

# MODULE 4: SPLICING

MEG LAAKSO

## Lesson Plan:

<b>Title</b>	<b>Removal of introns from messenger RNA by splicing</b>
<b>Objectives</b>	<ul style="list-style-type: none"> <li>• Examine splicing mechanism</li> <li>• Identify intron-exon boundaries</li> <li>• Identify splice donor and acceptor sites that are best supported by RNA-Seq and Exon Junction data</li> <li>• Identify alternative splicing patterns</li> </ul>
<b>Pre-requisites</b>	Modules 1-3
<b>Order</b>	<ul style="list-style-type: none"> <li>• Review mRNA processing; Investigation 1</li> <li>• Define isoform</li> <li>• Introduce consensus sequences; Investigation 2</li> <li>• Discuss pre-mRNA vs. mRNA</li> <li>• Explore how to find exon-intron junction using tra-RA as the example; Investigation 3</li> </ul>
<b>Homework</b>	Students will find intron-exon junctions for spd-2-RA
<b>Class instruction</b>	<ul style="list-style-type: none"> <li>• Review pre-mRNA processing (capping, polyadenylation, splicing) using appropriate figures from the textbook or Module 3</li> <li>• Introduce splicing and the spliceosome</li> <li>• Investigation 1: Students familiarize themselves with RNA-Seq data</li> <li>• Review consensus sequences for splice donor and splice acceptor</li> <li>• Investigation 2: Students find splice donor and acceptor for intron 1</li> <li>• Review RNA splicing of intron 1 using tra-RA as the example</li> <li>• Investigation 3: Students find remaining splice donor and splice acceptor for intron 2</li> <li>• Discuss length of pre-mRNA vs. length of spliced mRNA</li> <li>• Identify isoforms with different TSSs, or alternative splicing patterns</li> </ul>
<b>Associated videos</b>	<ul style="list-style-type: none"> <li>• RNA Seq and TopHat Video: <a href="https://youtu.be/gepVXEsfLMM">https://youtu.be/gepVXEsfLMM</a></li> </ul>

	<ul style="list-style-type: none"><li>• Genes and Isoforms Video: <a href="https://youtu.be/8jtTp_6vN4M">https://youtu.be/8jtTp_6vN4M</a></li></ul>
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## INVESTIGATION 1: EXAMINING RNA-SEQ DATA

We will continue to focus on isoform A of *transformer* (referred to as tra-RA). Here we will focus on data from experiments that assess the RNA population in cells. This data can be used to help us identify exons and introns for the gene under study.

All RNAs in the cell are collectively known as the 'transcriptome,' as almost all RNA is produced by transcription from a DNA template. (In some cases, RNA is made from an RNA template.) The transcriptome includes messenger RNAs, ribosomal RNAs, transfer RNAs, and other RNAs that have specialized functions in the cell.

RNA can be harvested from cells or a whole organism like *Drosophila* and converted to DNA, then sequenced to produce RNA-Seq (RNA Sequencing) data. First, extracted mRNA that has been fully spliced is copied back to DNA with the enzyme called **reverse transcriptase**. Short fragments of the copied or complimentary DNA are sequenced, and then these segments are mapped back to the genome. By analyzing the mapping data, it is possible to know which and how many messenger RNAs have been synthesized.

This is a powerful technique that allows us to see when and where different genes are expressed. This kind of information can help researchers and clinicians know which genes are expressed in different types of cancer, for example. Here and in the next modules we are going to use RNA-Seq data to explore how the *transformer* (*tra*) gene is expressed in male vs. female *Drosophila*.

RNA-Seq data can indicate where transcription occurs, to the exact nucleotide. The number of RNA-Seq fragments that map to a given site also tells us how many copies of the RNA are present in the sample. Remember from Module 3 that the initial RNA transcripts are quickly processed to remove the introns. Hence in total RNA from a cell, sequences from exons will be much more abundant than sequences from introns. We will use RNA-Seq data to help us find the exon-intron boundaries for tra-RA, the isoform of the *tra* gene that is expressed in female fruit flies.

1. Open a web browser on your computer. Internet Explorer, Mozilla Firefox, Safari, or Chrome will work for this investigation. Go to the GEP Mirror of the UCSC Genome Browser at <http://gander.wustl.edu>. Follow the instructions given in Module 1 to navigate to the contig1 project in the *D. melanogaster* 'July 2014 (Gene)' assembly.

**Reminder:** use the dropdown menus on the Genome Browser to select the following:

2. Under 'genome' field = "D. melanogaster"
3. Under "assembly" field = "July 2014(Gene)"
4. In the "search term" field = "contig1"
5. Then click 'submit' and the screen below will appear (Figure 1). As you will remember, this section of DNA is 11,000 base pairs long and is part of the left arm of chromosome 3, which is about 28,100,000 bp long. If your Browser window is showing other evidence tracks, reset by clicking on "default tracks."

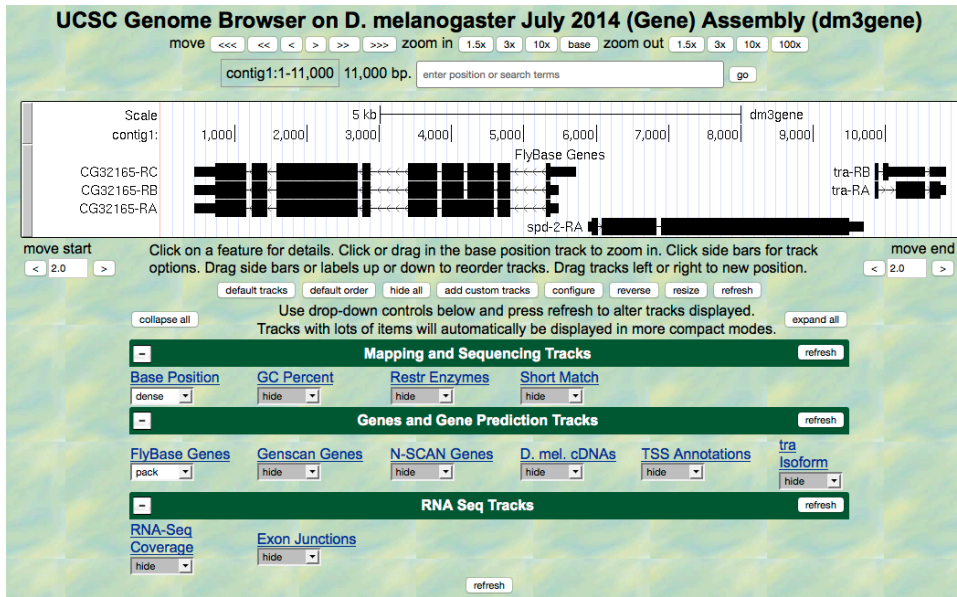


FIGURE 1 A SCREEN SHOT OF 'CONTIG1' PROJECT.

- Let's start by setting the evidence tracks we want to see. Click on the "hide all" button. Then open only the tracks that will provide data for this investigation.

**Base position "dense," then click "refresh."**

Note that you will not be able to see the DNA sequence until you "zoom in" at step 7.

**RNA-Seq coverage "full," then click "refresh."**

You will see blue and red histograms representing RNA-Seq data generated using RNA samples from adult females and adult males, respectively. This allows us to infer the pattern of RNA synthesis, and to look for similarities and differences between the sexes. Both similarities and differences are apparent!

- Zoom in to view only the *tra* gene by entering "contig1:9,800-10,900" in the "enter position or search terms" text box (Figure 2).

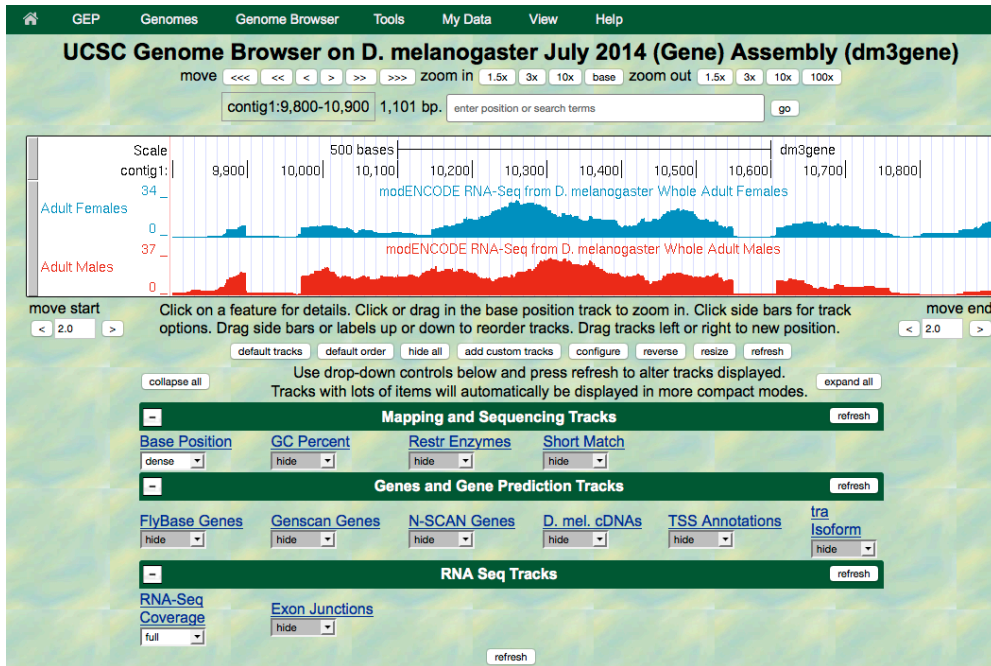


FIGURE 2. HISTOGRAMS REPRESENTING RNA-SEQ DATA IN TRANSFORMER (TRA).

8. We are now looking at the region of chromosome 3 where the *tra* gene is located. Compare the blue and red histograms for Adult Females and Adult Males. Note that there are numbers on the y-axis that show how many RNA reads (sequences from transcripts) map to that position.

Q1. List two ways in which the histograms are similar.

a. \_\_\_\_\_

b. \_\_\_\_\_

Q2. List one way that the histograms differ (other than color).

a. \_\_\_\_\_

To recap: The blue histogram represents the sequenced messenger RNA from female fruit flies, and represents the form of the *tra* gene referred to as isoform A (tra-RA). The red histogram represents the RNA-Seq data from male fruit flies. The mRNA in males is different from that in females, and this second isoform of the *tra* gene is called isoform B (tra-RB).

**Q3.** Recall that our other evidence (see Module 3) indicates that tra-RA extends from 9,851 to 10,846. How many gaps do you see in the histograms in this interval?  
\_\_\_\_\_

Each gap corresponds to an intron - there is very little RNA-Seq signal for that part of the gene because the intronic RNA was spliced out.

**Q4.** How many exons do you see? \_\_\_\_\_

Remember that the exon is the 'expressed' part of the gene and there will be either a sharp, or broad, peak in the RNA-Seq histogram.

**Q5.** Do females or males make more *transformer* mRNA or do they express it at about the same level? \_\_\_\_\_

9. Now that you've examined the evidence yourself, let's go back and review.

**Gaps (introns)** – Figure 3 is a screen shot of the genome browser with gaps circled. Note that there are 2 gaps in females, and 2 gaps in males. The first gap in females looks a little strange because it doesn't have clean boundaries, suggesting a mixed population of processed transcripts.

**Exons** – Brackets have been drawn underneath the RNA-Seq data for Adult Female flies, corresponding to the three exons that are expressed.

**Isoforms** – Note that isoform A and isoform B of the *tra* gene are the result of **alternative splicing**. For other genes, isoforms may have **different transcription start sites**. Isoforms of a gene always have different mRNA sequences, but they may have the same protein sequence. To learn more about isoforms and genes, watch the Genes and Isoforms video: [https://youtu.be/8jtTp\\_6vN4M](https://youtu.be/8jtTp_6vN4M)

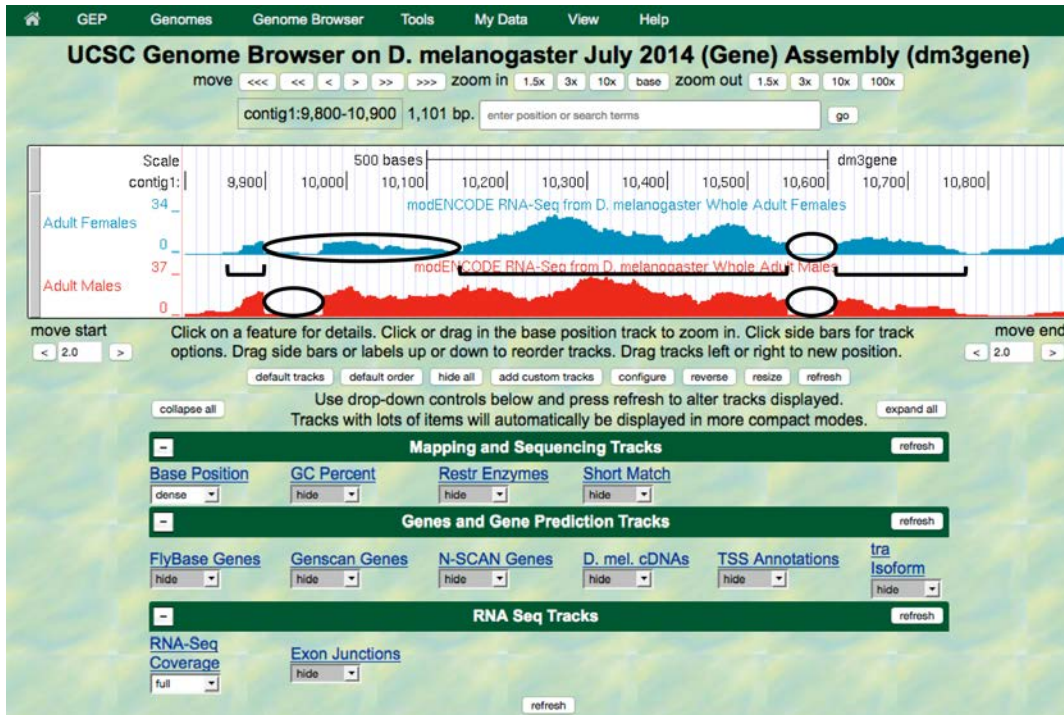


FIGURE 3 IN THIS SCREENSHOT OF THE *tra* GENE, INTRONS HAVE BEEN CIRCLED AND EXONS HAVE BRACKETS UNDERNEATH THEM.

**Q6.** Using the information you've gathered so far, make a diagram of the *tra-RA* (female specific) isoform with 3 exons and 2 introns. Represent exons as rectangular boxes and introns as lines connecting the boxes. Number each exon and intron (start from left with 'exon 1').

**Q7.** Where is the promoter in relation to the exons and introns? \_\_\_\_\_ . Mark the putative Transcription Start Site in your diagram with a bent arrow pointing in the direction of transcription.

## INVESTIGATION 2: IDENTIFYING SPLICE SITES

We will again focus on tra-RA to identify splice sites, that is, the exact nucleotides where splicing occurs to remove introns from the pre-mRNA.

### BACKGROUND:

Two software programs, called TopHat and Bowtie, use the RNA-Seq data to graphically represent the exon junctions. The resulting graphic on the genome browser coincidentally looks something like a little bowtie (two small boxes connected by a thin line) (Figure 4). The boxes represent the sequenced mRNA (the exons), and the line represents a gap (the intron). The exon junction can be inferred when the first part of a sequenced fragment from the RNA population matches (for example) DNA positions 50-100 and the second part of the same fragment matches DNA positions 200-250; the RNA between positions 101-199 must have been taken out of the middle!

Short sequences are present at the beginning and end of each intron that allow the spliceosome - the molecular machinery that cuts out introns - to precisely remove the intron, leaving only the exon sequences in the mature mRNA. The first two nucleotides of the intron are the splice donor site and almost always the nucleotides "GT". The last two nucleotides of the intron are the splice acceptor site, and usually the nucleotides "AG". (Recall your observations in Module 3.) For more information on RNA-Seq and the search for splice junctions, watch the RNA-Seq and TopHat video: <https://youtu.be/qepVXEsfLMM>

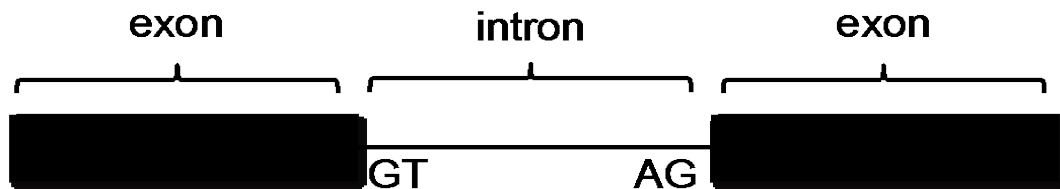


FIGURE 4. A DIAGRAM OF INTRON-EXON JUNCTIONS.

1. Using the same Genome Browser page, reset the Browser by clicking on "hide all." Then open the tracks that will provide the information we want for Investigation 2. *(note: If you stopped after Module 4: Investigation 1, then you may want to return to page 2 for a reminder of how to get the Genome Browser page.)*

**Base position "dense," then click "refresh."**

Note that you will not be able to see the DNA sequence until you "zoom in" at step 7.

**RNA-Seq coverage "full," then click "refresh."**

You will again see blue and red histograms representing the RNA-Seq data (indicating the amount of mRNA synthesized) in females and males, respectively. We will focus on the blue histogram (Adult Females) again. As we did in Module 3, let's customize the RNA-

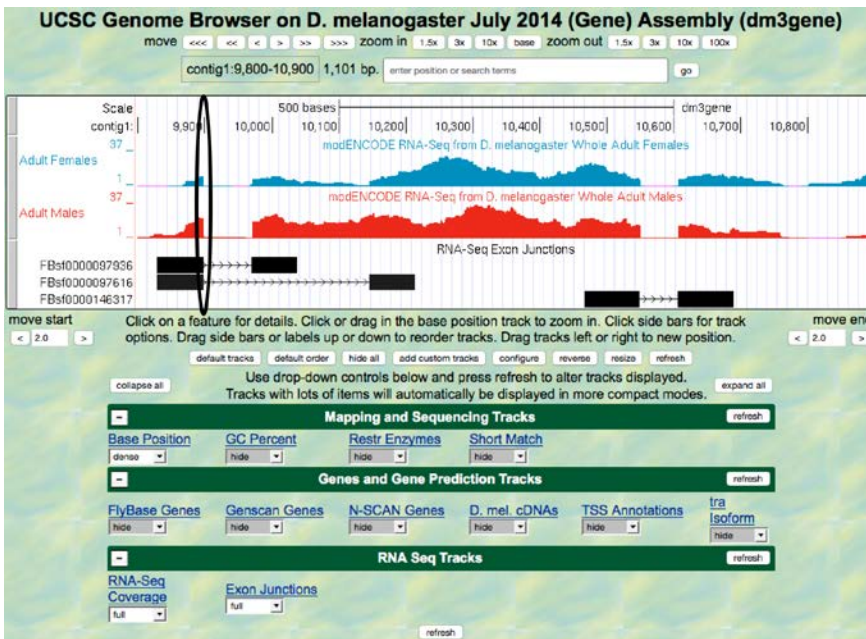


Seq track by setting the “Data view scaling” field to “use vertical viewing range setting” and the “max” field under “Vertical Viewing range” to 37. Remember that you gain access to these settings by clicking on the “RNA-Seq Coverage” link under the RNA Seq Tracks green bar.

**Exon junctions "full," then click “refresh.”**

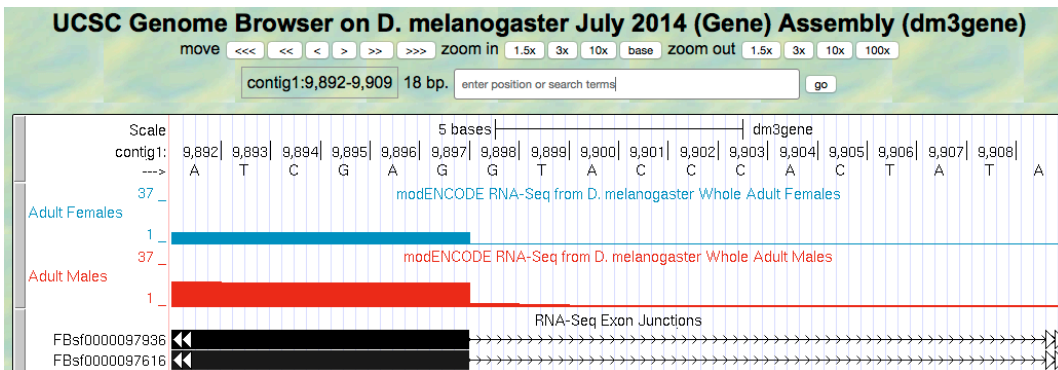
These rectangular boxes joined by a thin black line will help you identify the exon-intron boundaries.

Our graphical output viewer will look like the screen shot below (Figure 5).



**FIGURE 5. VIEW OF THE TRA RNA-SEQ DATA WITH THE SPLICE DONOR SITE IN INTRON 1 CIRCLED.**

2. Zoom in to the area that is circled - click and drag the cursor just above the numbers, or use zoom buttons.
3. Set the screen so that you can see about 15-20 nucleotides, as shown in the example below (Figure 6).



**FIGURE 6. TOPHAT DATA (IN BLACK) FOR ADULT MALES AND ADULT FEMALES INDICATING AN EXON JUNCTION.**

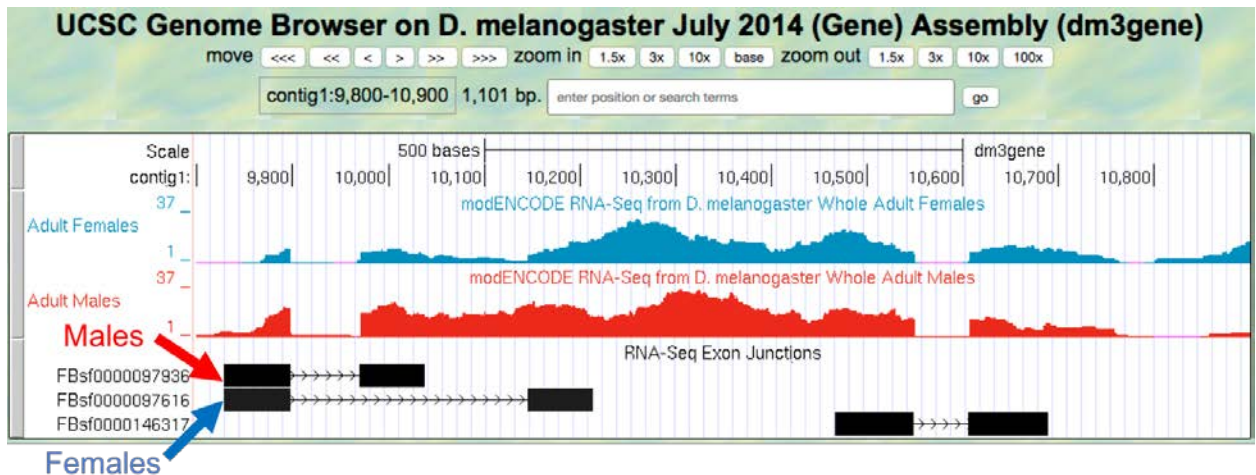
The blue histogram stops at the end of exon 1. The last three nucleotides of exon 1 are "G-A-G".

**Q8.** What is the coordinate of the last nucleotide in exon 1? \_\_\_\_\_

**Q9.** What are the first two nucleotides of intron 1? \_\_\_\_\_

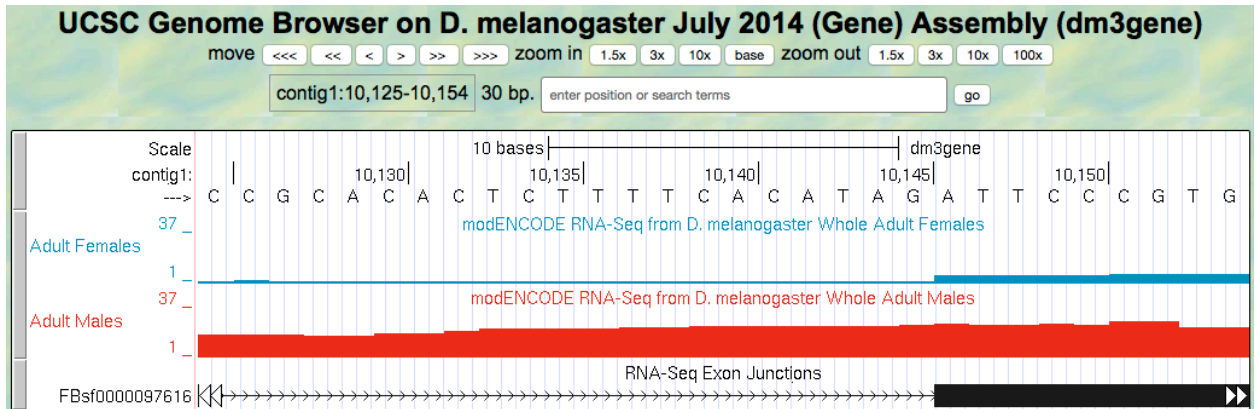
This is called the 5' splice site or 'splice donor site'.

- Zoom out so that you can see all of intron 1. Let's use TopHat to help us find the 3' end of the intron. Examine the Exon Junctions track. The first intron-exon junction predicted by TopHat (black) seems to align with the red histogram data from males; the second junction aligns better with the blue histogram data from females (Figure 7).



**FIGURE 7. TOPHAT AND RNA-SEQ DATA FOR MALES AND FEMALES.**

- Change the "enter position or search terms" field to contig1:10,140 and then click "go." Zoom out 10x and then 3x (Figure 8).



**FIGURE 8. GRAPHICAL VIEWER CENTERED ON THE JUNCTION BETWEEN INTRON 1 AND EXON 2 OF THE TRA-RA (FEMALE SPECIFIC) ISOFORM**

**Q10.** What are the last two nucleotides of the female tra-RA intron 1?

\_\_\_\_\_

This is called the 3' splice site or 'splice acceptor site'.

**Q11.** What is the coordinate of the first nucleotide in the female tra-RA exon 2?

\_\_\_\_\_

## INVESTIGATION 3: IDENTIFY THE 5' SPLICE DONOR AND 3' SPLICE ACCEPTOR SITES FOR INTRON 2

We can use the same approach to map the exon-intron boundaries of the second intron.

Review steps #2-5 in Investigation 2. Then repeat the process to answer the following questions about the 5' splice donor and 3' splice acceptor sites for the *tra-RA* (female specific) intron 2.

Zoom into the sequence surrounding the 5' splice donor for the *tra-RA* (female specific) intron 2

**Q12.** What are the last three nucleotides of the female *tra-RA* exon 2?

\_\_\_\_\_

**Q13.** What is the coordinate of the last nucleotide in the female *tra-RA* exon 2?

\_\_\_\_\_

**Q14.** What are the first two nucleotides of the female *tra-RA* intron 2?

\_\_\_\_\_

6. Zoom out as needed so that you can see all of intron 2. Use TopHat to find the end of intron 2.

7. Click and drag so that the end of intron 2 is centered in the viewer. Then zoom in so that you can see the nucleotide sequence.

**Q15.** What are the last two nucleotides of the female *tra-RA* intron 2?

\_\_\_\_\_

**Q16.** What is the coordinate of the first nucleotide in the female *tra-RA* exon 3?

\_\_\_\_\_

- Q17.** Using the information you've gathered so far, make a graphical picture of the tra-RA (female specific) isoform with 3 exons and 2 introns. Number each exon and intron at the corresponding DNA coordinates. Add the coordinates for first and last nucleotide of the exons that you have found so far. Add the sequences of the splice donor and splice acceptor sites at the appropriate locations.
- Q18.** Where do you think the promoter is located in relation to your gene model? What evidence do you have to support your idea, using the evidence tracks we have displayed (Base Position, RNA-Seq Coverage, Exon Junctions)?
- Q19. *Bonus question!*** Support your hypothesis by gathering additional data. Recall our explorations in Modules 2 and 3. You might want to open the tracks “D. mel. cDNAs,” and “TSS Annotations,” both in **full**. What type of evidence is shown by each of these tracks (refer to Module 2)? Finally, to see tra-RA as currently annotated in FlyBase, open the “tra Isoform” track on **full**, or to see both isoforms open the “FlyBase Genes” track in **pack**. Do these results support your model? Do any ambiguities remain?

**Questions:**

**Q1.**List two ways in which the histograms are similar.

a. \_\_\_\_\_

b. \_\_\_\_\_

**Q2.** List one way that the histograms differ (other than color).

c. \_\_\_\_\_

**Q3.** Recall that our other evidence (see Module 3) indicates that *tra*-RA extends from 9,851 to 10,846. How many gaps do you see in the histograms in this interval?

\_\_\_\_\_

**Q4.** How many exons do you see? \_\_\_\_\_

**Q5.** Do females or males make more *transformer* mRNA or do they express it at about the same level? \_\_\_\_\_

**Q6.** Using the information you've gathered so far, make a diagram of the *tra*-RA (female specific) isoform with 3 exons and 2 introns. Represent exons as rectangular boxes, and introns as lines that connect the boxes. Number each exon and intron (start from left with 'exon 1').

**Q7.** Where is the promoter in relation to the exons and introns? \_\_\_\_\_ . Mark the putative Transcription Start Site with a bent arrow, pointing in the direction of transcription.

**Q8.** What is the coordinate of the last nucleotide in the female *tra*-RA exon 1? \_\_\_\_\_

**Q9.** What are the first two nucleotides of the female *tra*-RA intron 1? \_\_\_\_\_

**Q10.** What are the last two nucleotides of the female *tra*-RA intron 1? \_\_\_\_\_

**Q11.** What is the coordinate of the first nucleotide in the female *tra*-RA exon 2? \_\_\_\_\_

**Q12.** What are the last three nucleotides of the female tra-RA exon 2?  
\_\_\_\_\_

**Q13.** What is the coordinate of the last nucleotide in the female tra-RA exon 2?  
\_\_\_\_\_

**Q14.** What are the first two nucleotides of the female tra-RA intron 2?  
\_\_\_\_\_

**Q15.** What are the last two nucleotides of the female tra-RA intron 2?  
\_\_\_\_\_

**Q16.** What is the coordinate of the first nucleotide in the female tra-RA exon 3?  
\_\_\_\_\_

**Q17.** Using the information you've gathered so far, make a graphical picture of the tra-RA (female specific) isoform with 3 exons and 2 introns. Number each exon and intron. Add the coordinates for first and last nucleotide of the exons that you have found so far. Add the sequences of the splice donor and splice acceptor sites at the appropriate locations.

**Q18.** Where do you think the promoter is located in relation to your gene model? What evidence do you have to support your idea, using the evidence tracks we have displayed (Base Position, RNA-Seq Coverage, Exon Junctions)?

**Q19. Bonus question!** Support your hypothesis by gathering additional data. Recall our explorations in Modules 2 and 3. You might want to open the tracks “D. mel. cDNAs,” and “TSS Annotations,” both in **full**. What type of evidence is shown by each of these tracks (refer to Module 2)? Finally, to see tra-RA as currently annotated in FlyBase, open the “tra Isoform” track on **full**, or to see both isoforms open the “FlyBase Genes” track in **pack**. Do these results support your model? Do any ambiguities remain?