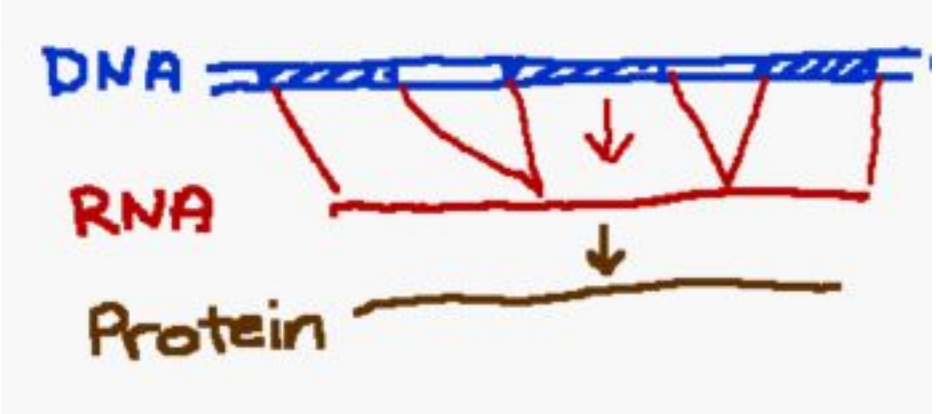
RECAP (1)



In eukaryotes, large primary transcripts are processed to smaller, mature mRNAs.

What was first evidence for this precursor-product relationship?

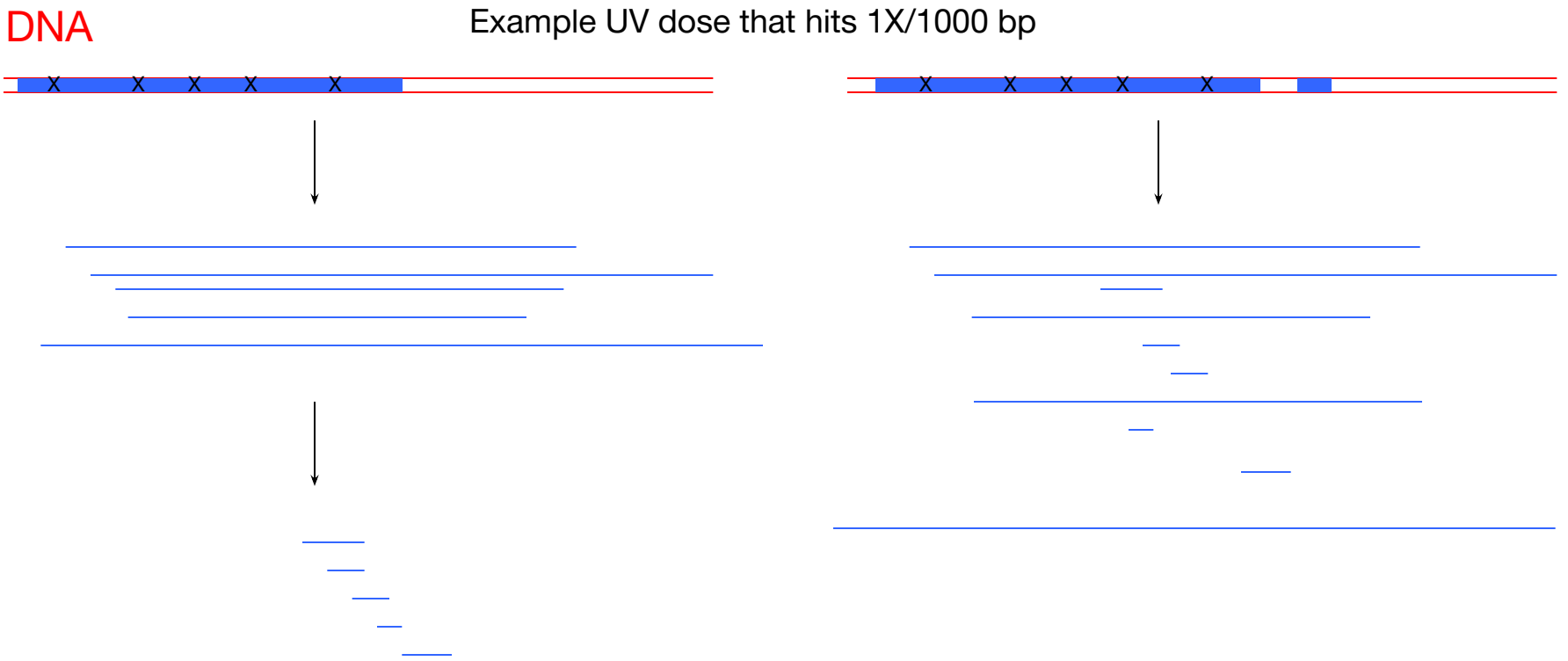
Observation:

Nuclear RNA pool consists of very high molecular weight species as well as lower molecular weight.

Darnell asked if there is a relationship between the high and low molecular weight RNAs

Experiment:

Treat cells with UV for varying periods of time. Thymidine dimers will form, blocking transcription. To assess the effects on the two pools of RNA, pulse cells with ^{3}H-Uridine and measure counts in each pool



If long RNAs are precursors then both long and short pools should exhibit comparable UV sensitivity

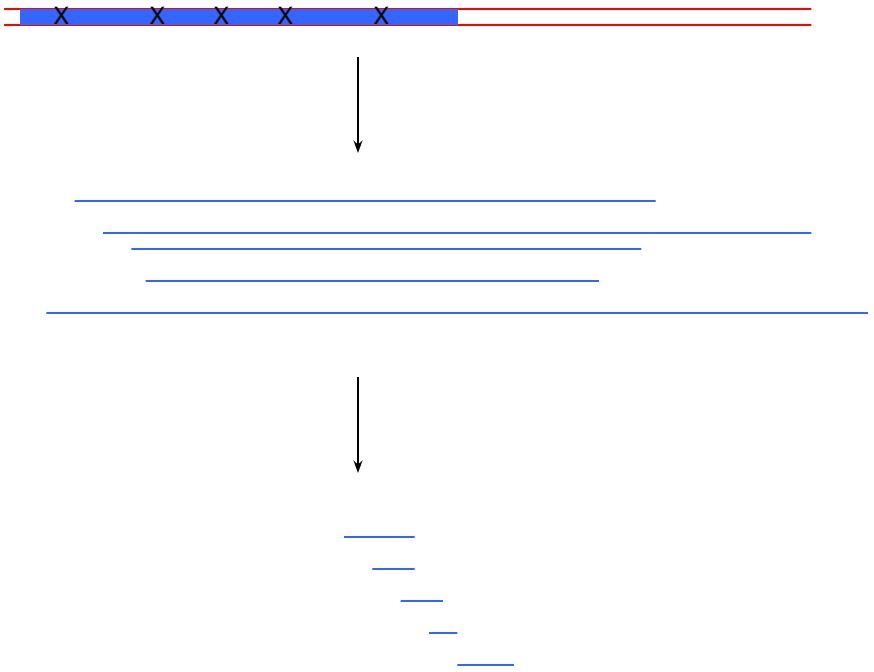
If long and short RNAs are independently transcribed, then they should exhibit different UV sensitivity

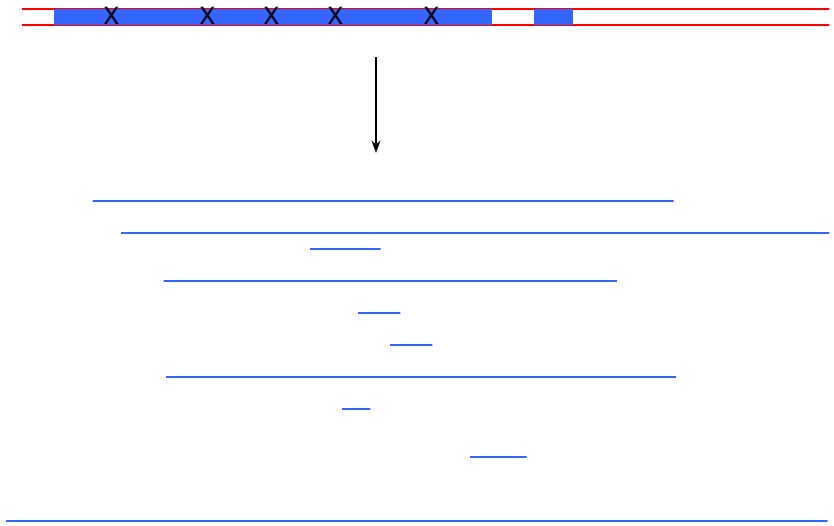
Experiment:

Treat cells with UV for varying periods of time. Thymidine dimers will form, blocking transcription. To assess the effects on the two pools of RNA, pulse cells with ^{3}H-Uridine and measure counts in each pool

DNA

Example UV dose that hits 1X/1000 bp

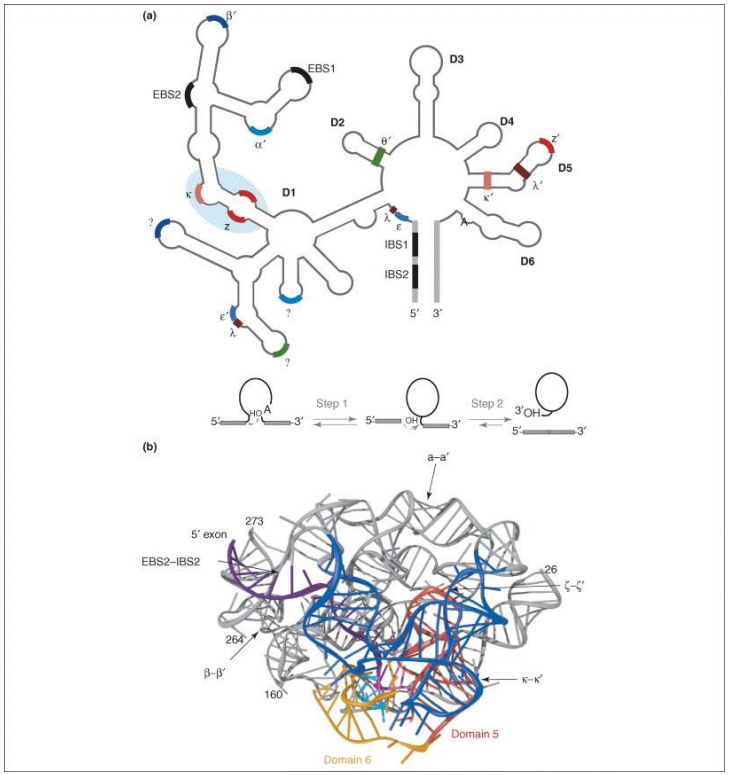




If long RNAs are precursors then both long and short pools should exhibit comparable UV sensitivity

If long and short RNAs are independently transcribed, then they should exhibit different UV sensitivity

RECAP (2)

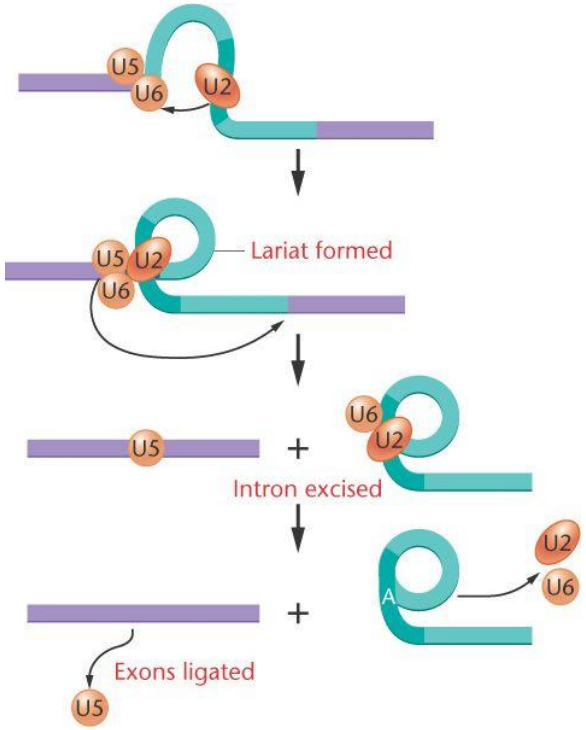


RNA is unstable – it can cleave itself.

RNA can fold into complex 3D structures.

Self-splicing introns utilize this suicidal tendency and contortionist ability to direct self-cleavage at precisely defined sites

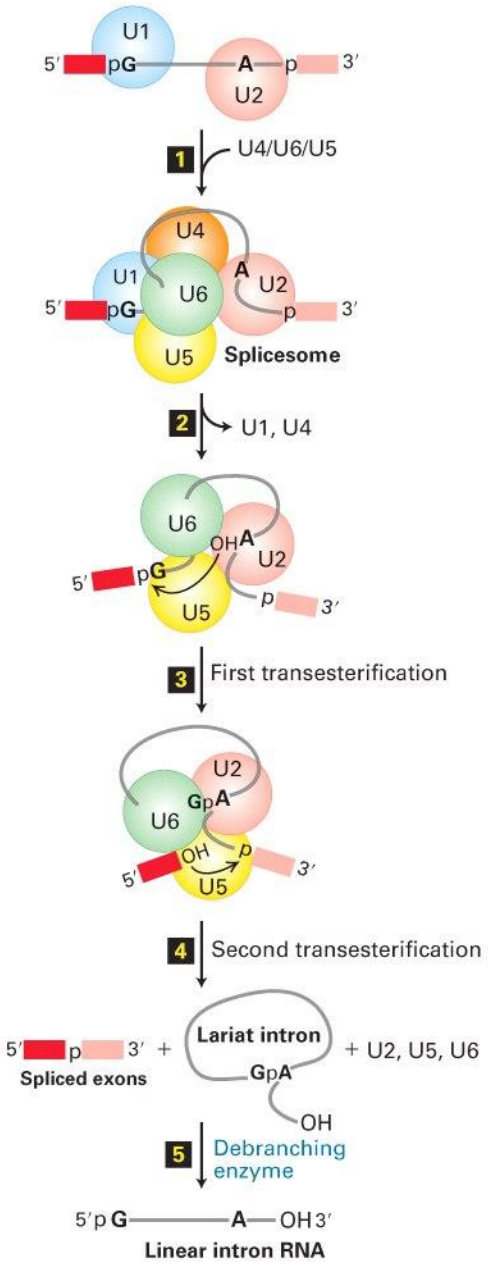
RECAP (3)



Splicing in eukaryotes probably relies on the same chemistry as self-splicing group II introns.

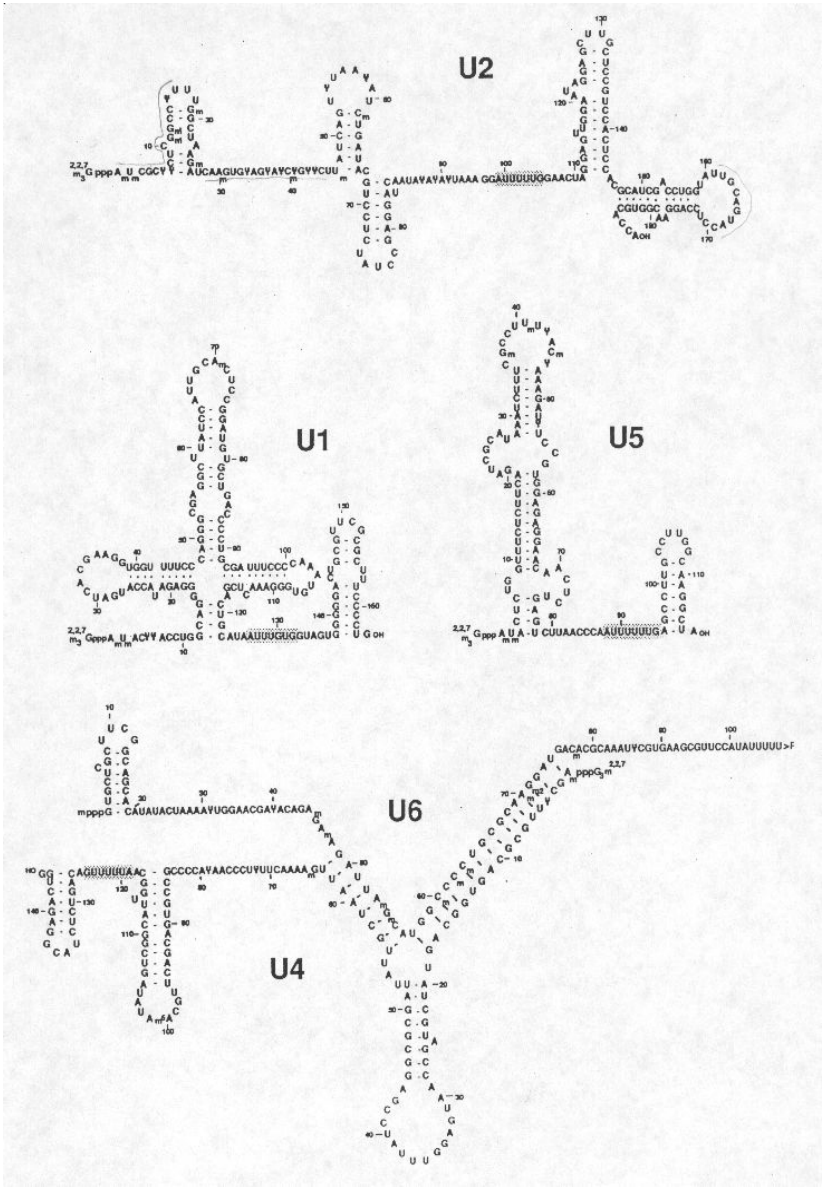
Splicing substrates in eukaryotes much more varied, and can't rely on 2º structure alone to define splice sites.

A complex RNA+protein machine is used to precisely define splice sites.



The spliceosome is made up of 5 small nuclear ribonucleoprotein subunits + > 100 proteins. These snRNPs are called: U1, U2, U4, U5, U6, and assemble in a stepwise pathway onto each intron. There are also many additional non-snRNP proteins in the spliceosome.

Structures of the Spliceosomal snRNAs



U1, U2, U4, U5

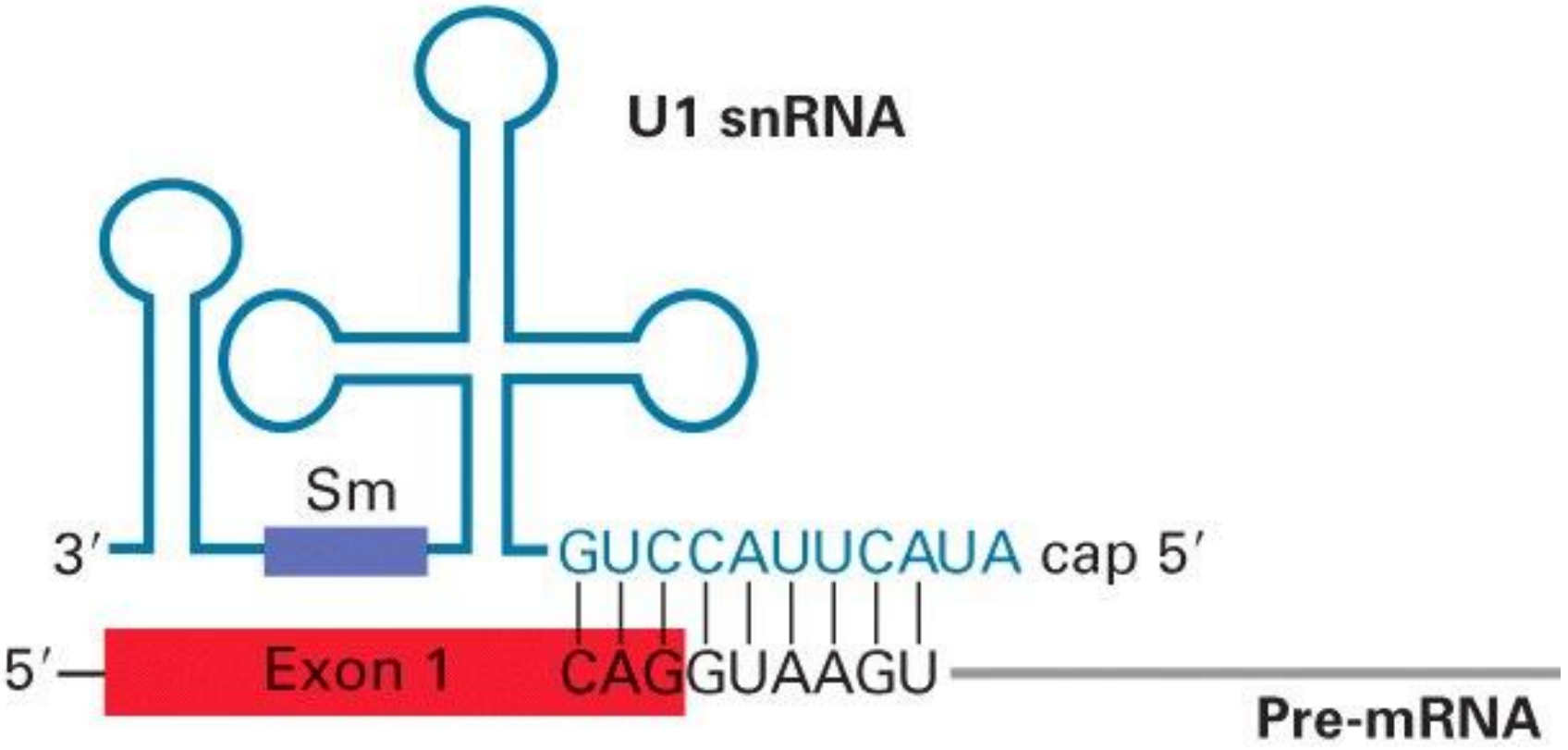
- RNA Pol II transcripts
- TriMethyl G Cap
- Bound by Sm Proteins

U6

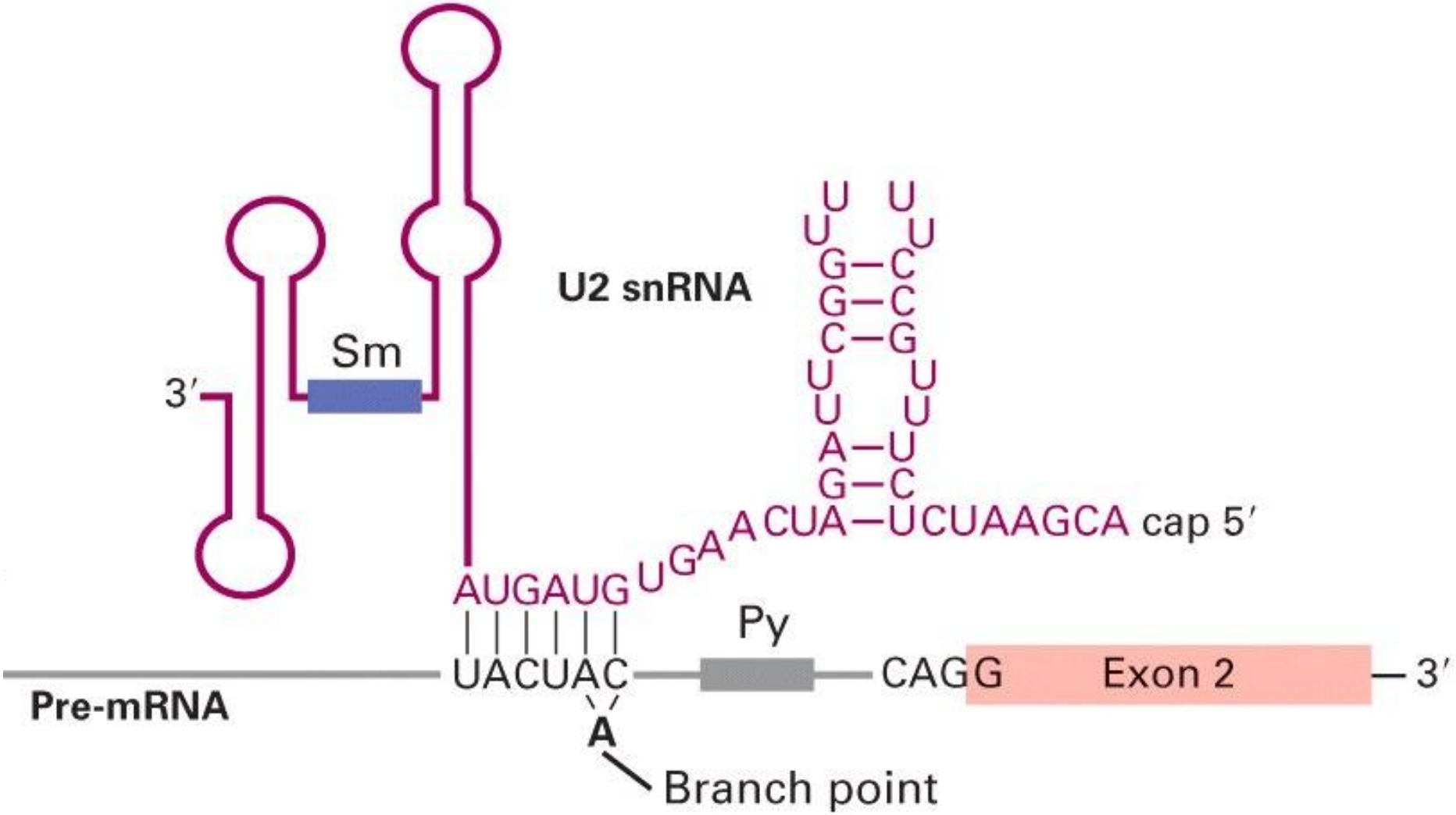
- RNA Pol III transcript
- Unusual Cap
- Not bound by Sm proteins

Each snRNA has a specific sequence and secondary structure and is bound by additional specific proteins

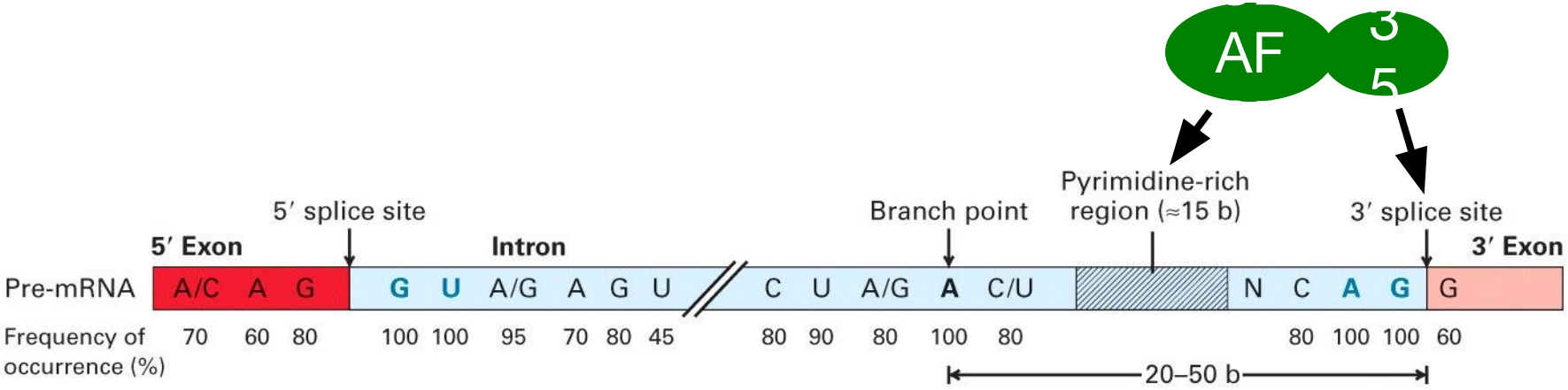
The earliest snRNP to bind to the pre-mRNA is U1, which uses its snRNA to base-pair to the 5' splice site.



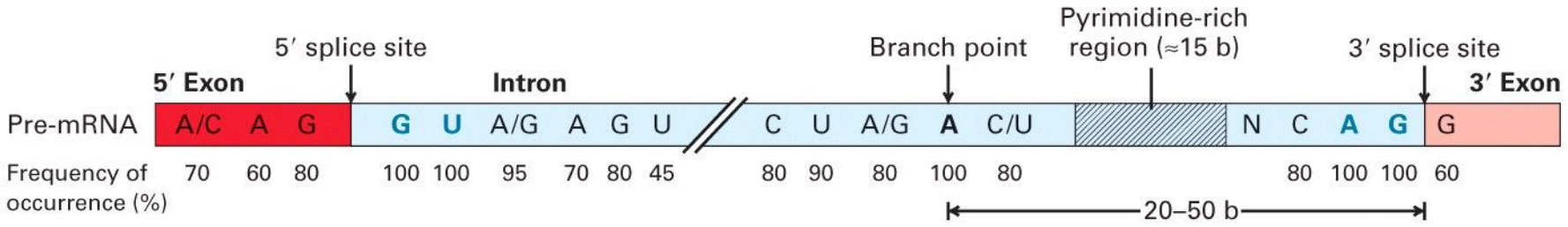
The U2 snRNP binds to the branchpoint via RNA/RNA base-pairs to create a bulged A residue. This forms the pre-spliceosomal "A" complex.

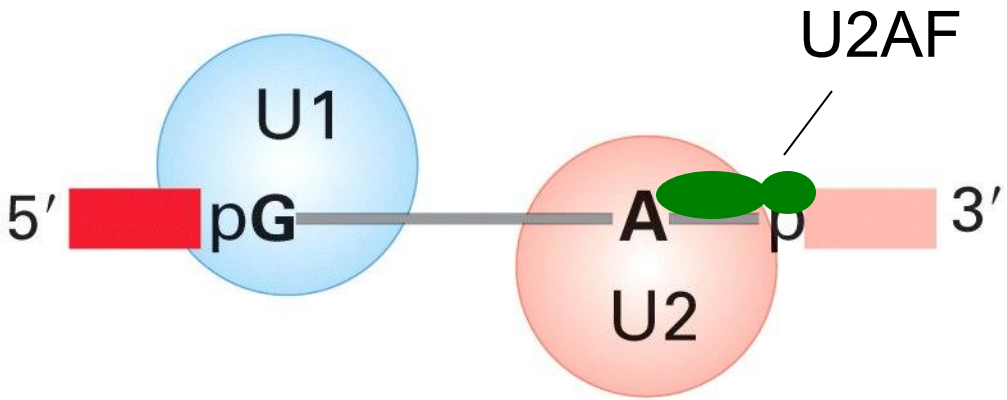


The protein U2AF (U2 Auxiliary Factor) binds to the Polypyrimidine tract and the AG of the 3’ splice site and helps U2 snRNP to bind to the branchpoint .

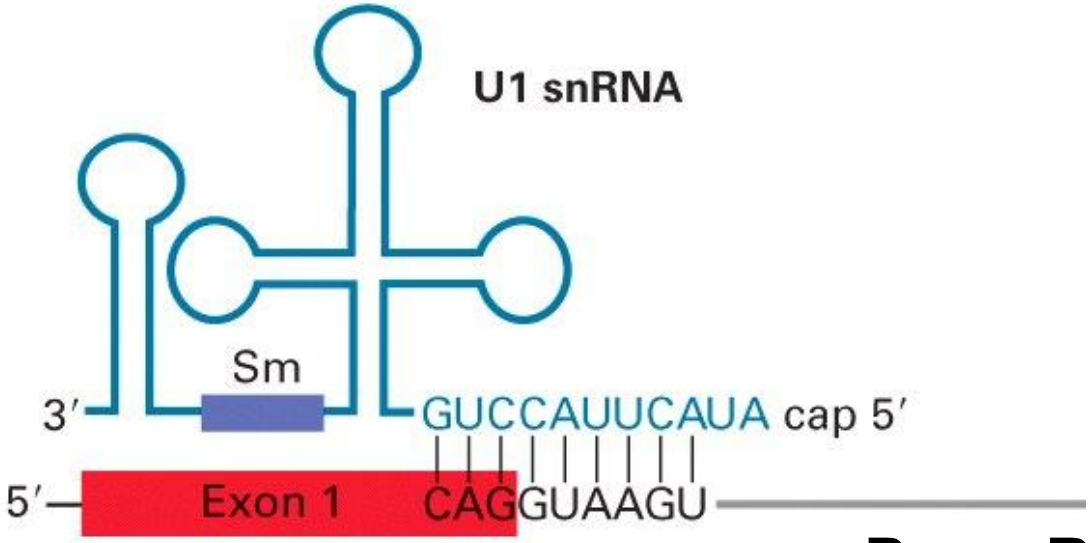


Splice sites do not always perfectly match the consensus sequences. Thus, the base-pairing interactions between the snRNAs and the pre-mRNA are not always the same.

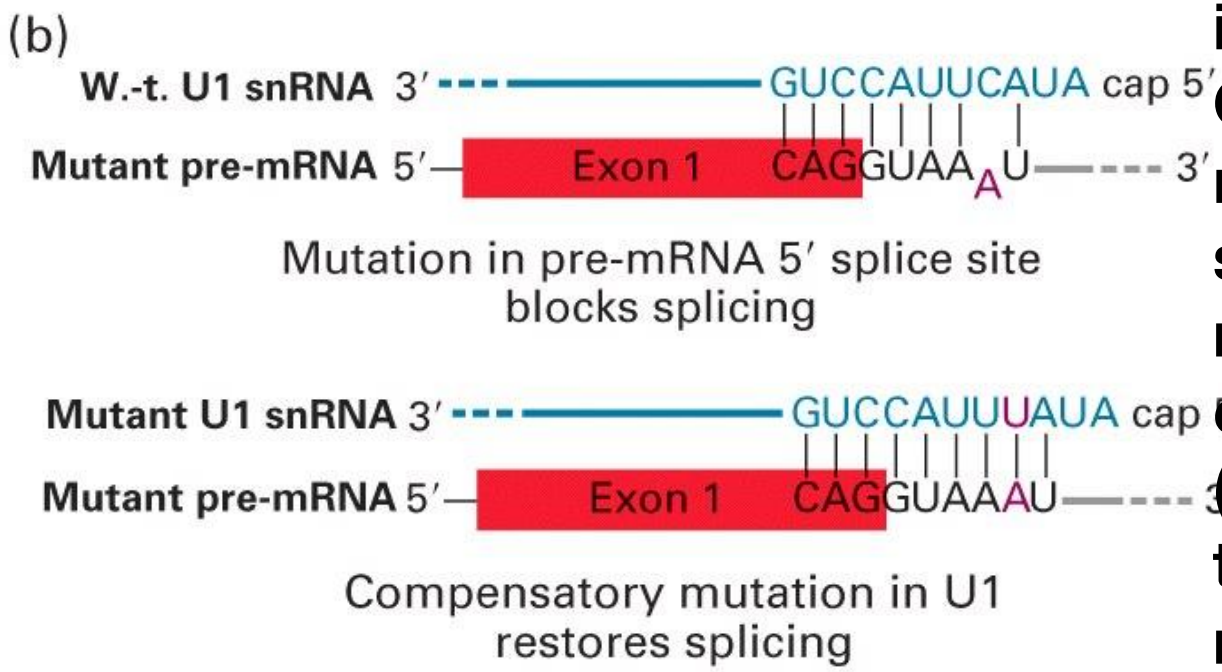




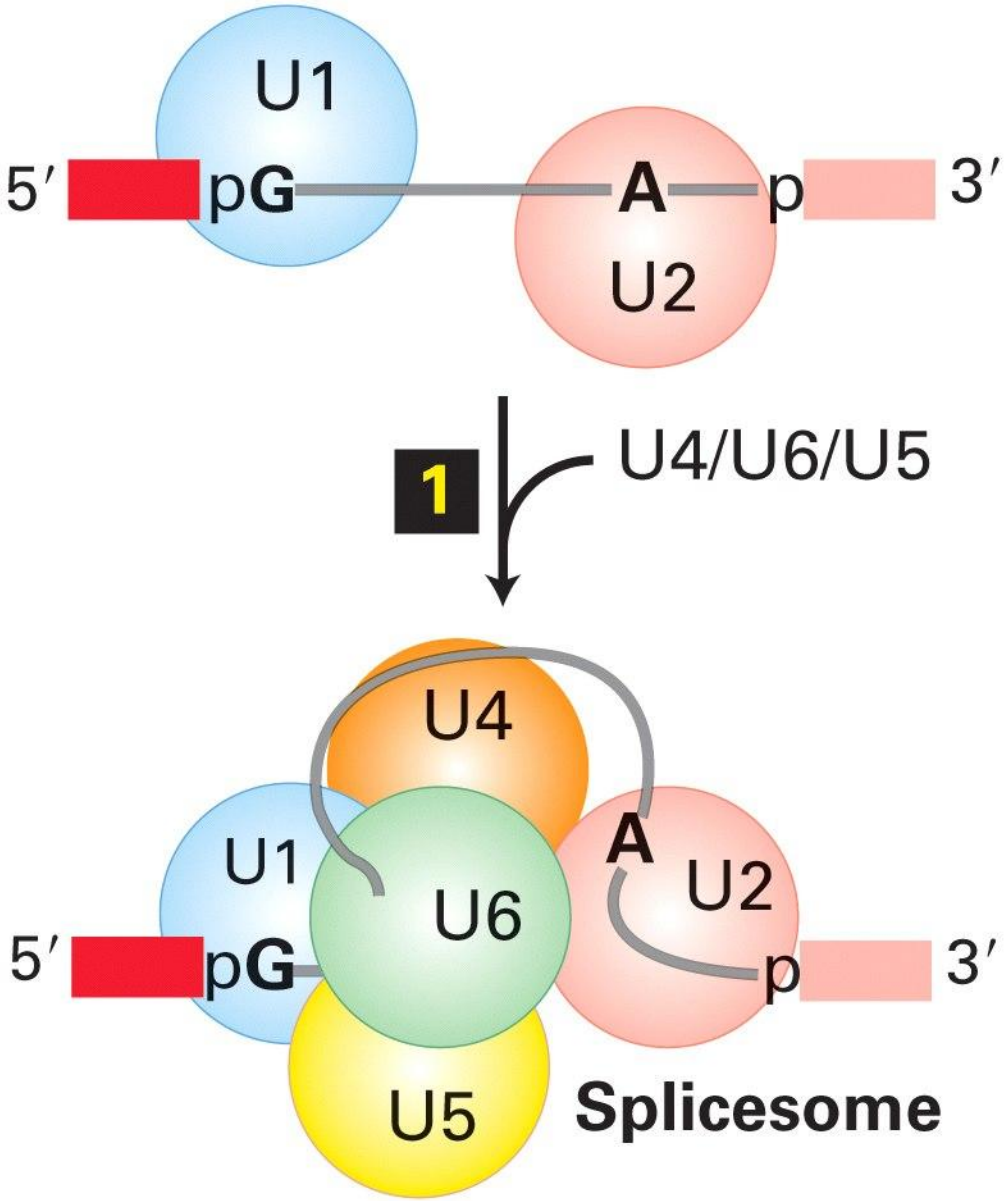
Pre-spliceosome



Pre-mRNA

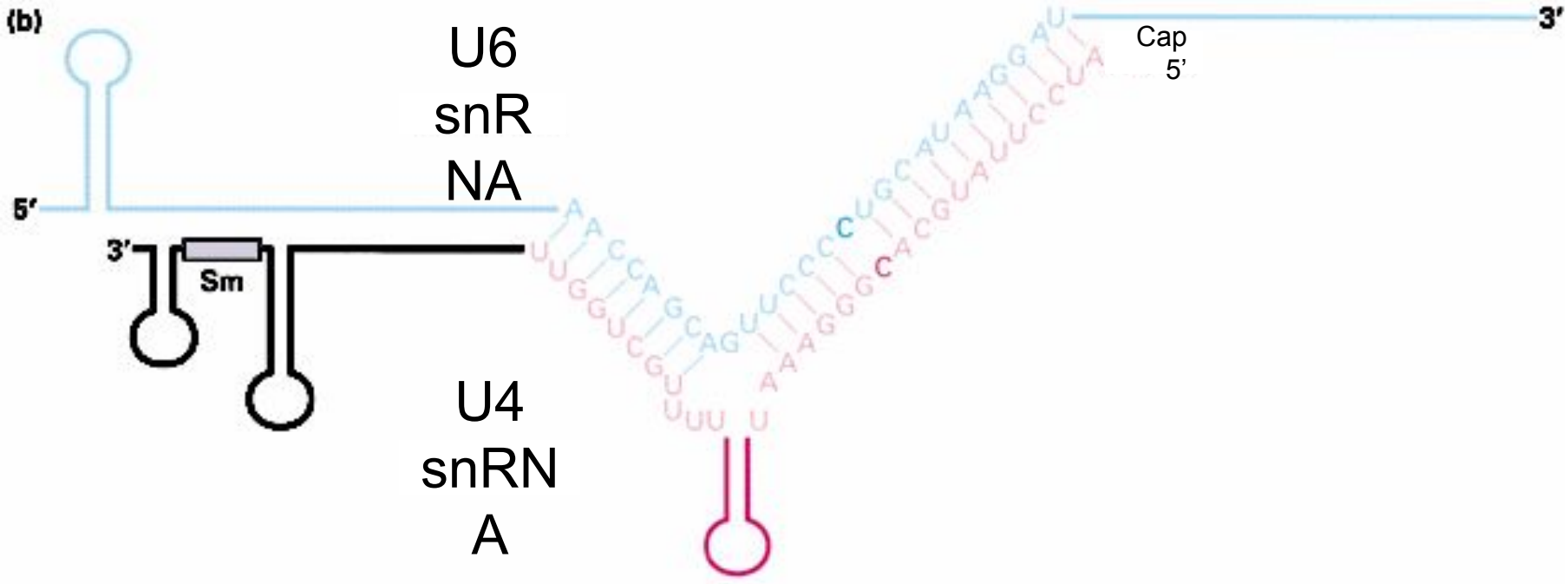


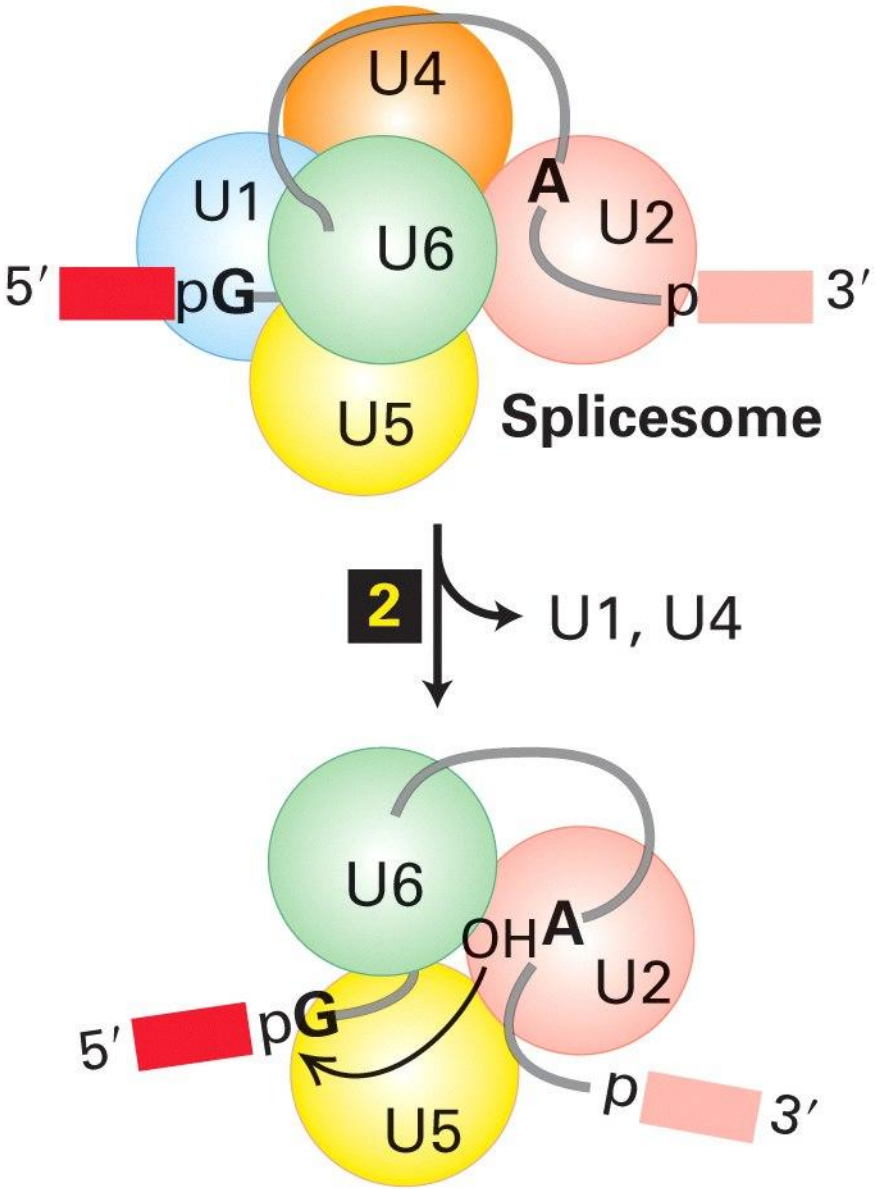
The interactions of U1 with the 5' splice site and U2 with the branchpoint were proven by creating mutant splice sites that bound the snRNA so poorly that splicing was inhibited. Compensating mutations in the snRNA that restored complementarity (base-pairing) with the splice site restored splicing.



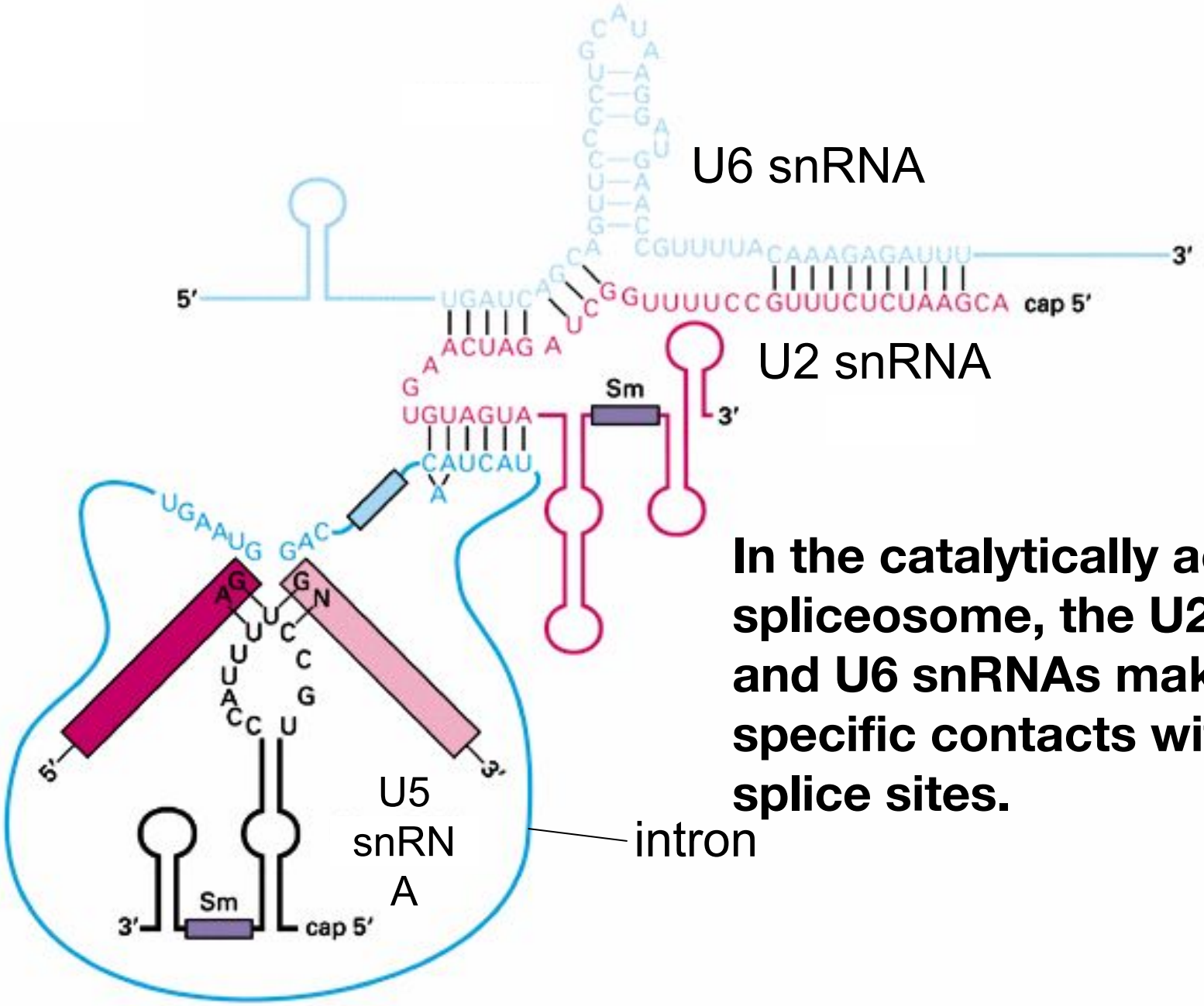
The full spliceosome is formed from the pre-spliceosome by the addition of the U4/U5/U6 Tri-snRNP.

In the U4/U6 Di-snRNP and the U4/U5/U6 Tri-snRNP, the U4 and U6 snRNAs are base-paired to each other. This interaction is later disrupted in the formation of the active spliceosome.

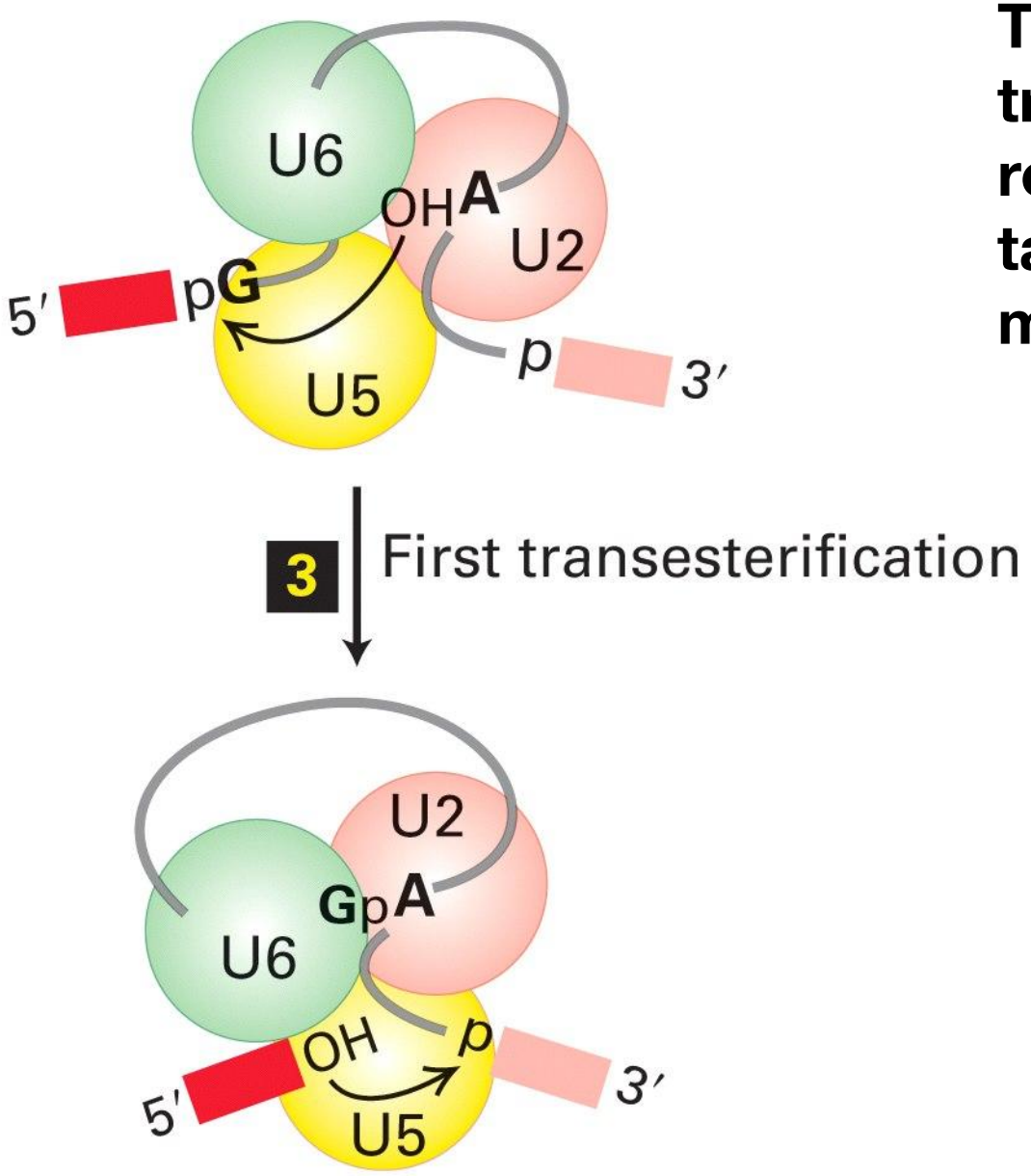




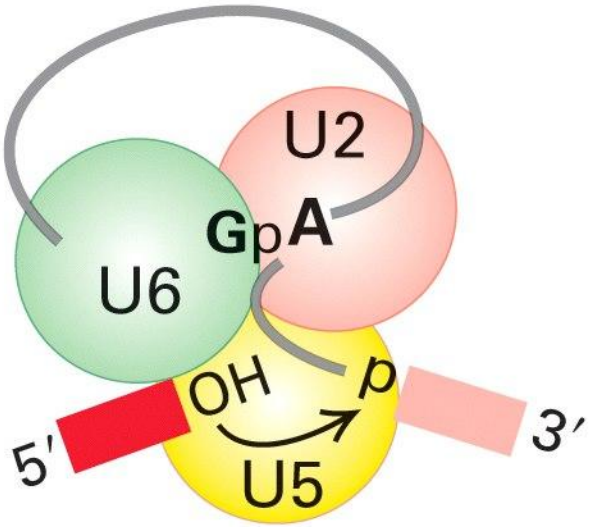
After the formation of the full spliceosome, the U1 and the U4 snRNPs are detached and the remaining U2, U5 and U6 snRNAs are rearranged. This conformational change creates the catalytic spliceosome.



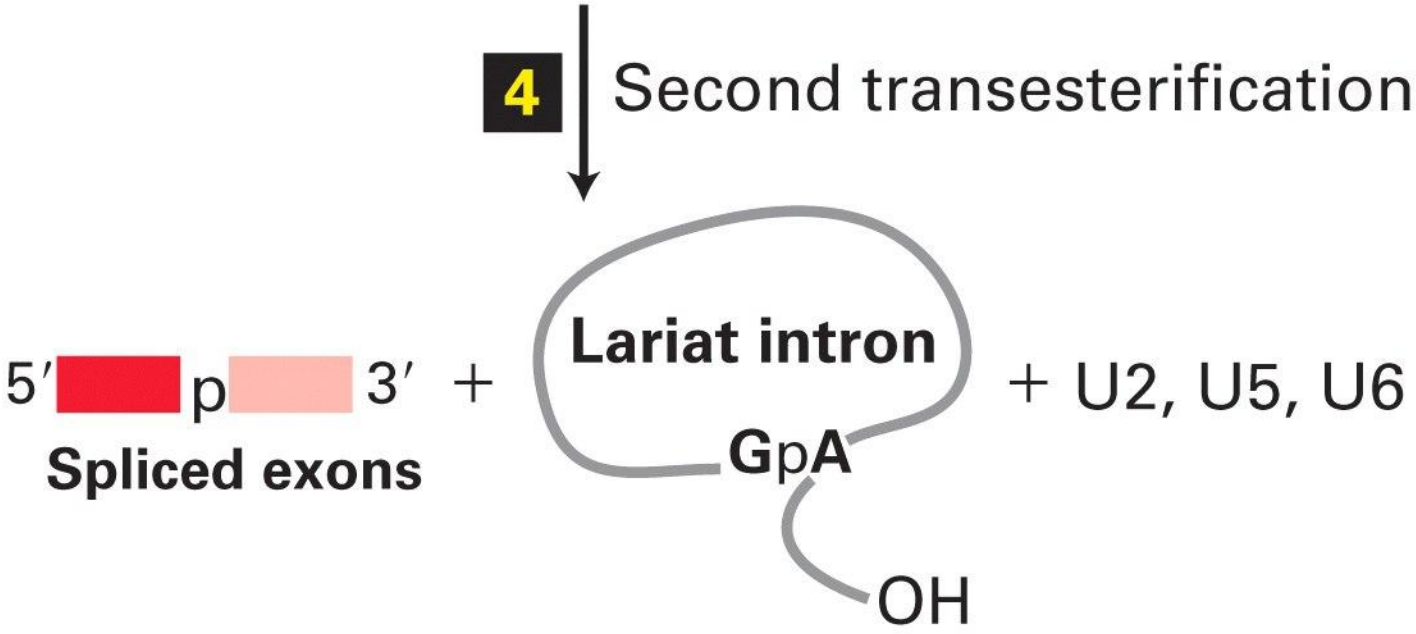
In the catalytically active spliceosome, the U2, U5 and U6 snRNAs make very specific contacts with the splice sites.



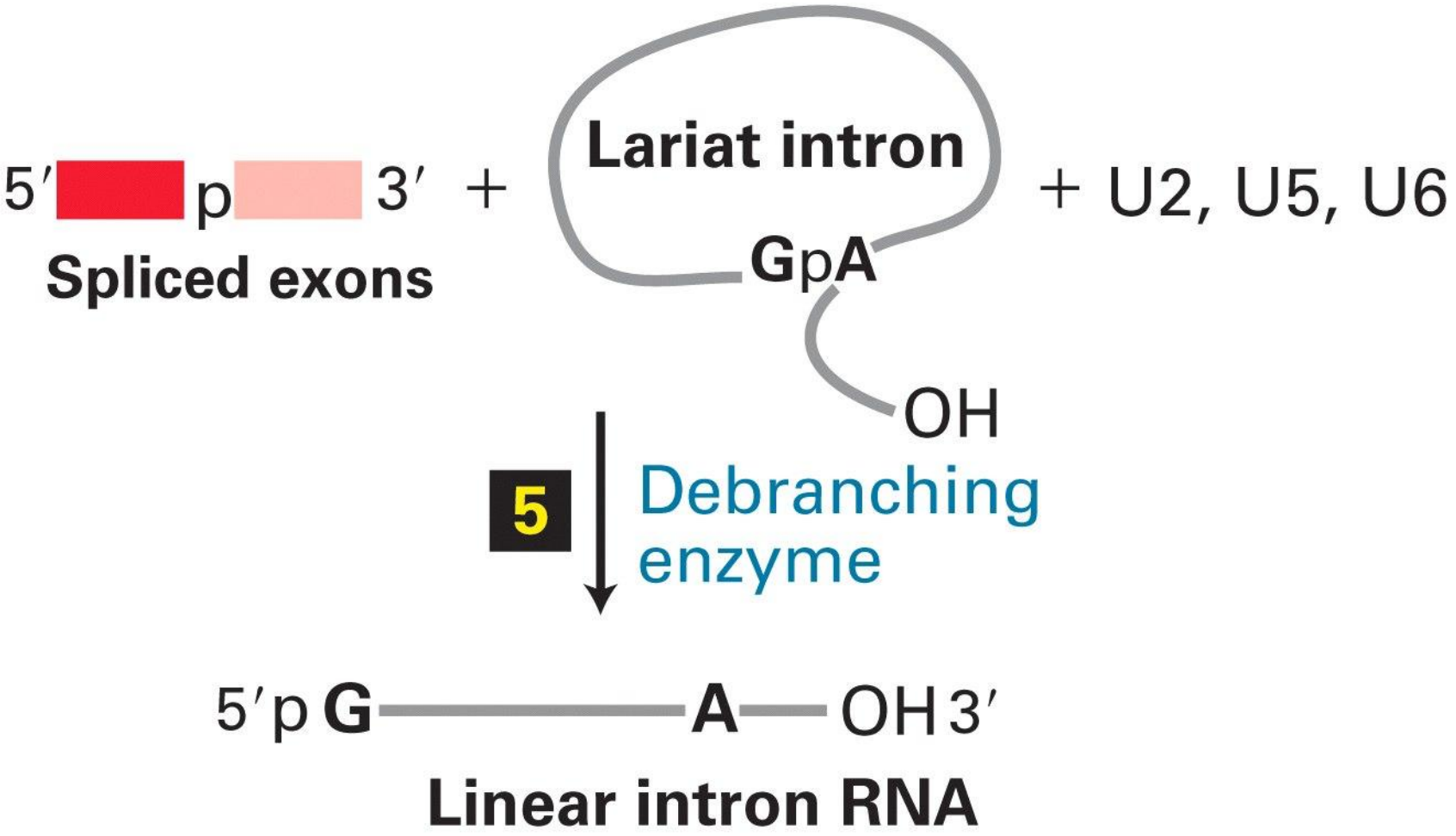
The two transesterification reactions of splicing take place in the mature spliceosome.



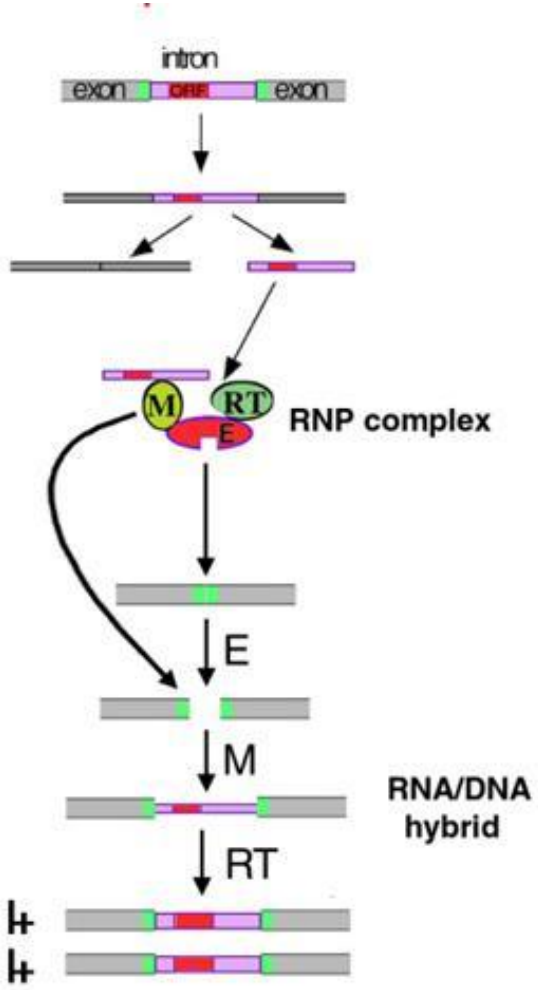
After the second transesterification reaction, the spliceosome comes apart. The snRNPs are recycled, and the spliced exons and the lariat intron are released.



The lariat intron is debranched by Debranching Enzyme returning it to a typical linear state. This linear intron is quickly degraded by ribonucleases.

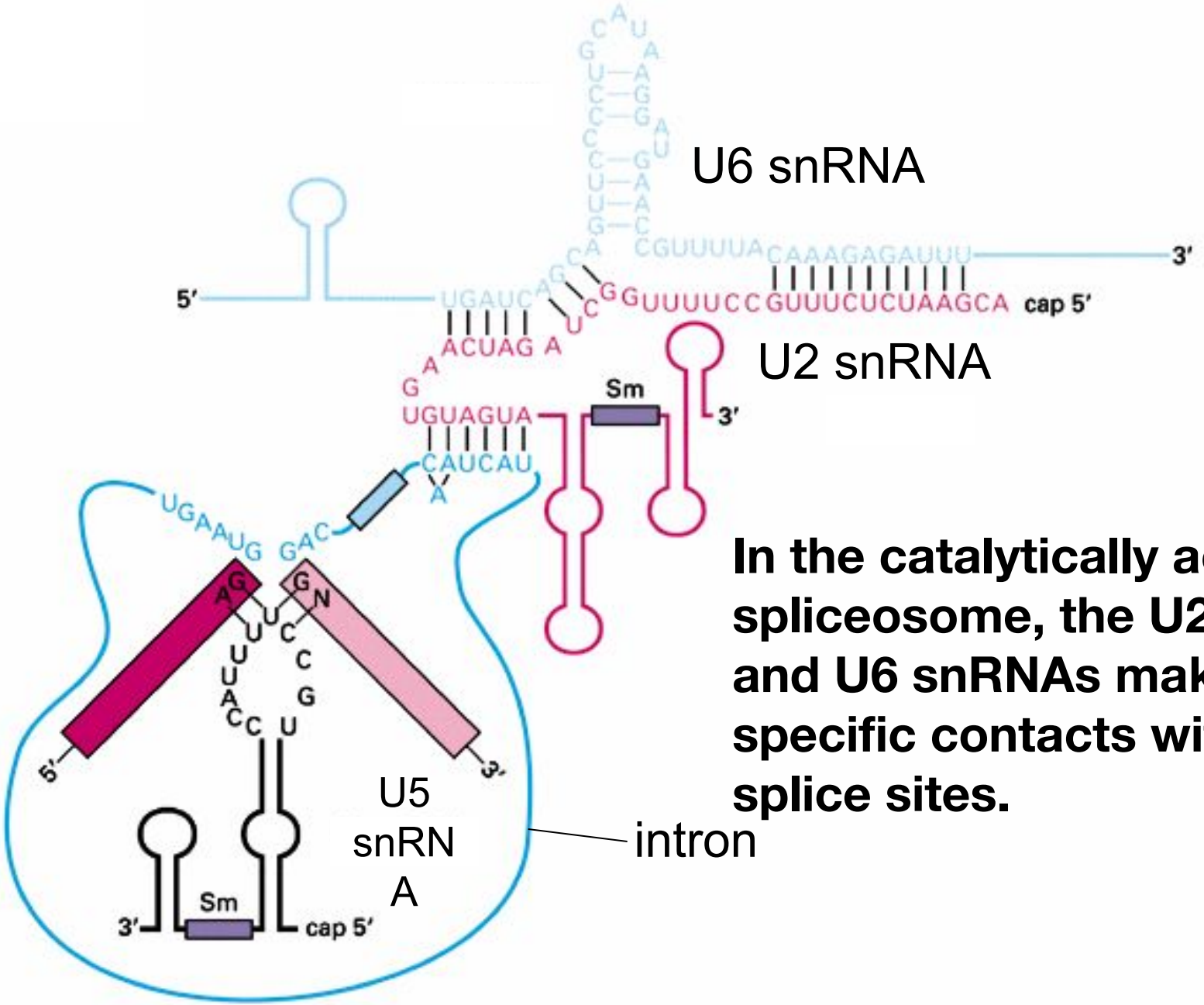


Mobile genetic elements provide an example of RNP complexes in which proteins and RNAs cooperate for specificity



group II self-splicing intron encodes an endonuclease (E) maturase (M) and reverse transcriptase (RT) that are used for integration of the mobile element back into the genome. The intron, E, M, and RT form an RNP and the 2'OH of the intron directs cleavage of the first strand of the target DNA.

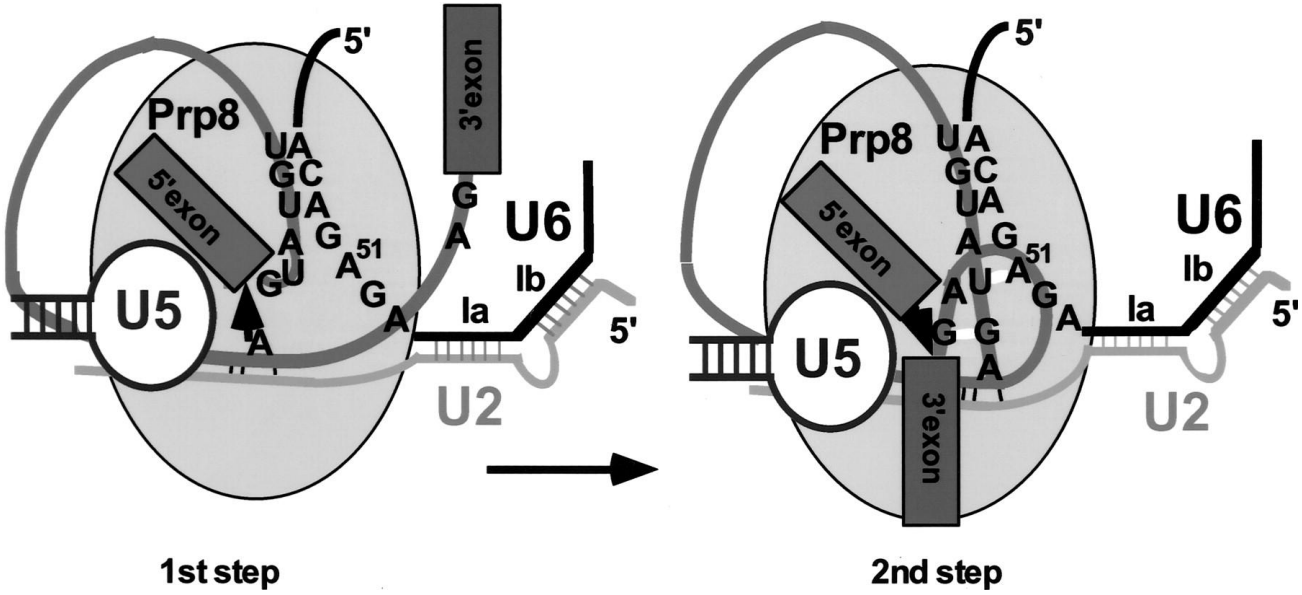
Group II self-splicing intron forms the core of an RNP that can direct cleavage of other nucleic acid polymers.



In the catalytically active spliceosome, the U2, U5 and U6 snRNAs make very specific contacts with the splice sites.

What are the proteins doing in catalysis?

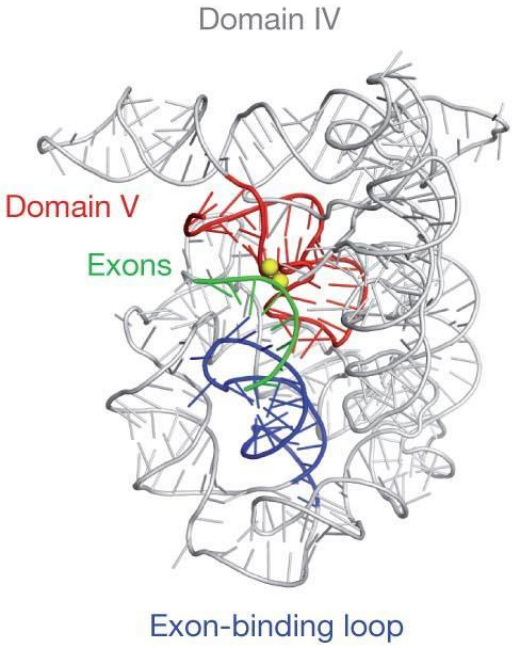
A tale of the U5 protein, Prp8.



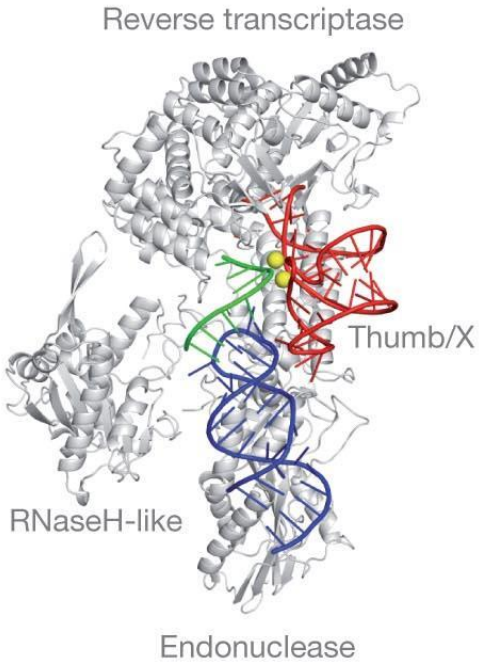
Prp8 mutants are splicing defective.
Many Prp8 mutations suppress splicing defects caused by 5'-SS, 3'-SS and branch point mutations.
Prp8 cross links to crucial U5, U6, 5'-SS, 3'-SS and branch point residues.
Prp8 interacts with Brr2 and Snu114, which unwind U4/U6 and allow U2 to pair with U6

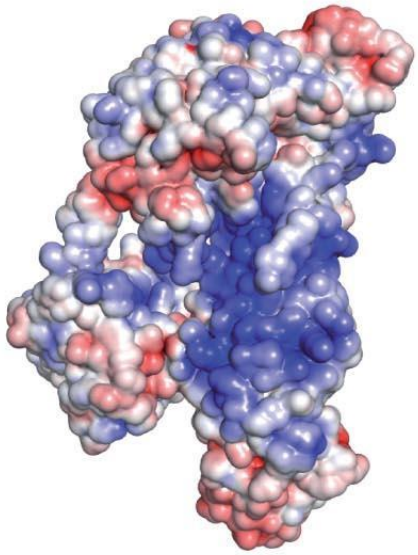
Crystal structure of Prp8 reveals a cavity of appropriate dimensions to position spliceosomal RNAs for catalysis.

Group II intron



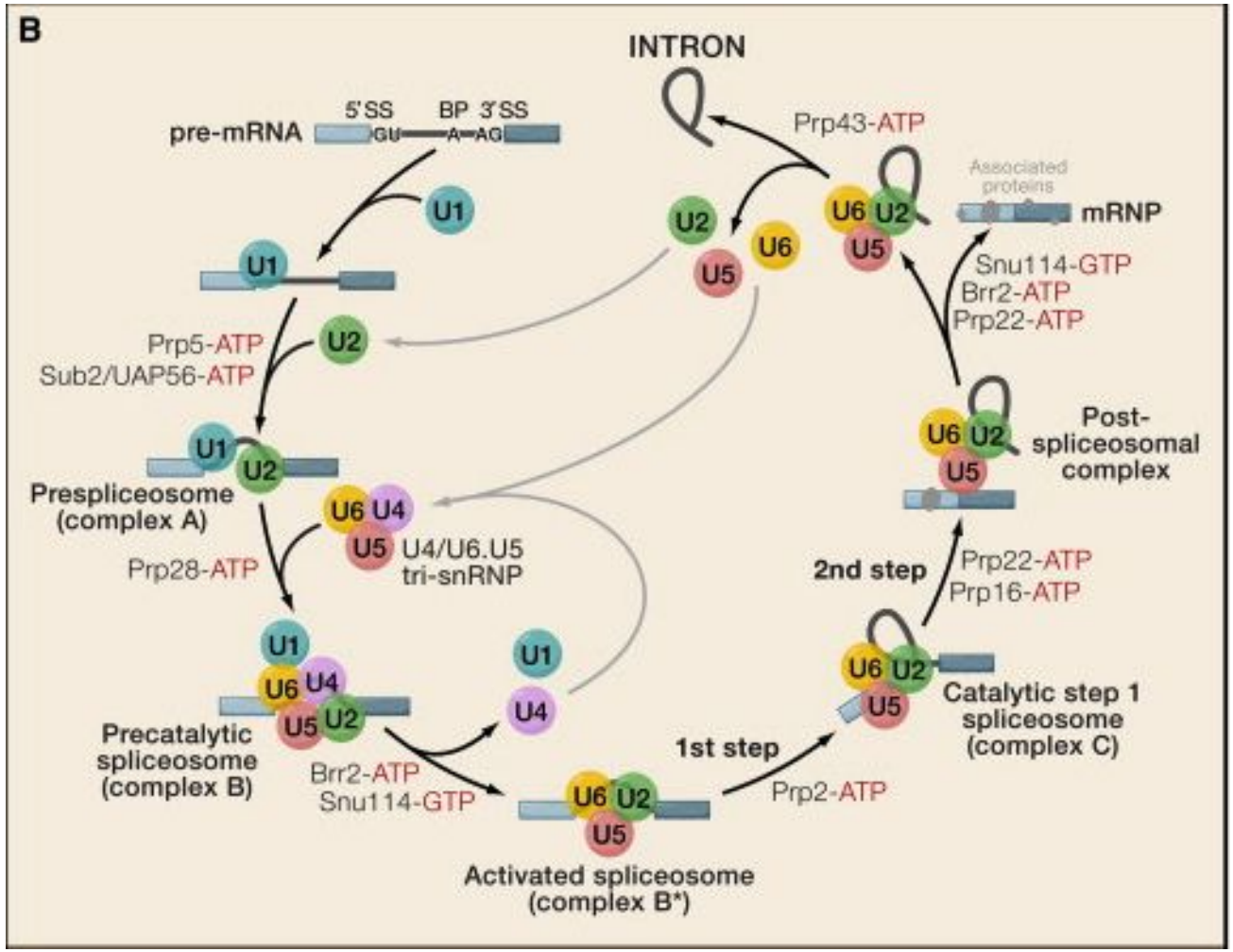
Prp8



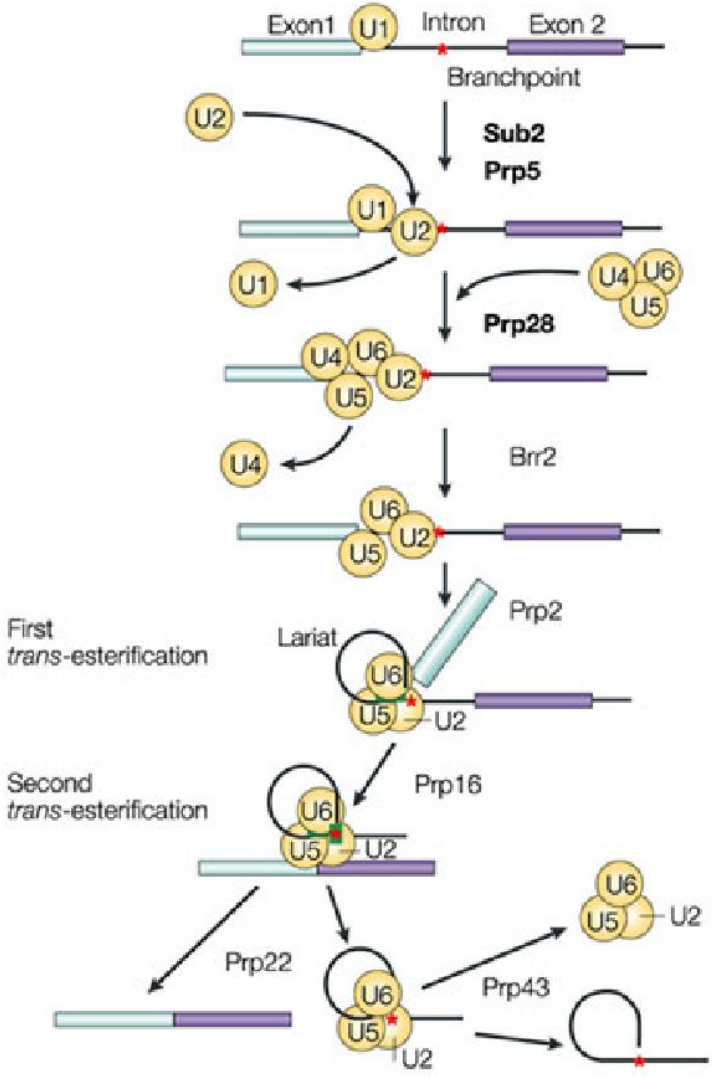


Structural domains of Prp8 (endonuclease, reverse transcriptase) suggest ancient evolutionary origins as a homing endonuclease.

Splicing is dynamic, with sequential regulated alterations in RNA:RNA and RNA:protein interactions

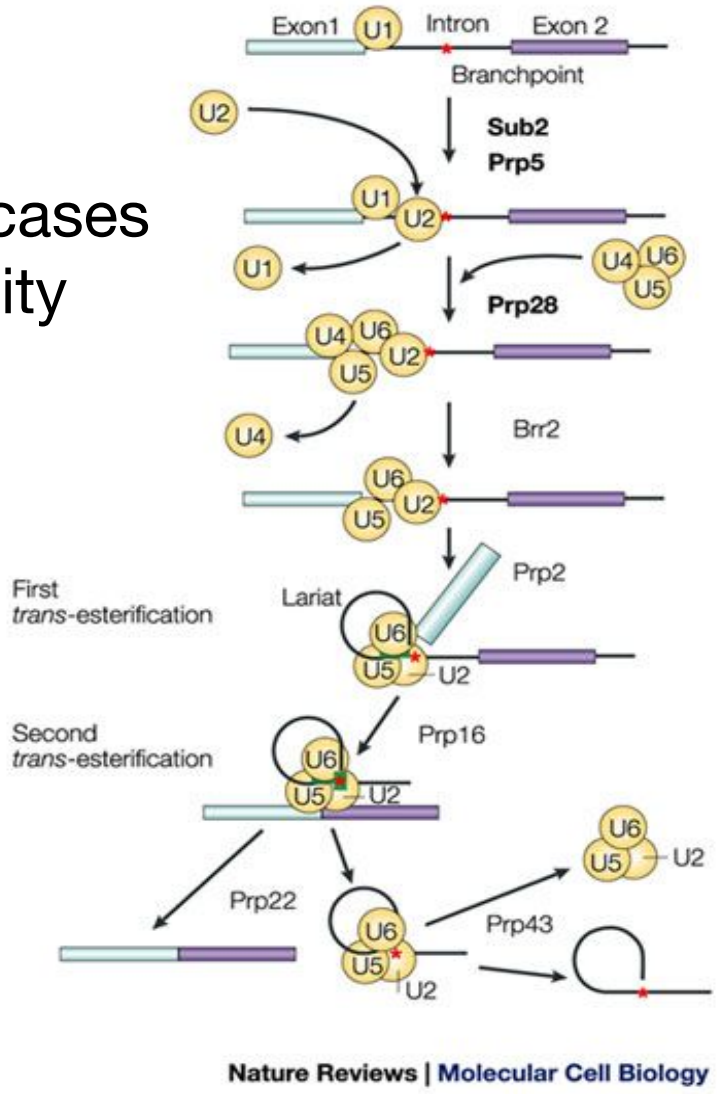


DEAD-box helicases found at every step



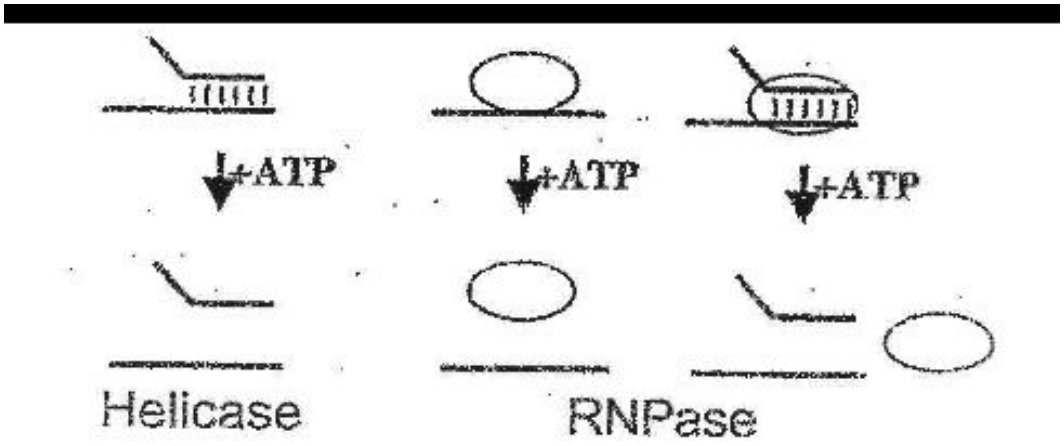
Splicing error rates range from 1 in 1000 to 1 in 100,000

DEAD-box RNA helicases implicated in quality control



Transitions regulated by DEAD-box ATPases

Monomeric (vs. “AAA” ATPases)
RNA-dependent ATPases
~300 aa domain with 7 signature motifs (e.g. eponymous tetrapeptide)
2 RecA-like folds bind ATP, RNA (“closed form”)
Conformation opens upon ATP hydrolysis (i.e. switch-like)



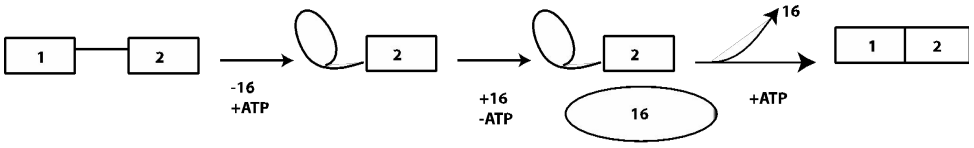
8 ***essential <u>spliceosomal</u>*** DEAD-box ATPases in yeast (more in mammals)

<u>In vitro</u>:
Most catalyze RNA-dependent ATP hydrolysis (**<u>ATPase</u>**)
Some catalyze ATP-dependent RNA unwinding (“**<u>helicase</u>**”)
<u>In vivo</u>????
Likely most are “**<u>RNPases</u>**”, destabilizing RNA:protein complexes

The story of one helicase: PRP16

Prp16 is required for the second chemical step:

- Immunodeplete Prp16, inc. extract w ATP, P-32 substrate -> LI
- Now deplete ATP, then add back rPrp16 + ATP -> Exon ligation
- Instead, add back rPrp16 – ATP -> No splicing, but Prp16 bound



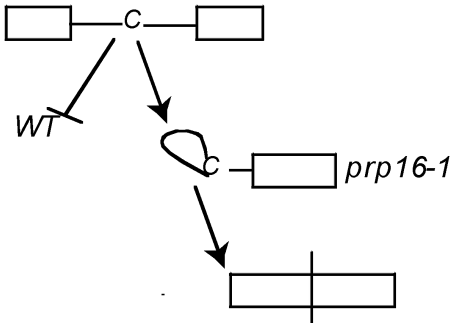
Conclude:

Prp16 can bind to LI but requires ATP hydrolysis for release and promotion of the second chemical step

The story of one helicase: PRP16

Prp16-1 mutant was identified in a screen for reduced-fidelity mutants:

Mutate branchpoint A to C in a splicing reporter
Mutagenize cells ->Select for improved splicing of reporter



Repeat selection by mutagenesis of cloned PRP16 gene ->

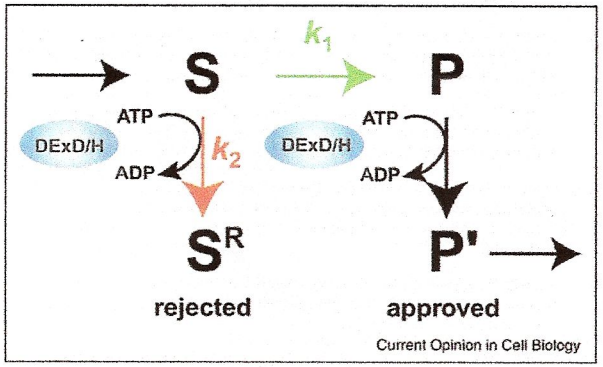
- New suppressors all map to the conserved DEAD-box domain
- In vitro, mutant Prp16 proteins have **reduced** ATPase activity

Conclude:

Prp16 modulates the fidelity of splicing by an ATP-dependent mechanism

The story of one helicase: PRP16

Hypothesis: Prp16 promotes fidelity

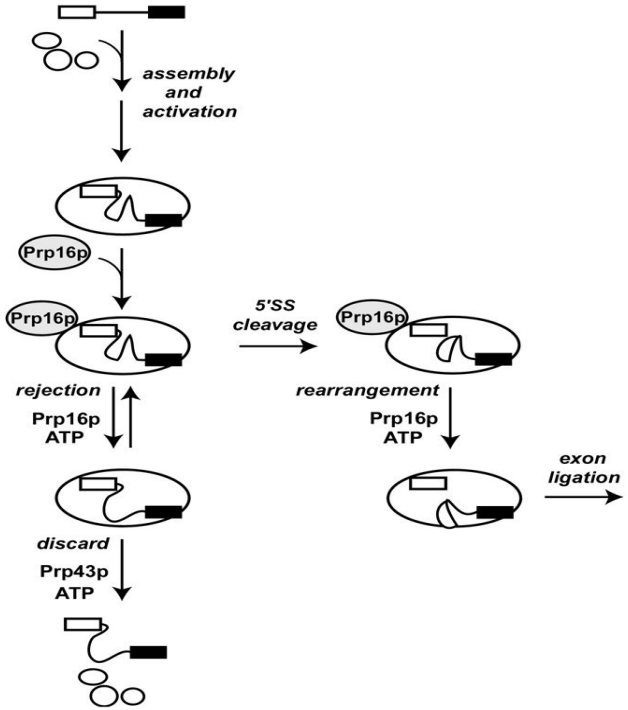


1) *branchpoint* mutations -> slow conformational rearrangement -> rejection

2) *suppressor* mutations in Prp16 -> more time

The story of one helicase: PRP16

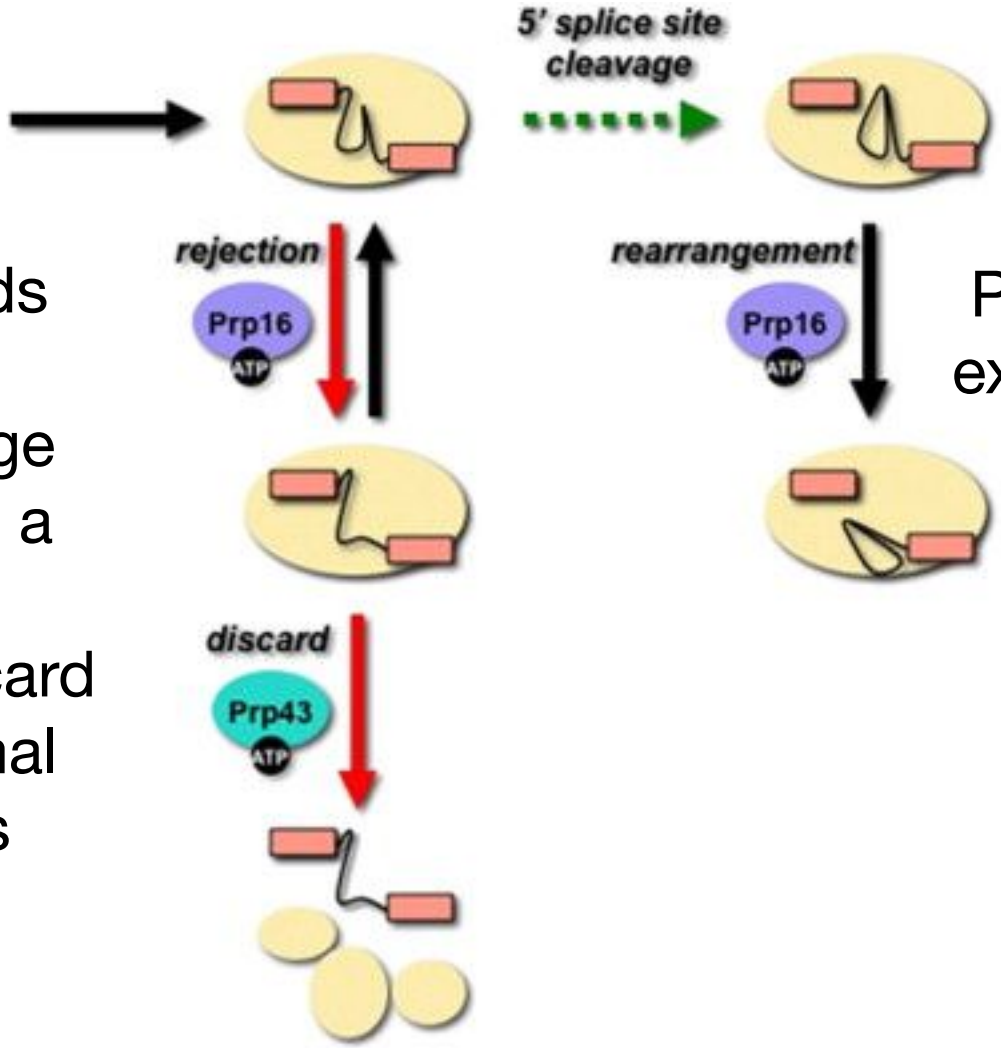
How to discriminate between “correct” vs. “incorrect”?
A “slow” spliceosome -> ATP-dependent rejection of WT substrate.



Conclusion:
ATPases promote specificity by discriminating against slow substrates

PRP16: functions at 2 steps

PRP16 binds before 5’ss cleavage and acts as a sensor to promote discard of suboptimal substrates



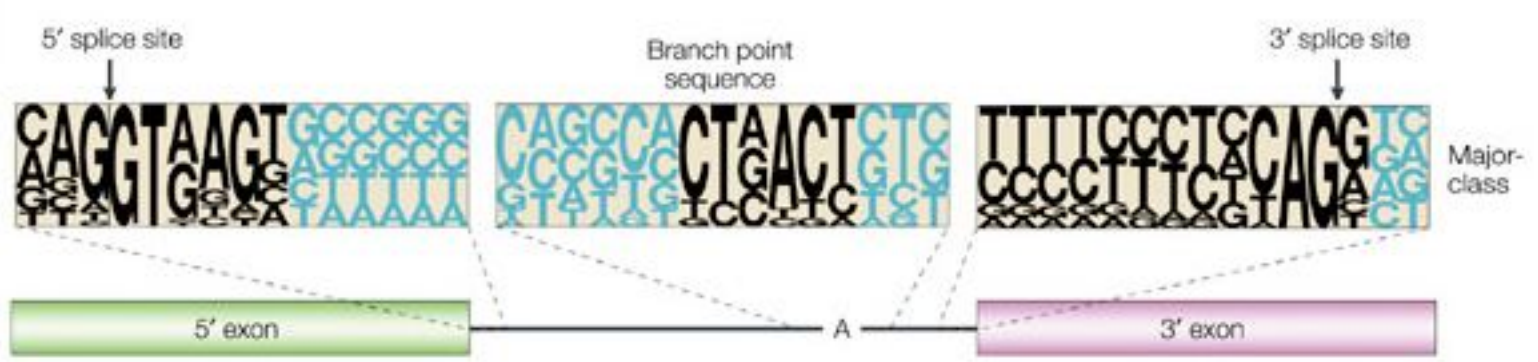
PRP16 promotes exon-exon ligation

Questions

How are the intervening sequences removed?

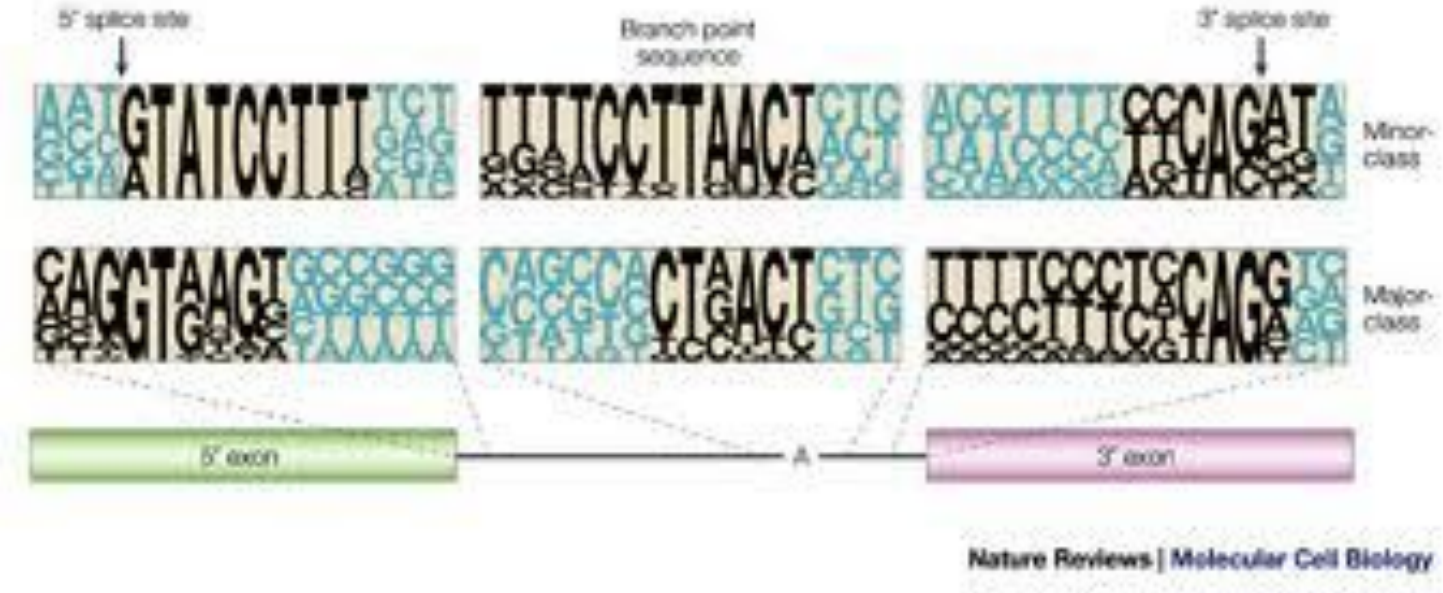
How are the splice sites identified?

How are the splice sites identified?



In higher eukaryotes, there isn't much sequence information encoded in the 3'ss, 5'ss, or branch point

How are the splice sites identified?



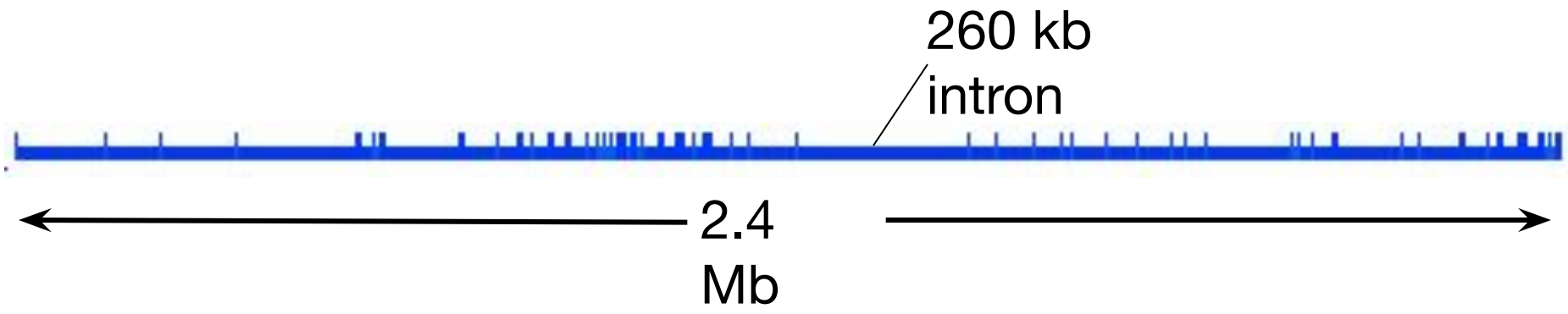
Minor spliceosome, consists of U11, U12, U4atac, U6atac, and U5

About 100-fold less abundant than major spliceosome

Splices ~ 0.2% of introns in vertebrates

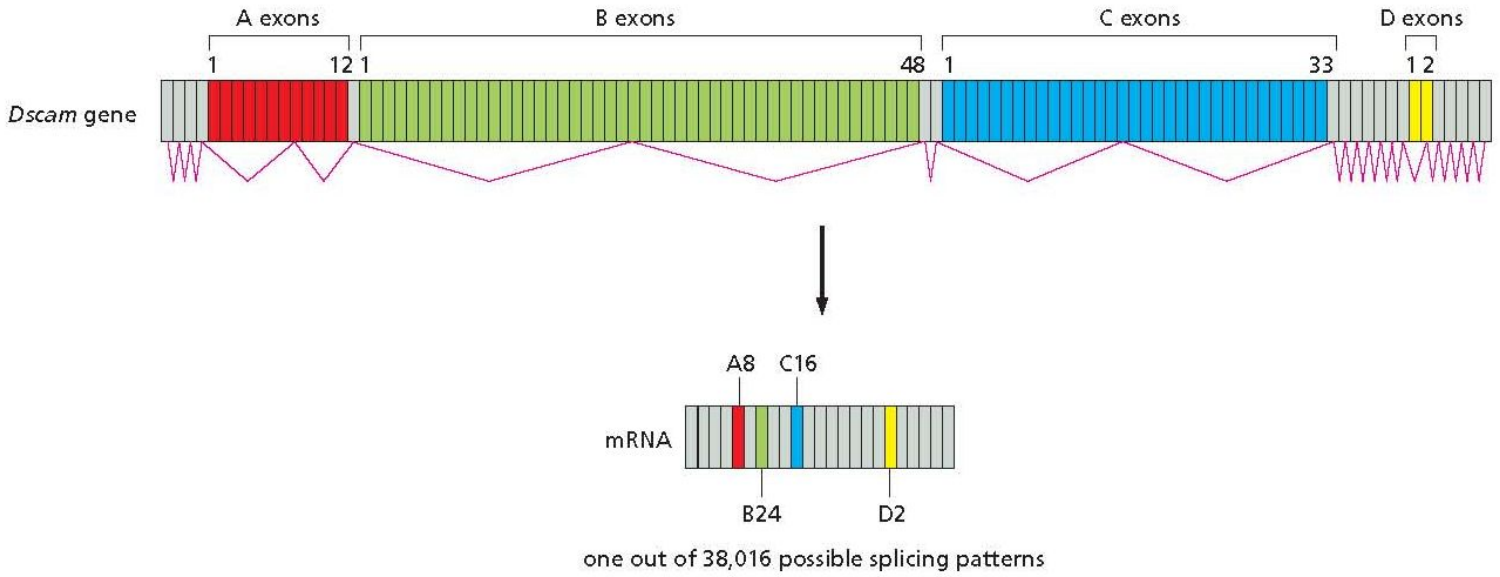
How are the splice sites identified?

Human Dystrophin gene



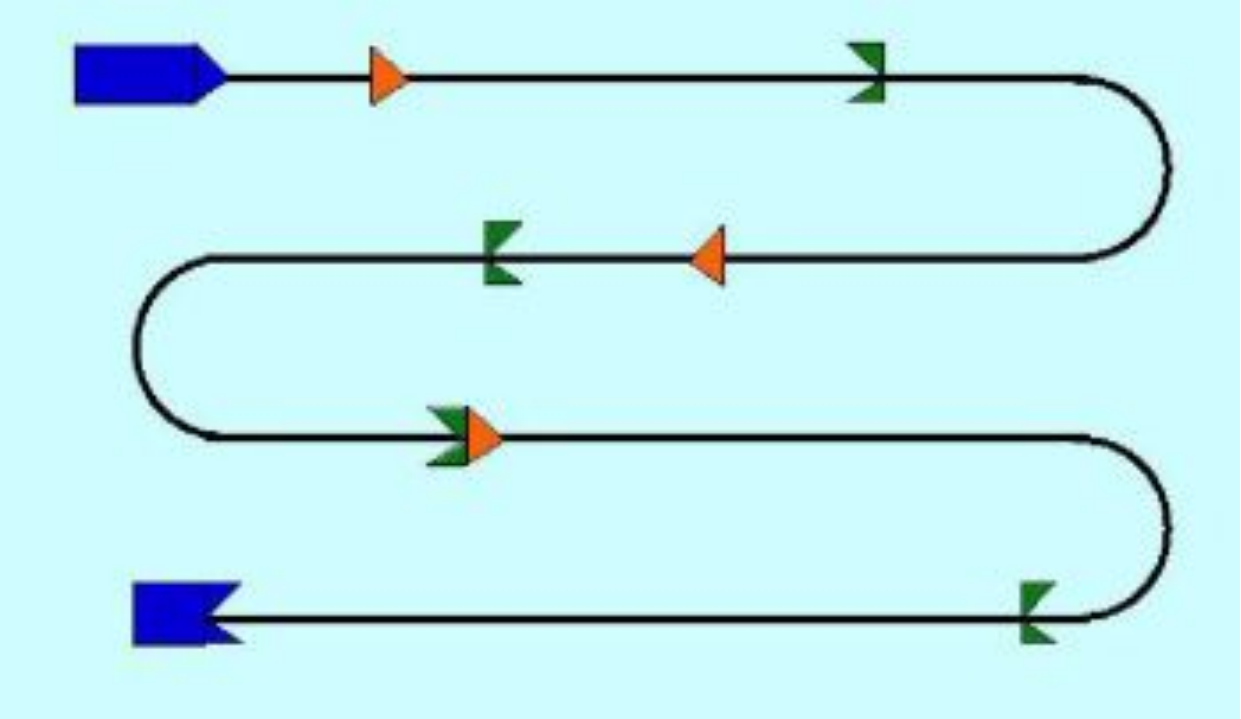
Genes in higher eukaryotes have many exons and introns can be very large

How are the splice sites identified?



The same primary transcript can be spliced many different ways (estimated 90% of genes experience alternative splicing)

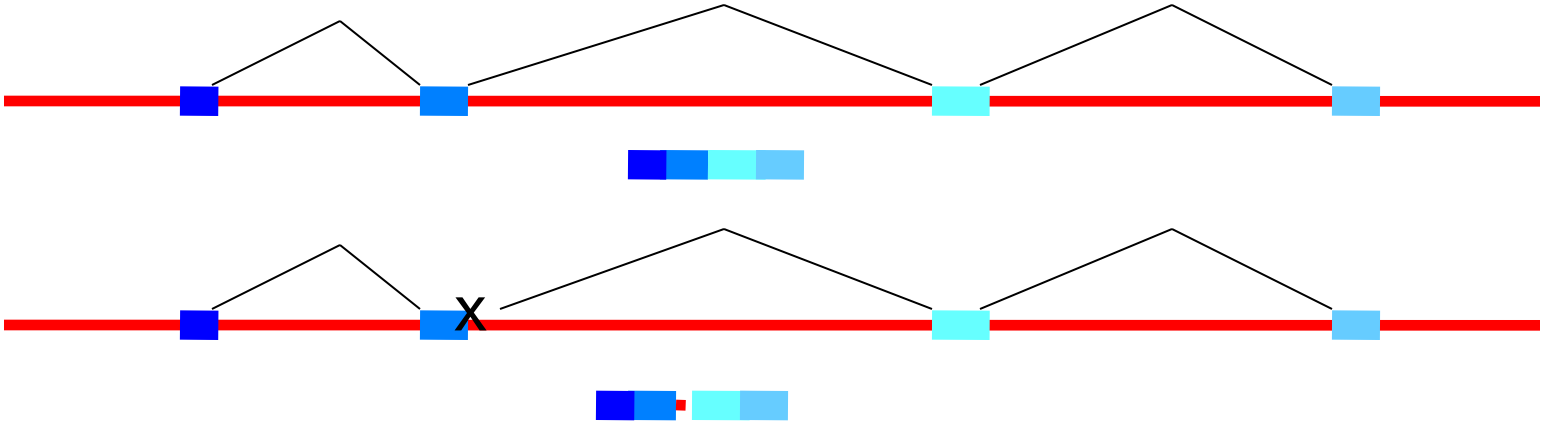
How are the splice sites identified?



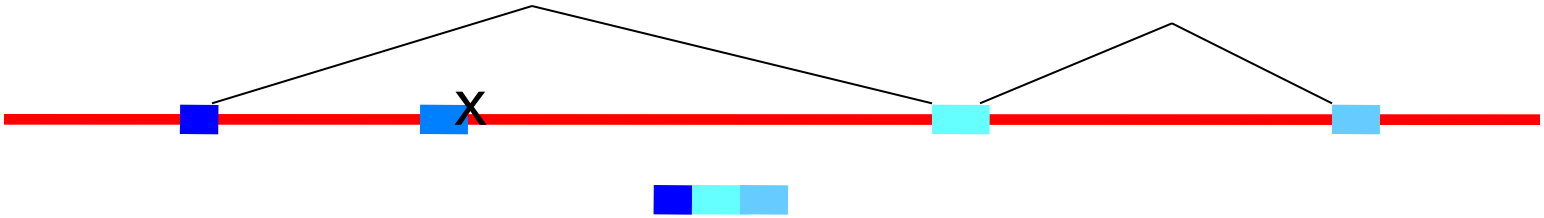
Because of the intron length and lack of specificity of splice sites, most introns contain numerous cryptic splice sites in addition to bona fide alternative splice sites.

How are the splice sites identified?

outcomes of 5’ ss mutants

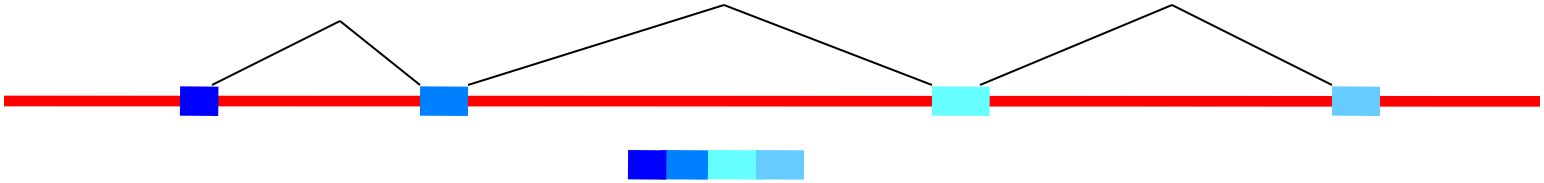


1. activates cryptic 5’ ss, but only if there is one within 100-300 bp of original 5’ ss

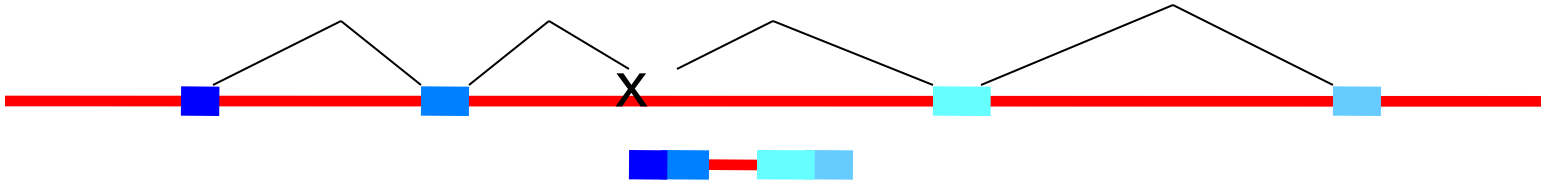


2. skip the exon altogether and ignore perfectly good 3’ and 5’ ss of the upstream intron

How are the splice sites identified?

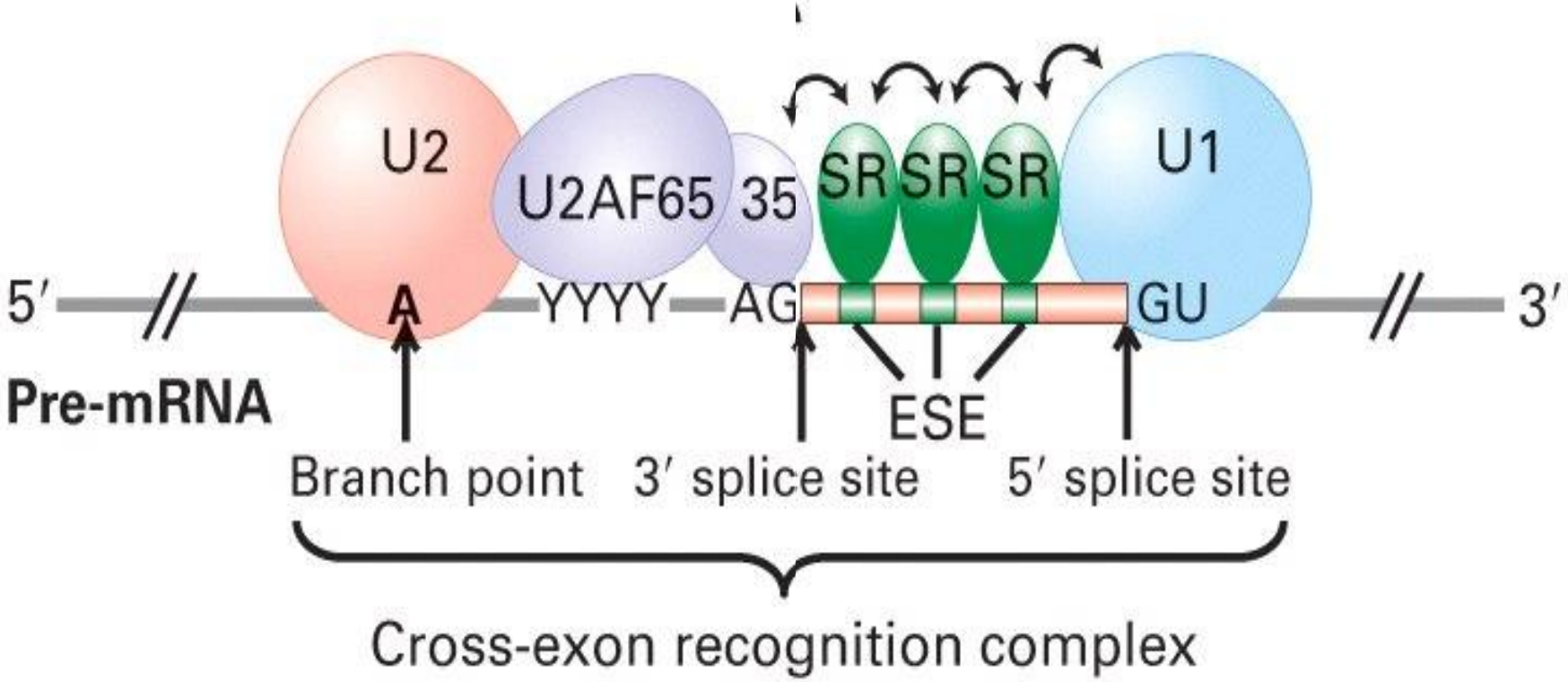


beta-globin mutants that create a new 3’ ss within an intron:



also create a new exon???

In multicellular organisms, exons are recognized as units prior to assembly of the spliceosome across the long introns. This “exon definition” step involves interactions between the splice sites across the exon and special sequences in the exon called Exonic Splicing Enhancers (ESE).

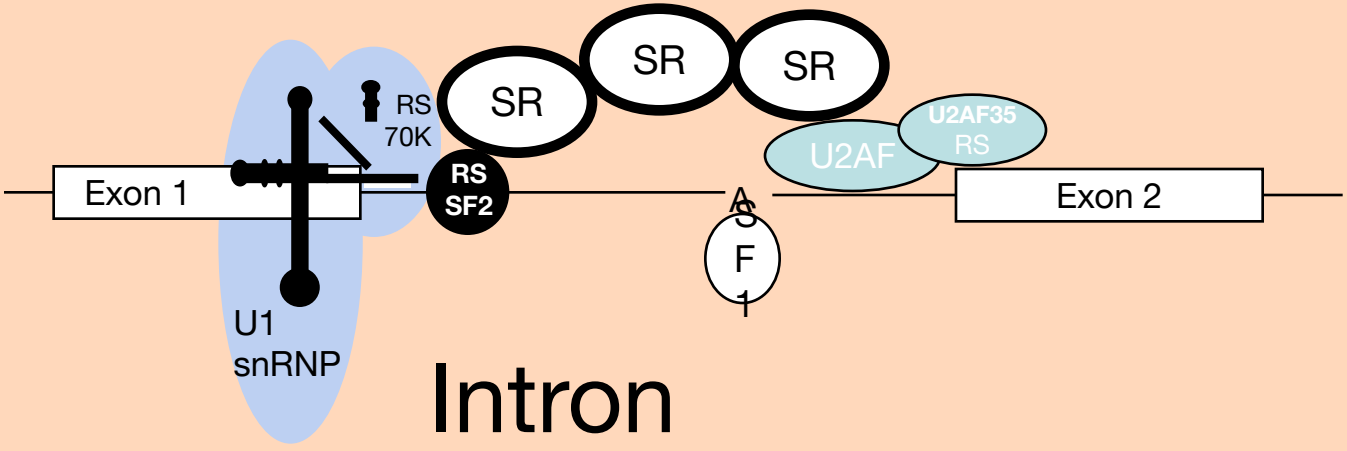


The sequences in exons are selected to not just code for particular peptide sequences, but also for binding of regulatory proteins to ESE’s.

How are the splice sites identified?

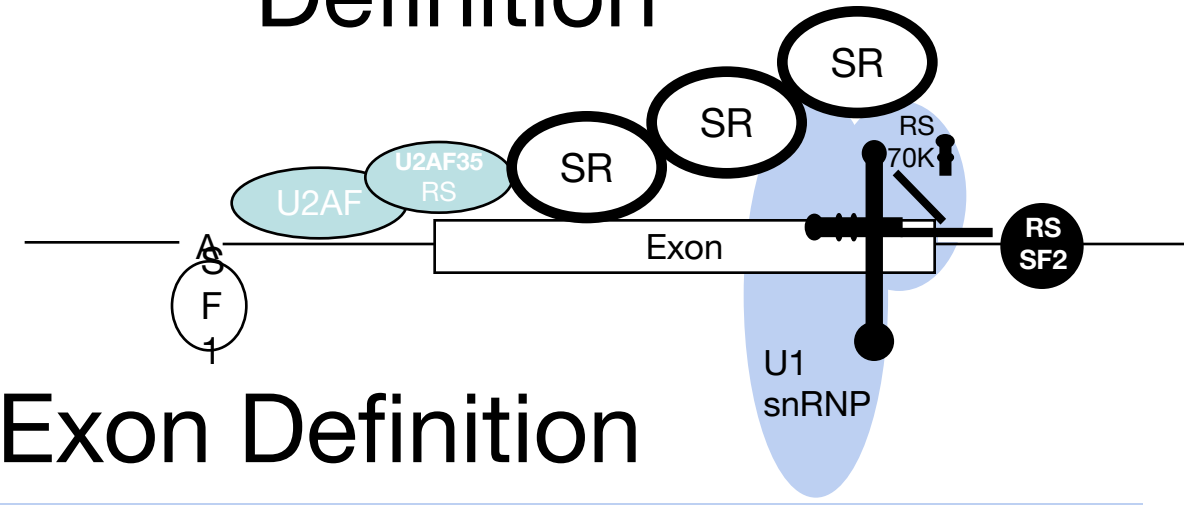
Boundaries between introns & exons are recognized through its interaction with multiple proteins either across exon or intron

Intron definition:
Uses intron as the unit of recognition mechanism. Complex forms through stabilized protein interactions across the intron



Intron Definition

Exon Definition:
Complex can easily form stabilized protein interactions across the exon. Excises out the flanking introns

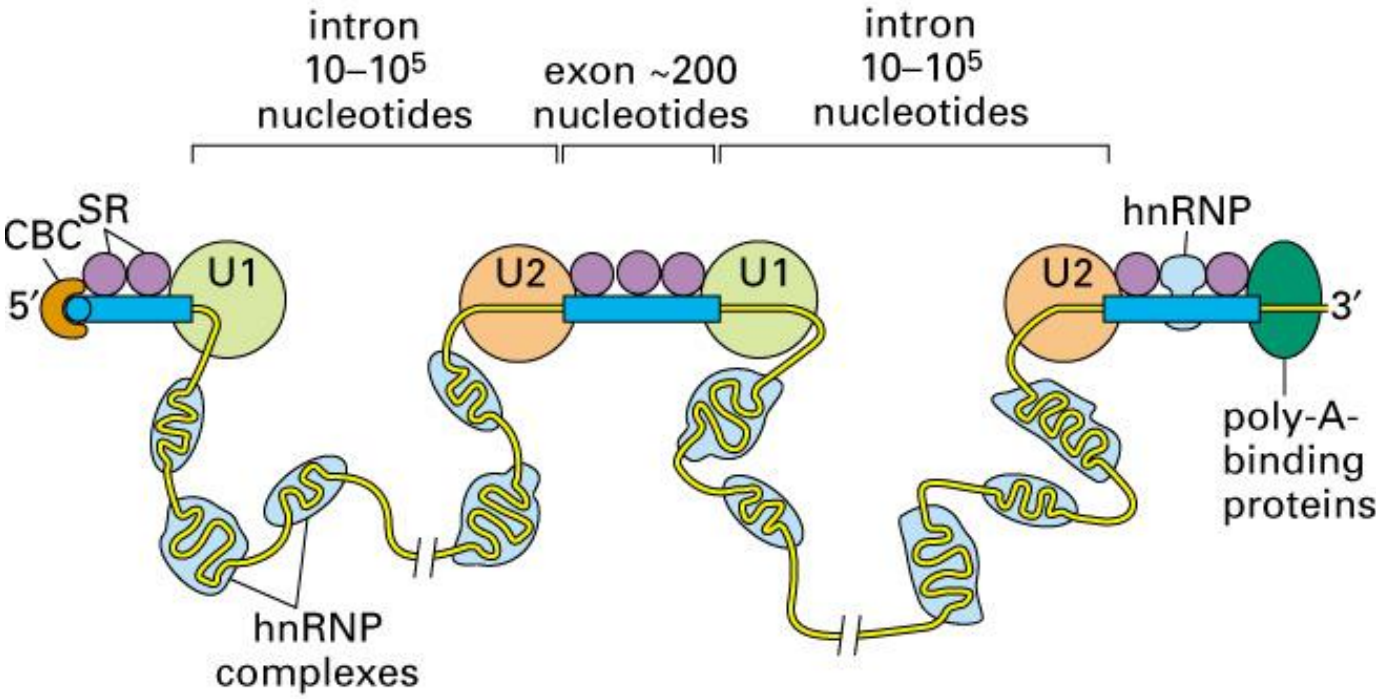


Exon Definition

(Cote, Univ. of Ottawa)

Stable interaction confirms accuracy of splice site choice

Why are exons preferentially recognized?



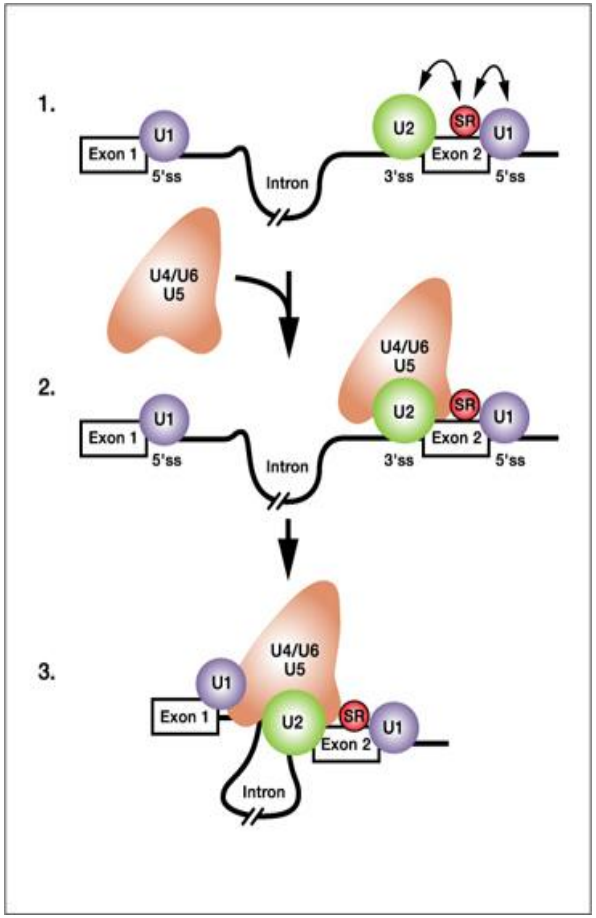
Differential size distributions of exons (~50 to 300 nt) vs. introns (<100-100,000 nt)

- SR protein - preferentially binds to exon sequences
  - mark the 5' & 3' splicing sites in conjunction w/ U1 & U2 during transcription

- hnRNP - heterogenous nuclear ribonucleoproteins (twice the diameter of nucleosome)
  - consists at least eight different proteins
  - compacts introns, thereby masking cryptic splicing sites
  - preferentially binds to introns, but also bind to exons, although less frequently

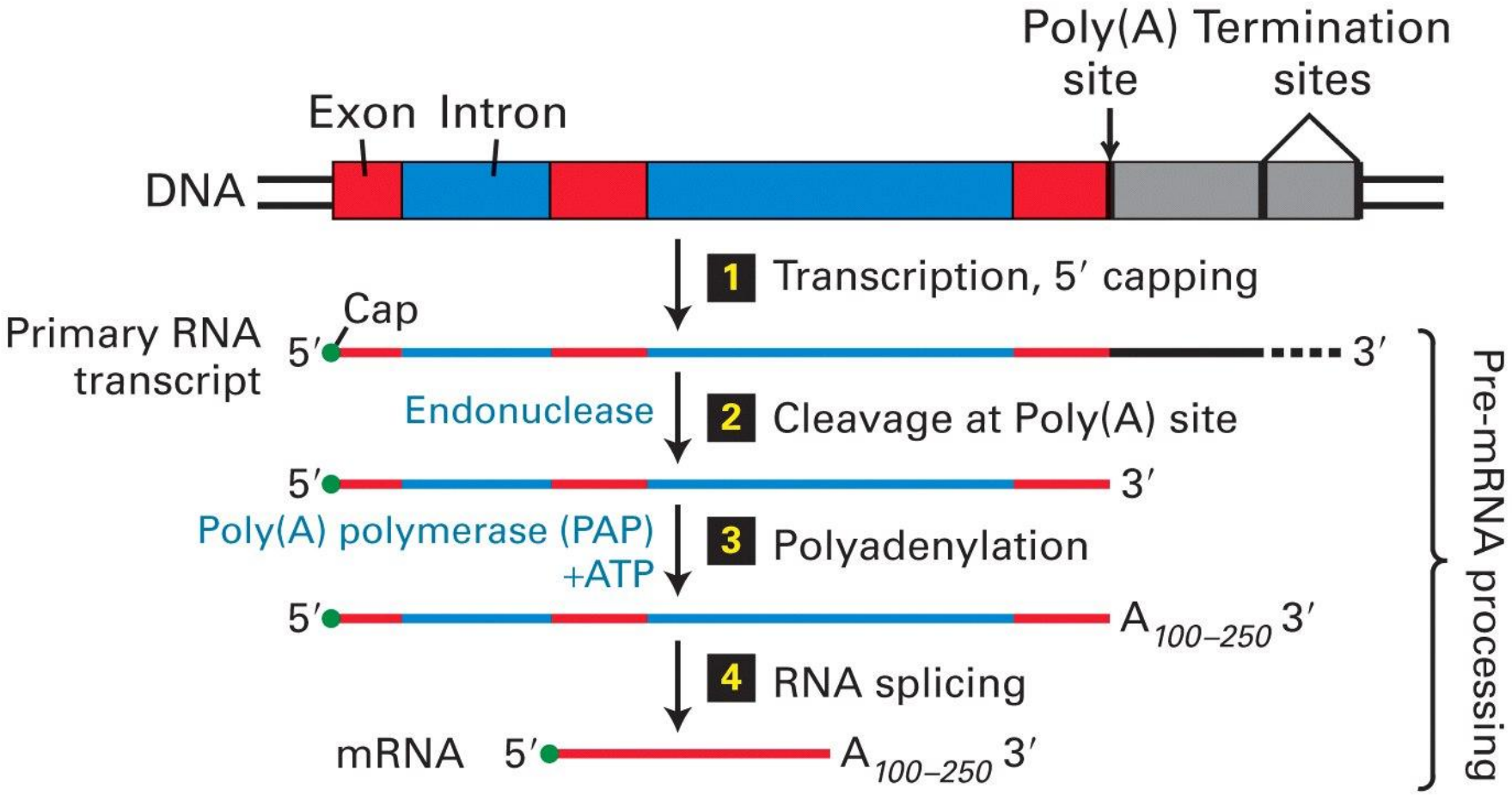
Cross-exon bridging interactions involve SR domains of U2AF, U170K
And 1 or more SR-family proteins

- ~12 in mammals (and # AS isoforms!)
- Tissue-specific differences in concentration
- RRMs vary in degree of sequence preferences

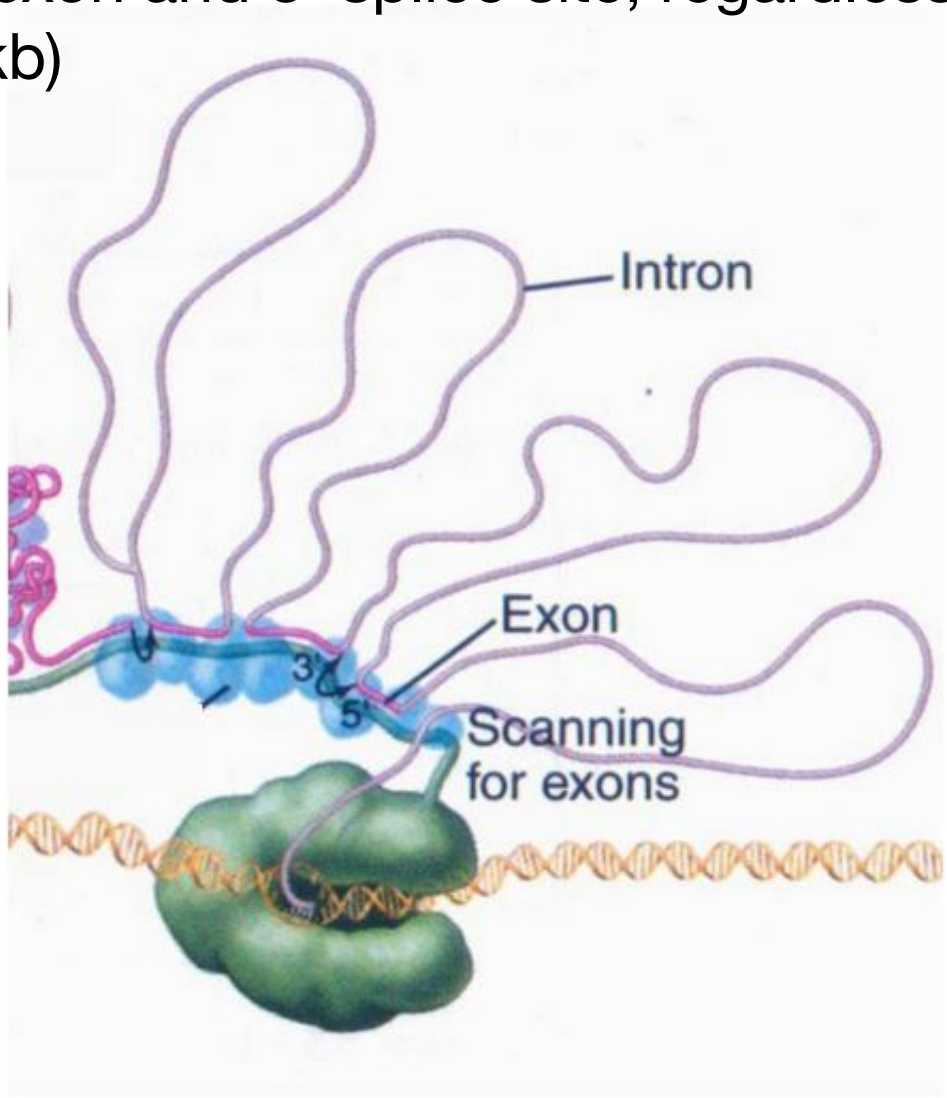


Outstanding question:
What triggers the switch from Exon- to Intron-Defined interactions?

Vertebrate external exons



Splicing is co-transcriptional and all introns assayed are spliced within 5-10 minutes of transcription of the downstream exon and 3' splice site, regardless of intron size (1 kb or 240 kb)



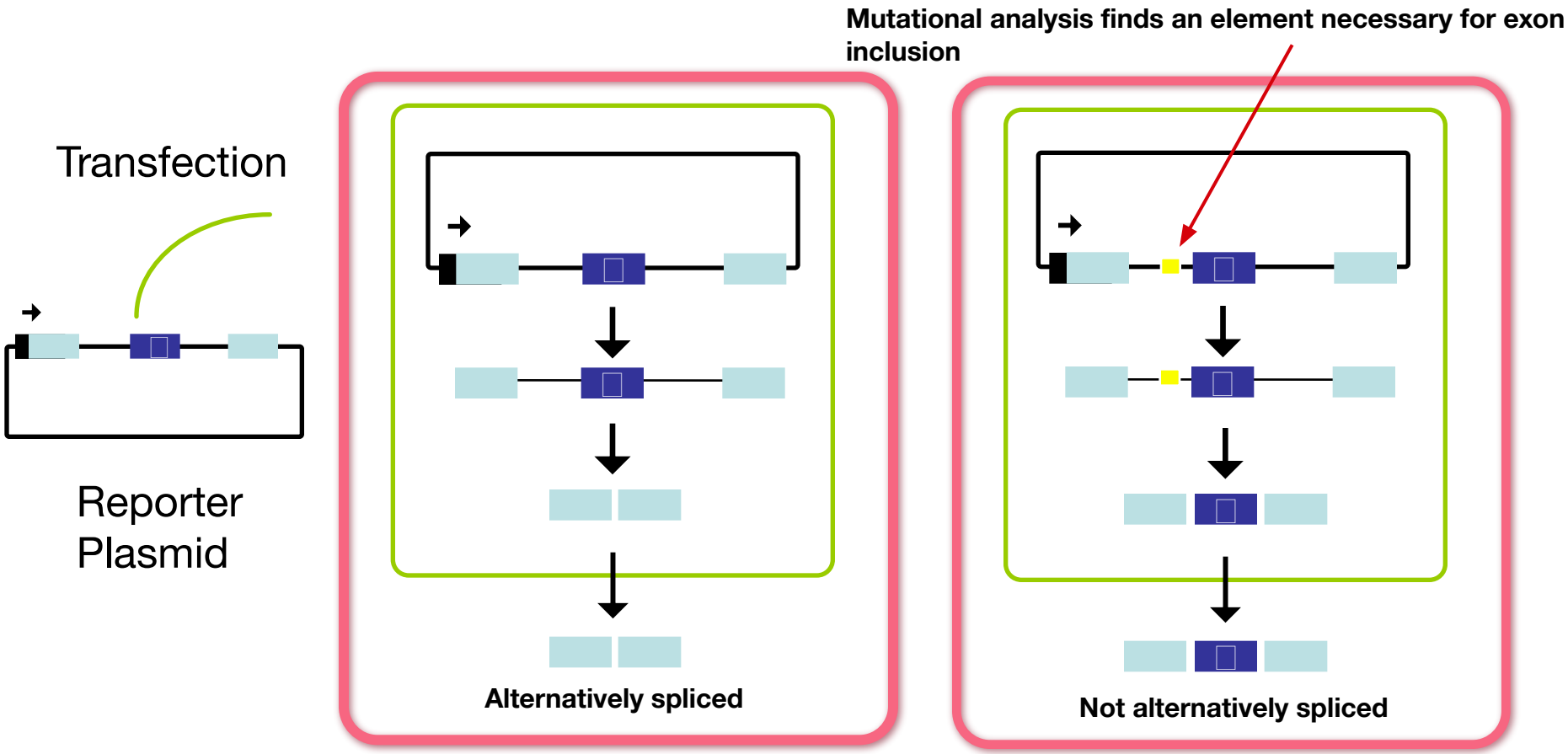
Defining an exon involves the specific *stabilization* or *destabilization* of splice site recognition

Stabilization: exon inclusion
Destabilization: exon skipping

Regulation of alternative splicing involves the specific *stabilization* or *destabilization* of splice site recognition

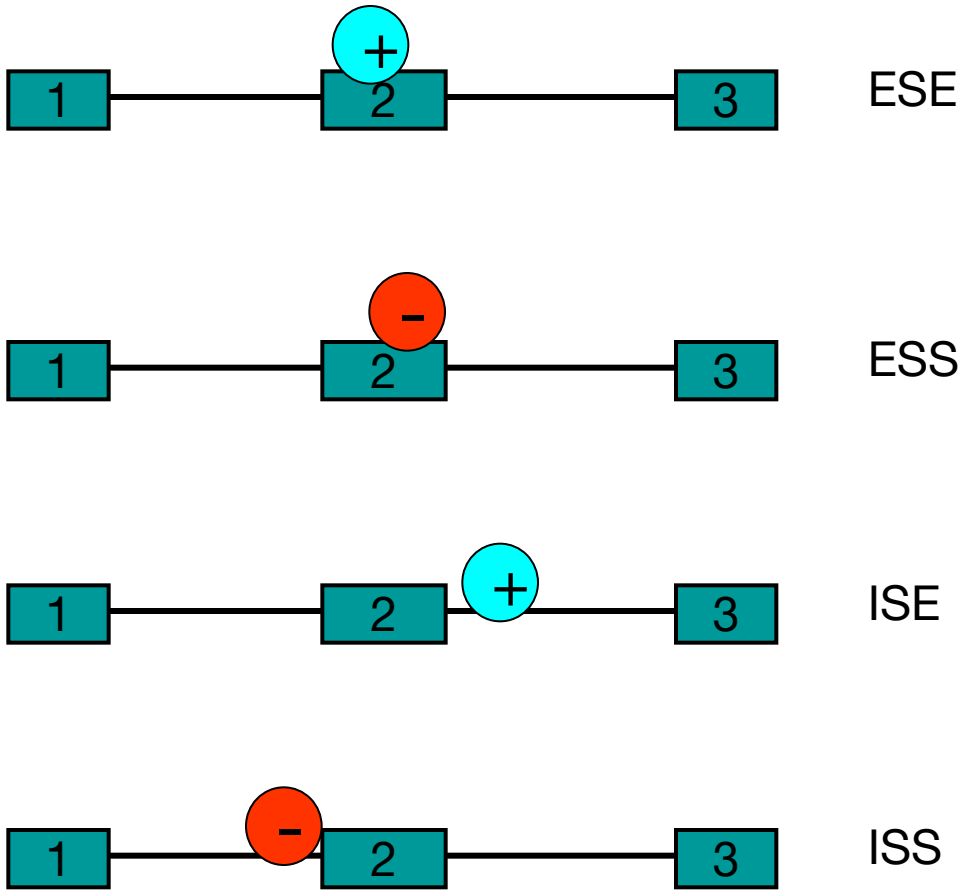
Stabilization: exon inclusion
Destabilization: exon skipping

How would you identify *cis*-regulatory sequences responsible for alternative splicing ?

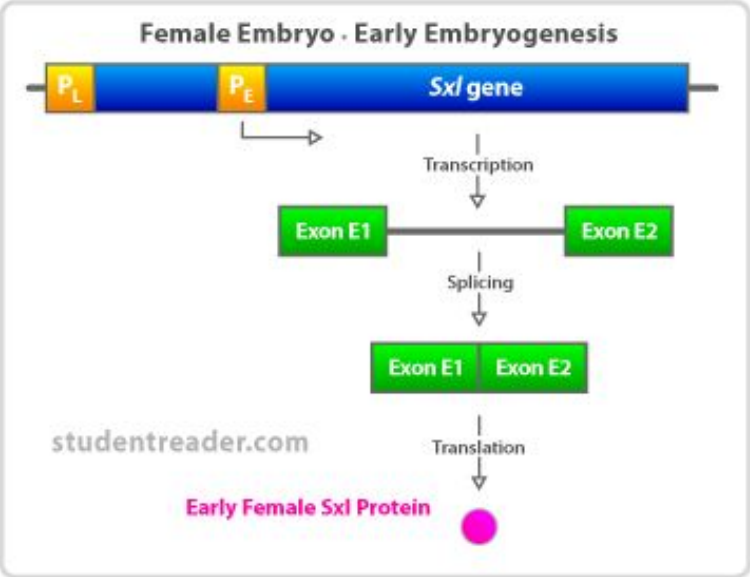


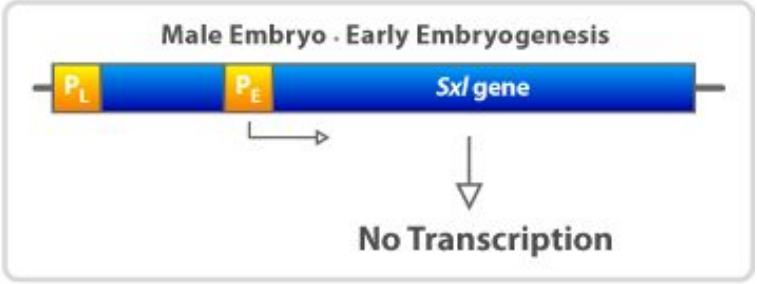
Examine RNA Splicing of Transfected Splicing Reporters to identify cis-regulatory regions

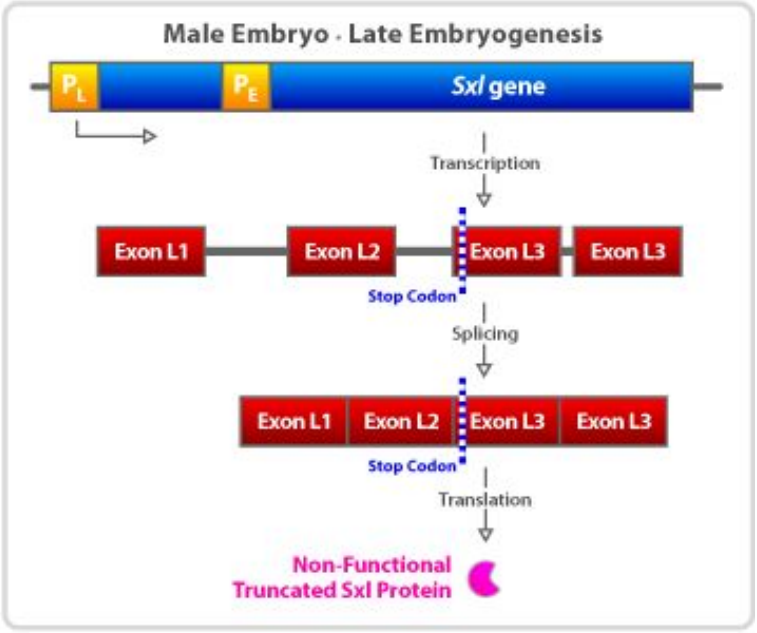
Four classes of splicing regulatory elements: Exonic Splicing Enhancers, Exonic Splicing Silencers (ESS), Intronic Splicing Enhancers (ISE), and Intronic Splicing Silencers (ISS).

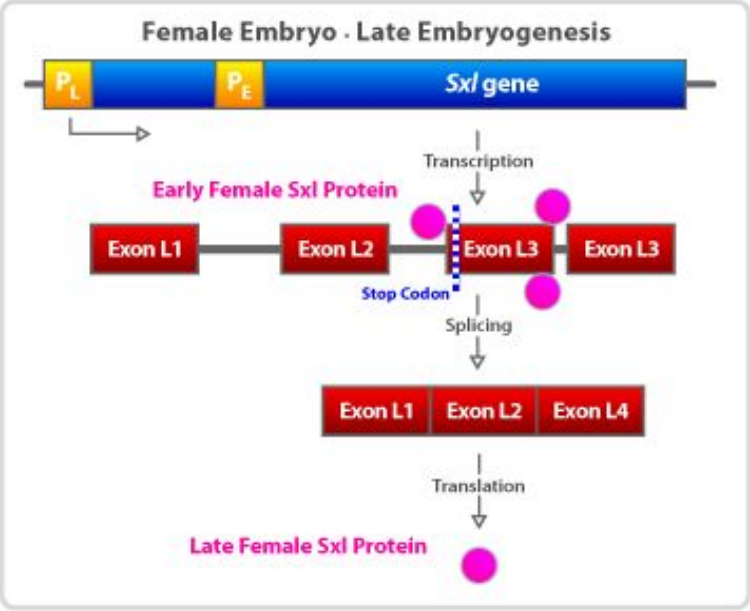


How would an Intronic Splicing Silencer work



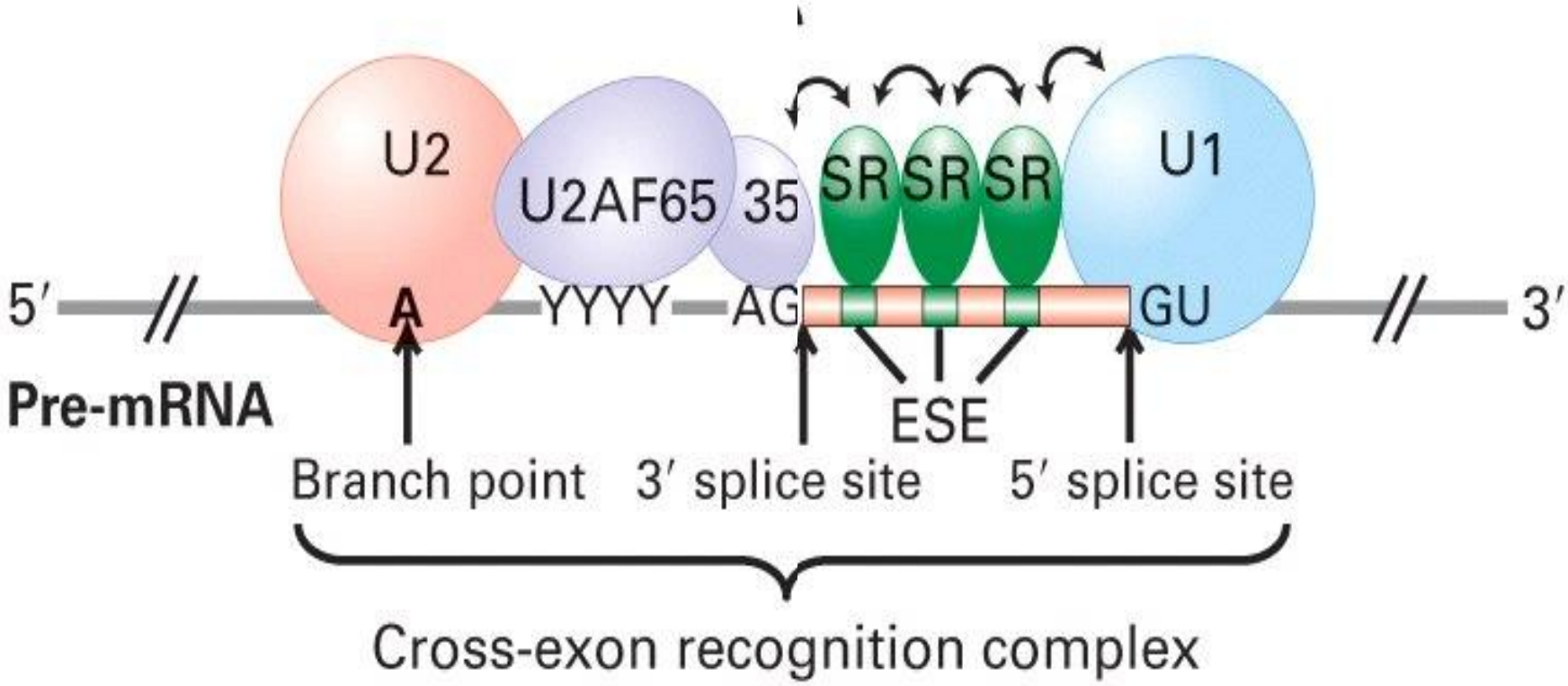






Early female Sxl binds near Exon L3, thus blocking U2AF from binding the 3' splice site. This causes skipping of exon 3 in female flies. A functional late female Sxl protein is transcribed from L1, L2 and L4. Late Sxl protein feeds back on itself and late female Sxl is produced throughout female fly development.

SR proteins generally bind ESE, ESS, ISE, and ISSs



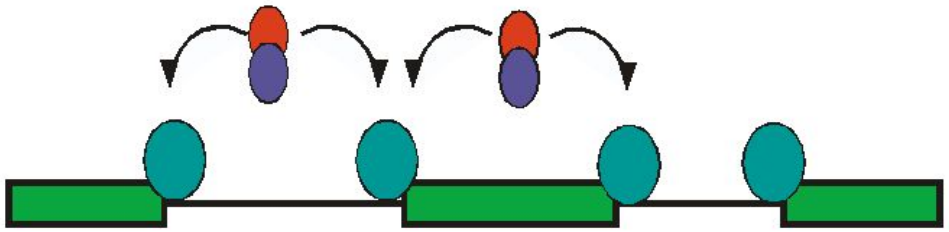
SR Proteins

Labs of Fu, Krainer, Manley, Roth and others.

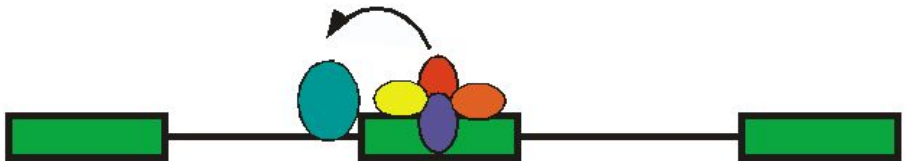
Required splicing factors and effectors of splice site choice.

1 or 2 RNP Domains

C-terminal SR Domain containing multiple SR Dipeptides



And / Or

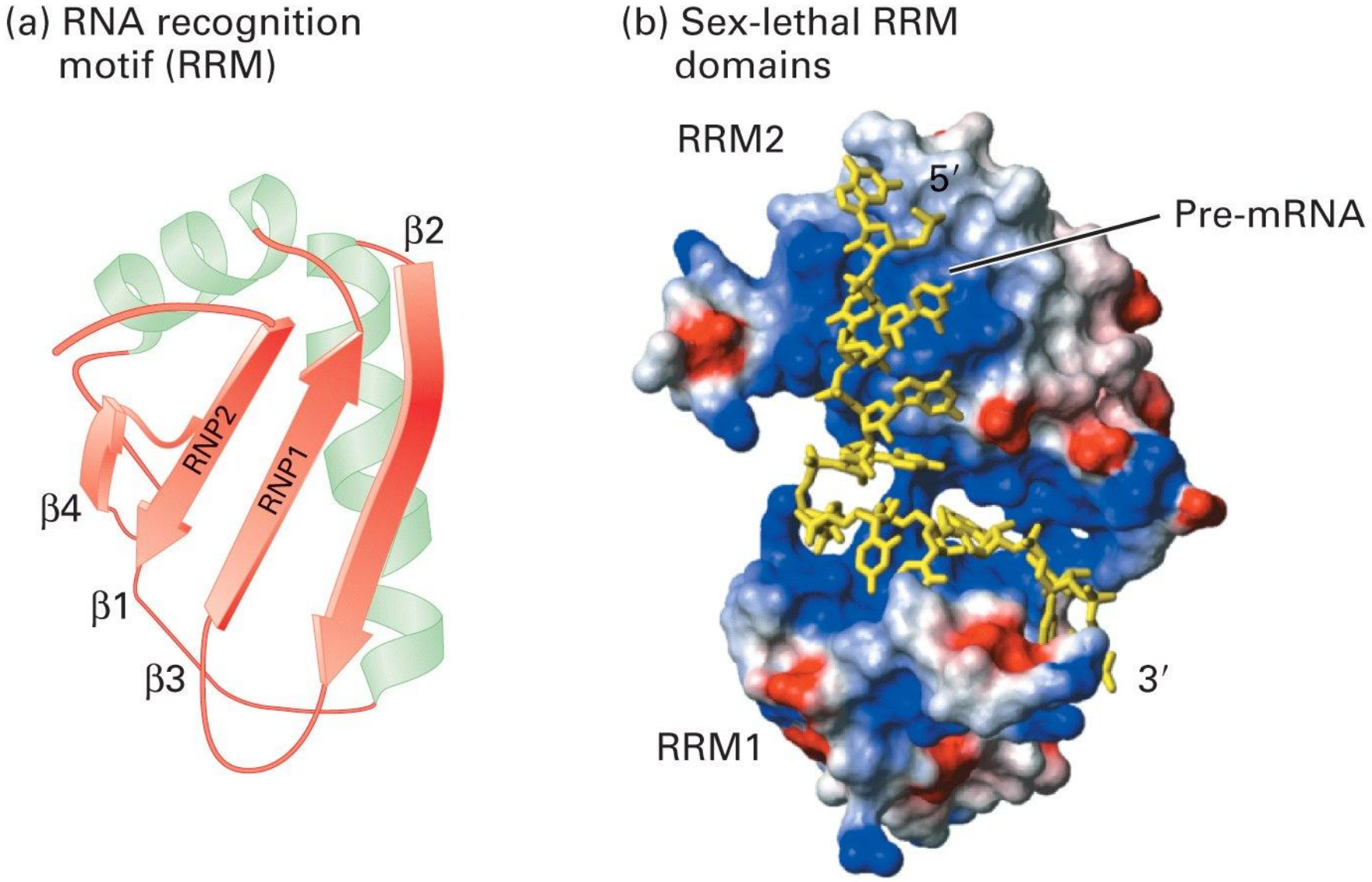


The SR Proteins are a family of proteins with a common domain structure of 1 or 2 RNP RNA binding domains (also called RRMs) and a C-terminal domain rich in SR dipeptides.

These proteins are involved in many aspects of splicing, but most significantly they bind to Exonic Splicing Enhancers (ESEs) and stimulate spliceosome assembly at the adjacent sights.

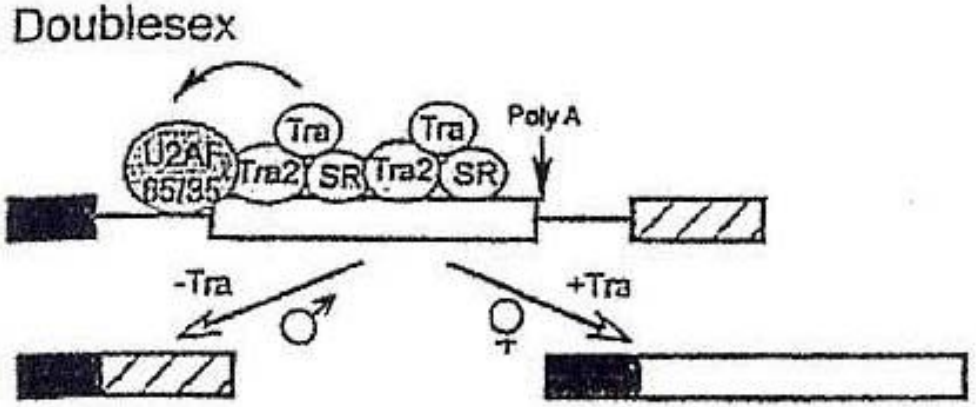
It is thought that most exons carry ESE's and require SR proteins for exon recognition.

SR Proteins bind to specific RNA elements using their RNA binding domains similar to those in the Sex-Lethal protein.



Characterization of an ESE and SR protein in flies

- Sex differentiation in flies controlled by AS Cascade
- Dsx: weak 3'SS next to female-specific exon
- Tra/Tra2 (females) promotes recruitment of U2AF

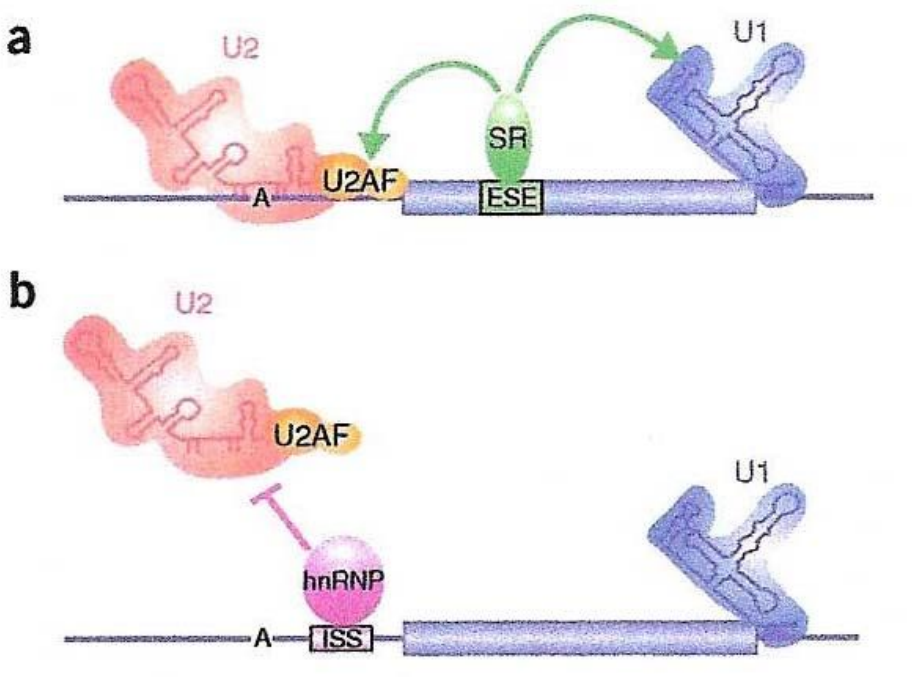


- Sequence-specific RRM -> binds 13-nte. Repeats
- RS domain interacts w U2AF RS domain

Proof of concept: Convert ESE to MS2 binding site -> activated by MS2:RS

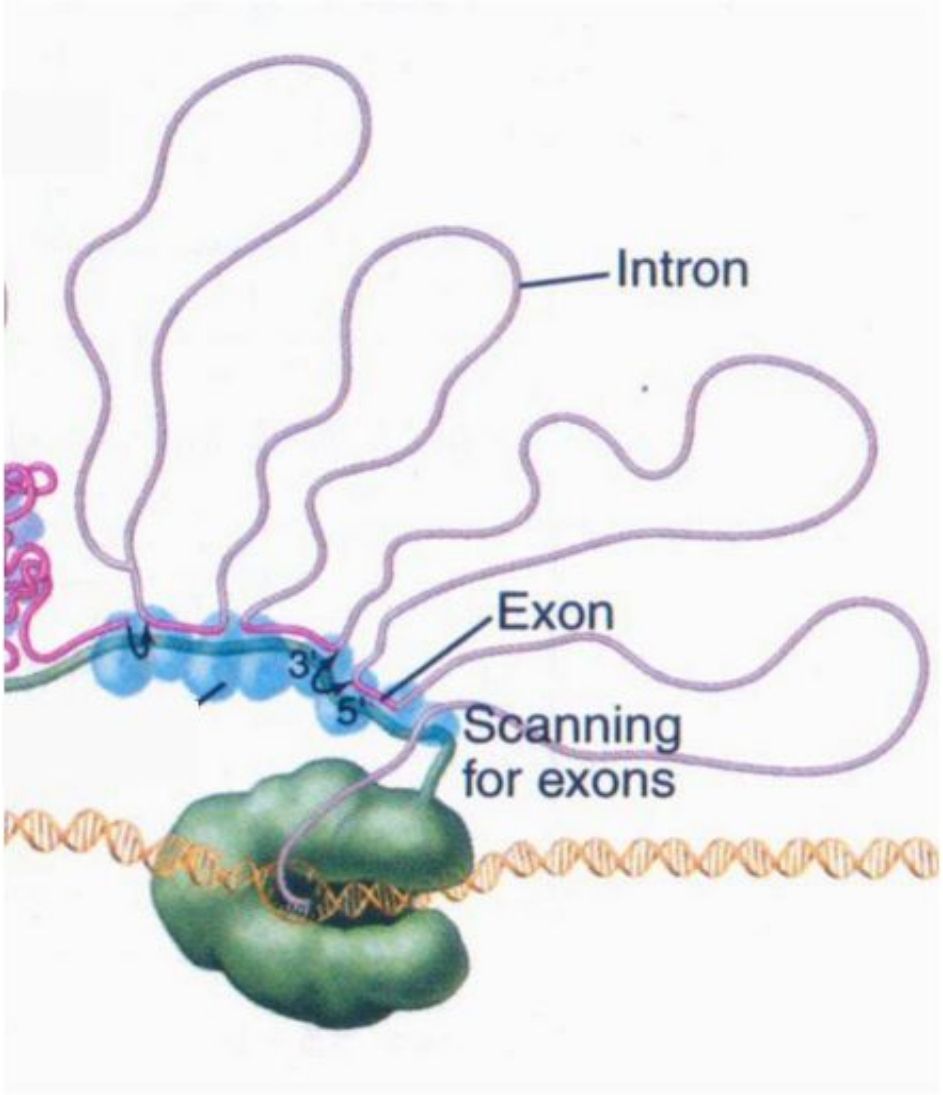
hnRNP function at ISSs

hnRNP contain RRMs but not SR domain

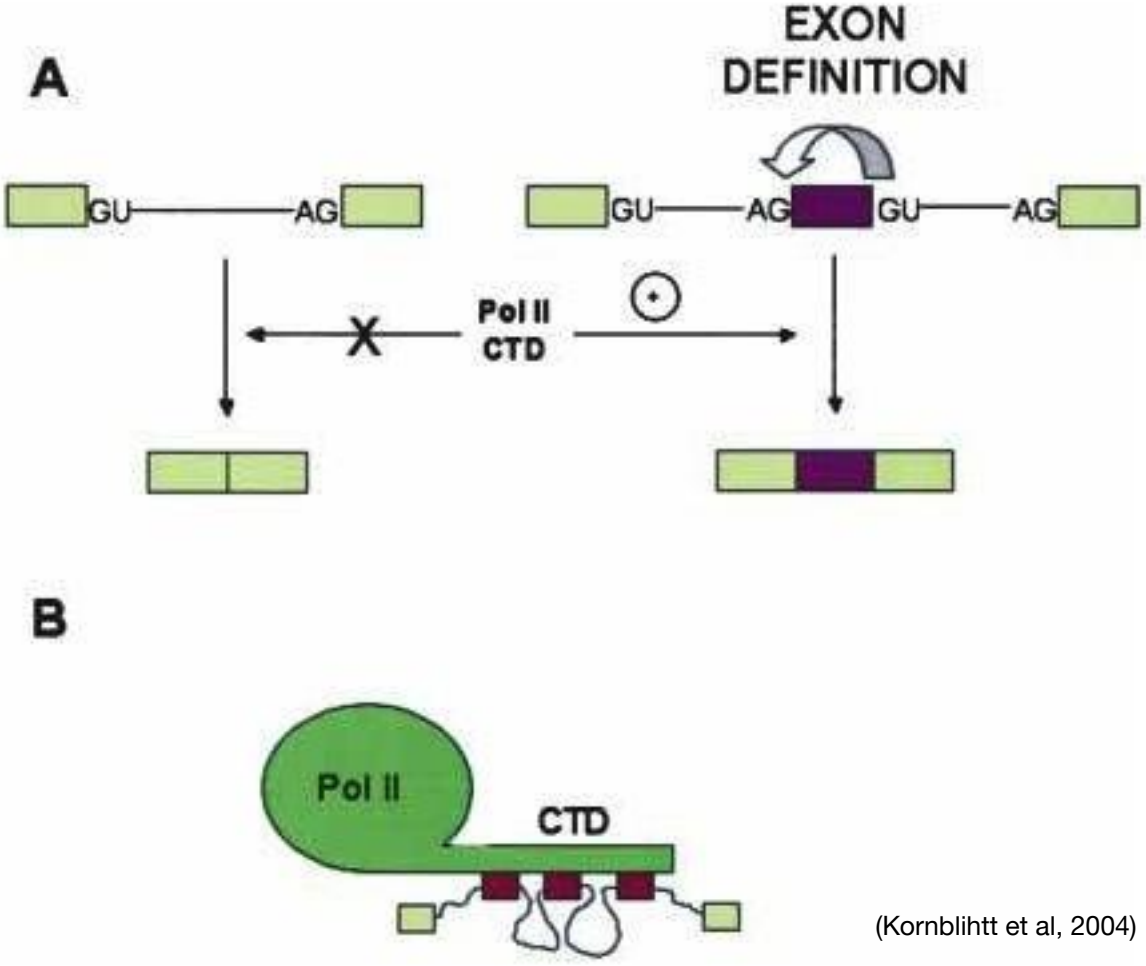


Can block sterically, tighter binding affinity than U2AF

SR Proteins bind to CTD of polII: promote co-transcriptional splicing?

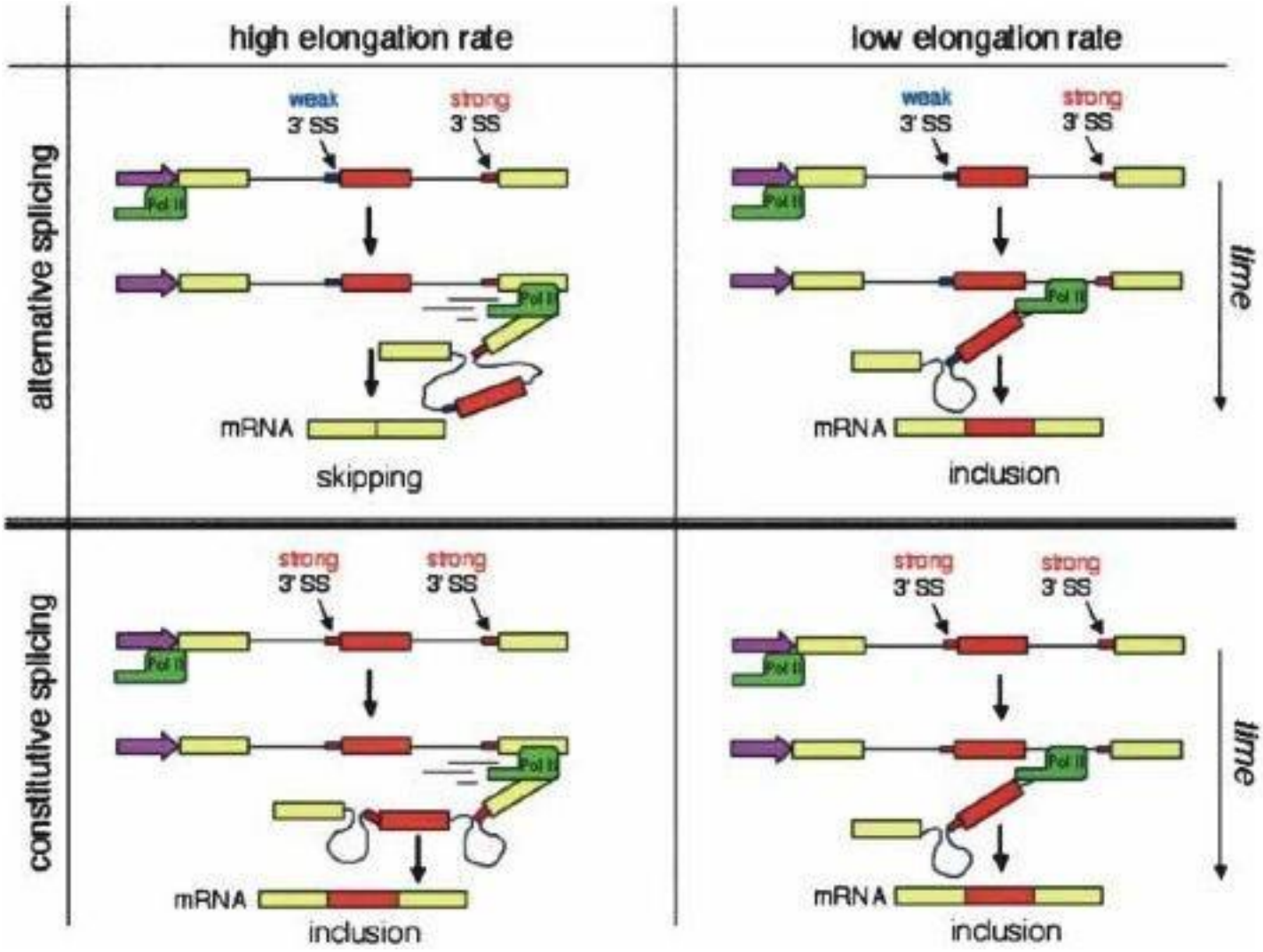


CTD of RNA pol II plays important role in pre-mRNA splicing

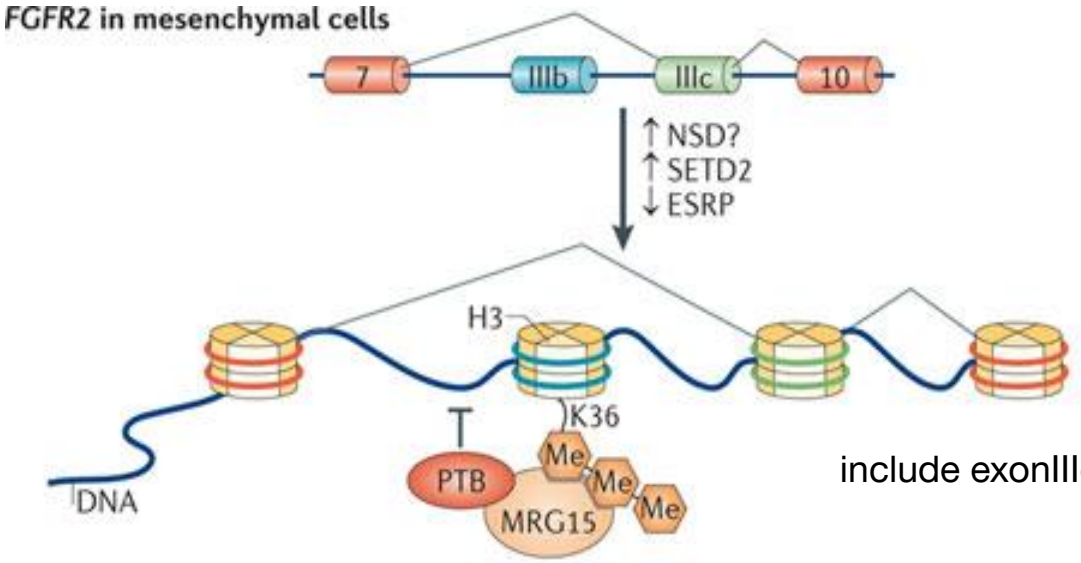


(Kornblihtt et al, 2004)

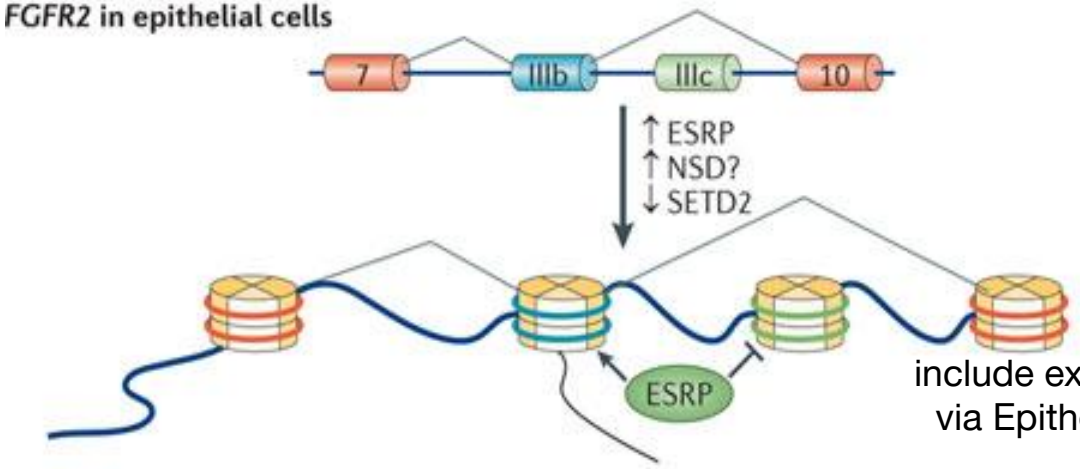
Does splice site strength affect alternative splicing?



A connection between chromatin and splicing



include exonIIIc by repress exonIIIb

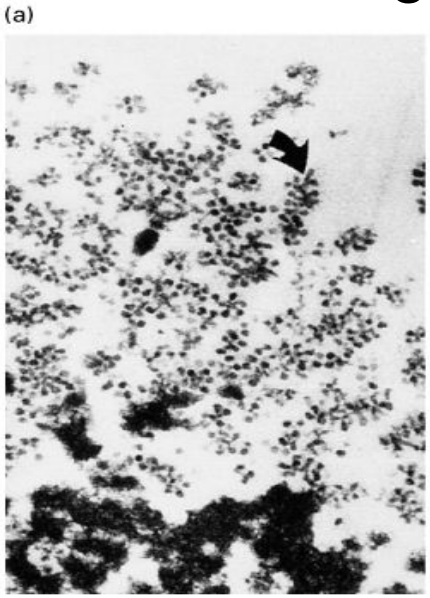


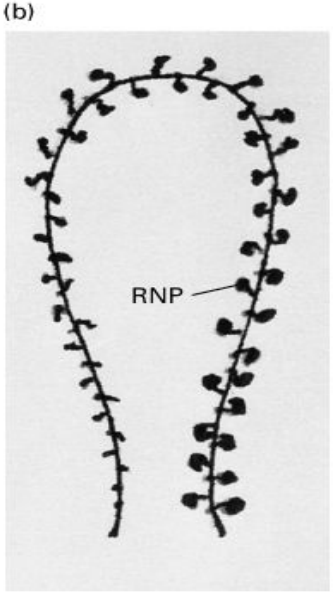
include exonIIIb, repress exon IIIc, via Epithelial splicing regulatory protein

Nature Reviews | Molecular Cell Biology

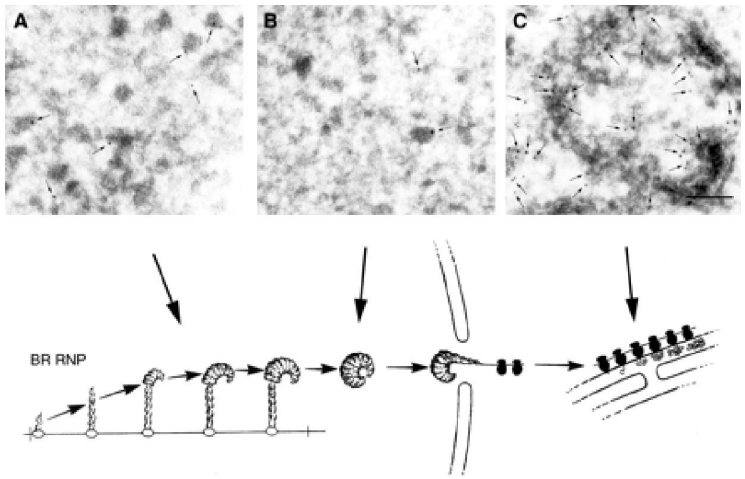
mRNA export - formation of an export competent mRNP

Balbiani Rings (Chironomus tentans)





Sees formation of mRNP as transcription commences



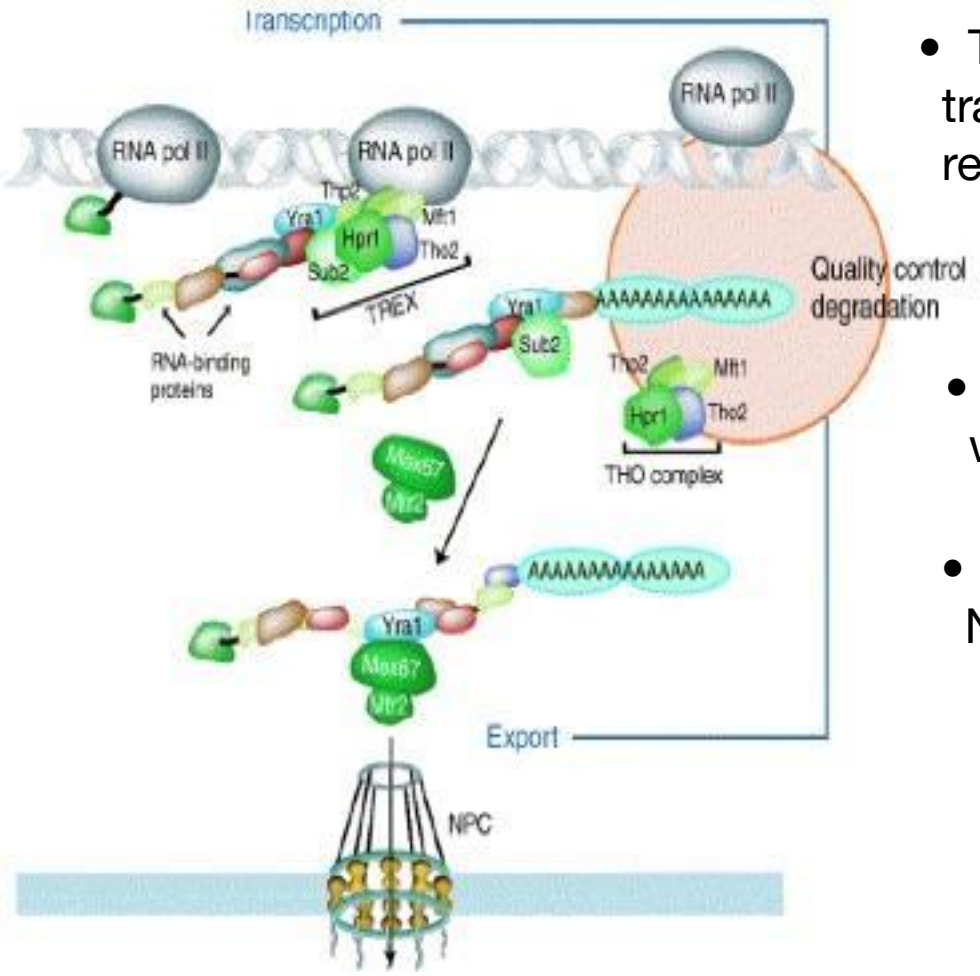
Follow mRNP through NPC

- Why export as a protein/DNA complex? RNAs are too big and lack the signals to interact w/ nuclear export receptors

- Specific “adaptor” proteins must first bind to the RNA and chaperone this molecule to the export receptor, which, in turn, guides the RNA across the NPC

Factors involved in mRNA export are co-transcriptionally recruited

Model from yeast:



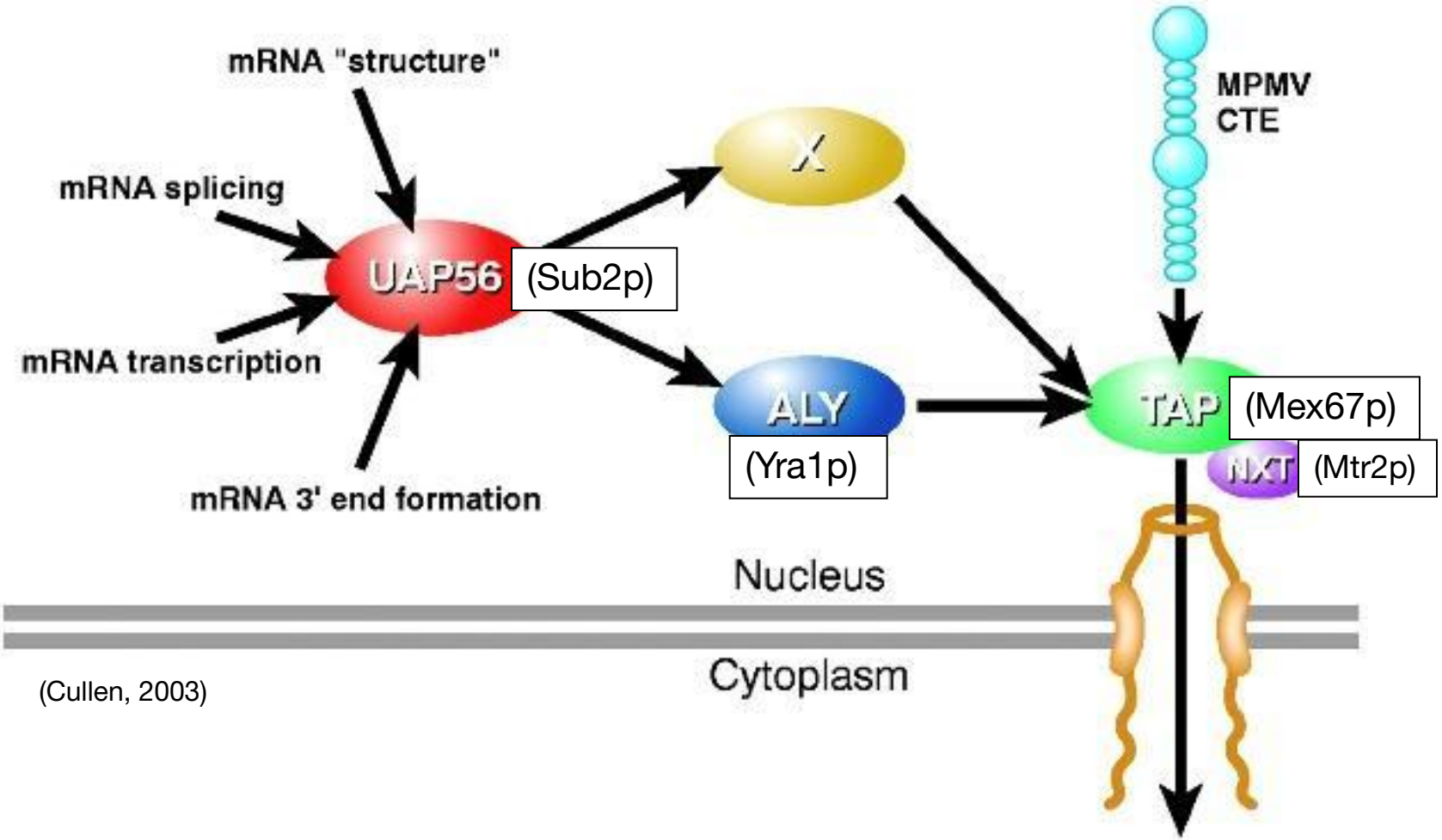
- THO complex: major role in transcriptional elongation and recruitment of mRNA export factors

- Yra1 - mRNA export factor, interacts with Mex67

- Mex67 - promotes translocation across NPC

(Stutz & Izaurralde,2003)

Proteins involved in the nuclear export of mRNAs



(Cullen, 2003)

(yeast homolog is indicated in parentheses)

Path of transporting mRNA to the nuclear pore complex

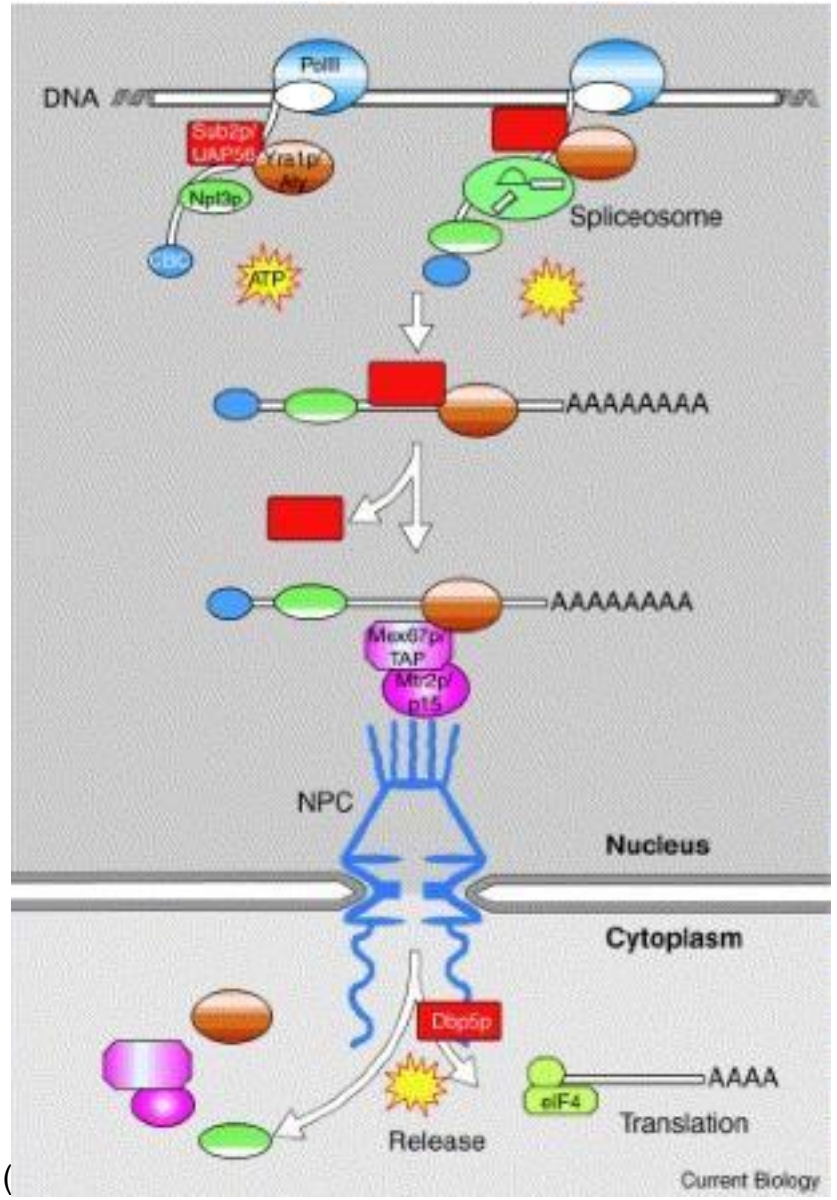
- Sub2, Yra1p and hnRNP proteins such as Npl3p associate co-transcriptionally with the mRNA in yeast.

- In mammalian cells, Aly/REF(Yra1) and UAP56(Sub2) are part of the exon-junction complex (EJC) on the spliced mRNA (not shown). UAP56 is replaced by the TAP-p15 (Mex67-Mtr2 in yeast) heterodimers

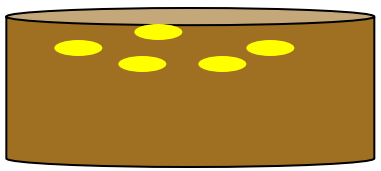
- The Mex67-Mtr2 heterodimers mediate the interaction of the mRNP with components of the nuclear pore complex (NPC).

- The DEAD box protein Dbp5p is required for release of mRNP on the cytoplasmic side of the NPC.

- DEAD box-mediated ATPase activities important for mRNA export are indicated by stars.



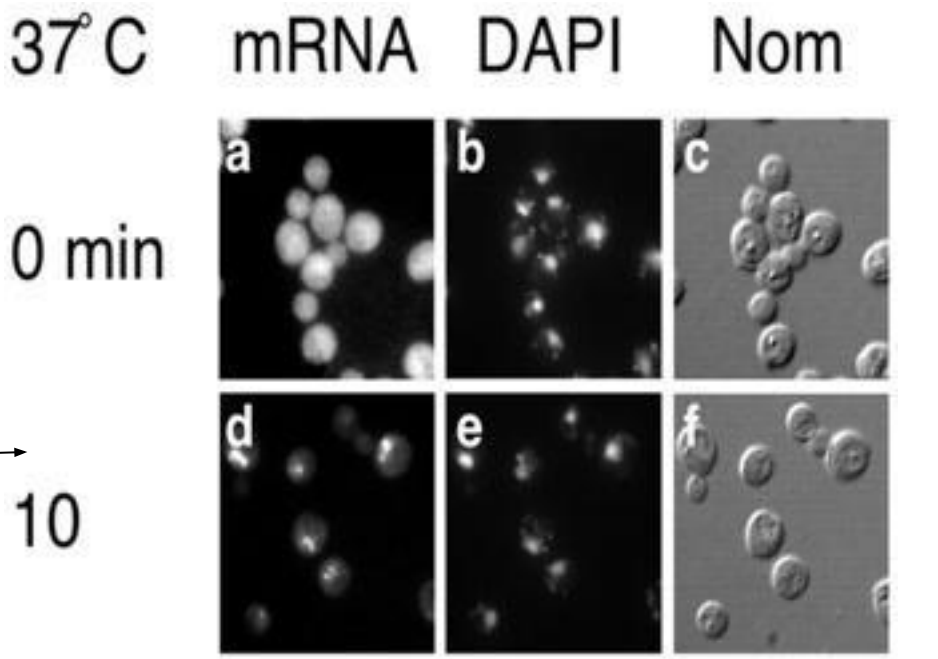
Genetic approach to identify genes involved in mRNA export process



Mutagenized cells or collection of non-essential gene KOs

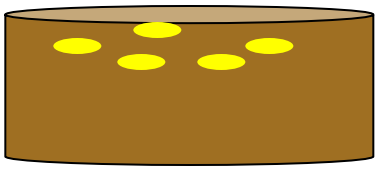
Non-essential genes

RNA FISH w/ oligo dT

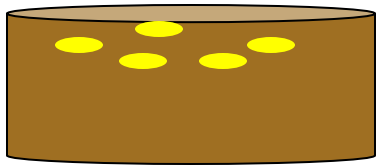


(Lei et al, 2003)

essential genes



Growth at permissive temperature

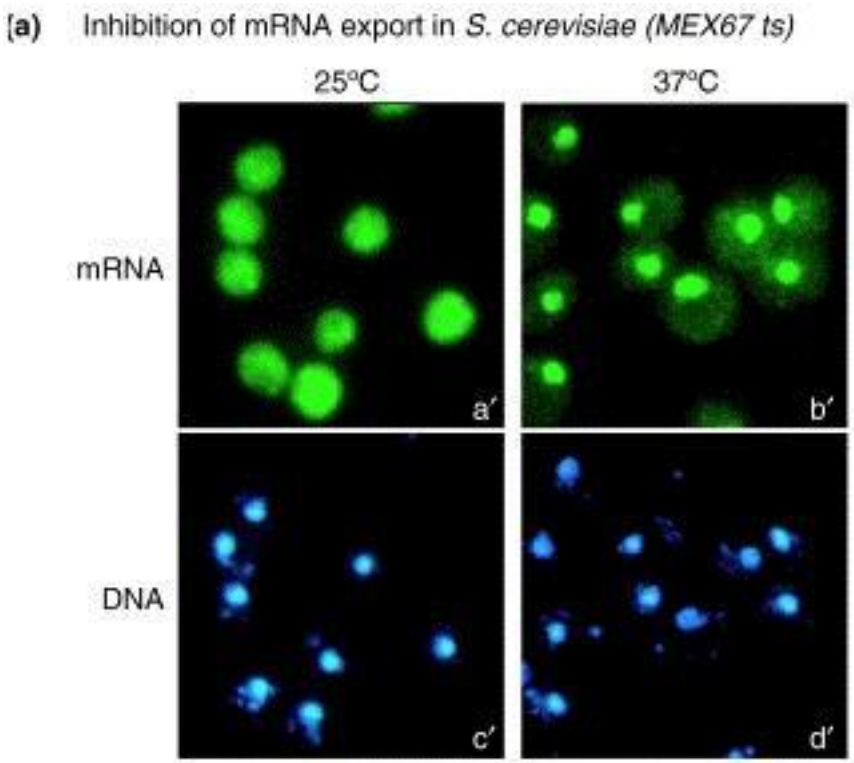


Shift to non-permissive temperature

mtr2-142

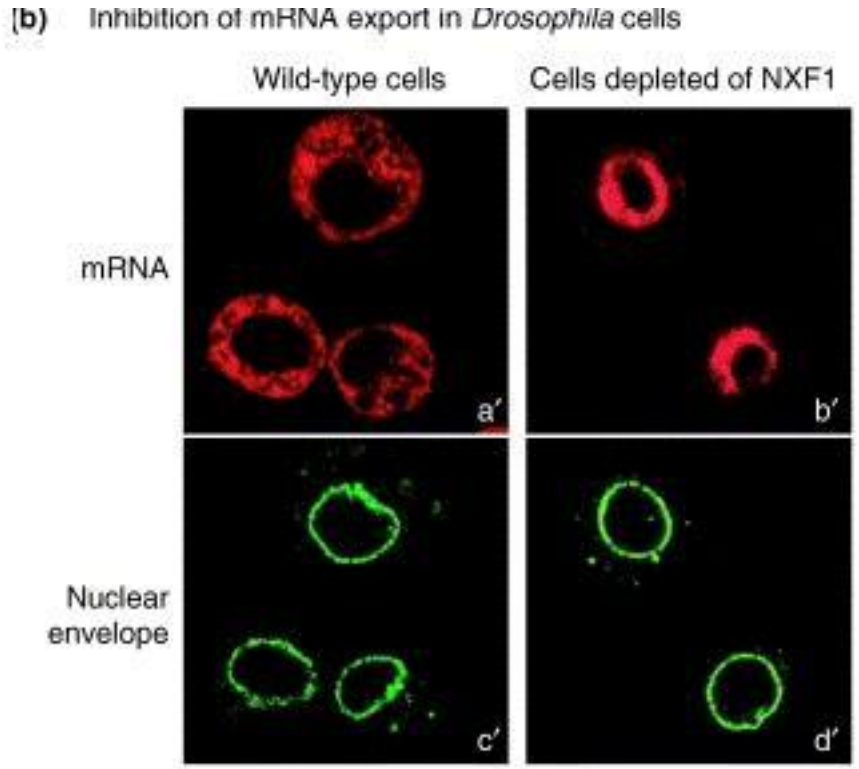
RNA FISH w/ oligo dT

Mex67(yeast) and NXF1(Drosophila) are essential genes involved in mRNA export



Nuclear mRNA accumulation is observed after shifting mex67 TS mutant to the restrictive temperature (37°C)

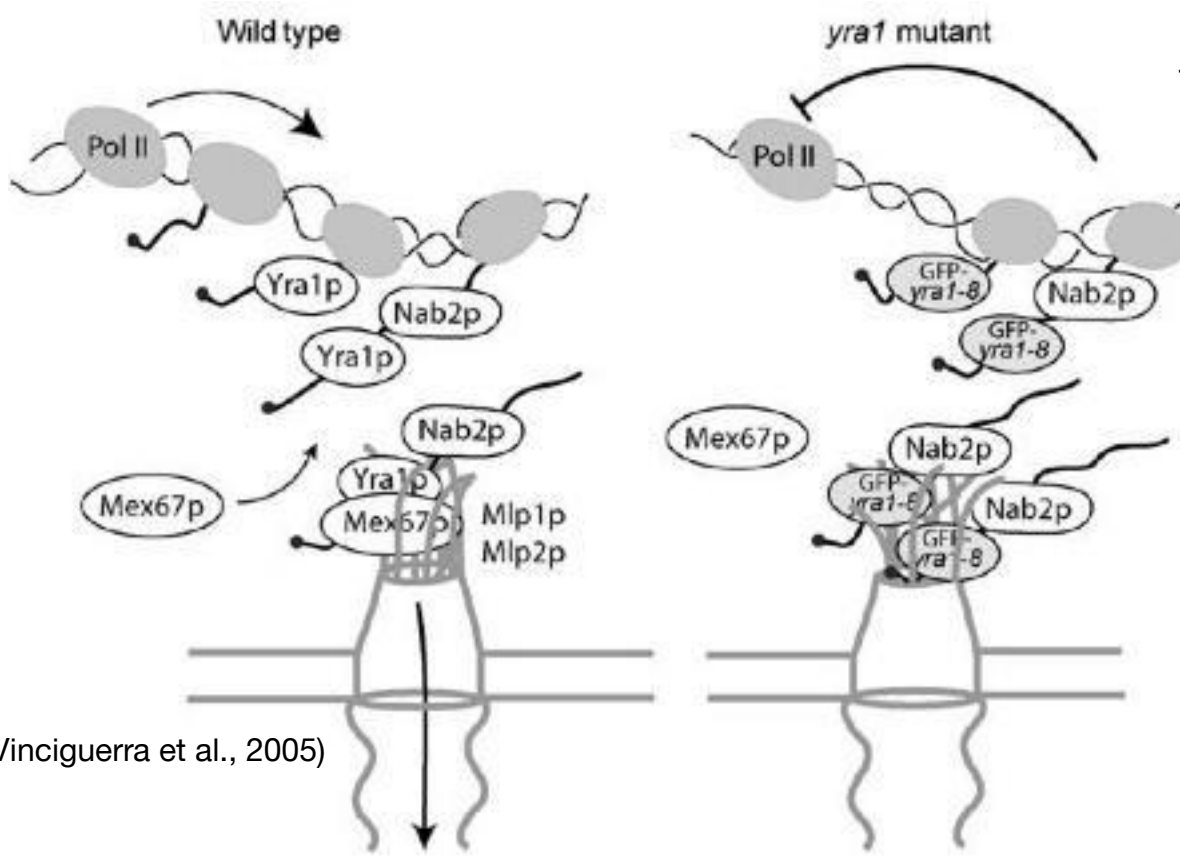
(Stutz & Izaurralde, 2003)



Depletion NXF1 by double stranded RNA inhibition results in nuclear mRNA accumulation.

Visualization of poly(A) mRNA is accomplished by in situ using fluorescently-labeled oligo-dT probe

Linking mRNA biogenesis with mRNA export: Mlp proteins

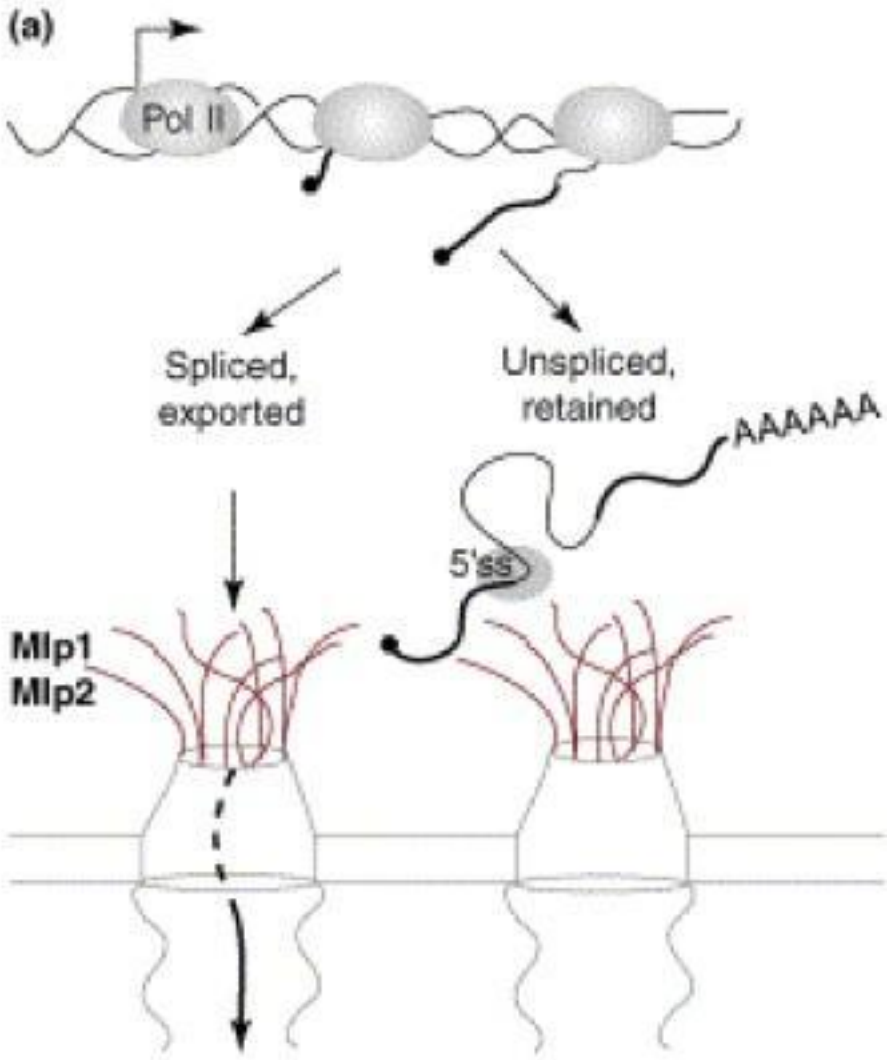


(Vinciguerra et al., 2005)

Mlp proteins: filamentous proteins on the nuclear side of NPC

- Yra1p and Nab2p are essential for mRNP docking to the Mlp export gate at the nuclear periphery.
- mRNP complexes produced in the GFP-yra1-8 mutant strain are retained by the Mlp selective filter.
- mRNP stalling negatively feeds back on mRNA synthesis.
- Loss of Mlp1p or Mlp2p alleviates the negative effect on mRNA synthesis and allows a fraction of transcripts to reach the cytoplasm.

Mlp proteins act as selective filters at NPC entrance



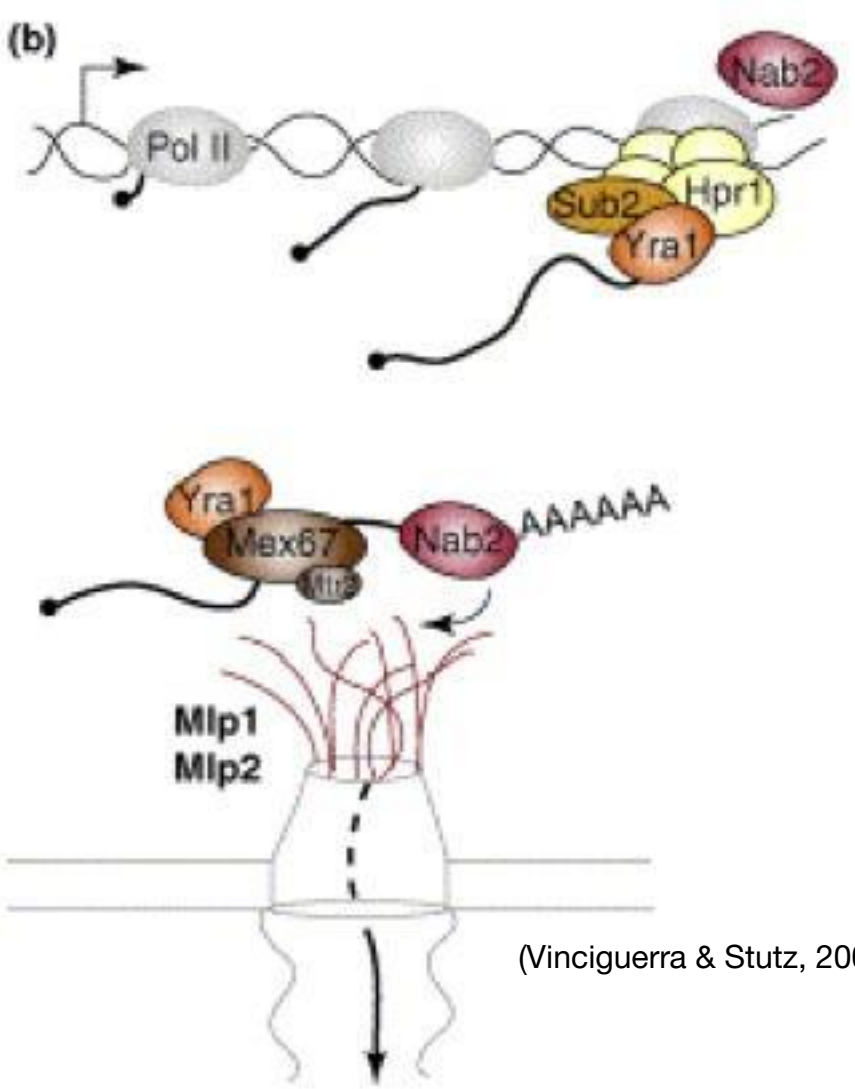
(Vinciguerra & Stutz, 2004)

- The perinuclear Mlp1p protein contributes to mRNP surveillance by retaining unspliced transcripts within the nucleus
- This is achieved possibly via recognition of a component associated with the 5´ splice site.

Nab2 is responsible for the docking of mRNPs to Mlp

- Nab2p, a shuttling mRNA binding protein involved in polyA tail length regulation, directly interacts with Mlp proteins.

- Possible mechanism: by signaling proper 3´ end formation.



(Vinciguerra & Stutz, 2004)