Kit. Green = RAD6 gene, RED = Cherry (RFP) gene, Blue = MTT1 promoter, Light green = RPL29 5’ and 3’ NTS.
The gel shows that pBMFH6-RAD6 clones #2 and #4 were digested to produce bands of 4500, ~2100 and ~1100 consistent with the predictions of GCK. There are several other bands present that likely indicate partial digestion of the plasmid. These results confirm that pBMFH6-RAD6 clones #2 and #4 contain the correct plasmid. Figure 8 shows the gel that was loaded with the digestion products of pBSMmCherry-RAD6. The gel shows bands at 6000, 2500 and 600 bp consistent with the GCK predictions. This indicates that all four clones of pBSMmCherry-RAD6 are correct.

Following confirmation of the tagging plasmid identities, a larger plasmid isolation was performed to obtain enough DNA to transform into *T. thermophila*. The plasmid DNA was isolated using the lysosyme boiling method and was purified using phenol-chloroform extraction. To transform the construct into *T.t.* it first had to be linearized. Restriction enzymes were chosen for each construct that would leave the gene plus the...
targeting sequences flanking it. In the case of the RAD6-FH6 construct the restriction enzymes SacI and KpnI were chosen. Digestion with these enzymes was predicted to produce fragments of 4333 and 2876 base pairs. The RAD-RFP construct was digested with EarI and BlpI. This digestion was predicted to produce bands of 6853, 1804, 637 and 353 base pairs. Figure 9 shows that the digestion of the RAD6-RFP plasmid produced the correct sized products as well as what appears to be undigested plasmid. The smallest product is not visible in the image but this may be a result of poor staining in that portion of the gel. The RAD6-FH6 plasmid shows the appropriate sized products as indicated by the arrows as well as some undigested and overdigested products. Following this confirmation that the constructs were correct and had been linearized properly they were transformed into *T. t.* using biolistic transformation.

The transformation of the tagging constructs into *T. t.* was confirmed first by drug selection. In the case of the RAD6-FH6 construct drug selection was performed using paclitaxel. Incorporation of the construct into the beta tubulin 1 locus affords paclitaxel survivability to the otherwise sensitive strain. Ten cells lines were shown to have drug resistance. Three of these were tested for tagged protein

![Figure 9: 1% agarose gel stained with ethidium bromide. Ladder = 1 kb ladder (NEB). RAD6-RFP was digested with BlpI and EarI, RAD6-FH6 was digested with SacI and KpnI. Stars indicate general location of predicted products. Ladder markers have units of kbp.](image)
using Western blotting. Antibodies against Flag were used to detect expression of FH6 tagged RAD6. The Western blot in Figure 10 confirms that the each cell line tested possessed RAD6-FH6. The RAD6-RFP construct was targeted to the Rpl29 locus. Integration of construct results in cyclohexamide resistance. Only one cell line showed resistance to cyclohexamide following transformation of RAD6-RFP. This construct will be confirmed using fluorescence microscopy at a later date.

![Western blot image](image)

**Figure 10:** Western blot confirmation of RAD6-FH6 expression at the protein level in the background strain Cu522. 80 μg of protein was loaded on each lane. Primary antibody: monoclonal anti-Flag, secondary antibody: anti-mouse conjugated to horseradish-peroxidase. A: 5 second exposure of blot. B: 20 minute exposure of blot.

**Expression of RAD6:** RAD6 is known in other species to play a role in post replication repair. To confirm that this role is conserved in *T.t.* the expression of RAD6 was analyzed via qPCR. Prior to the actual expression experiment primers were made to specifically amplify
RAD6 (see Table 2). These primers were tested at multiple annealing temperatures using conventional PCR with the GoTaq reagent. Four annealing temperatures were used to amplify either gDNA or cDNA. The annealing temperatures were chosen based off of the calculated annealing temperatures of the primers. Figure 11 shows the results of this PCR. The gel shows that each annealing temperature produced the same amount of product. Based off of this information an annealing temperature of 54 °C was chosen for future experiments. The gel also shows that the product is ~200 bp, consistent with the calculated length of 193 bp.

![Figure 11: PCR amplification of RAD6 at multiple annealing temperatures.](image)

To further confirm the capabilities of the RAD6 primers, a trial experiment of qPCR was performed. Four reactions were setup, two samples with gDNA at concentrations of 100 and 10 ng (Std. 1 and Std. 2), a cDNA sample and one sample with no DNA. Figure 12 shows the amplification curve of the experiment produced by the Real-Time PCR. The curve shows that each sample amplified product above threshold. This confirms that the RAD6 primers are capable of being used in qPCR. The amplification of something in the No DNA sample indicates the water used in the reaction was likely contaminated. This was further confirmed by another experiment that showed similar results (data not shown).
The qPCR protocol used in this experiment included a melt curve procedure. The melt curve can indicate the production of different sized products. Figure 13 shows the melt peak from this experiment. The graph shows that the melting temperature of each product was 77.50 °C. This indicates that all of the products were the same.

Following confirmation of the RAD6 primers ability to be used in qPCR an experiment was setup to determine RAD6 expression following DNA damage and in growing or starved
cells. The DNA damage used was ultra violet light. The RAD6 primers discussed previously were used in this experiment as well as primers specific for HHP1. HHP1 is a “housekeeping” gene that has been shown to have constant expression (data not shown). The HHP1 results were used to normalize the expression of RAD6. Figure 14 shows the amplification curve from this experiment. To make the data more interpretable an expression graph was produced, Figure 15. The values in this graph have been normalized to HHP1 and are set relative to the log growing cells. The expression graph shows high expression of RAD6 early after UV treatment. This will be discussed further in the Discussion. Figure 16 shows the melt-peak from this experiment. Consistent with the previous experiment all of the products had a melting temperature of 77.50 °C, meaning they are the same.

Figure 14: Amplification curve of RAD6 expression profile. Colors: STD1 (RED), STD2 (YELLOW), STD3 (BRIGHT BLUE), Log growing cells (DARK GREEN), Starved cells (TEAL), No UV damage (BRIGHT GREEN), 0hr after UV (ORANGE), 1hr after UV (GREY), 2hr after UV (PINK), 3hr after UV (DARK BLUE), and 4hr after UV (PURPLE). RFU = relative fluorescent units.
Figure 15: Expression profile of RAD6. UV treatment was 100 J/cm². Values indicate the starting quantity of RAD6 cDNA as calculated through qRT-PCR normalized using HHP1 and set relative to “No Treatment”.

Figure 16: Melt peak produced in the expression profile of RAD6 experiment. Colors: STD1 (RED), STD2 (YELLOW), STD3 (BRIGHT BLUE), Log growing cells (DARK GREEN), Starved cells (TEAL), No UV damage (BRIGHT GREEN), 0hr after UV (ORANGE), 1hr after UV (GREY), 2hr after UV (PINK), 3hr after UV (DARK BLUE), and 4hr after UV (PURPLE). RFU = relative fluorescent units.

Sequencing of pENTR-RAD6: In order to substantiate future research using the constructs previously described. The cloning plasmid pENTR-RAD6 was sequenced at the University of Missouri DNA Core Facility. The sequencing was carried out using M13 forward and reverse primers. These primers bind regions that flank RAD6 within the pENTR plasmid. The sequencing results shown on the next two pages were analyzed using BLAST. The BLAST analysis compared the sequence results with the predicted sequence of pENTR-RAD6 between
the M13 F and M13 R sites and the RAD6 coding sequence. The BLAST results showed 100% identity between the experimental sequence and the predicted sequence. Confirming that pENTR-RAD6 contains RAD6 without any mutations.
Discussion:

Bioinformatic analysis of T.t. RAD6 and its homologues gave some indication of the role of RAD6 in T.t. ExPASy Prosite analysis showed that T.t. RAD6 contains an ubiquitin conjugating domain, indicating that the protein may function as an E2 enzyme. Phylogenetic analysis showed that T.t. RAD6 is most closely related A.t. E2 RAD6. The A.t. othologue of RAD6 has been shown to be involved in ubiquitination of histone H2B (Xu et al., 2009). This may indicate a potential role for RAD6 in T.t.

To further characterize the role of RAD6 in T.t. two tagging constructs were produced, RFP and FH6. The RAD6-RFP construct will be used in future research to determine the localization of RAD6. While the FH6 construct will be used to determine protein interactions of RAD6 via coimmunoprecipitation. Both of these constructs were produced from the pENTR-RAD6 construct, Figure 3. This construct was sequenced and was found to contain RAD6 without any mutations. This will substantiate further research using these constructs. Western blotting has confirmed that the RAD6-FH6 construct was successfully inserted into the T.t. genome. Fluorescence microscopy will be used to confirm the RAD6-RFP construct.

Real-time PCR was also used in this project to characterize RAD6 role in T.t. RAD6 expression was determined in log growing cells, starved cells and cell treated with UV. Exposure of DNA to UV can produce cyclobutane pyrimidine dimers and 6,4-photoproducts. These types of damage are normally repaired though nucleotide excision repair. RAD6 was shown to have high expression following UV treatment. This could indicate that RAD6 may play a role in nucleotide excision repair. However, RAD6 may be involved in bypassing this damage during
replication, consistent with the role of other RAD6 orthologues. The Real-time PCR will need to be repeated several more times to confirm these results.

In conclusion, this project has laid the framework for the characterization of RAD6 in *Tetrahymena*. 
References:


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