# Functional relevance of sequence changes: not forgetting the impact of splicing-affecting mutations

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# Introduction

- newly emerging data: many disease causing mutations affect the event of **splicing**

# Introduction

- subjects of this presentation:
  - importance of splicing
  - splicing regulation
  - splicing affecting mutations
  - testing of splicing mutations in hyper IgE patients

# Mutations or polymorphisms?

- genetic diagnostics reveal many new sequence variants
  - their effect on disease uprise and progression is not always clear

# Mutations or polymorphisms?

- common diagnostic criteria:

  according to the location of a mutation in the gene

  - in **protein coding region** - according to amino acid change
  - premature stop codons are usually considered disease-causing
  - missense mutations (changes for different amino acid) are classified according to the type of amino acids changed
  - null mutations (coding for the same sense codon) are ranked as **„unclassified variants“ = UVs**

# Mutations or polymorphisms?

- common diagnostic criteria:

  according to the location of a mutation in the gene

  - at the **exon-intron borders:** pathogenicity is predicted only if a mutation changes consunsual dinucleotide of the splice site
  - in the **introns** - rarely found mutations, difficult predictions
  - many **„unclassified variants“ = UVs**

# Unclassified sequence variants

- there is a growing evidence that many sequence variants affect diasese development and/or severity by an **alteration of pre-mRNA splicing**

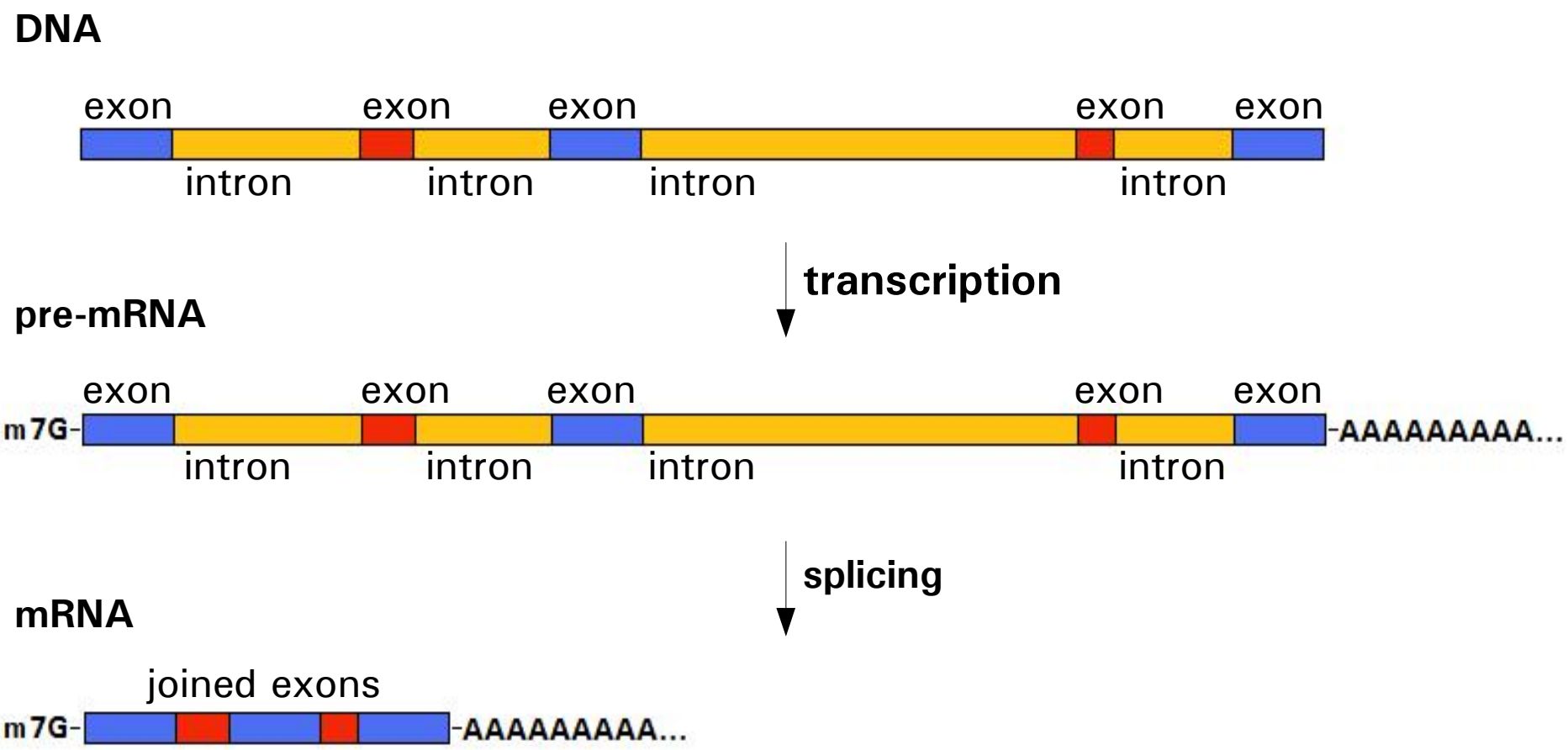
  estimations of proportion of splicing-affecting mutations among all disease causing mutations vary between **15 and 50 %**

# Splicing

- primary transcript of protein coding genes contain both **exons** (protein coding sequences) and **introns** (non-coding sequences)

- intronic sequences must be removed from pre-mRNA prior to the translation = the process of **splicing**

# Splicing


DNA
exon
exon
exon
exon
exon
intron
intron
intron
intron
transcription
pre-mRNA
exon
exon
exon
exon
exon
m7G-
-AAAAAAAAA...
intron
intron
intron
intron
splicing
mRNA
joined exons
m7G-
-AAAAAAAAA...

# Splicing

- constitutive splicing:

1 pre-mRNA

↓

1 mRNA

↓

1 protein

# Splicing

- most of human genes are subjected to **alternative splicing**

# Splicing

- alternative splicing:

1 pre-mRNA

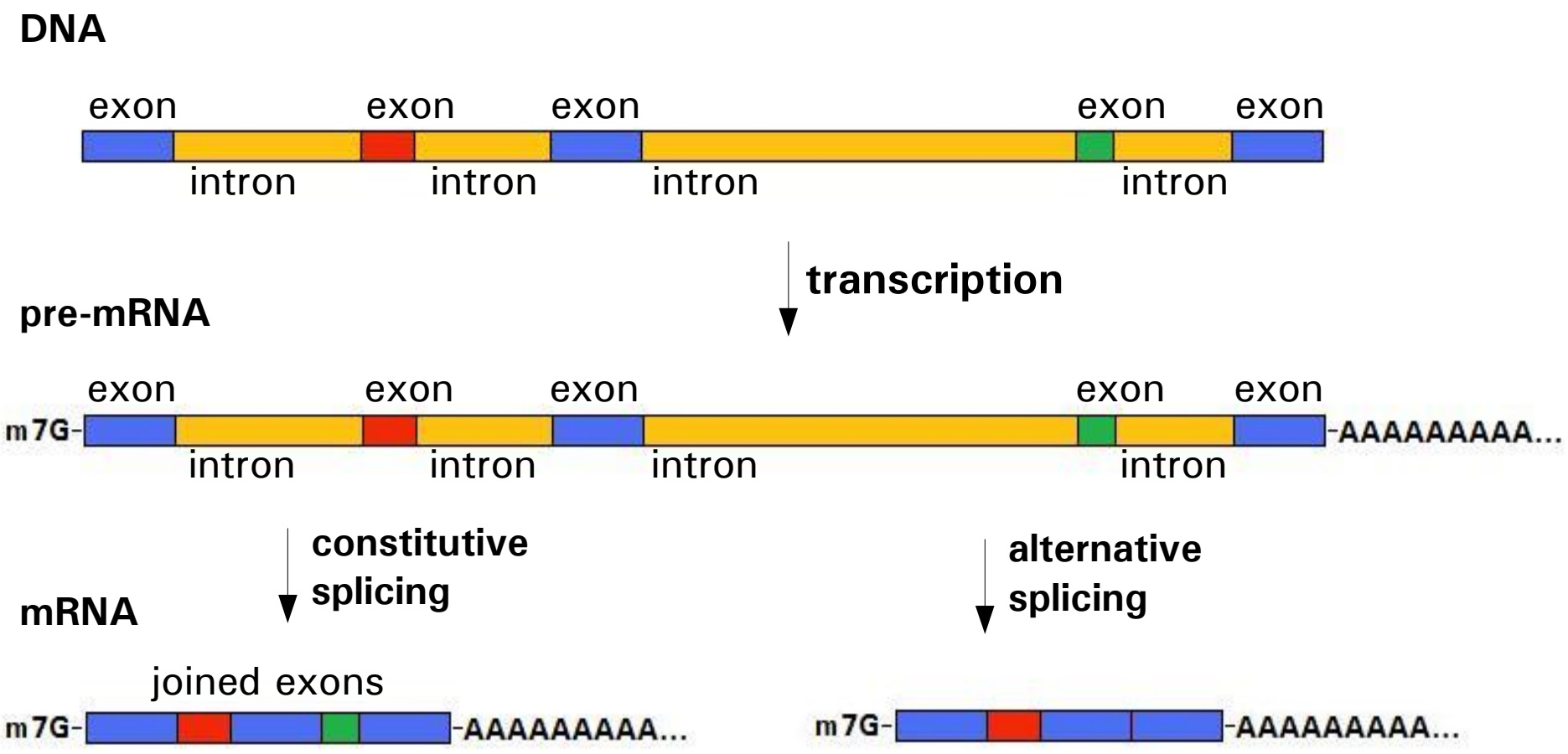
↓

> 1 mRNA

↓

several different protein isoforms

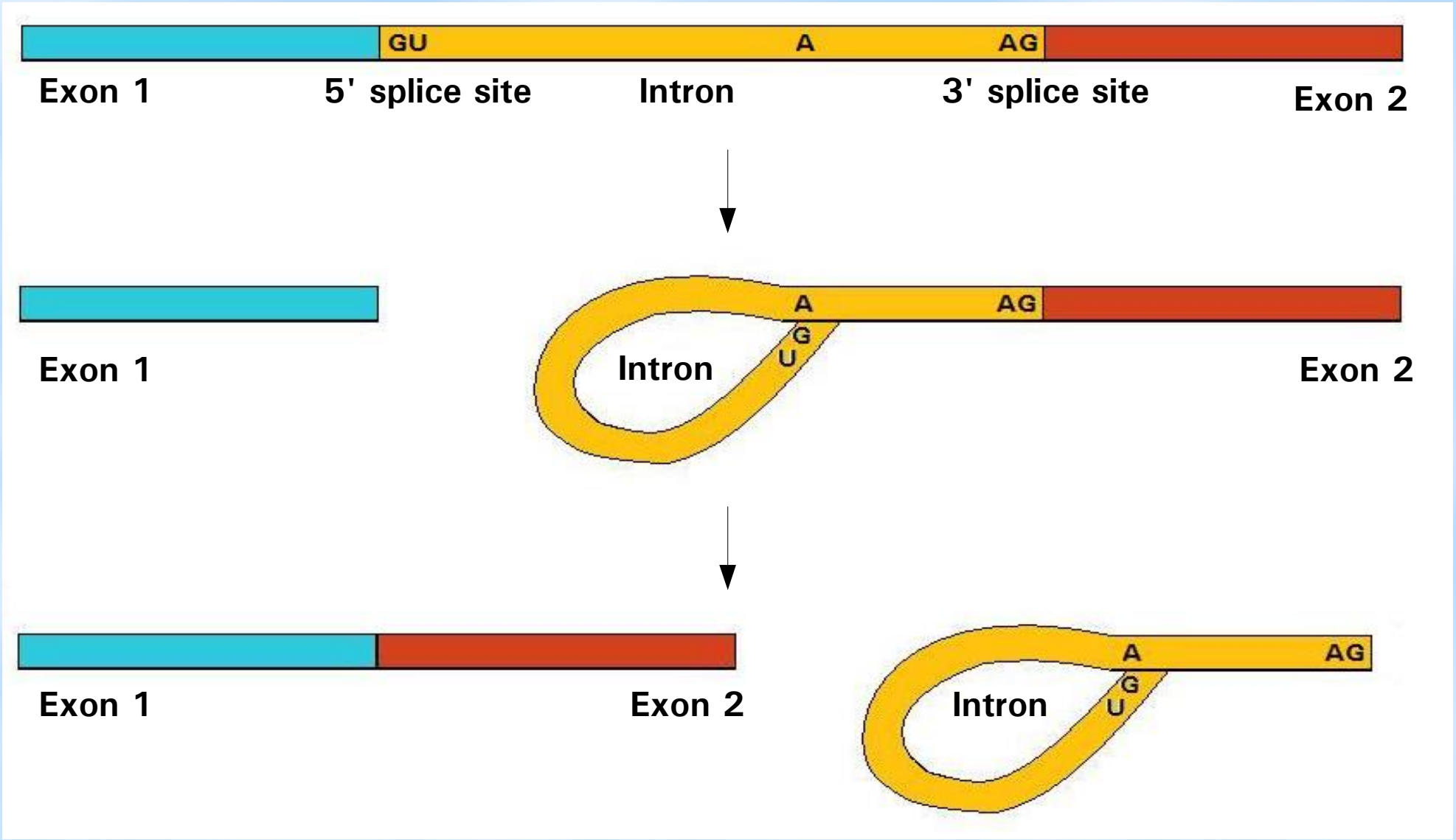
# Splicing


DNA
exon
exon
exon
exon
exon
intron
intron
intron
intron
transcription
pre-mRNA
exon
exon
exon
exon
exon
m7G-
-AAAAAAAAA...
intron
intron
intron
intron
constitutive
splicing
alternative
splicing
mRNA
joined exons
m7G-
-AAAAAAAAA...
m7G-
-AAAAAAAAA...

# Splicing

- splicing reaction: two consecutive transesterifications

# Splicing


GU
A
AG
Exon 1
5' splice site
Intron
3' splice site
Exon 2
Exon 1
A
AG
G
U
Intron
Exon 2
Exon 1
Exon 2
A
AG
G
U
Intron

# Splicing

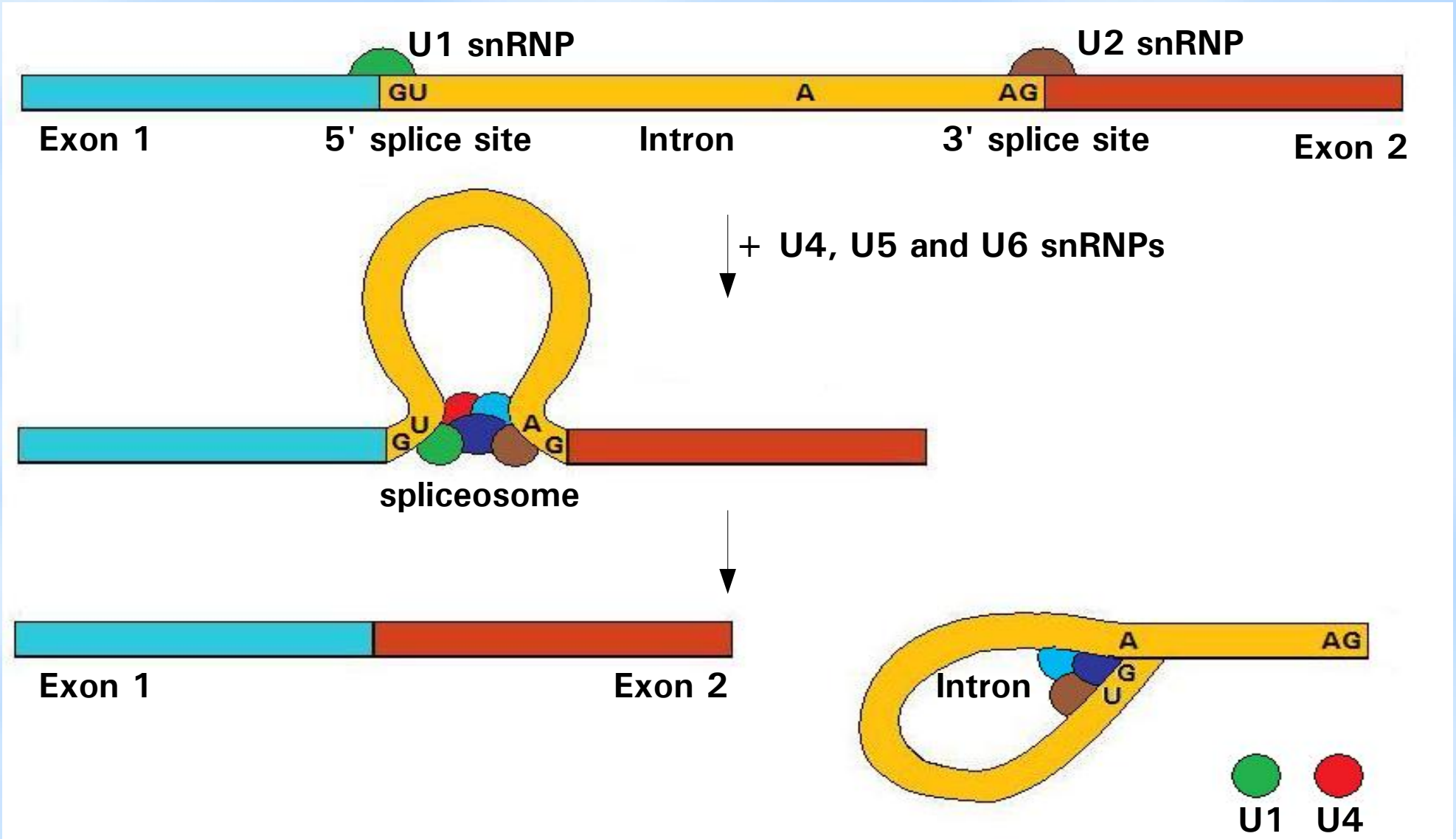
- splicing reaction: two consecutive transesterifications catalyzed by complex of snRNP - **spliceosome**

- spliceosome recognises borders of exons - splice sites

# Splicing

- splice site
  - several nucleotides around the borders of exon and intron
  - conserved sequence: GU at the 5' end of intron
    AG at the 3' end of intron
  - other nucleotide positions are rather degenerate

# Splicing


U1 snRNP
U2 snRNP
GU
A
AG
Exon 1
5' splice site
Intron
3' splice site
Exon 2
+ U4, U5 and U6 snRNPs
U
A
G
G
spliceosome
Exon 1
Exon 2
A
AG
Intron
G
U
U1
U4

# Splicing regulation

- computational DNA analysis: many sequences that ressemble classical splice sites but are never used = **pseudo splice sites**

- distinguishing pseudo splice sites from real splice sites?

**auxiliary *cis*-elements** aid in the recognition of exones

# Splicing regulation

- auxiliary *cis*-elements: splicing enhancers and silencers located both in exons and introns
  - they regulate both constitutive and alternative splicing
  - exon splicing enhancers (**ESE**)
  - exon splicing silencers (ESS)
  - intron splicing enhancers (ISE)
  - intron splicing silencers (ISS)

# Splicing mutations

- mutations disrupting splicing enhancers or silencers can cause human diseases

  effect of these mutations: mainly **exon skipping**

  - exon skipping often disturbs reading frame of the gene, thus changing the sense of all downstream codons
  - frameshift often induces premature stop codon into mRNA
  - mRNA with premature stop codon is subjected to degradation by a mechanism called **nonsense-mediated decay (NMD)**

# Splicing mutations

- mutations disrupting short sequence of splice site cause human diseases

  effect of these mutations:

  - exon skipping
  - retention of intron in mRNA
  - introduction of new splice site within an exon or intron

# Splicing mutations

- both types of above described splicing mutations can:
  - activate preexisting pseudo splice site
  - affect fine balance of alternatively spliced isoforms

# Splicing mutations

- however, splice sites, SEs and SSs are **highly degenerate**
  - problem with designating splicing-affecting mutations just from inspection of DNA
  - importance of splicing mutations is probably underestimated
  - even silent mutations (at protein level) can cause disease through disruption of splicing
  - significant fraction of exonic mutations that cause diseases are most probably unrecognized splicing mutations

# Splicing mutations

- test for splicing mutations:
  - direct analysis of patients mRNA
  - in vitro analysis by use of minigenes

# Splicing mutations

- test for splicing mutations:

  direct analysis of patients mRNA: RT-PCR

  - patients RNA is not always available for laboratory
  - not always accessible since the splicing is often tissue-specific
  - aberrant splicing often induces frameshift and premature stop codon in mRNA molecule which makes it a target for NMD, thus preventing us from its detection

# Splicing mutations

- test for splicing mutations:

  in vitro analysis by use of minigenes

  - testing for splicing events in a short fragment of exon and adjacent intronic sequences (or intron and adjacent exons)
  - will be further described together with a new project of ours concerning examination of *STAT3* gene mutations found in two hyper IgE (HIE) patients

# HIE patients: inspecting possible splicing mutations

- we found two patients suffering from HIE whose mutations found in *STAT3* gene appears to possibly affect splicing

# HIE patients: inspecting possible splicing mutations

- patient A bears mutation N581N, g. AAC>AAT in exon 19 of *STAT3* gene
  - computational predictions revealed that the mutation disrupts potentional ESE
  - *in silico* analysis also predicted that the splice site at 5' end of exon 19 is of moderate strength
  - potential exon 19 skipping would cause a frameshift that would induce a premature stop codon into mRNA → NMD?

# HIE patients: inspecting possible splicing mutations

- patient B bears mutation L284V, g. CTG>GTG in exon 9 of *STAT3* gene
  - amino acid change is computionally predicted as benign
  - computational predictions revealed that the mutation disrupts potentional ESE
  - *in silico* analysis also predicted that the splice site at 5' end of exon 9 is rather weak (score 0,34; maximum is 1)
  - potential exon 9 skipping would not cause a frameshift, but a deletion of 53 amino acids

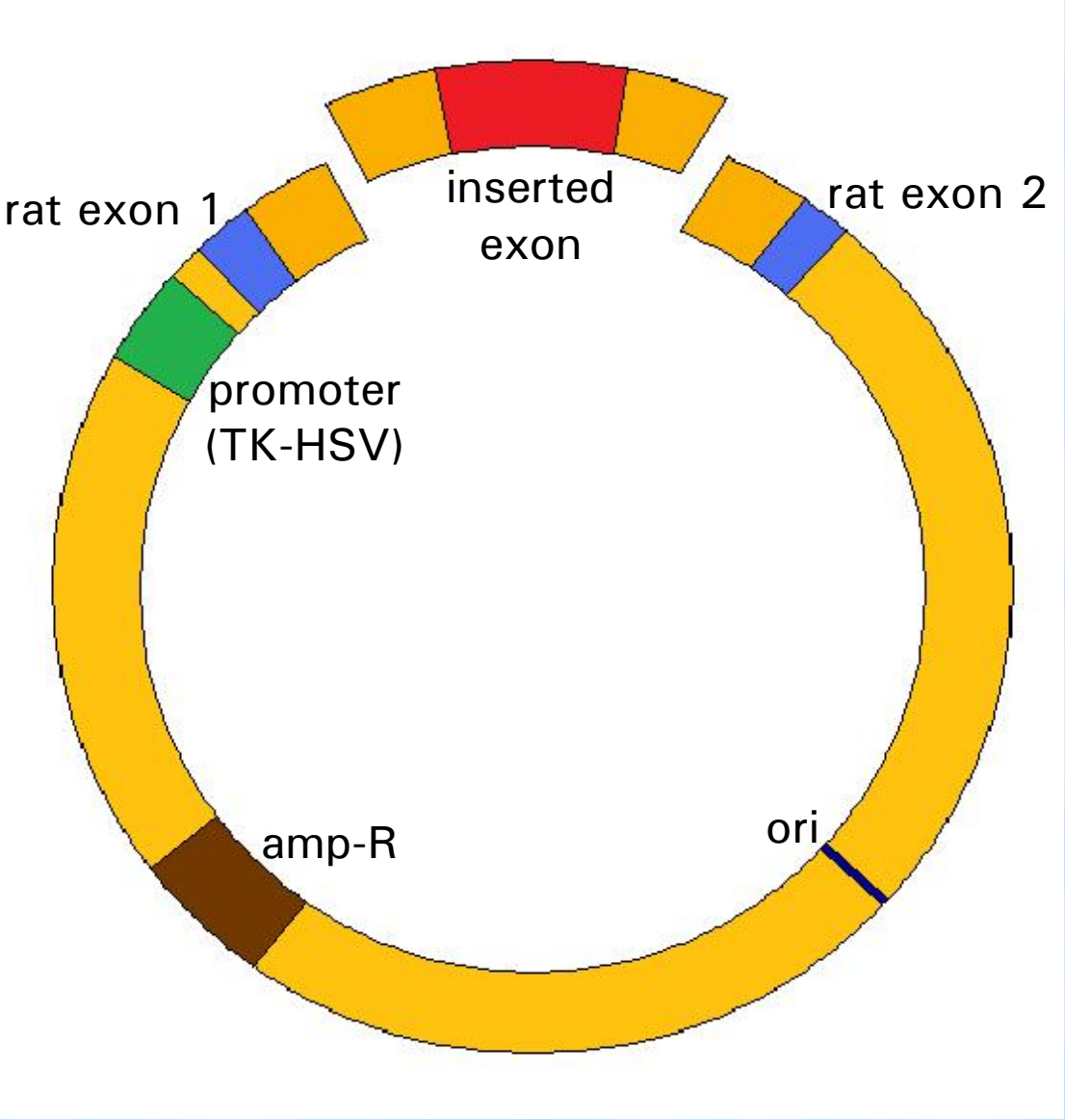
# HIE patients: inspecting possible splicing mutations

- for both mutations we designed
  - RT-PCR and
  - splicing minigene assay

# HIE patients: inspecting possible splicing mutations

- splicing minigene assay:
  - exon under inspection with adjacent introns is inserted into a plasmid right into an intron region between two exons of non-human gene (e.g. rat insulin gene)

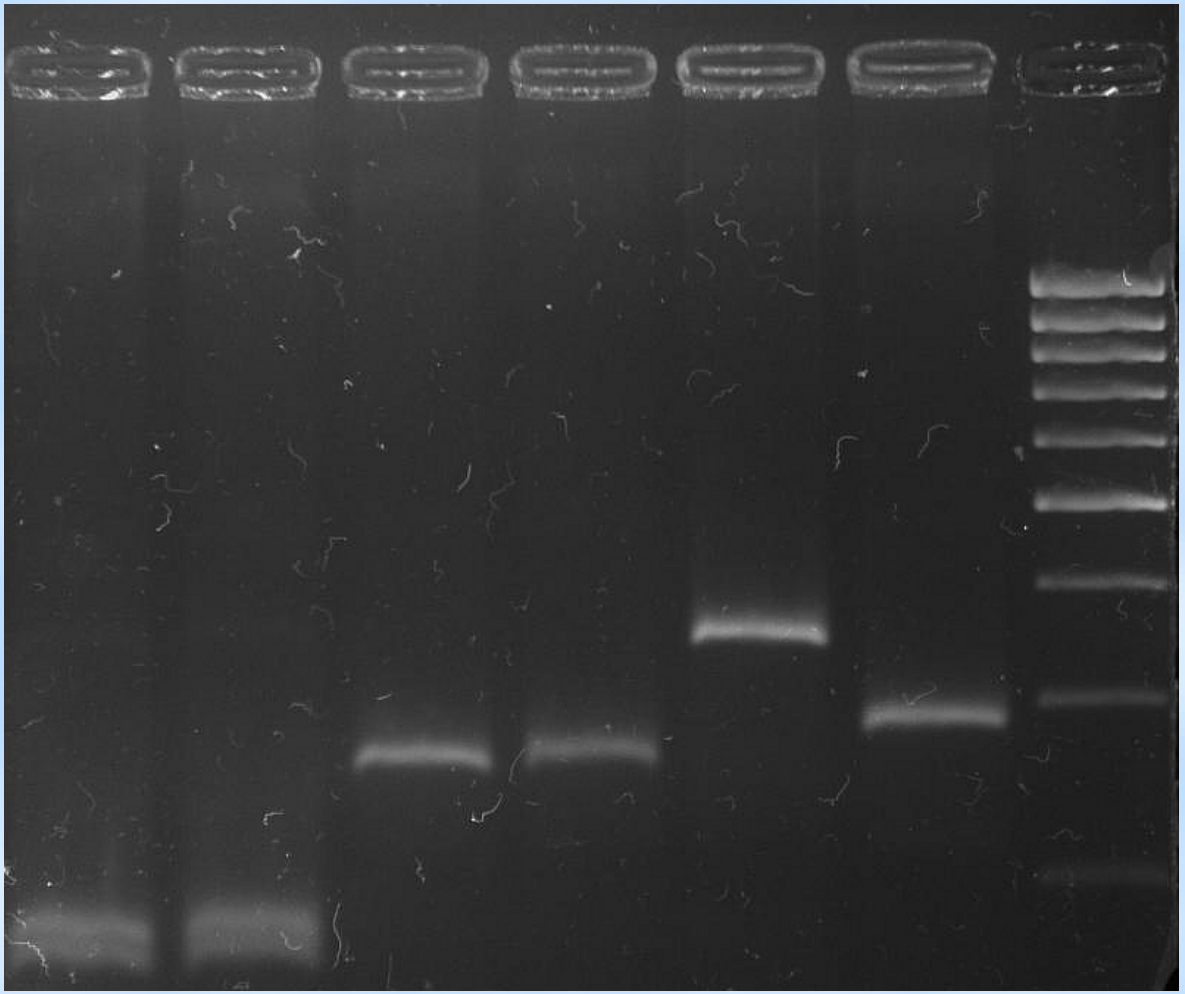
# HIE patients: inspecting possible splicing mutations



# HIE patients: inspecting possible splicing mutations

- splicing minigene assay:
  - exon under inspection with adjacent introns is inserted into a plasmid right into an intron region between two exons of non-human gene (e.g. rat insulin gene)
  - plasmid construct is transfected into human cell line
  - RNA from the cells is analysed by RT-PCR with primers specific to the rat gene
  - PCR product analysis: gel electrophoresis

# HIE patients: inspecting possible splicing mutations



Thank you for your attention.