

# **Can Stalling of RNA Polymerase Influence Alternative Splicing?**

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## **ABSTRACT**

**Transcription of messenger RNA (mRNA) is accomplished by RNA polymerase II, a complex enzyme that requires many factors. Many mRNAs require processing, for example introns must be spliced out in order to join the exons, and some of this processing occurs as the mRNA is being made by RNA polymerase. We are studying whether controlling the rate of RNA polymerase II transcription through specific regions of a gene can influence the pattern of splicing. Evidence indicates that slow transcription through an exon leads to inclusion of the exon, while fast transcription can lead to skipping of the exon. To understand if stalling of RNA polymerase II contributes to nearby exon inclusion we are creating a test gene containing a specific pause site called an artificial arrest sequence. If the arrest sequence leads to pausing (but not complete termination) of RNA polymerase II, then inclusion of an extra exon should be observed. Because inclusion of the exon makes an mRNA for a copper resistance protein whereas skipping of the exon makes a nonfunctional mRNA, we can easily monitor the pausing-splicing event by testing for copper resistance. This experiment should allow us to study the role of RNA polymerase pausing in alternative splicing.**

## **INTRODUCTION**

A model has been developed that suggests that the rate of RNA polymerase is a factor in determining exactly how a gene will be spliced (Howe, Kane, and Ares 2003). When RNA polymerase is transcribing slow through a particular exon that exon gets included in the spliced mRNA. Inclusion of the exon is seen because splicing decisions concerning the first intron could be made before the second intron has time to be synthesized. In particular, the U2 snRNP recognizes the branch point of the first intron before the branch point of the second intron is synthesized. This allows for the pairing of the U2 snRNP and the U1 snRNP to initiate the start of splicing (Woolford 1989). When RNA polymerase is transcribing quickly through a particular exon that exon could be skipped in the mature mRNA. This might occur because the second intron is synthesized before the decisions leading to commitment to splice the first intron are made. This creates a competition between the branch point of the first intron and the branch point of the second intron (Howe et al. 2003)

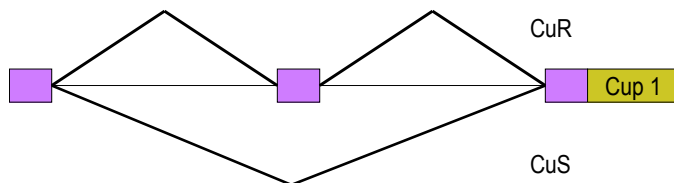
To test this model we inserted an artificial arrest site (ARTAR sequence (Dmitry Kulish and Kevin Struh 2001)) into the second intron of a reporter gene. This sequence has been shown to reduce RNA read through of a gene and appears to create a paused polymerase complex. If true, this should increase the chance that the first intron will be recognized, allowing for the inclusion of the second exon. If the second exon is included we should see copper resistance from the reporter gene we constructed. Our hypothesis is that alternative splicing can be regulated by controlling the stalling of RNA polymerase. We inserted a pause site into the second intron of a reporter gene to measure if exon inclusion increases. If regulation of alternative splicing can be achieved it will give us more insight as to exactly how the cell turns on and off its alternative splicing machinery.

## MATERIALS AND METHODS

### Designing the Reporter Gene

In order for the reporter to accurately report what kind of splicing is occurring, we needed the reporter to have a low amount of inclusion of the second exon, confer copper sensitivity when transformed into our model organism, *Saccharomyces cerevisiae* or budding yeast, and possess a convenient cut site

for insertion of the artificial arrest sequence. The original gene, GL8a-1 had none of these characteristics. The GL8a-1 gene consistently spliced the second exon in; producing copper resistant strains of yeast. This gene had to go through extensive modifications, the first of which was a mutation in the intron's secondary structure (Howe et al. 1997). Under normal circumstances, the secondary structures of each intron paired with itself and create a hairpin structure which promotes inclusion of the second exon. In order to knock out this secondary structure, mutations were made that promoted pairing of the introns with each other instead of with themselves. This mutation was called 6171A and effectively knocked down inclusion of the second exon but it did not completely knock it out (Howe et al. 2003). The next step was to create deletions of the regulatory regions within the introns. Removal of the regulatory regions within the introns also created a convenient site to put the artificial arrest sequence. After the regulatory regions of the introns were deleted, the gene was named 6171A $\Delta$ X. To test if the reporter gene was, in fact, skipping the second exon more frequently we performed three experiments: plating transformed yeast on various concentrations of copper, reverse transcriptase PCR, and primer extension.



**FIG 1.** When the second exon is included, the CUP1 gene is expressed leading to copper resistance. When the second exon is excluded the CUP1 gene is not expressed leading to copper sensitivity.

## **Copper Resistance and Splicing Patterns**

We transformed yeast with a plasmid containing the 6171A $\Delta$ X gene or the 6171A CUP fusion gene as well as a uracil marker that allowed us to select for yeast that have taken up the plasmid (Chun-Ming Lin and Daniel J. Kosman 1990). We then plated the yeast on copper plates with various concentrations of copper and incubated them overnight (Fig 3). The copper plate with a 0.3mM concentration had neither strain growing on it, suggesting that the copper concentration was too high even with the Cup1 gene being expressed. The copper plate with a concentration of 0.1mM looked just as we had expected: the colonies containing 6171A $\Delta$ X gene had not survived while the colonies transformed with the 6171A gene had. The control for this experiment was plating both types of yeast on 0mM copper plates and the result was that both colonies grew indicating that both colonies are able to grow and it is only the copper that is inhibiting growth of the yeast transformed with the 6171A $\Delta$ X gene. From this experiment we concluded that the gene containing intron deletions, 6171A $\Delta$ X, successfully excludes the second exon therefore not expressing the CUP1 gene and not conferring transformed yeast with copper resistance.

## **RT-PCR and Primer Extension**

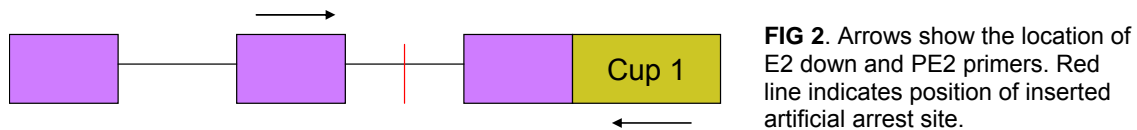
The results from the copper plating indicated that there was inclusion of the second exon in the 6171A gene while there was skipping of the second exon in the 6171A $\Delta$ X gene. In order to quantify these findings, a primer extension was performed. During primer extension, a radioactive DNA primer that is annealed to an mRNA transcript, reverse transcriptase is added and a cDNA is made. Analysis of the size of the cDNA through gel electrophoresis will indicate the types of splicing that occurred on a gene within a cell. The results of this experiment indicated that in the 6171A gene there was about 53% inclusion of the second exon, whereas in the 6171A $\Delta$ X gene there was only about 7% inclusion of the second exon (Fig 4).

We then decided to do an RT-PCR to be absolutely certain that our reporter gene was in fact splicing the second exon out. In RT-PCR, mRNA is reverse transcribed into cDNA then amplified using the polymerase chain reaction. The results are then run on an agarose gel using gel electrophoresis. The results from our experiment indicated that there was a greater amount of inclusion of the second exon in the 6171A gene and a greater amount of exclusion of the second exon in the 6171A $\Delta$ X gene (Fig 5). These two experiments led us to believe that we had successfully reduced inclusion of the second exon.

## **Constructing the Reporter Gene**

Now that we knew our reporter gene was working correctly, it was time to insert the artificial arrest sequence into the second intron of the gene. To do this we needed to pick a restriction site that is only present in the second intron and nowhere else on the gene, we found an Xho site that fit these parameters and used it for a restriction digest. We ran the digest on an agarose gel to make sure that the plasmid was cut and then we gel purified the linear DNA using the fragment purification method (Ausubel et al. 1993).

We then ligated the artificial arrest sequence to the plasmid by kinasing the ends of the sequence and then ligating it to the open plasmid. The artificial arrest sequence was constructed using a series of GC and A repeats which we hypothesized would pause RNA Polymerase without allowing it to completely fall off. We checked to see if the sequence was inserted by using colony PCR (after transforming the plasmid into bacteria). The primers used were E2 down, which complements the second exon and PE2, which complements the Cup1 gene. The results of the PCR showed that the ARTAR sequence had been successfully inserted into the gene (Fig 6).



**FIG 2.** Arrows show the location of E2 down and PE2 primers. Red line indicates position of inserted artificial arrest site.

The problem with our method is that the artificial arrest sequence can be inserted into the gene backwards or in multiples as well as in a single, correctly oriented copy. In our experiment, we were only interested in how one correctly oriented artificial arrest sequence affects splicing. To make sure the sequence was inserted in the correct direction and no multiples were inserted, the resulting plasmid was sent away to be sequenced. The sequencing results showed that only one artificial arrest sequence had been inserted into the plasmid but some plasmids had the sequence inserted backwards. We decided that although some sequences were inserted backwards, they represent a kind of control, and we can still investigate the effect they have on splicing.

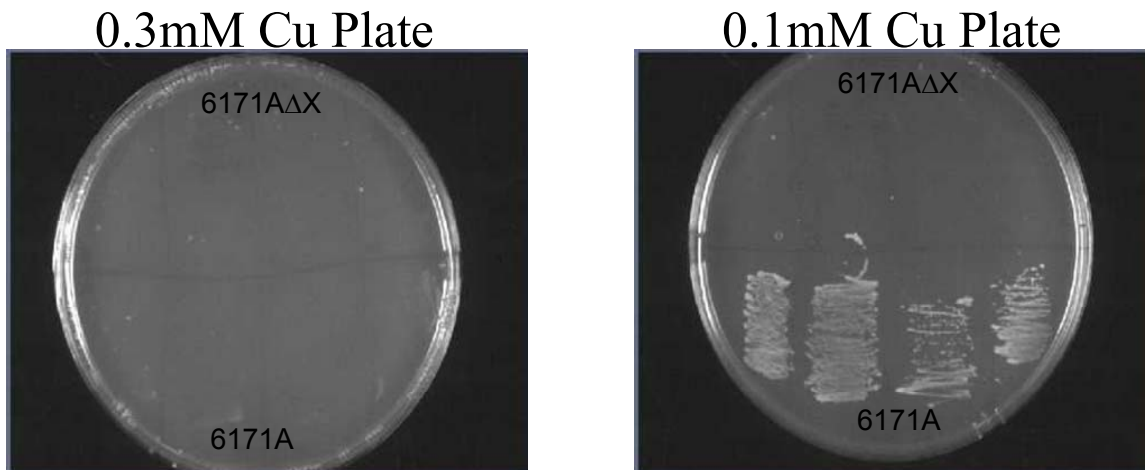
## SUMMARY

Using both reverse transcriptase PCR and measuring levels of radioactive mRNA, we came to the conclusion that our reporter construct was working properly. The wild type reporter gene spliced the second exon in about 50% of the time, but the reporter gene with intron deletions spliced the second exon in only about 7% of the time leading us to believe that we successfully increased the skipping of the second exon.

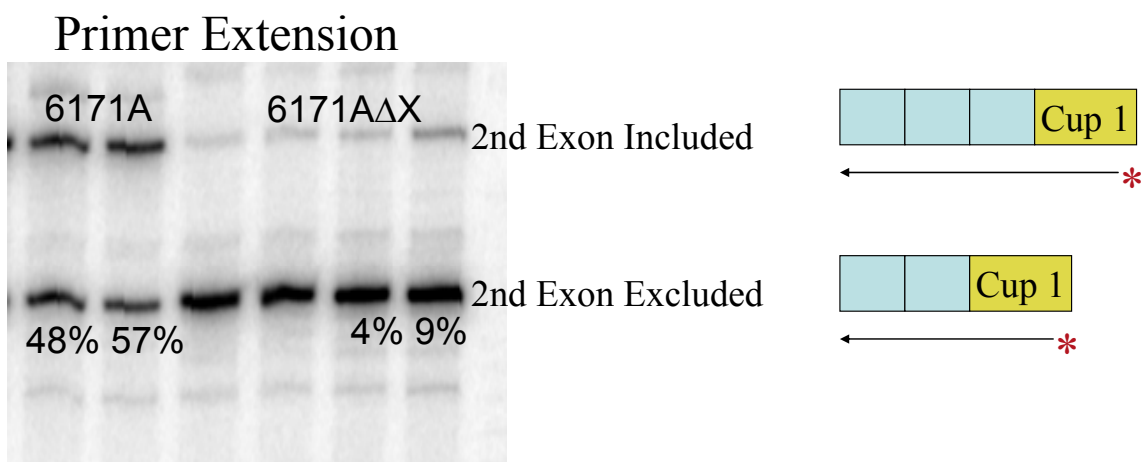
When we sequenced the results of the colony PCR we found that our construct had only one insert per plasmid. We also found that some plasmids had the artificial arrest sequence inserted backwards. We can now determine (1) whether the arrest sequence reduces downstream transcription by pausing RNA polymerase and (2) whether pausing affects the efficiency of exon inclusion.

## FUTURE EXPERIMENTS

Now that the construct has been successfully completed the next step is to transform the construct into *S. cerevisiae*. Yeast are the simplest eukaryotic organisms and their entire genome has been sequenced allowing us to take advantage of their splicing machinery and observe the affects of the artificial arrest sequence. We will promote transcription of the reporter gene to see if the artificial arrest sequence will induce pausing of RNA polymerase and therefore inclusion of the second exon. We then need to extract the yeast's RNA and analyze using both primer extension and copper plating.



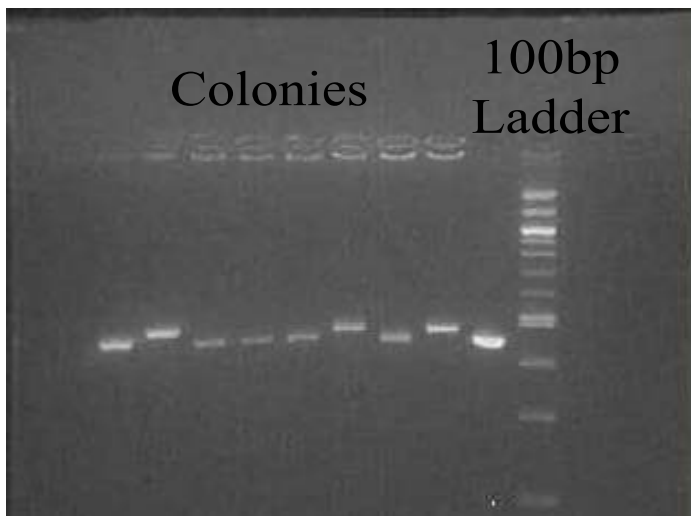
**FIG 3.** Yeast plated on copper plates. The 6171A gene, when spliced, includes the second exon. The 6171A $\Delta$ X gene, containing intron deletions, leads to exclusion of the second exon. Copper resistance is only conferred to yeast containing mRNA which includes the second exon. The 0.1mM copper plate shows that 6171A $\Delta$ X is copper sensitive leading to the conclusion that the second exon is being excluded.



**FIG 4.** Primer extension experiment. The 6171A gene includes the second exon about 50% of the time. When regulatory regions of the introns were deleted resulting in gene 6171A $\Delta$ X, the amount of inclusion of the second exon went down to only about 7%.



**FIG 5.** Reverse transcriptase PCR results. (1) 6171A $\Delta$ X (2) 6171A (3) 100 base pair ladder. There is more inclusion of the second exon in the 6171A gene and more exclusion of the second exon in the 6171A $\Delta$ X gene.



**FIG 6.** Colony PCR. The raised bands contain the 6171A $\Delta$ X gene plus the artificial arrest sequence. The band closest to the ladder is just the 6171A $\Delta$ X plasmid which was used as the control. All bands that are lined up with the control do not contain the ARTAR insert. This gel was used to confirm the presence of the artificial arrest sequence in the 6171A $\Delta$ X plasmid.

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