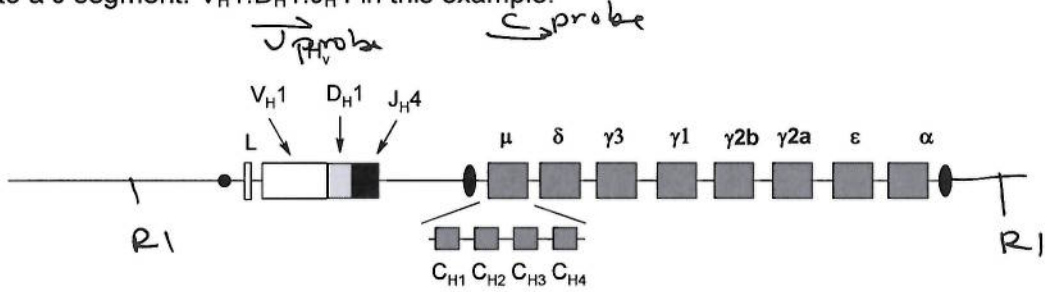
Lecture 9: Antibody Diversity, Checkpoints, Allelic Exclusion, B-Cell Act. Ch 7 & 8.

Key Points:

- Joining specificity – RSS 1+2, 2+1
- Joining mechanism – coding joint
- P-nucleotides
- Exonuclease deletion
- N-nucleotides, TdT
- H&L pairing via C_L&C_{H1}
- HC and LC checkpoints
- Allelic exclusion
- Self-tolerance
- Receptor editing
- Anergy
- CD40-CD40L, b7-CD28

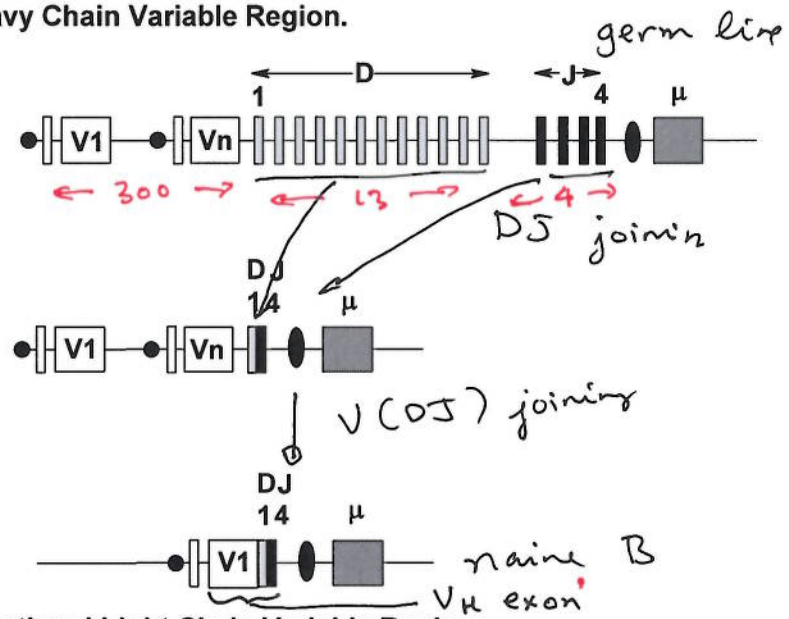
Changes in Genomic DNA: The cDNA sequence from a **naïve B-cell** was found to be the following. The V_H domain is encoded by a single exon that was formed by the joining of a V to a D to a J segment: V_H1:D_H1:J_H4 in this example.



VDJ Joining Generates a Functional Heavy Chain Variable Region.

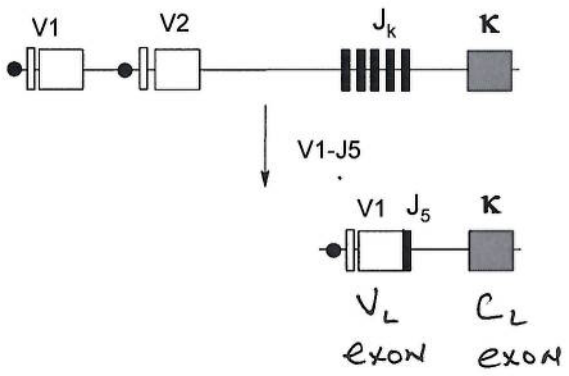
The sequences of events that occur to generate a viable heavy chain gene are as follows:

1. One of the 13 D segments joins to one of the four J regions, generating a DJ junction. **This forms CDR3 of the heavy chain variable region.**
2. One of the ~300 L-V segments joins to new DJ segment, creating a functional exon for the V_H part of the HC gene.
3. The constant regions are added via mRNA splicing.
4. Since every possible combination of VDJ joining is generally possible, the total number of different heavy chains that can be assembled is **300 x 4 x 13 = 15,600**



Light Chain: VJ Joining Generates A functional Light Chain Variable Region:

The light chain genes show a similar arrangement of cassettes. The κ-chain locus consists of a series of approximately 300 variable regions (with a leader exon), indicated by 'L $V_κ1$', followed by 23 kb of DNA, five short segments of DNA called J segments, 2.5 kb of DNA and then DNA the encodes the constant region of the κ light chain. Only four of the five J segments are functional, the middle one is actually a pseudogene which has become non-functional. To form a kappa light chain, one of the 300 L-V segments joins, at random, to one of the 4 J segments, leading to altered chromosomal DNA. **The total number of possible light chains: 300 x 4 =1200**.



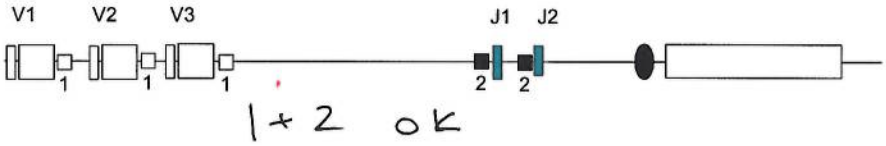
The above is insufficient to account for the 10^{10} different sequences that are produced in the bone marrow (of which only ~1-5% are self-tolerant).

pair any heavy chain any light chain

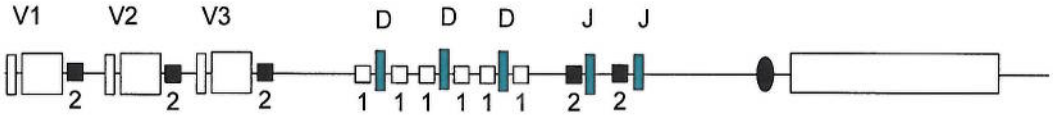
$10^4 \times 10^3 = 10^7$

Specificity of Segment Joining: During the recombination events that lead to the final form of the light and heavy chains there is a need to insure that the correct segments are joined. For example, you would not want to join two J segments together instead of a VJ joining event. The correct segments are joined because of two conserved **recombination signal sequences (RSS)** found directly adjacent to the V, J, and D regions in immunoglobulin genes. One is called a one-turn and the other a two-turn RSS.

The position of RSS in the kappa light chain is as follows:



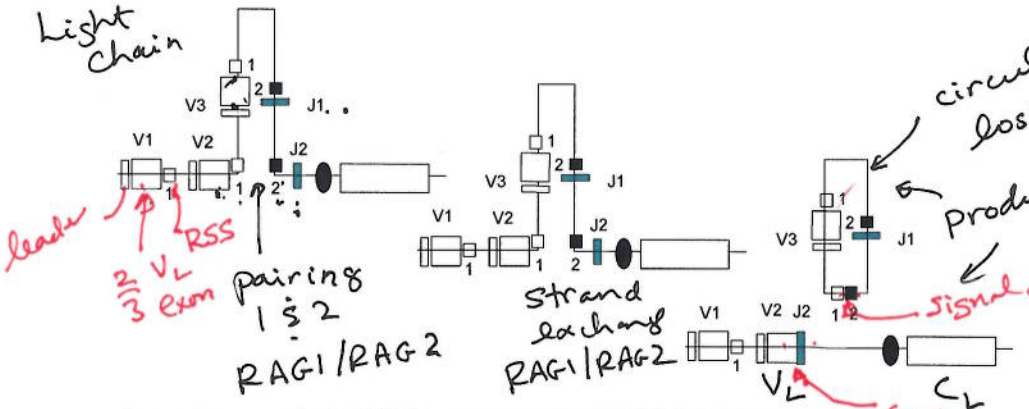
The position of the RSS in the heavy chain segment is as follows:



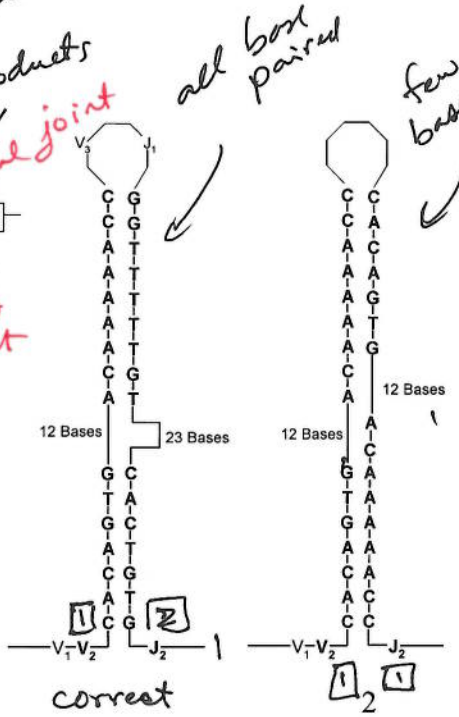
Predict the Pairing Rules for Correct Joining:

Joining Mechanism: The actual joining of DNA segments is catalyzed by two enzymes: **RAG-1 and RAG-2** (Recombination Activating Genes). Steps are:

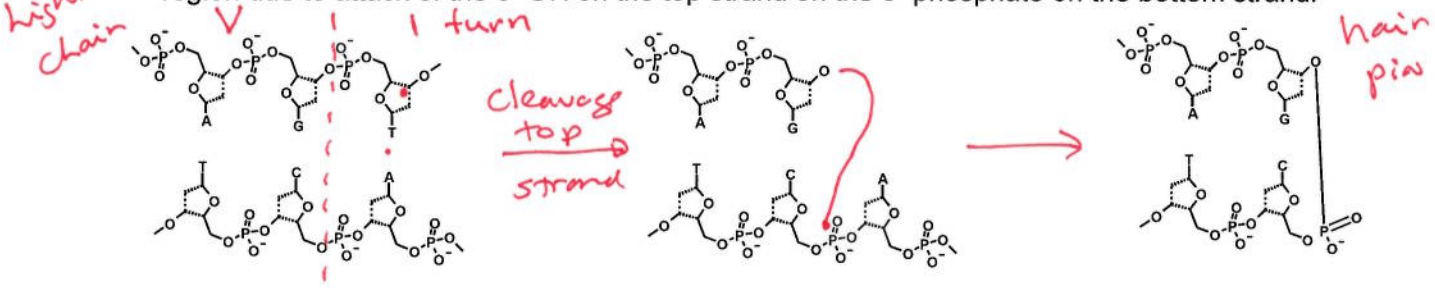
1. Alignment of the V/J junction via the RSS
2. Precise cleavage at the boundary of the coding and RSS on one strand
3. Formation of a hairpin structure at the ends of the V and J segments (see below)
4. Resolution of hairpin and joining of V and J, generating a **coding junction.**
5. Joining of the ends of the RSS, creating a signal joint. The circular DNA is lost from the cell.



One-turn sequence	CACAGTG - 12 bp - ACAAAAACC GTGTCAC - 12 bp - TGTTTTTGG
Two-turn sequence	GGTTTTTGT- 23 bp - CACTGTG CCAAAAACA- 23 bp - GTGACAC

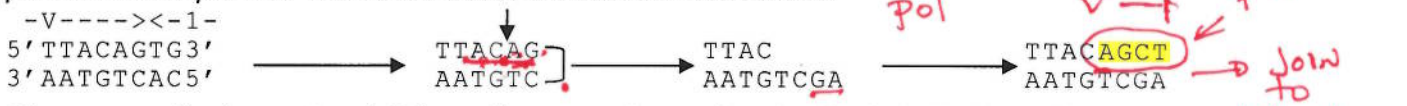


Hairpin formation: Cleavage at the RSS produces a hairpin structure at the end of each coding region due to attack of the 3'-OH on the top strand on the 5'-phosphate on the bottom strand.

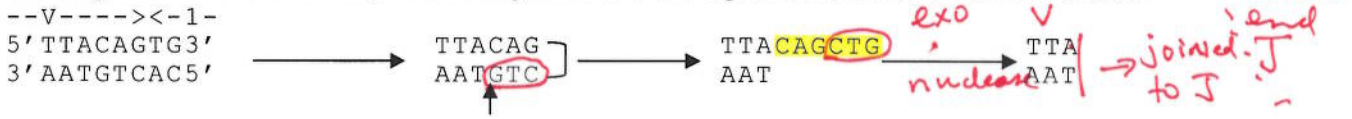


Crossover Uncertainty (Junctional diversity) & P-nucleotides: The end points of the RSS are the nominal points for joining heavy and light chains. However, there is some uncertainty (up to 5 bases) where the rejoining of fragments occur. This leads to the loss/gain of codons, and subsequent amino acid changes in the third hypervariable loop. Frame shifts are also possible.

Cleavage on the top strand, followed by repair synthesis adds additional bases, generating palindromic sequence. The added bases are called P-nucleotides.



Cleavage on the lower strand, followed by exonuclease digestion leads to the loss of bases:



Example of Crossover Uncertainty: Sequences *A, B, or C* are possible sequences after rearrangement of germ line to generate light chains (germ line sequence is shown in middle).

A

AGT CGC TTA CCT GCT GCT TTT
TCA GCG AAT GGA CGA CGA AAA
Ser Arg Leu Pro Ala Ala Phe

B

AGT CGC TTA CCT GCT TTT
TCA GCG AAT GGA CGA AAA
Ser Arg Leu Pro Ala Phe

deletion

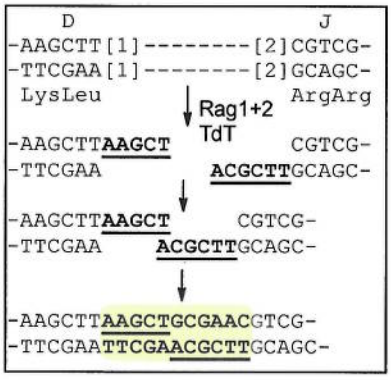
V_3-region
(germ line) **AGT CGC TTA CCT**1-----2**GCT GCT TTT** J_1-region
TCA GCG AAT GGA1-----2**CGA CGA AAA**
Ser Arg Leu Pro---------*Ala Ala Phe*

C

AGT CGC TTA CT GCT TTT
TCA GCG AAT GA CGA AAA
Ser Arg Leu

frame shift
∴ not functional

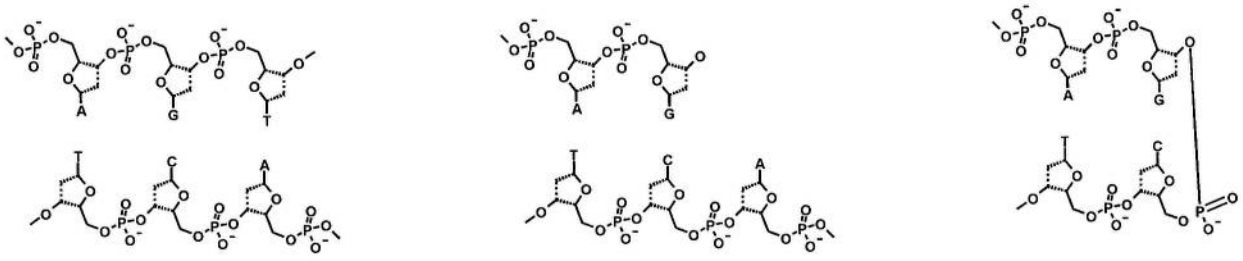
N-Nucleotides: Addition of up to 15 nucleotides, by the enzyme terminal transferase (**TdT**). This enzyme adds bases to hypervariable loop 3 of only the *heavy* chain at each joining event. The added nucleotides are referred to as N nucleotides. The expression of TdT is very low when the light-chain begins rearrangement; consequently N-nucleotides are rare in the light chain.



Summary of Diversity:

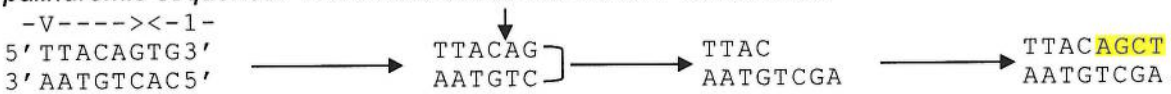
Mechanism of Diversity	Heavy Chain	Light Chain
Combinatorial V-D-J and V-J:	$300\times12\times4=1.4\times10^4$	$300\times4=1.2\times10^3$
P base (V-D-J) ,(V-J) (x3/joint)	× 9	× 3
Junctional Diversity (x3/joint)	×9 (VDJ)	×3 (VJ)
N-base addition (TdT) (V-D-J)	× 9	× 1
# Chains	**~1.0 × 10^7**	**~1.0 × 10^4**
Estimated Diversity	**1.0 × 10^{11}**	

Hairpin formation: Cleavage at the RSS produces a hairpin structure at the end of each coding region due to attack of the 3'-OH on the top strand on the 5'-phosphate on the bottom strand.

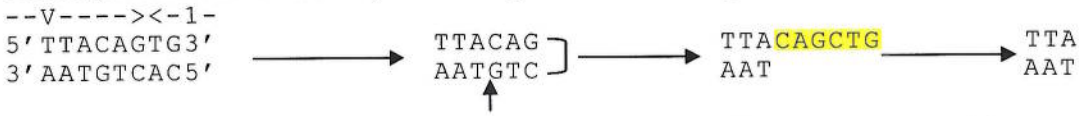


Crossover Uncertainty (Junctional diversity) & P-nucleotides: The end points of the RSS are the nominal points for joining heavy and light chains. However, there is some uncertainty (up to 5 bases) where the rejoining of fragments occur. This leads to the loss/gain of codons, and subsequent amino acid changes in the third hypervariable loop. Frame shifts are also possible.

Cleavage on the top strand, followed by repair synthesis adds additional bases, generating palindromic sequence. The added bases are called P-nucleotides.



Cleavage on the lower strand, followed by exonuclease digestion leads to the loss of bases:



Example of Crossover Uncertainty: Sequences *A, B, or C* are possible sequences after rearrangement of germ line to generate light chains (germ line sequence is shown in middle).

A

AGT CGC TTA CCT GCT GCT TTT
TCA GCG AAT GGA CGA CGA AAA
Ser Arg Leu Pro *Ala Ala Phe*

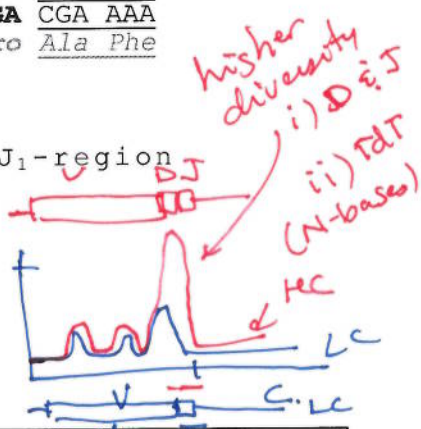
B

AGT CGC TTA CCT GCT TTT
TCA GCG AAT GGA CGA AAA
Ser Arg Leu Pro *Ala Phe*

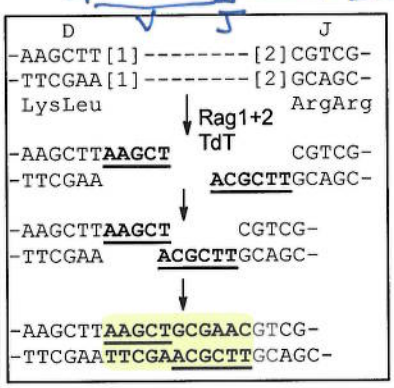
V_3-region
(germ line) **AGT CGC TTA CCT**[1]------[2]**GCT GCT TTT** J_1-region
TCA GCG AAT GGA[1]------[2]**CGA CGA AAA**
Ser Arg Leu Pro--------*Ala Ala* Phe

C

AGT CGC TTA CT GCT TTT
TCA GCG AAT GA CGA AAA
Ser Arg Leu

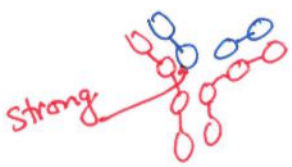


N-Nucleotides: Addition of up to 15 nucleotides, by the enzyme terminal transferase (**TdT**). This enzyme adds bases to hypervariable loop 3 of only the *heavy* chain at each joining event. The added nucleotides are referred to as N nucleotides. The expression of TdT is very low when the light-chain begins rearrangement; consequently N-nucleotides are rare in the light chain.



Summary of Diversity:

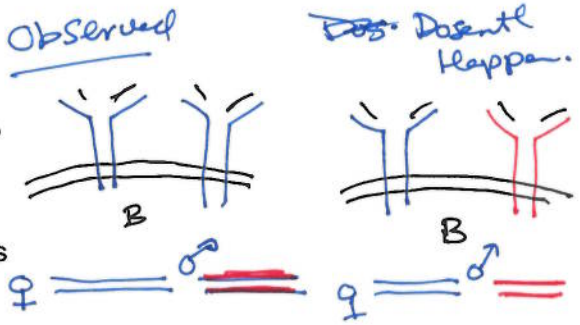
Mechanism of Diversity	Heavy Chain	Light Chain
Combinatorial V-D-J and V-J:	$300\times12\times4=1.4\times10^4$	$300\times4=1.2\times10^3$
P base (V-D-J) ,(V-J) (x3/joint)	× 9	× 3
Junctional Diversity (x3/joint)	×9 (VDJ)	×3 (VJ)
N-base addition (TdT) (V-D-J)	× 9	× 1
# Chains	**~1.0 × 10^7**	**~1.0 × 10^4**
Estimated Diversity	**1.0 × 10^{11}**	



any light chain can pair with any heavy chain. pairing driven (stabilized) by C_H C_L interactions.

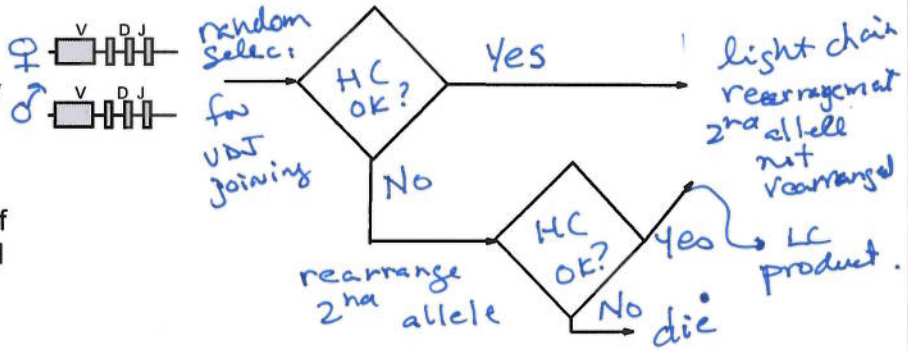
HC & LC Checkpoints & Allelic Exclusion – Production of homogeneous antibodies.

1. Cell attempts to make a functional heavy chain, using both alleles if necessary, if this fails the cell dies.
2. Integrity of heavy chains assessed by the ability to complex with surrogate light chains, Vpre-B & λ5.
3. Once one heavy chain allele is successfully rearranged, VDJ joining of the other heavy chain allele is inhibited (**Allelic Exclusion**). Allelic exclusion is important because it provides for a single specificity and reduces the chance of a self-reactive B, which would result in loss of most B-cells.



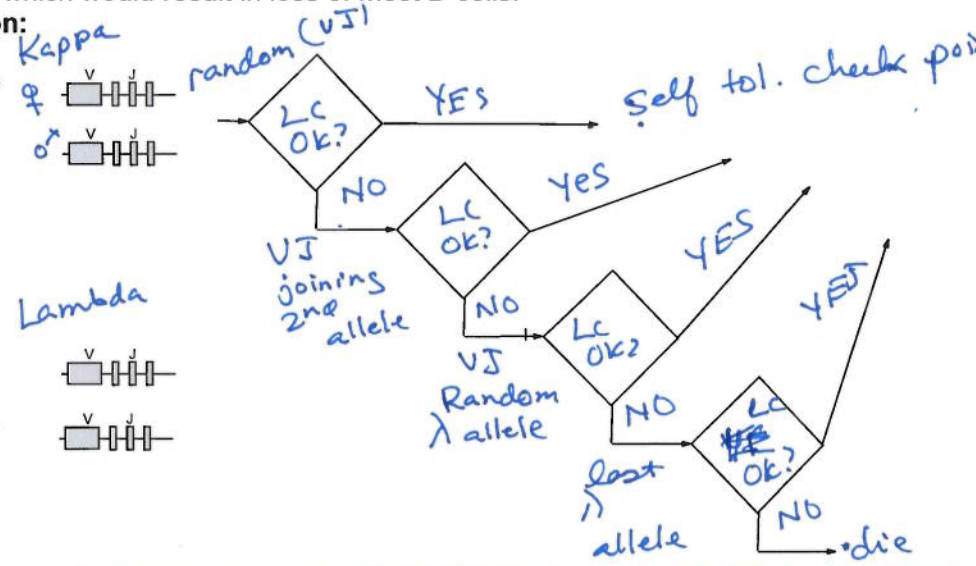
Heavy-Chain Allelic Exclusion:

1. Cell attempts to make a functional heavy chain, using both alleles if necessary, if this fails the cell dies.
2. Integrity of heavy chains assessed by the ability to complex with surrogate light chains, Vpre-B & λ5.
3. Once one heavy chain allele is successfully rearranged, VDJ joining of the other heavy chain allele is inhibited (**Allelic Exclusion**). Allelic exclusion is important because it provides for a single specificity and reduces the chance of a self-reactive B, which would result in loss of most B-cells.



Light chain Allelic Exclusion:

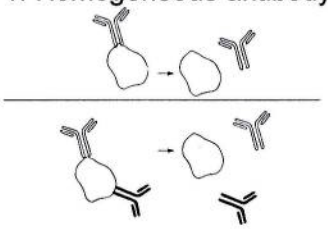
1. Successful rearrangement of one κ-chain inhibits rearrangement of the other κ allele.
2. Unsuccessful rearrangement of both κ-chains prompts rearrangement of λ-chains.
3. Unsuccessful rearrangements of both λ-chains causes B-cell death.
4. Consequently, only one specificity is presented in the BCR. The other alleles are silenced.



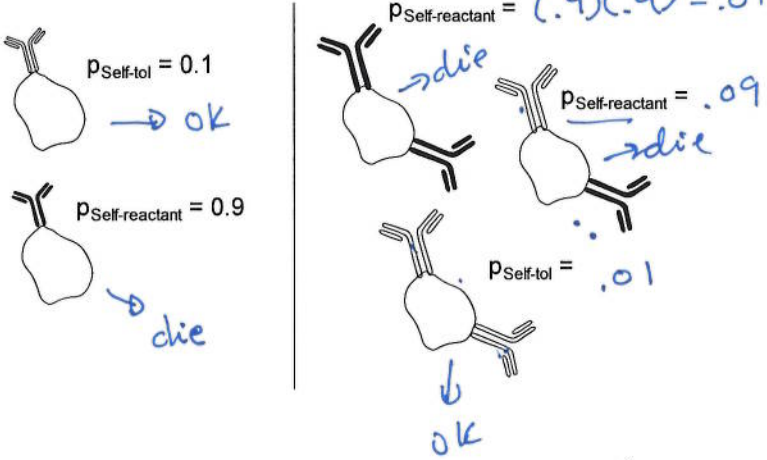
HC → LC → Self tolerance

Allelic exclusion is important:

1. Homogeneous antibody:



2. Lower probability of self-reactive Ab:

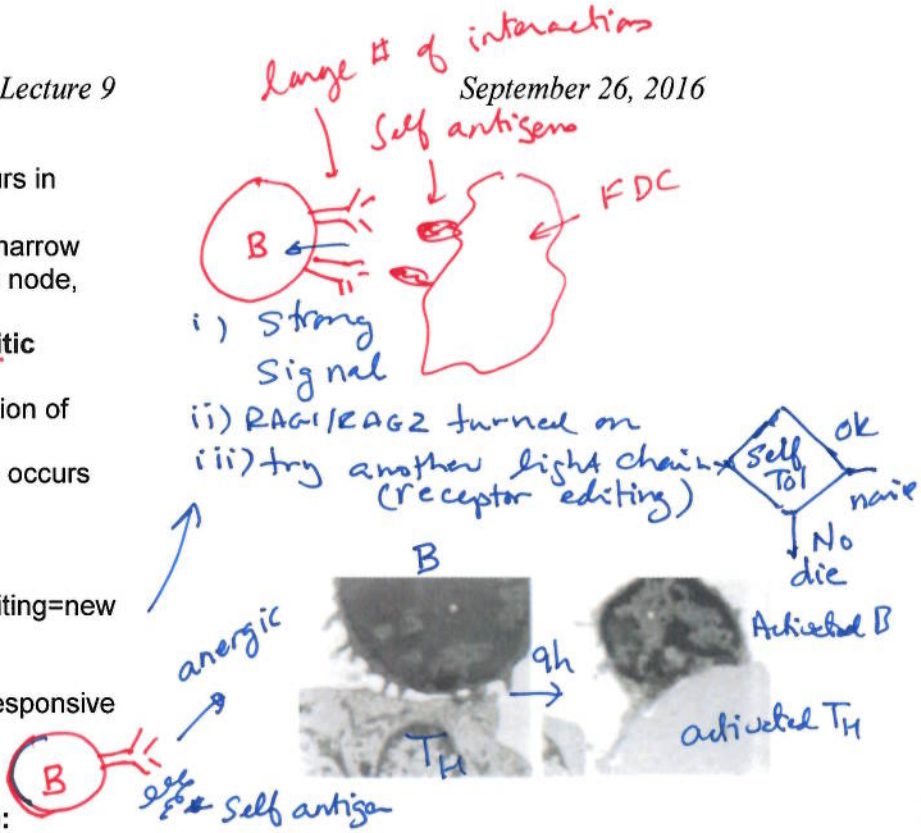


Self-Tolerance Checkpoints:

- 1st self-tolerance checkpoint occurs in bone marrow.
- Immature B cells from the bone marrow home to secondary tissue (lymph node, spleen)
  - Interact with **Follicular dendritic cells** (FDC) in 2° organ.
  - Complete maturation, expression of IgD.
  - Self-tolerance checkpoint also occurs here.
- Mature B-cell exits into blood.

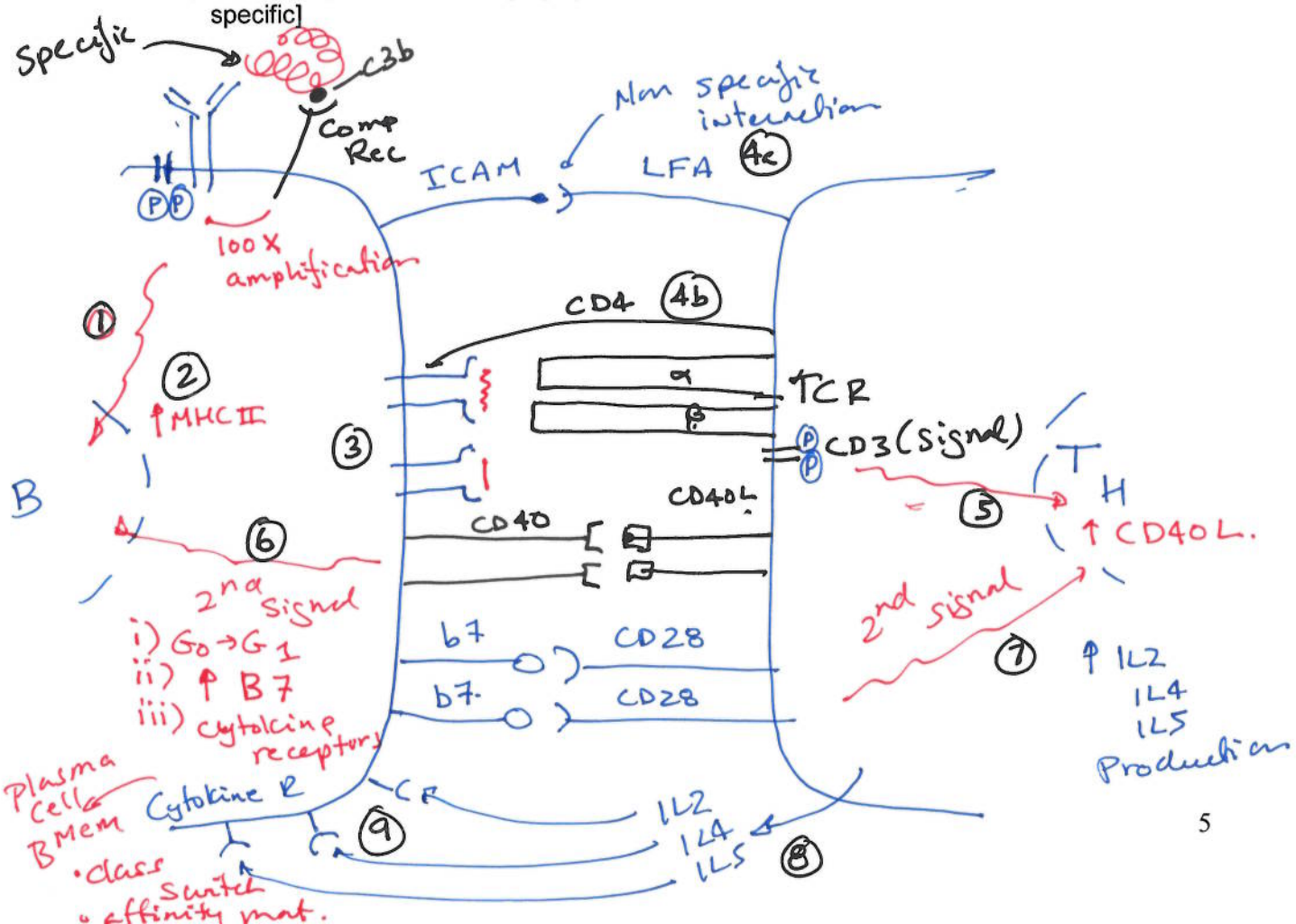
i) Cell surface antigens (receptor editing=new LC):

ii) Soluble antigens (→ anergy, unresponsive B-cell)

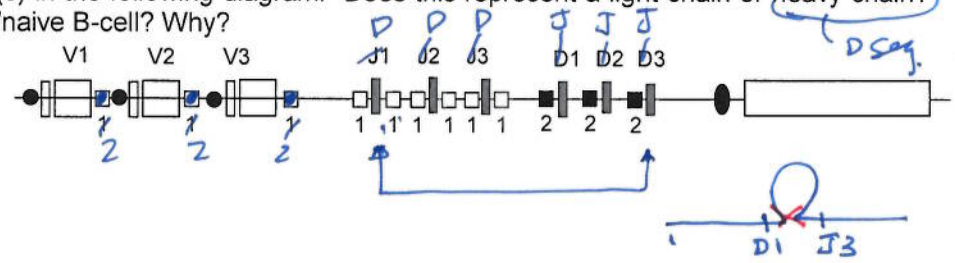


B-Cell Activation (T-cell Dependent):

1. **1st B-cell signal:**
   a) Antigen binds to BCR [Highly specific]
   b) Signal enhanced by complement receptor (CR2) = co-receptor
   c) Phosphorylation of BCR signaling chains.
2. Signaling: ↑ MHC II expression on B-cell.
3. Antigen processing & presentation.
4. **1st T-cell signal**
   a) ICAM & LFA [Non-specific]
   b) MHC-II-Peptide-TCR/CD4 [Highly specific]
5. ↑ CD40L on T_H.
6. **2nd B-cell signal.** CD40-CD40L interaction:
   a. $G_o \rightarrow G_1$, b. ↑↑ B7, c. ↑ cytokine receptors.
7. **2nd T-cell signal.** B7 binds to CD28 on T_H:
8. T_H releases cytokines: IL-2, IL-4. IL-5
9. B-cell: $G_1 \rightarrow S$, proliferation (clonal expansion), class switch & affinity maturation

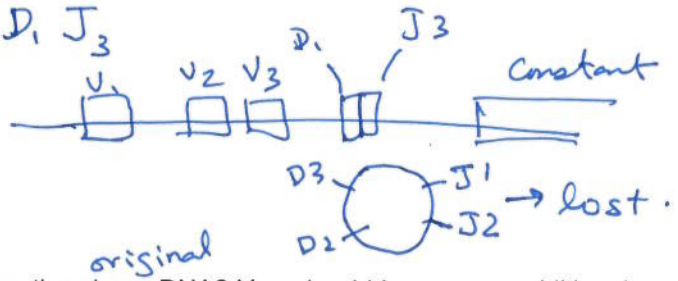


1. Correct the mistake(s) in the following diagram. Does this represent a light chain or heavy chain? Germ-line, or mature/naive B-cell? Why?



No joining yet

2. Sketch **all** of the DNA products that would occur after recombination at D1 and J3 (using the corrected diagram)



original

3. How many different chains could be made from the above DNA? You should ignore any additional diversity that is generated during the joining process.

$3_{(V)} \times 3_{(D)} \times 3_{(J)} = 27$

4. The following is the DNA sequence of part of a V-segment contained in the kappa light chain locus and part of a J-segment from the same locus.

germ line

RSS 12bp RSS 23 bp

```
----V-segment---|                                          |---J-segment
AGTCGCTTACCTCACAGTGATG-n-ACAAAAACC----GGTTTTTGT-m-CACTGTGGCTGCTTTT
TCAGCGAATGGAGTGTCACTAC-n-TGTTTTTGG----CCAAAAACA-m-GTGACACCGACGAAAA
SerArgLeuPro                                               AlaAlaPhe
```

1 turn 2 turn

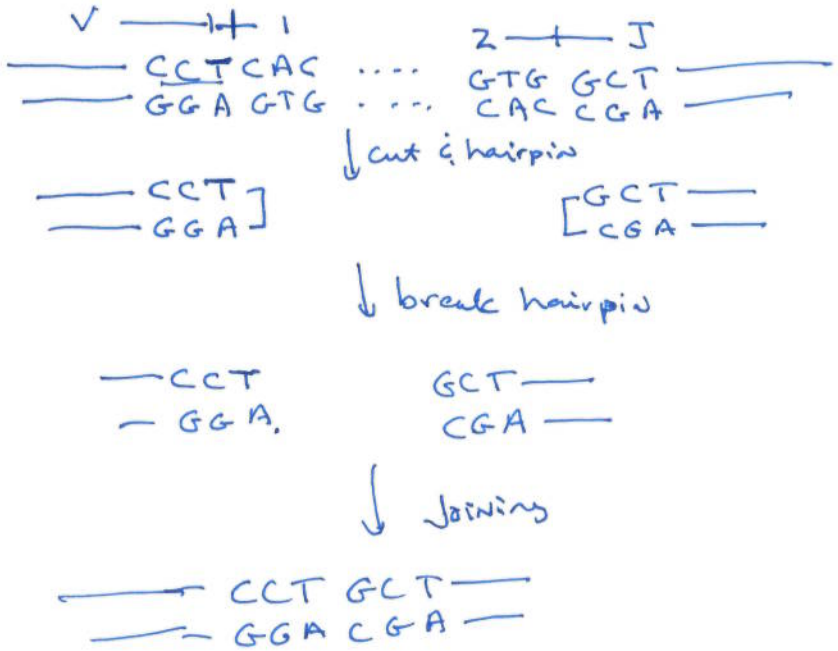
i) Identify the recombination signal sequences (RSS).

ii) What are the values for "*n*" and "*m*", in basepairs?

iii) Draw out the molecular events that gave rise to this product (precise joining)

```
AGT CGC TTA CCT GCT GCT TTT
TCA GCG AAT GGA CGA CGA AAA
Ser Arg Leu Pro Ala Ala Phe
```

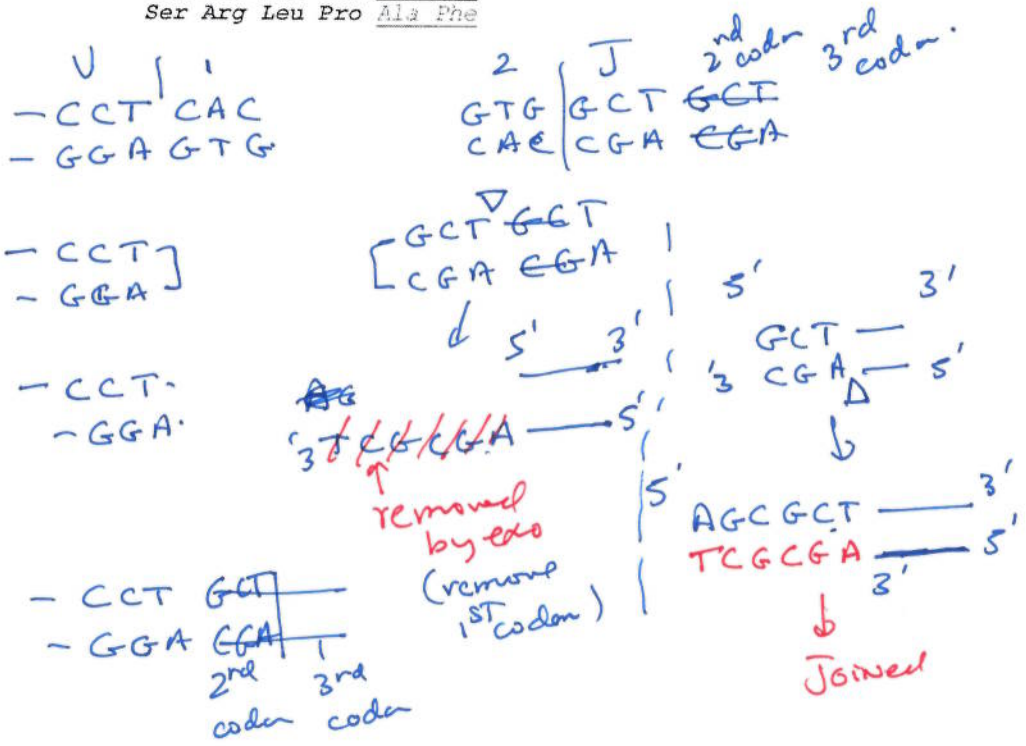
VJ in naive B-cell



```
AGT CGC TTA CCT CACAGTGATG-n-ACAAAAACC----GGTTTTTGT-m-CACTGTG GCT GCT TTT
TCA GCG AAT GGA GTGTCACTAC-n-TGTTTTTGG----CCAAAAACA-m-GTGACAC CGA CGA AAA
Ser Arg Leu Pro                                           Ala Ala Phe
```

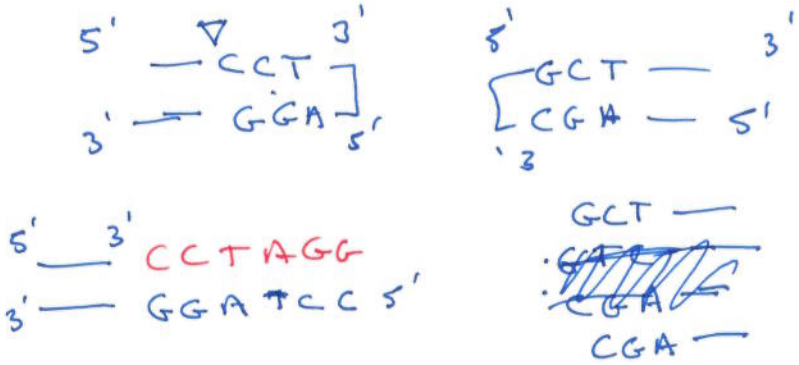
iv) Draw out the molecular events that gave rise to this product (imprecise, with loss of a J-codon)

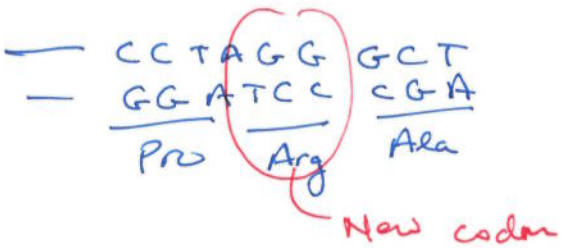
AGT CGC TTA CCT GCT TTT
TCA GCG AAT GGA CGA AAA
Ser Arg Leu Pro *Ala Phe*



v) Draw out the molecular events that gave rise to this product (imprecise, with gain of codon)

AGT CGC TTA CCT AGG GCT GCT TTT
TCA GCG AAT GGA TCC CGA CGA AAA
Ser Arg Leu Pro Arg *Ala Ala Phe*





No frame shift
- cleave hairpin
- at codon boundaries

Frame shift if cleave within a codon