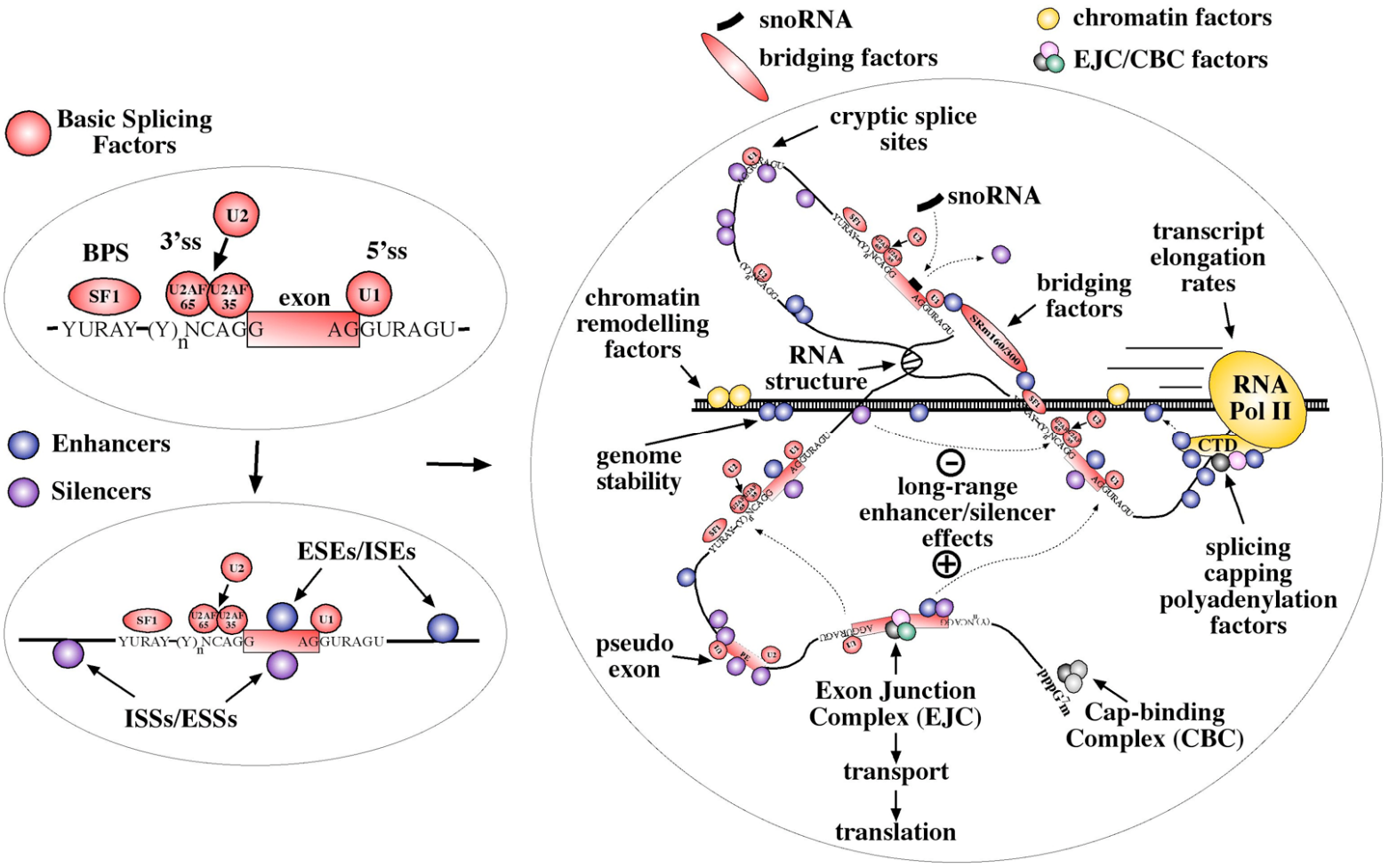
International Centre for Genetic Engineering and Biotechnology. Trieste (Italy)

# Splicing and Diagnostic Methodologies

# When should I suspect a splicing mutation?




**The result of all this complexity is that identifying splicing spoilers from a background of harmless polymorphisms has become very difficult. All that can really be emphasized is the need to consider any genomic variation, even those that occur deep within intronic regions , as a potential splicing mutation.**

# What are the most common consequences of a splicing mutation?

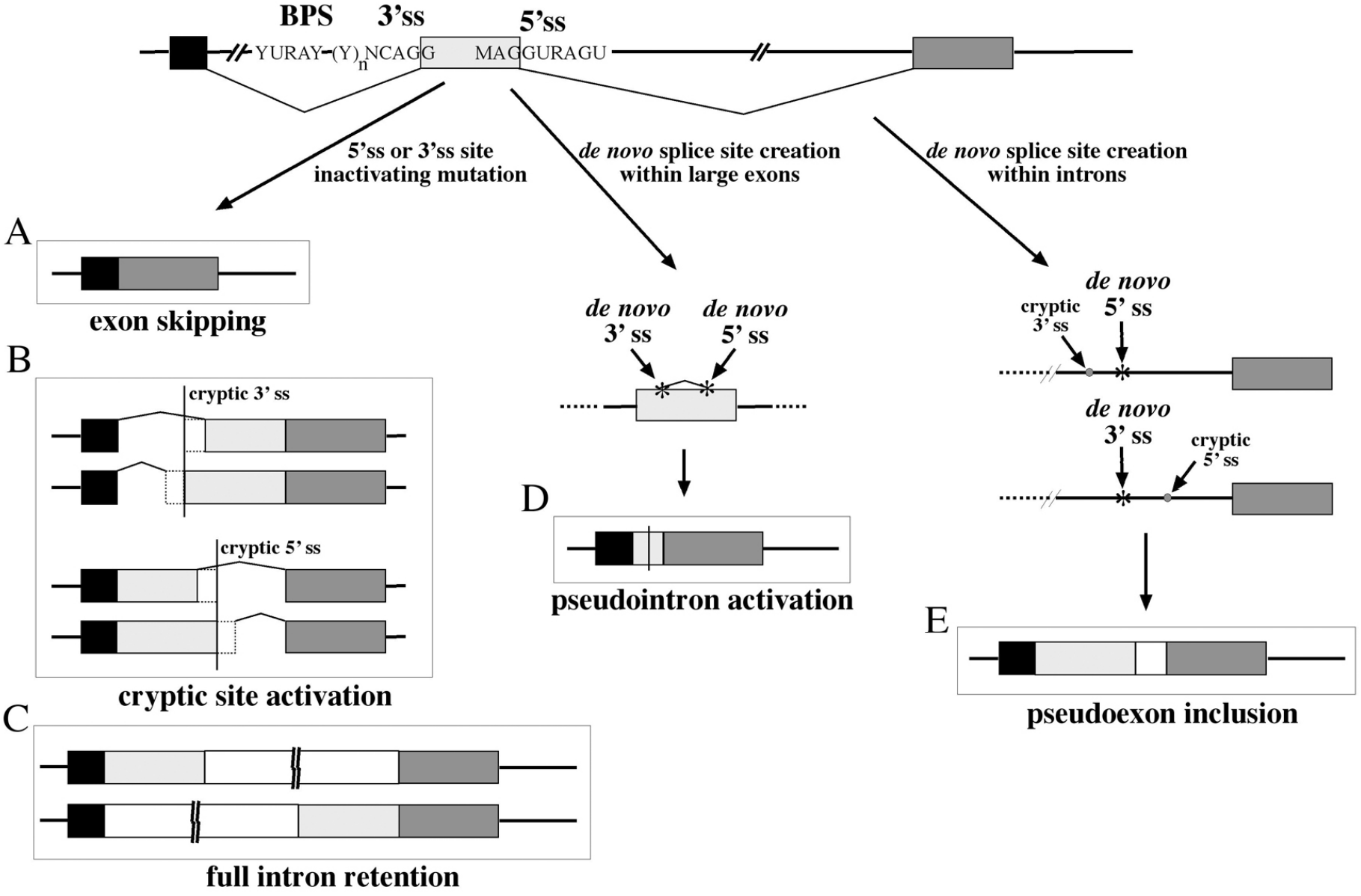



Fig.1

**Experimental approaches in the study of alternative splicing and disease can be divided in two major classes:**

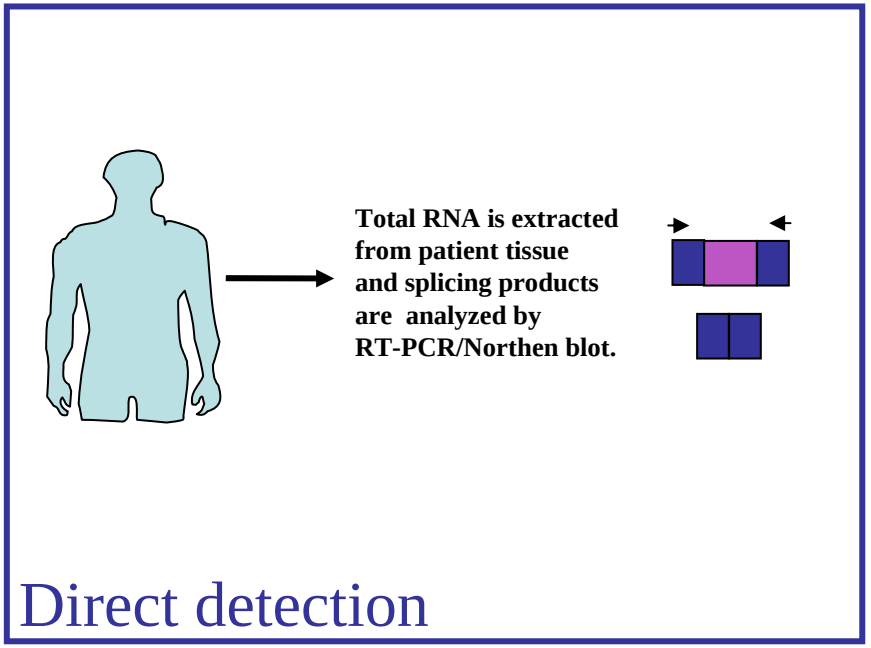
**Detection and validation of splicing mutations:**

**-Direct amplification of spliced transcripts.**
**-Minigene splicing systems.**
**-In vitro splicing systems.**

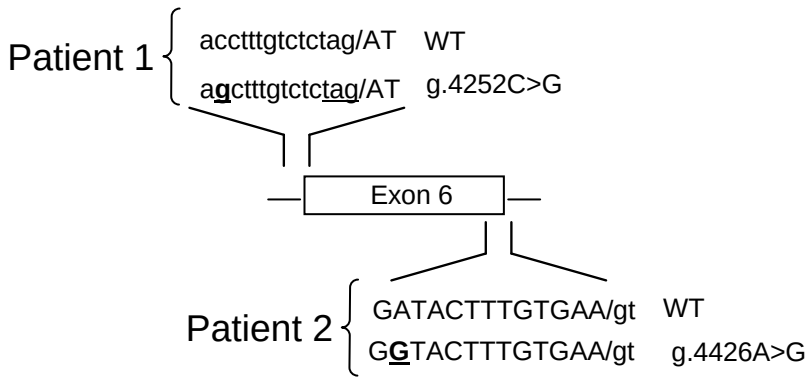
**Experimental methods to define the functional effects of the mutation:**

**-RNA-protein interactions.**
**-snRNP-RNA interactions.**
**-RNA secondary structures.**

# Northern/RT-PCR direct detection



**We studied two unrelated Italian patients affected by type 1 Gaucher disease. Gaucher disease is the most frequent lysosomal storage disorder caused by an autosomal recessive deficiency of acid beta-glucosidas that in turn leads to the accumulation of glucocerebroside. The disease was discovered in 1882 by Philipe Charles Ernest Gaucher and in the worldwide population has a frequency of 1:40000 to 1:60000**



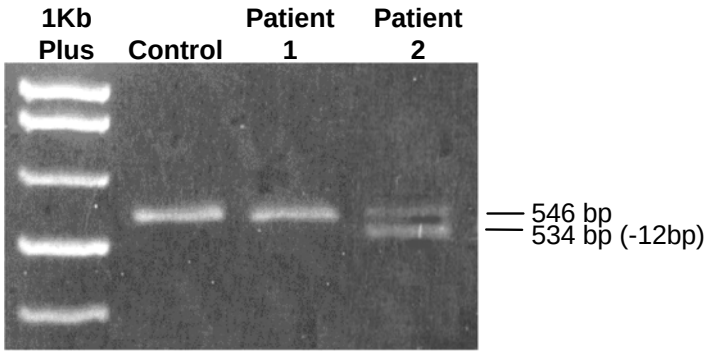

Patient 1 revealed the presence of the g.4252C>G in heterozygosis with the known mutation p.R170C ,

Patient 2 presented the g.4426A>G in heterozygosity with the frequent mutation p.N370S

**Allele composition represents an important information when looking directly at transcripts**

**Dominissini et al. HumMut 2006 27: 119.**

# Transcript analysis in patient fibroblasts



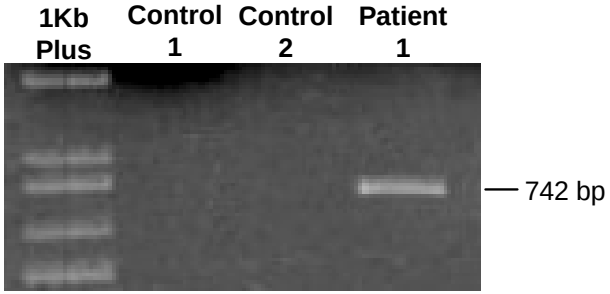

In order to better define the alleles of origin of these transcripts, RT-PCR fragments from exon 3 to 9 were purified, cloned in pGEM, and >25 colonies screened.

In Patient 1, all the colonies carried the p.R170C mutation and neither the normal nor the mutant transcript from the allele carrying the g.4252C>G substitution could be detected.

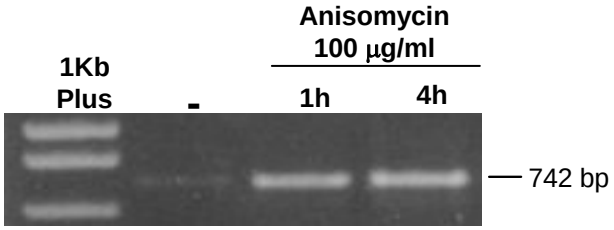
In Patient 2 almost all colonies carried either the p.N370S mutation or the mutant transcript produced from the g.4426A>G allele, lacking 12 bp. of exon 6. Interestingly, less than 25% of the colonies carried the wild-type allele suggesting that in vivo the natural 5'ss near to the g.4426A>G mutation has a residual activity

Dominissini et al. HumMut 2006 27: 119.

# Missing transcript in Patient 1 is degraded by NMD. It can then be detected using a RT-PCR specific approach coupled with inhibition of NMD with anisomycin.




- In Patient 1, the transcript derived from the allele carrying the g.4252C>G substitution can be detected using a primer specific for usage of this 3'ss.




- Treatment with anisomycin of the patients' fibroblasts demonstrates that this transcript is rapidly degraded by NMD

Dominissini et al. HumMut 2006 27: 119.

**Direct detection:**

**Advantages:**

**1) the RNA is analyzed directly from the patient.**

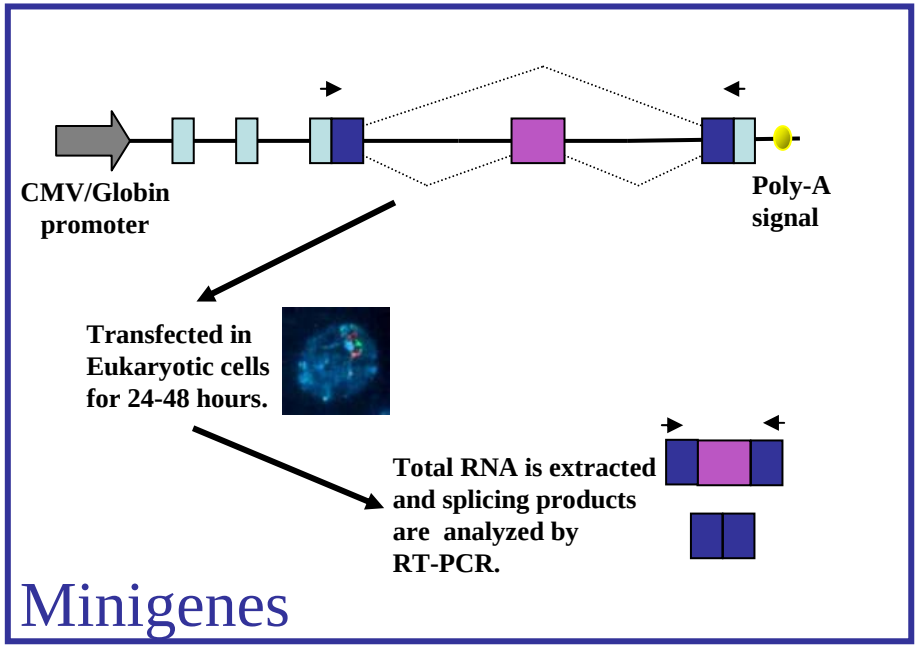
**Drawbacks:**

**1) patients or RNA samples from affected tissues are not always available.**

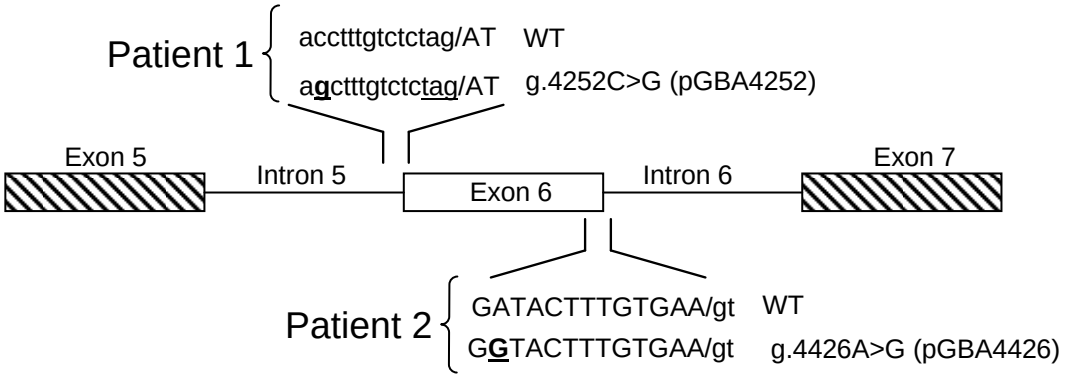
**2) the gene of interest may not be expressed in easily available tissues.**

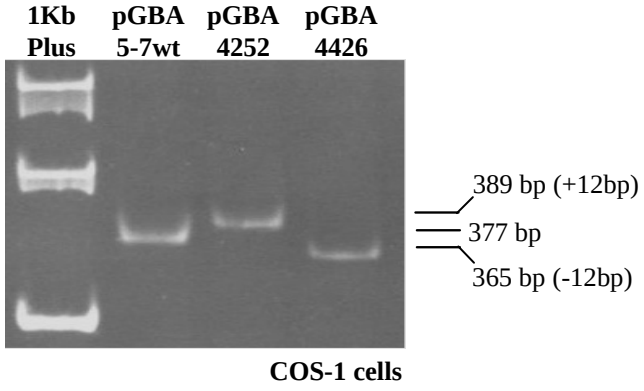
**3) often, unless the suspected splicing mutation is present in both alleles, care must be taken when evaluating results.**

# Minigene systems



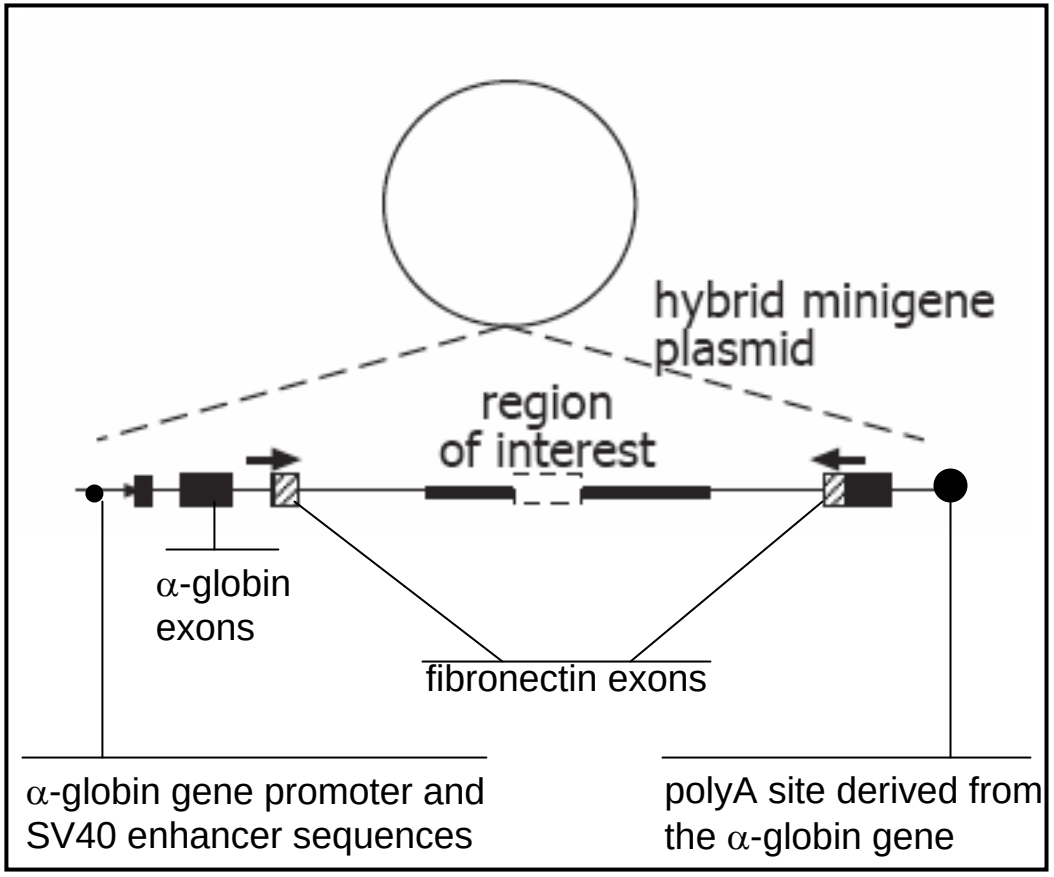
**Minigene analysis has confirmed the severity of both Gaucher mutations. In both cases, only the newly created acceptor and donor sites are exclusively used in the splicing reaction.**




Dominissini et al. HumMut 2006 27: 119.

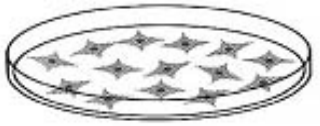
**Our minigene system is based on a plasmid constructs that contain all the elements required for the formation of a "spliceable" mRNA.**




Hybrid minigenes transfection in different cell types

0.5 μg DNA
300.000 cells

Cotransfection with plasmids codifing for splicing factors

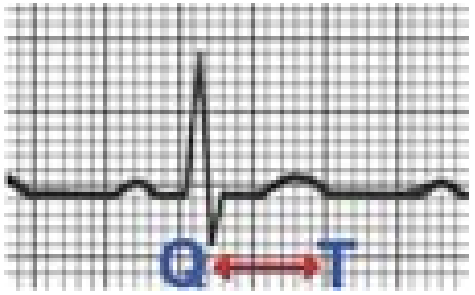


24 hours

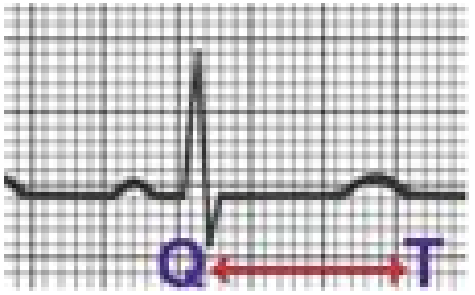
RT-PCR analysis of splicing products using specific oligonucleotides

**The long QT syndrome (LQTS) is a heart condition associated with prolongation of repolarisation (recovery) following depolarisation (excitation) of the cardiac ventricles. It is associated with fainting and sudden death. Hereditary long QT syndrome (LQTS) is caused by over 250 mutations in five genes**

**Normal QT** (390-410 msec)




**Prolonged QT** (> 440 msec)




**Genes involved in Long QT syndrome:**

- KCNQ1 (KVLQT1, LQT1)
- KCNH2 (HERG, LQT2)
- KCNE1 (mink, LQT5)
- KCNE2 (MiRP1, LQT6)

} Potassium channel subunits

- SCN5A (LQT3)

Cardiac sodium channel gene

**We have studied a patient that presented an IVS7+6T>C mutation in the HERG gene.**

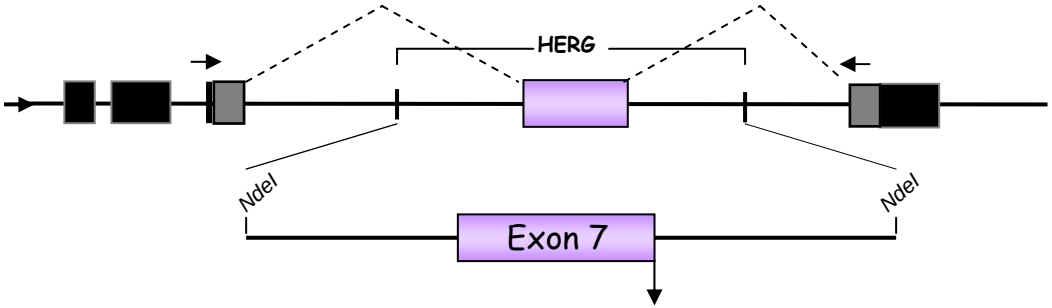
- **One of the most important events in 5’ splice site definition is represented by base pairing of the U1snRNA component of U1snRNP with the 5’ splice site consensus sequence.**
- **A “T” in the +5 position is rather loosely conserved:**

| | -3 | -2 | -1 | +1 | +2 | +3 | +4 | +5 | +6 |
|---|---|---|---|---|---|---|---|---|---|
| A | 32 | 56 | 8 | 0 | 0 | 38 | 70 | 5 | 13 |
| C | 38 | 15 | 4 | 0 | 0 | 4 | 9 | 5 | 21 |
| G | 19 | 15 | 80 | 100 | 0 | 56 | 14 | 86 | 25 |
| U | 11 | 14 | 8 | 0 | 100 | 2 | 7 | 4 | 41 |
| | C | A | G | G | U | R | A | G | U |

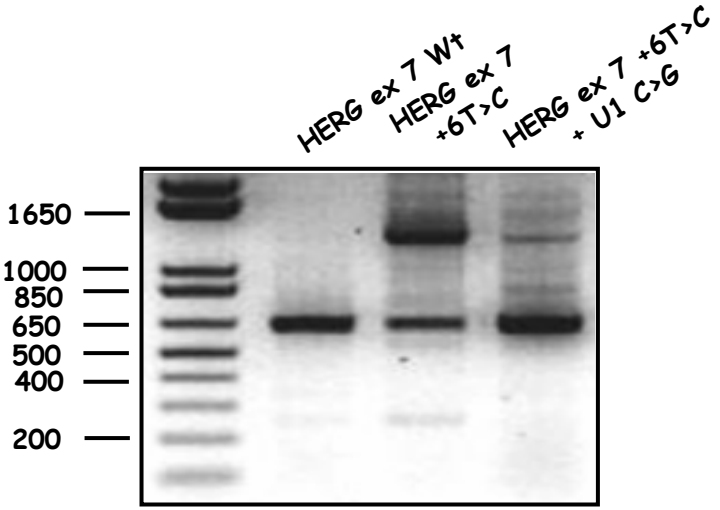
Zhang et al., 1998

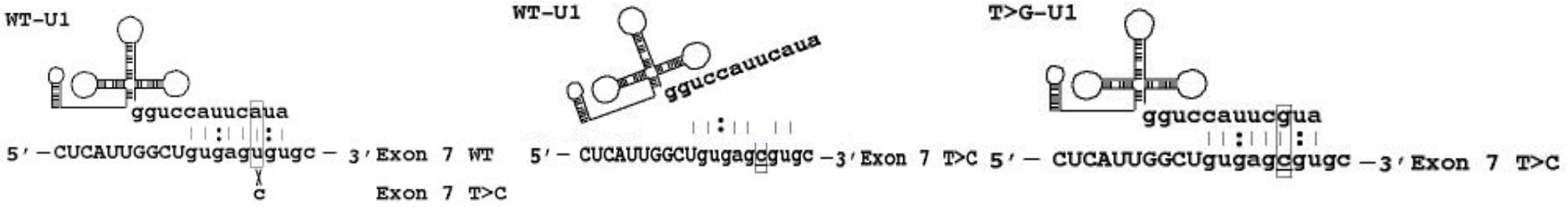
- **Is a +6T>C change capable of affecting splicing?**

# The +6T>C is a mutation that perturbes the snRNP U1 interaction with the 5'ss and in a minigene system causes intron retention




Exon 7 Wt CTCATTGGCT/gtgagtgtc
Exon 7 +6T>C CTCATTGGCT/gtgagcgtc




Zhang et al., 2004. J Am Coll Cardiol; 44(6):1283-91

**Neurofibromatosis Type 1 (NF1) is an autosomal dominant genetic disorder that causes tumours to grow on the covering of the nerves anywhere in the body at any time. The disorder affects 1 in 3,000 males and females of all races and ethnic groups. The NF-1 gene is located on chromosome 17.**

## NF-1 symptoms:

### *Ophthalmologic*

- Optic pathway tumor
- Lisch nodules
- Glaucoma (rare)

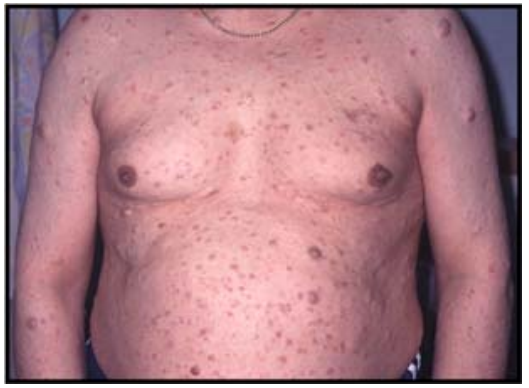
### *Musculoskeletal*

- Speniod wing dysplasia (5-10%)
- Long bone narrowing (2-5%)
- Scoliosis (20-30%)
- Short stature (25-35%)
- Relative Macrocephaly

### *Cardiovascular*

- Hypertension (2-5%)
- Congenital heart defect (2%)

### *Tumours*

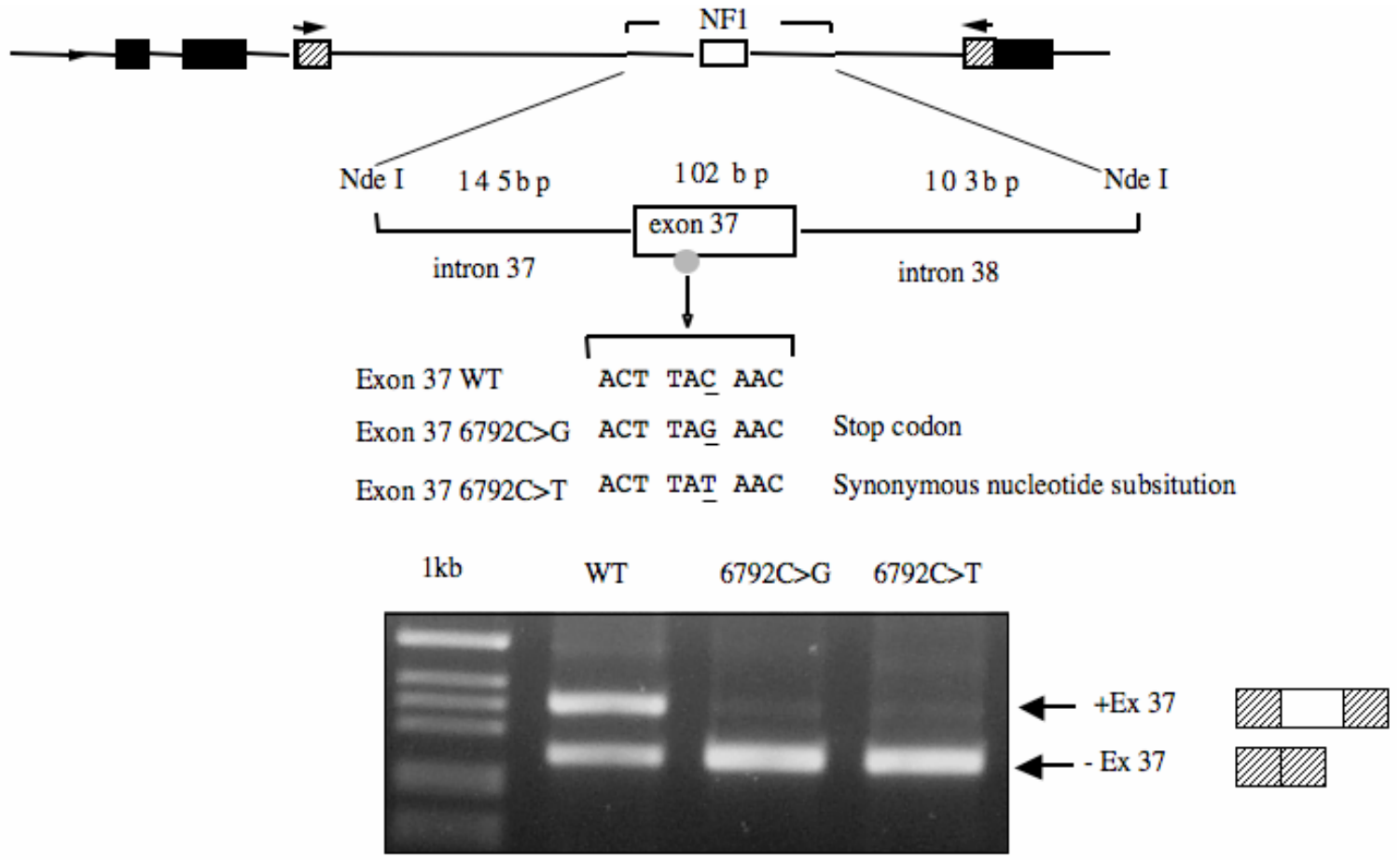


### *Neurological*

- Hydrocephalus (5%)
- Seizures (6-7%)
- Educational difficulty (40-60%)
- Sensorineural hearing loss (5%)
- Precocious puberty (2-5%)

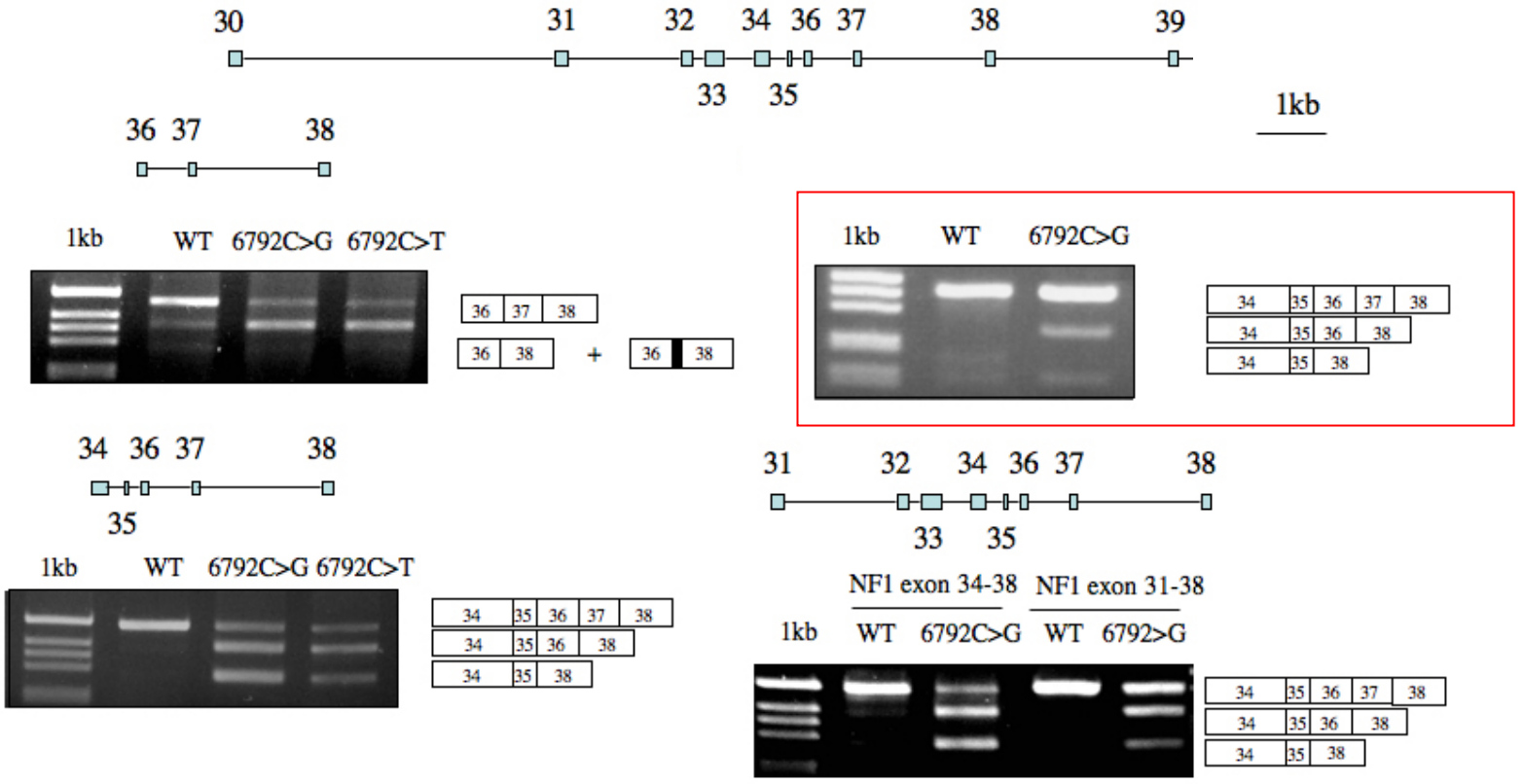
Ricardi 1992, Huson and Hughes 1994, Friedman and Birch 1997.

**In NF-1 exon 37 a practical example of overlap between a coding and splicing mutation is represented by the C6792G mutation that for a long time was thought to be a translational mutation.**



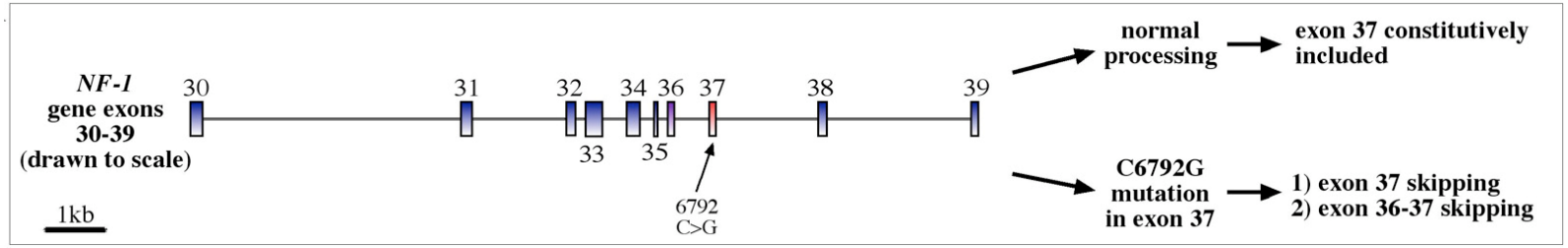

Baralle et al. FEBS Lett. 2006 580: 4449-4456.

**Using as a reference the results obtained from a patient's lymphoblasts carrying the C6792G mutation we have investigated the importance of genomic context on reproducing the effects of splicing of this mutation:**




Baralle et al. FEBS Lett. 2006 580: 4449-4456.

# The importance of genomic context:

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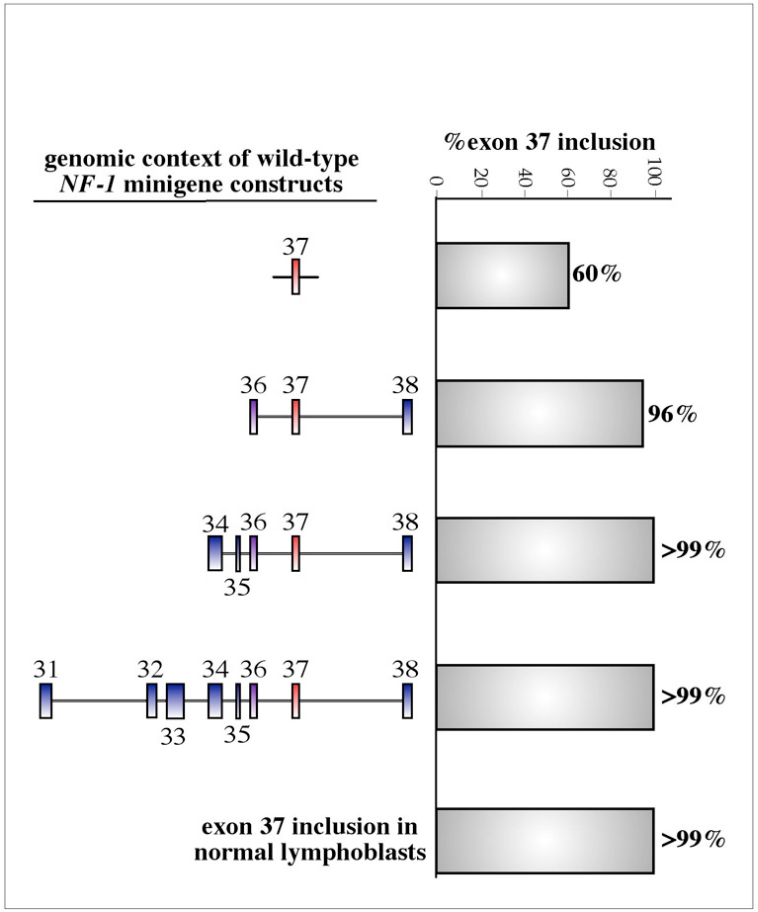
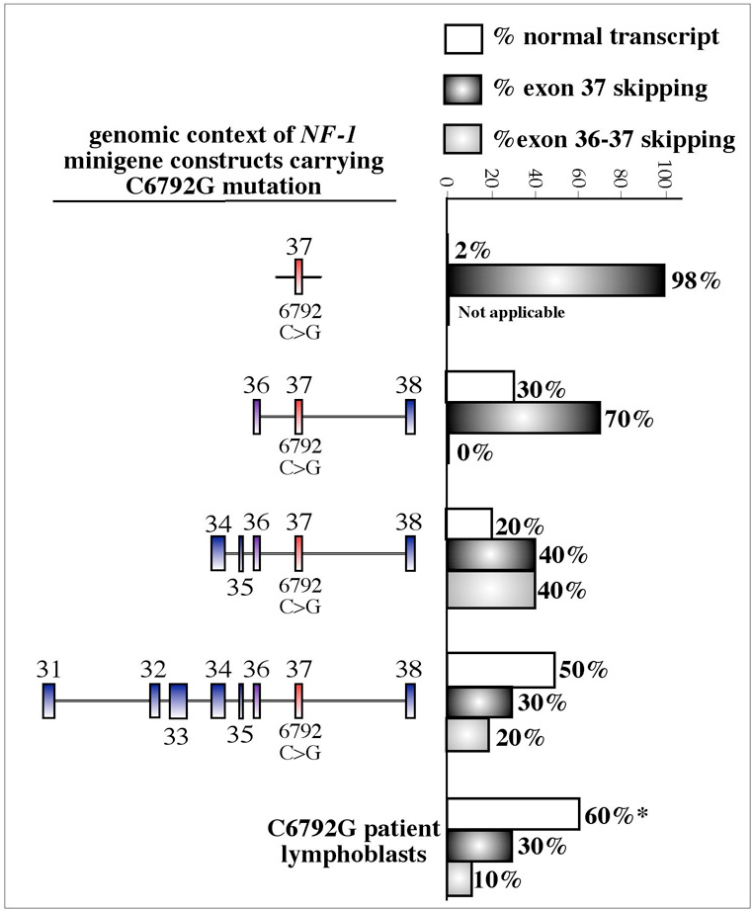



Baralle et al. FEBS Lett. 2006 580: 4449-4456.

Buratti et al. NAR 2006 34: 3494-3510.

## Minigene systems

## Advantages:

**1) the RNA is processed “in vivo”, taking into account the complexity of the cellular environment.**

**2) is recommended to analyze complex exonic/intronic regions.**

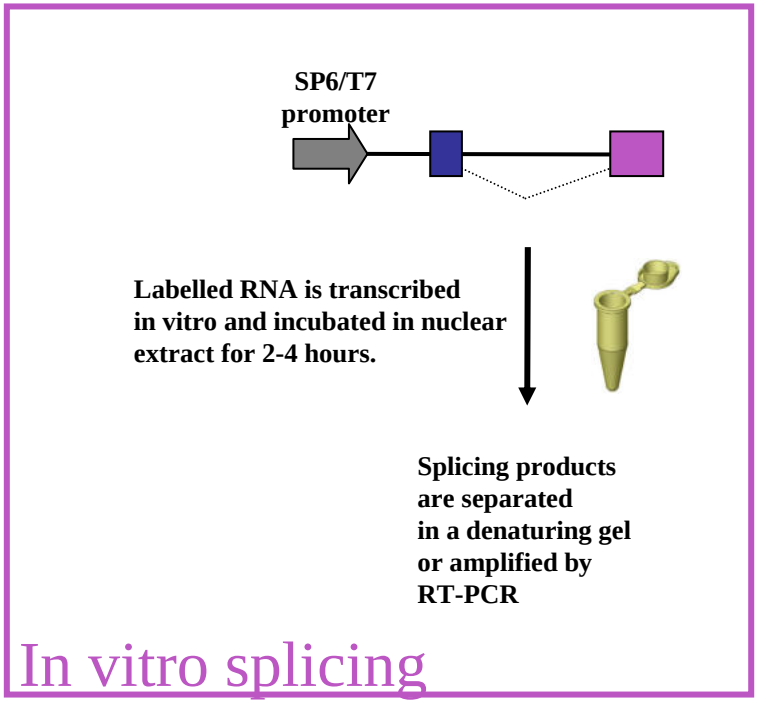
**3) can be adapted to visualize the the splicing of single pre-mRNA molecules or for fluorescent imaging of alternative splicing regulation “in vivo”.**

## Drawbacks:

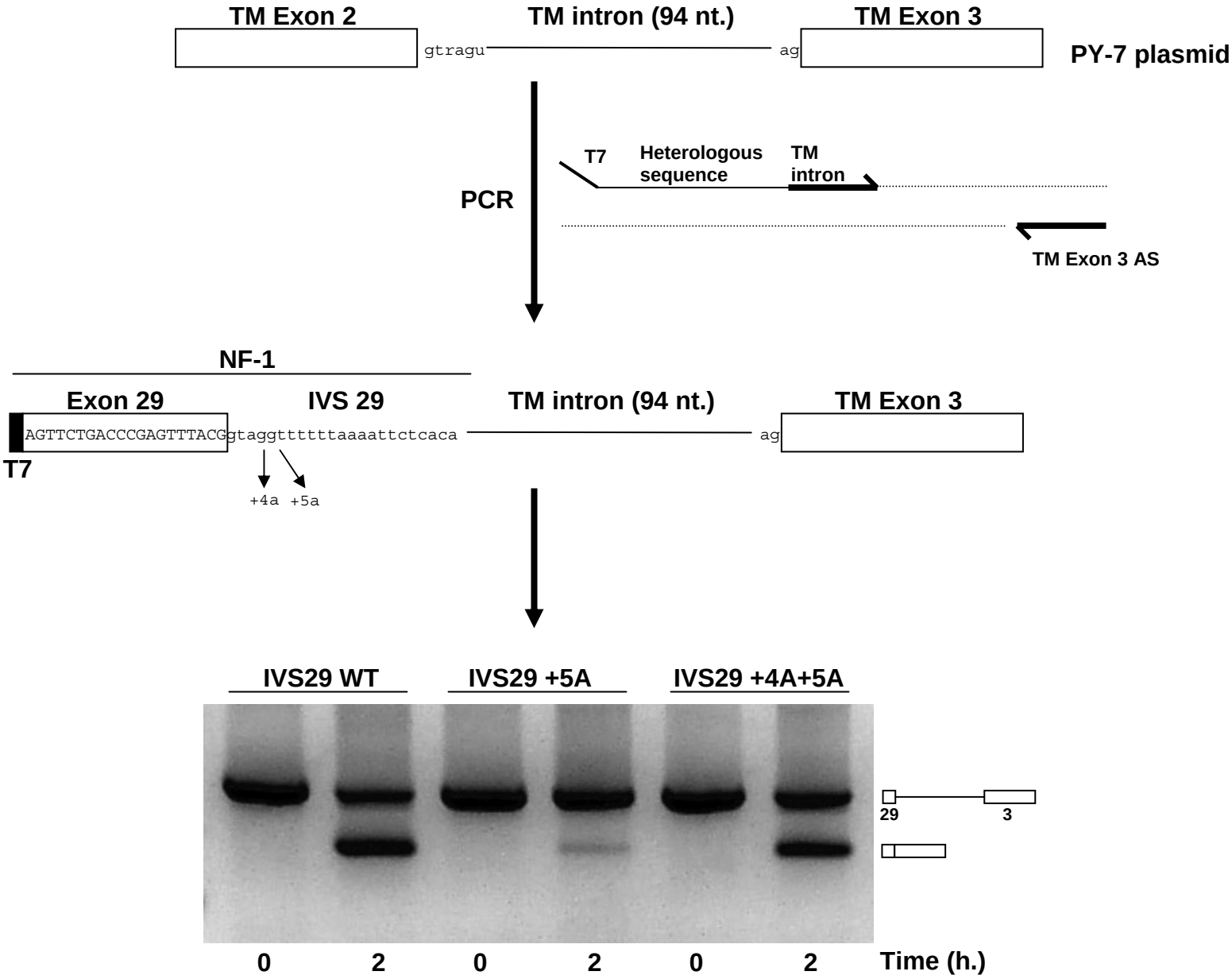
**1) it does not provide (easily) information regarding splicing intermediates or kinetics.**

**2) it is rather difficult to act on the cellular environment and remove/replace selected factors.**

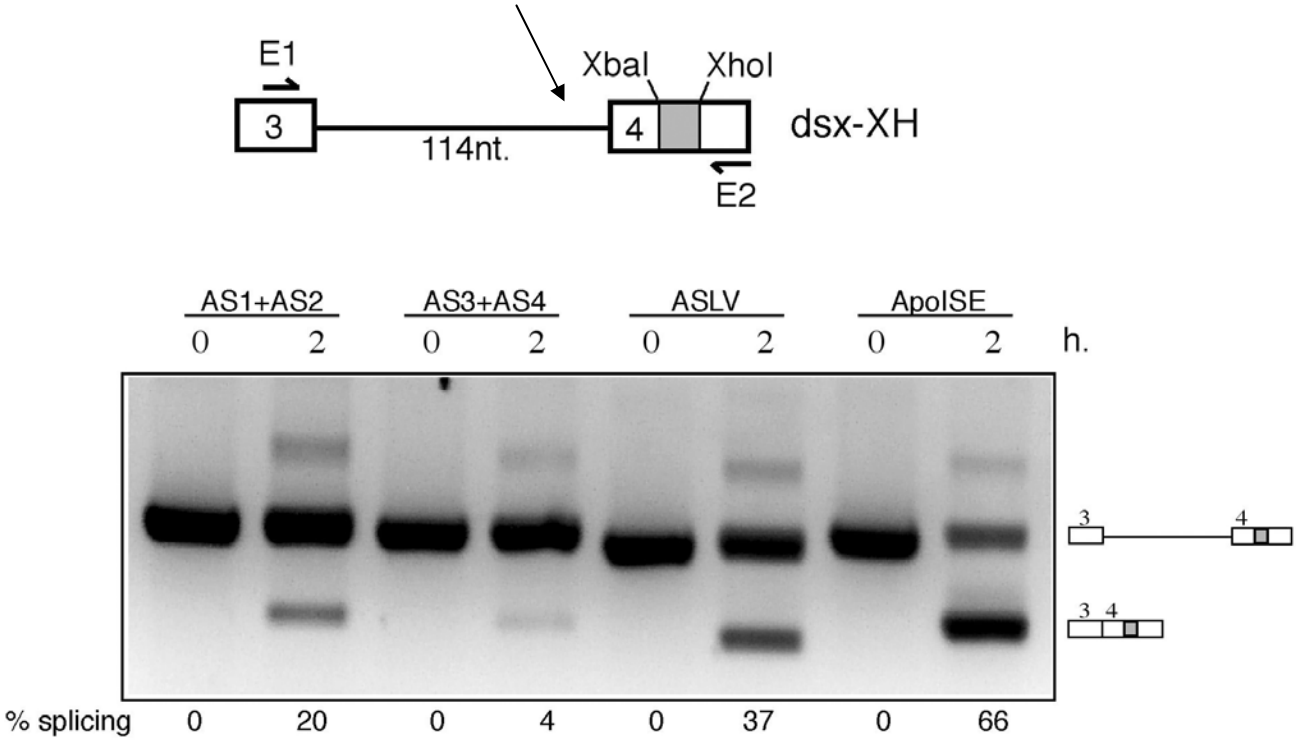
# In vitro splicing systems



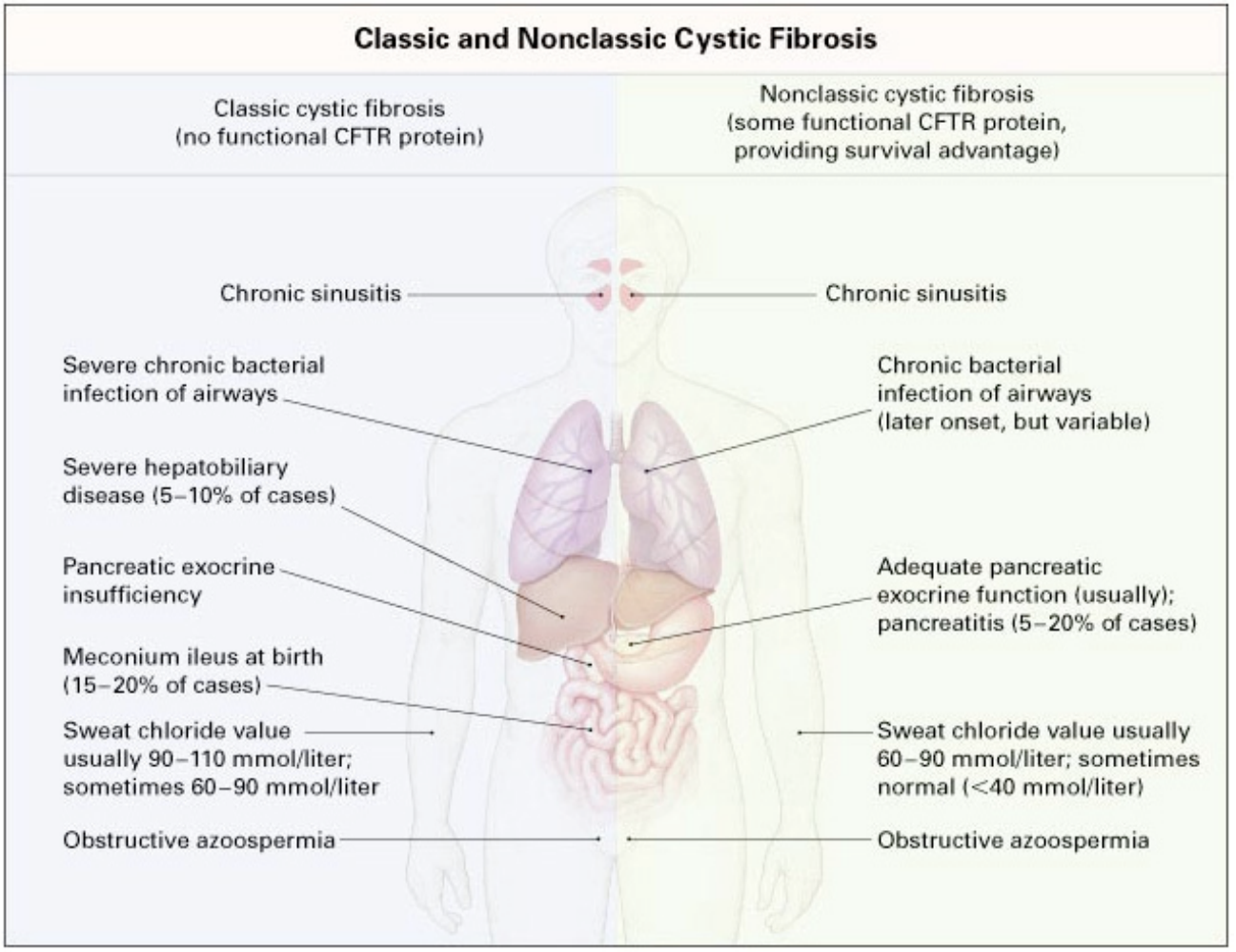
# In vitro splicing can be easily adapted to screen for donor site mutations:



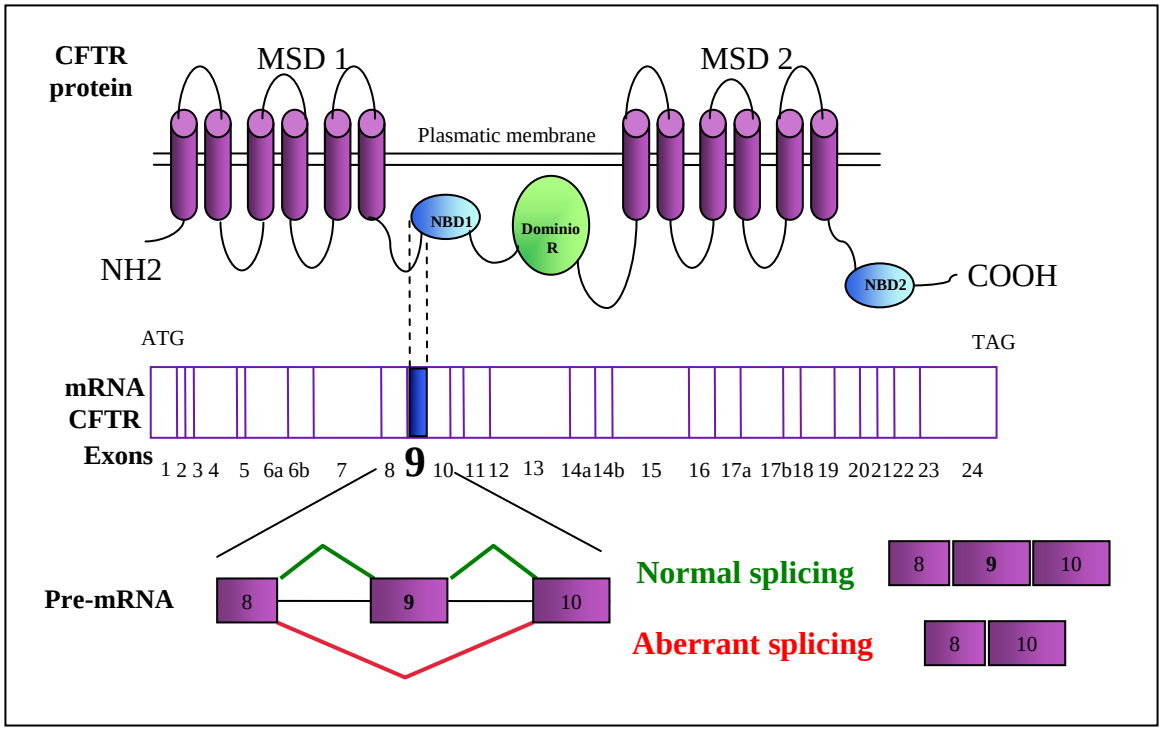
**It can also be used to screen for mutations in enhancer elements using particular substrates (ie the dsx-HX system) where the enhancer properties are required for proper processign of the intron:**



# Classical and non classical CF



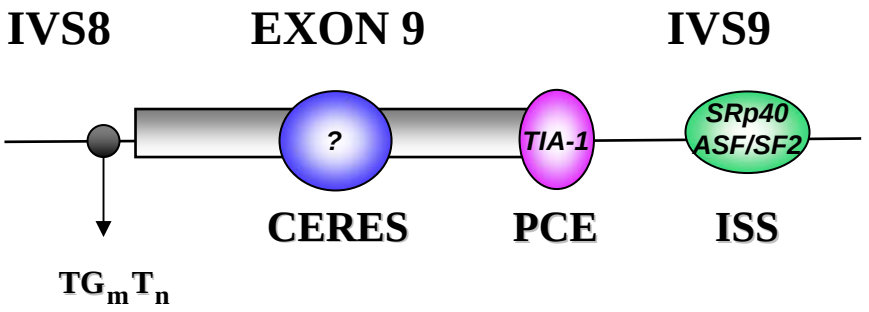
# CFTR EXON 9 ABERRANT SPLICING



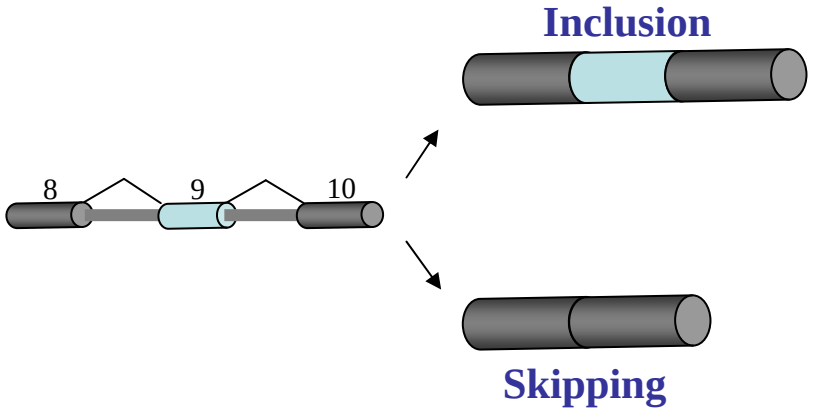

- **Skipping of exon 9 is associated to monosymptomatic forms of CF (Congenital Bilateral Aplasia Vas Deferens, bronchiectasia, pancreatitis) and produce a non functional protein**
- **Presence of polymorphic variants in humans at the polypirimidine tract at the 3' end of intron 8 (TG repeats and T9, T7 T5)**
- **Not evolutionary conserved**
- **Amplification of exon 9 related sequences in the human genome (Rozmahel et al 1997)**

## Exon 9 with the exonic and regulatory sequences:

1. A polymorphic tract (TG)m(T)n in IVS8
2. CERES elements in exon 9
3. A PCE element at the donor site of exon 9
4. An Intronic ISS element in IVS9

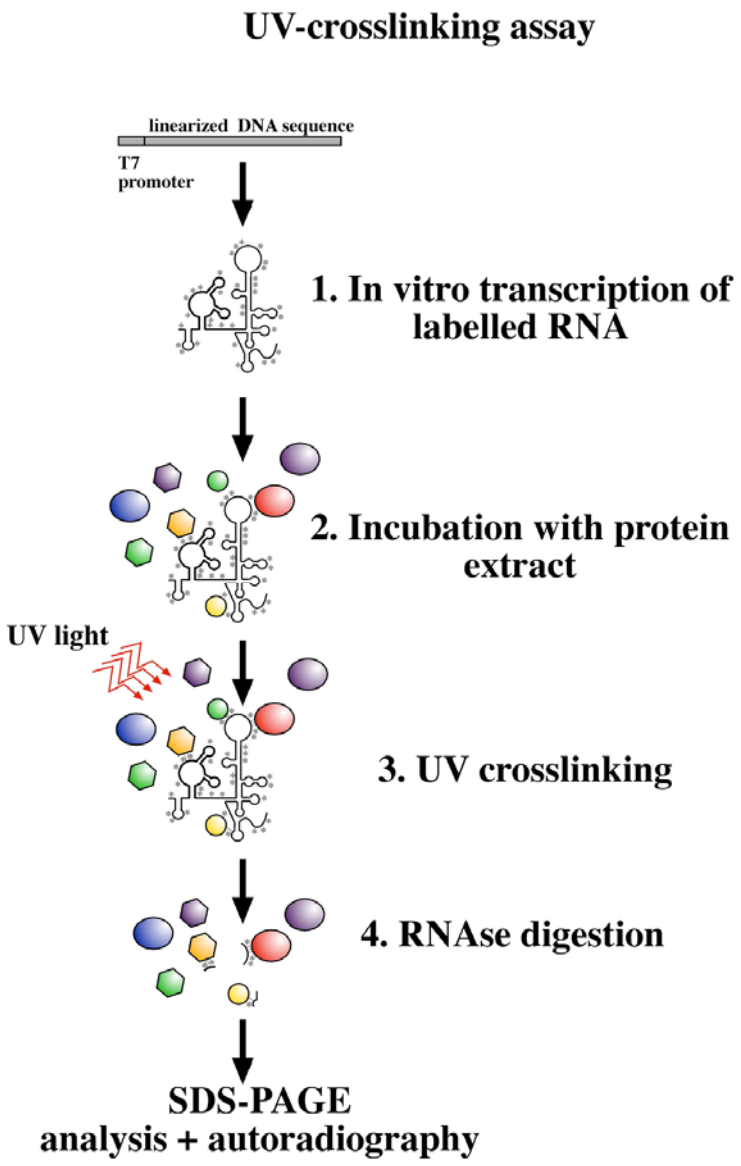


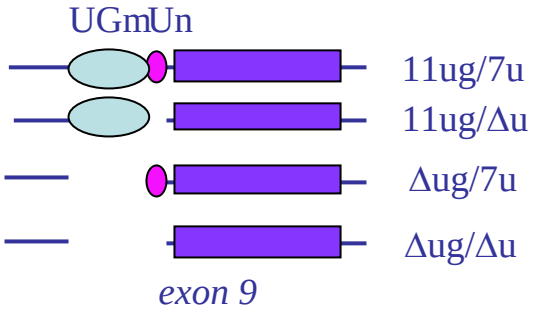

## CFTR exon 9 gene variants and phenotypes associated with different TGmTn polymorphism

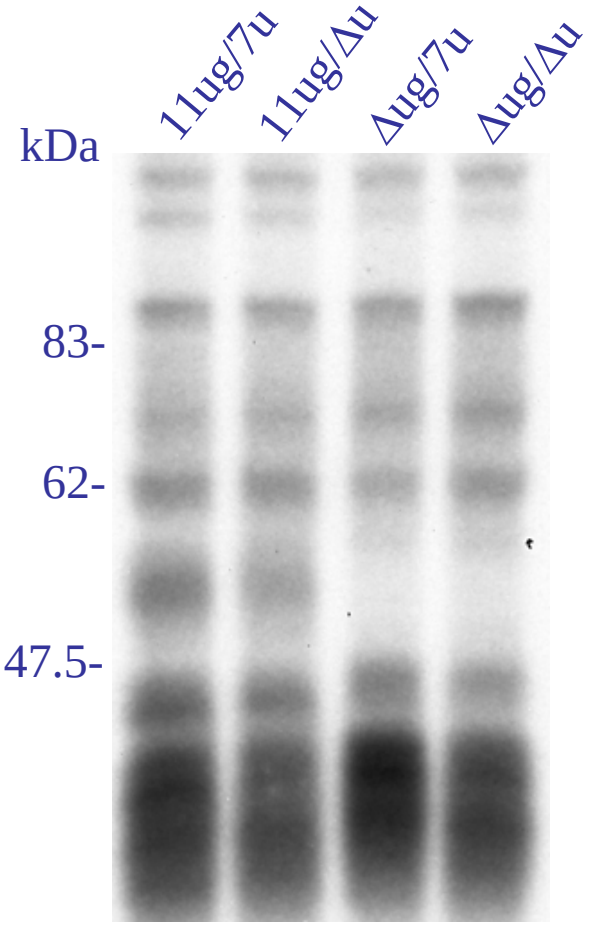



| Clinical phenotype | CFTR genotype (TG)m | (T)n |
|---|---|---|
| Normal | TG9 | T9 |
| | TG10 | T7 |
| | TG11 | T5 |
| Non-classical CF | TG12 | T5 |
| Cystic Fibrosis | TG13 | T3 |

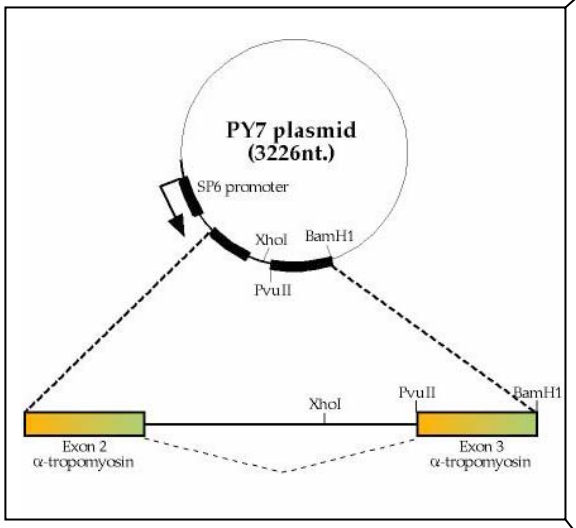
# Identification of RNA binding proteins by UV-crosslinking



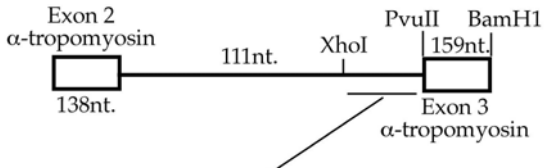



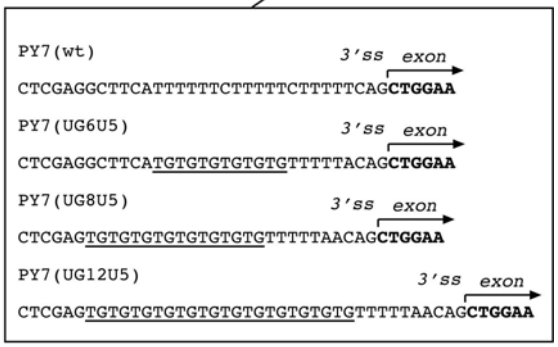



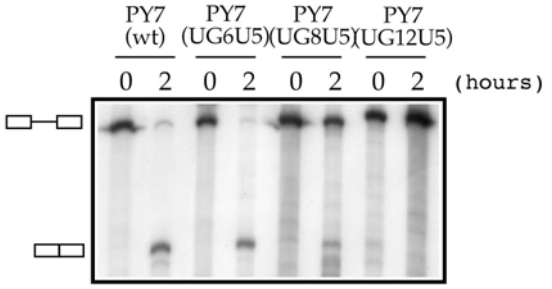
- **In vitro splicing systems can be used to test the importance of ug-repeats on 3'ss recognition**

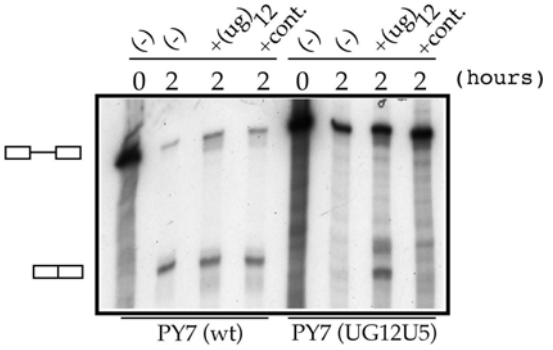



Buratti E. et al. AJHG, 2004, 74 :1322-1325

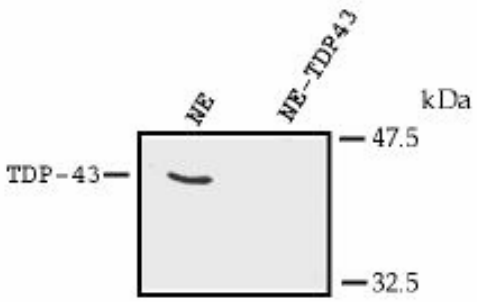


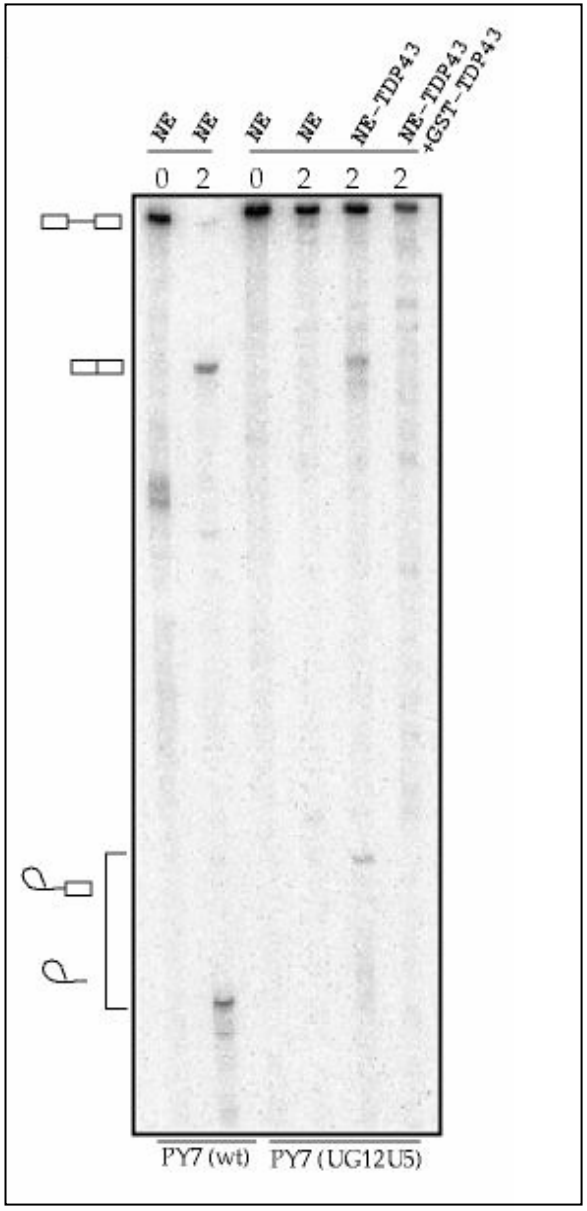



- **Nuclear extract depleted of TDP43 (with the use of antibodies against this protein) can be used to test the importance of this protein in the splicing process and also in add-back experiments to confirm its inhibitory potential.**




Buratti E. et al. AJHG, 2004, 74 :1322-1325

## In vitro splicing systems

**Advantages:**

**1) splicing intermediates can be observed and kinetics followed easily.**

**2) splicing factors can be removed and replaced (also with mutants) to assess exactly their activity.**

**3) they are especially useful in dissecting the work of regulatory elements outside their original context.**

**4) No cell culture facility is required**

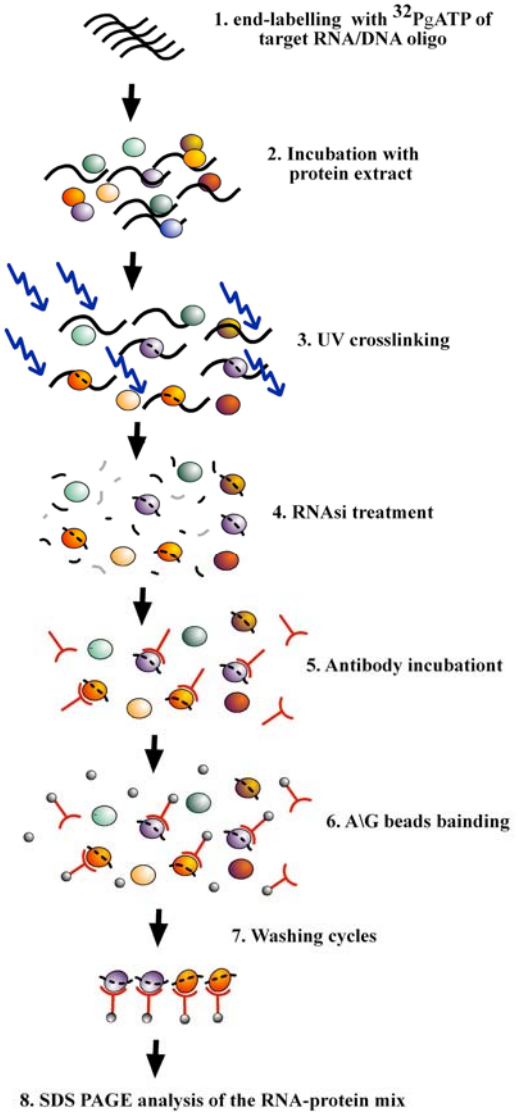
**Drawbacks:**

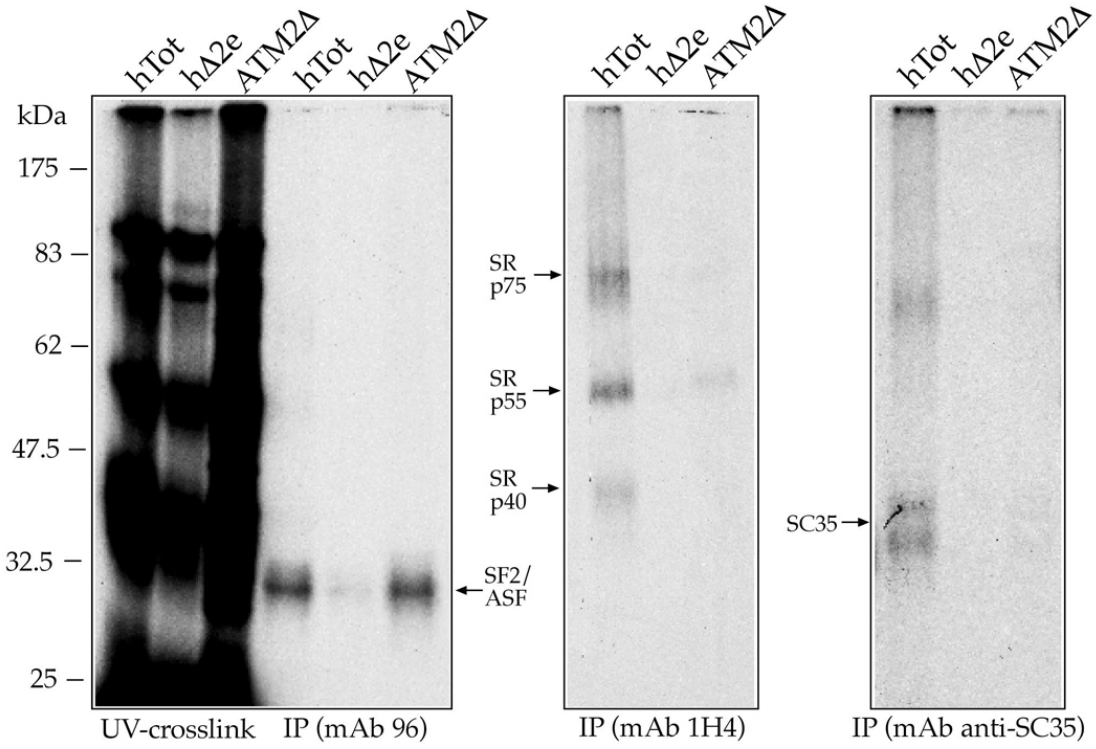
**1) the RNA is transcribed “in vitro”before the splicing reaction (although new protocols have tried to address this issue).**

**2) there is a limit to the length of the pre-mRNAs that can be used (approx. 2000 nt.).**

# RNA-protein interactions

**Important caveat: ESEfinder results are “in silico” predictions and its output has always to be checked by experimental analysis, for example, by immunoprecipitation**



# U1snRNP detection methods

(ie. the ATM gene)

- U1snRNP involvement can be confirmed using a variety of techniques:

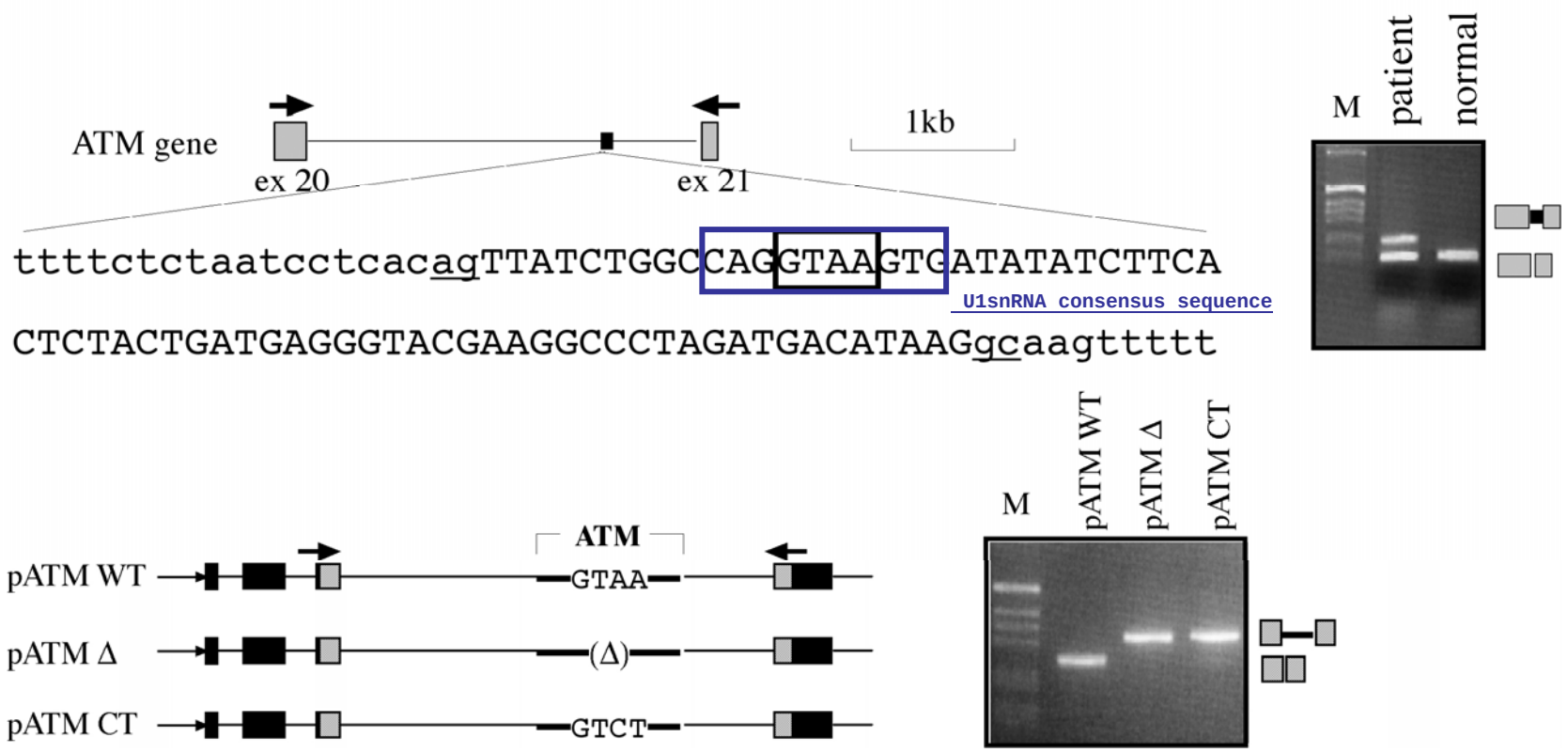
  1) Supershift analysis using a specific antibody

  2) RNAse H degradation of the 5′ end of U1snRNA

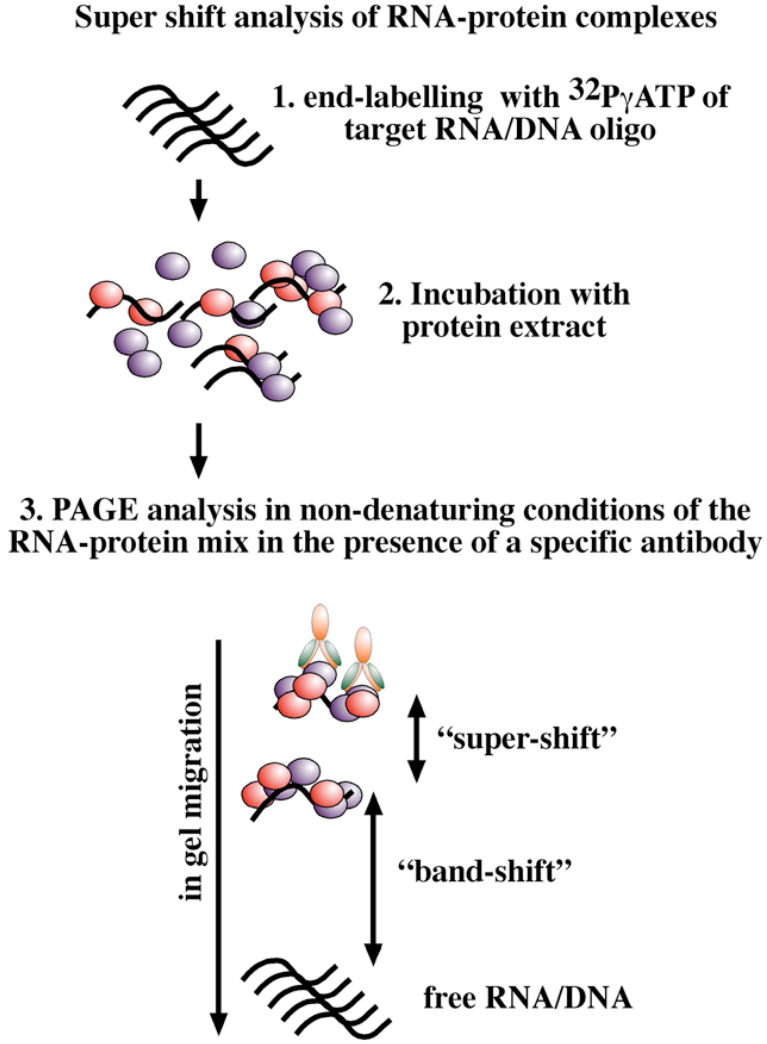
  3) Direct UV-crosslinking between U1snRNA and target RNA

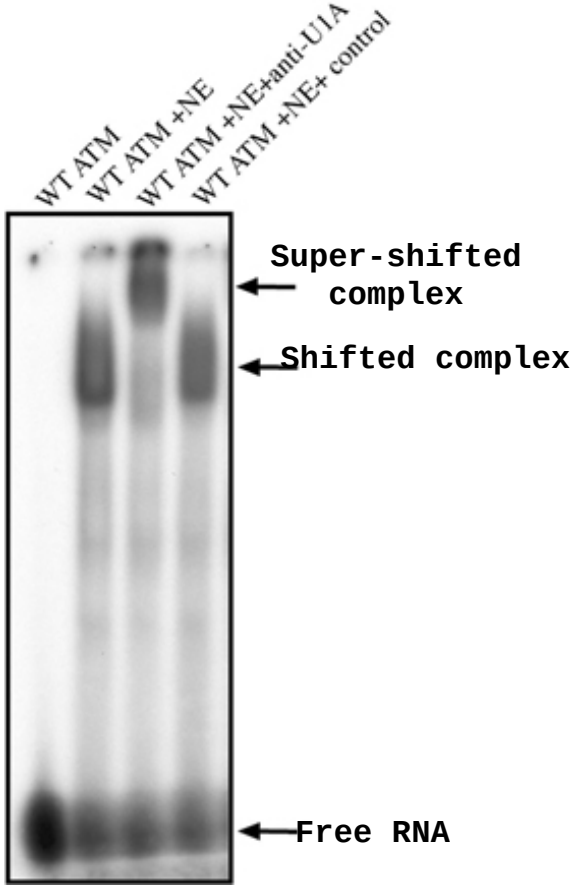
  4) Pulldown analysis using RNA-derivatized beads

In a patient affected by ataxia-telengectasia the deletion of four GTAA bases deep in ATM intron 21 activates a 65 bp. cryptic exon using distant 3’ and 5’ splice sites. RT-PCR amplification from total RNA derived from normal and patient cell lines shows the 65bp aberrant splicing product. This effect can be reproduced in hybrid minigene experiments.

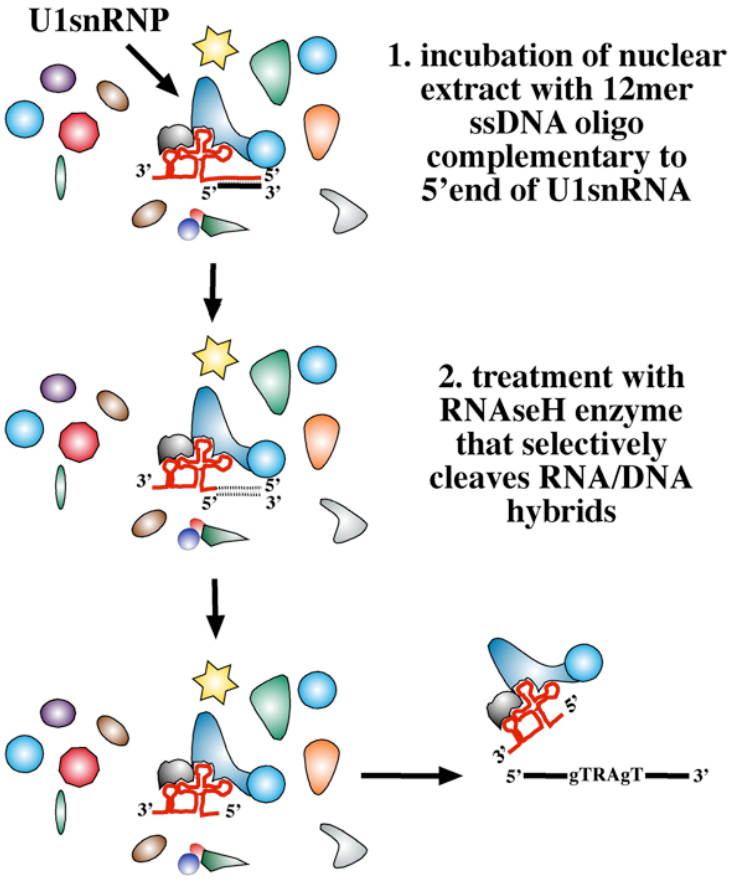


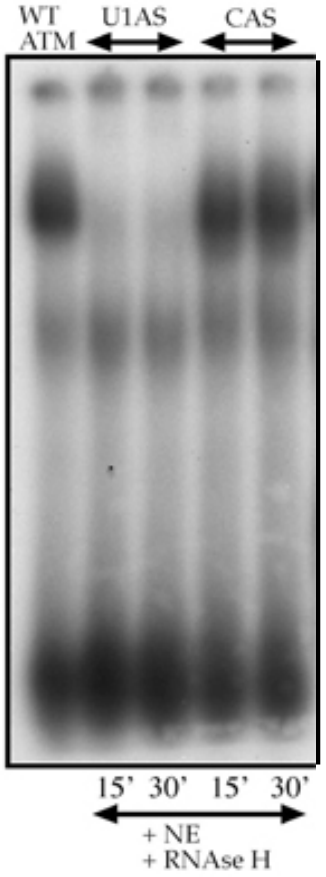
## 1) super-shift analysis using small RNA oligos and an anti U1-A monoclonal antibody



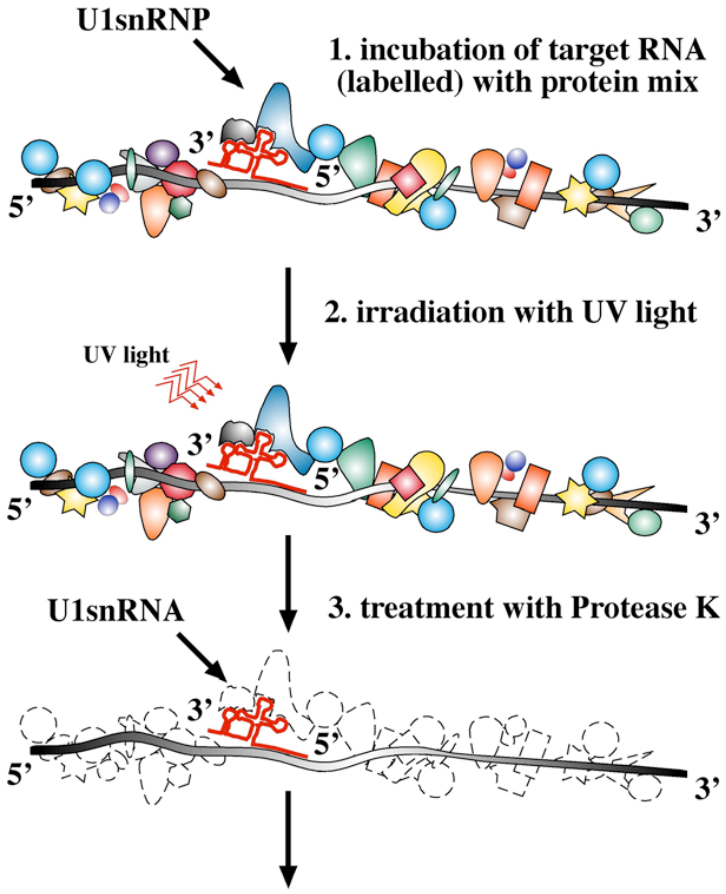



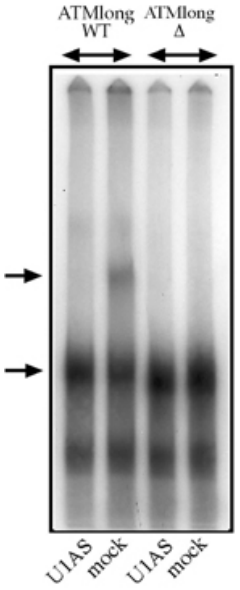
## 2) Inactivation of U1snRNA 5’end by digestion using RNAse H and a complementary oligo



# 3) U1snRNA-target RNA UV-crosslinking following RNAse H treatment.




taactgcaacagtggtaataataatcatttattgaatt

ccacaatagaagctagacttttacgtttatttctcta

3'ss
atcctcacagTTATCTGGCCAGGTAAGTGATATATCTT

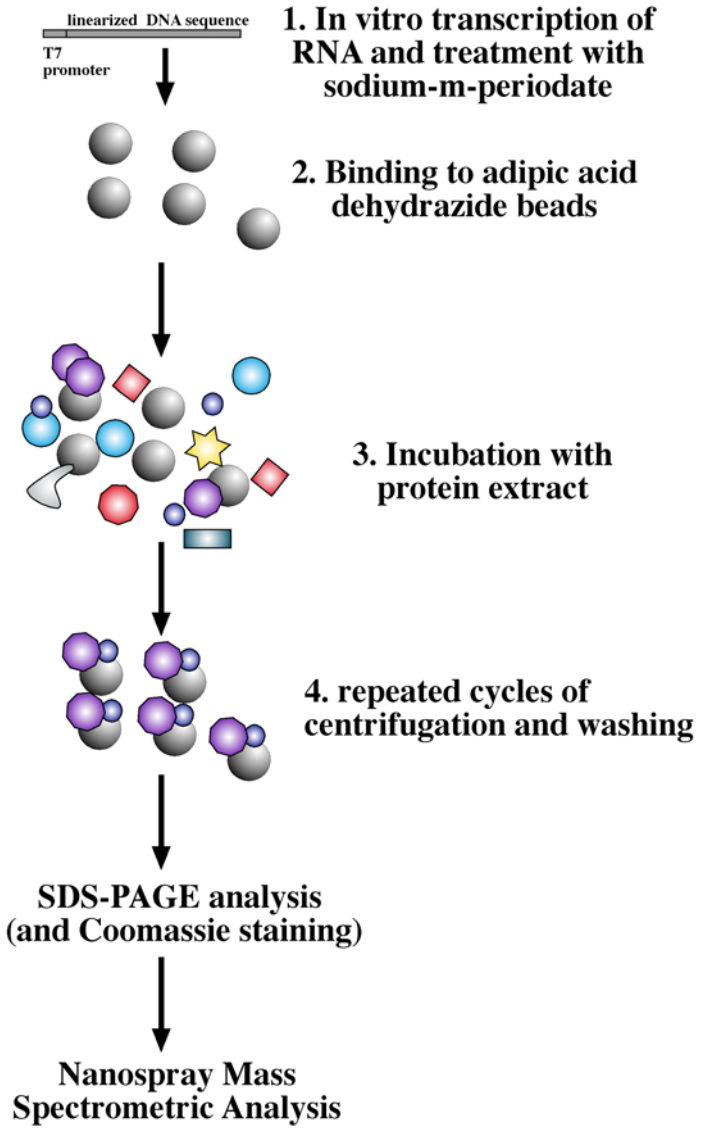
CACTCTACTGATGAGGGTACGAAGGCCCTAGATGACAT

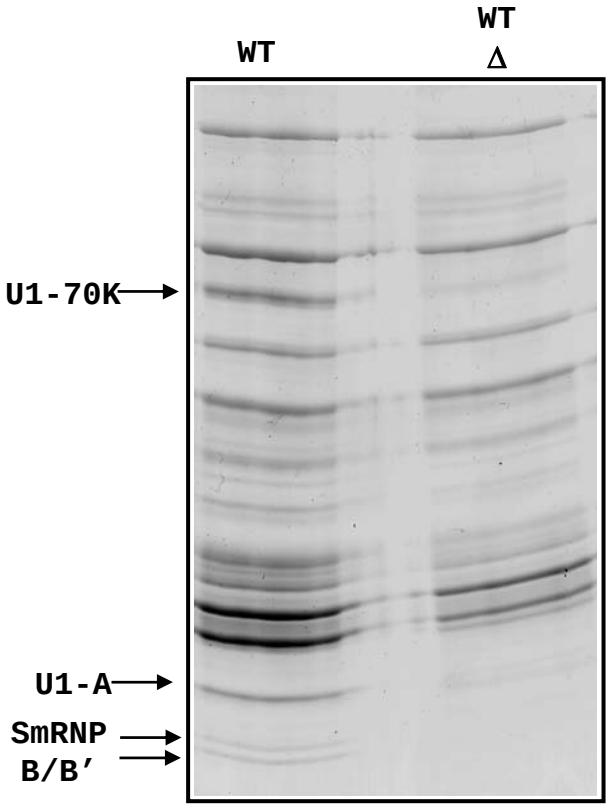
5'ss
AAGgcaagtttttttaccagaaaagttaaggctgtggta

ctgggatttgaattttctgtttgacttcaaagcctttt

gtttttactatacacct

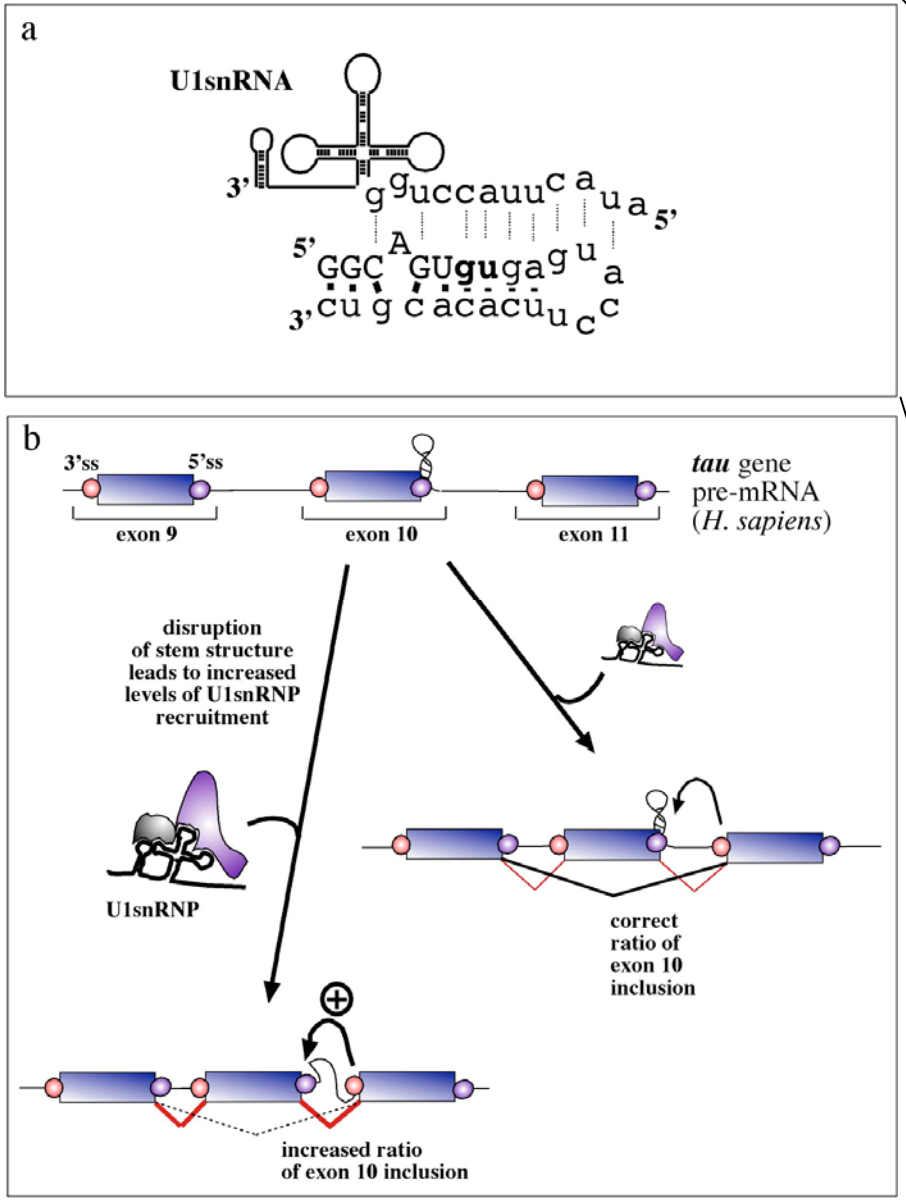
# Pull-down analysis of U1 snRNA using adipic acid dehydrazide beads

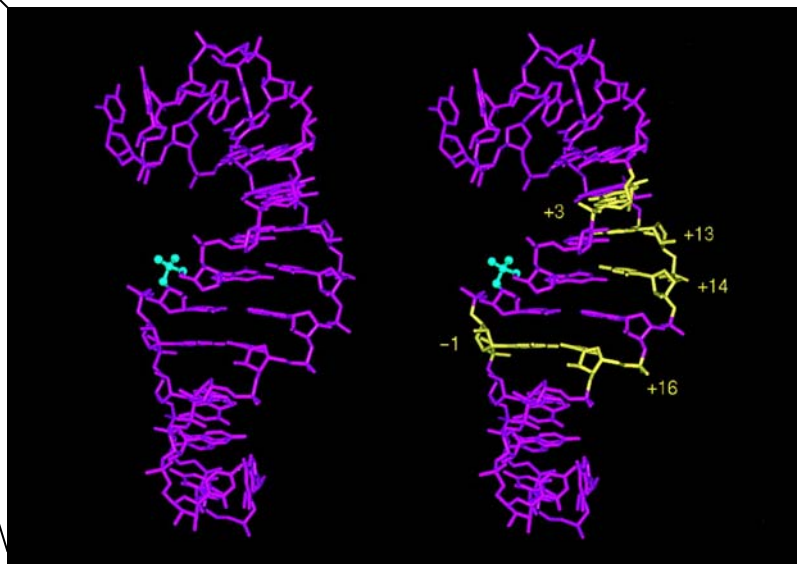


# RNA secondary structure

**In the tau gene RNA secondary structure has been proposed to be responsible for the occurrence of fronto-temporal dementia and Parkinsonism.**

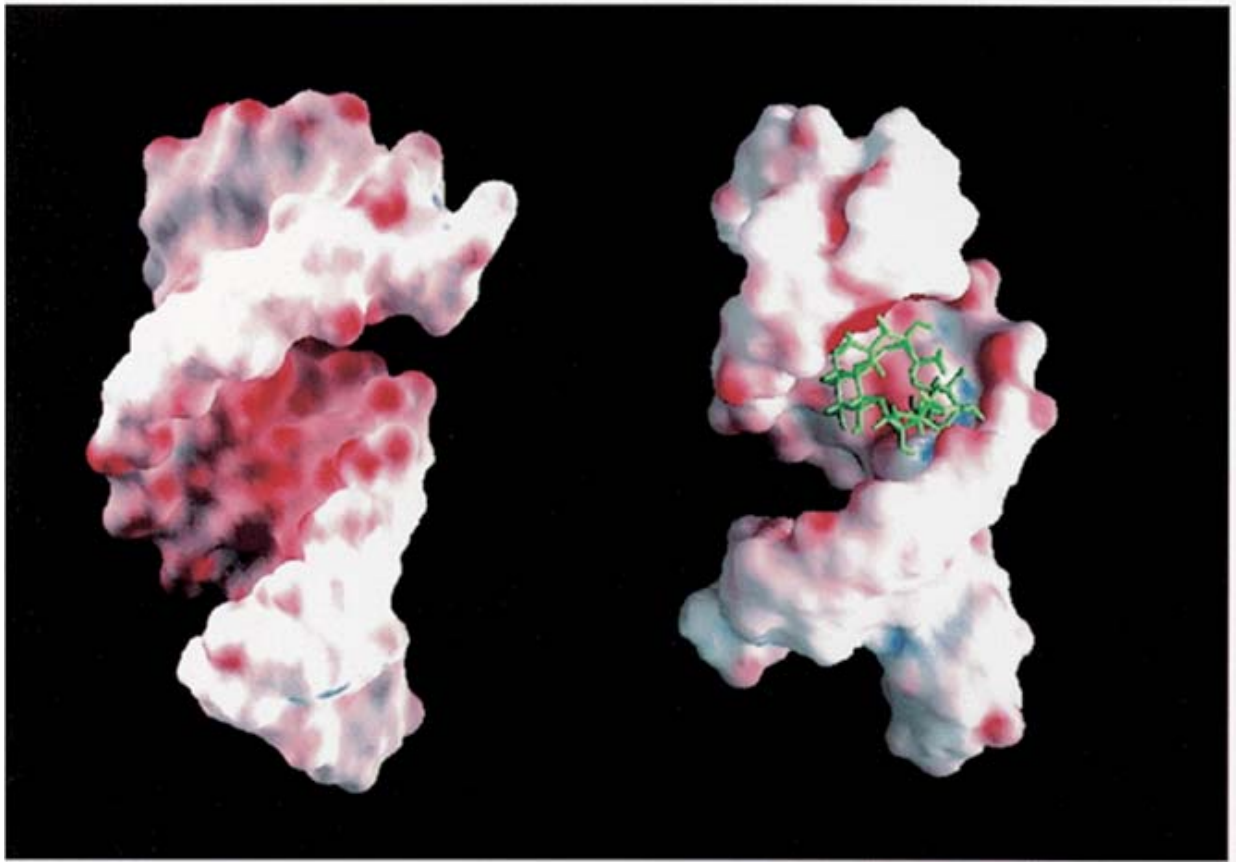



Varani, L et al. (1999) Proc. Natl. Acad. Sci. USA 96, 8229-8234

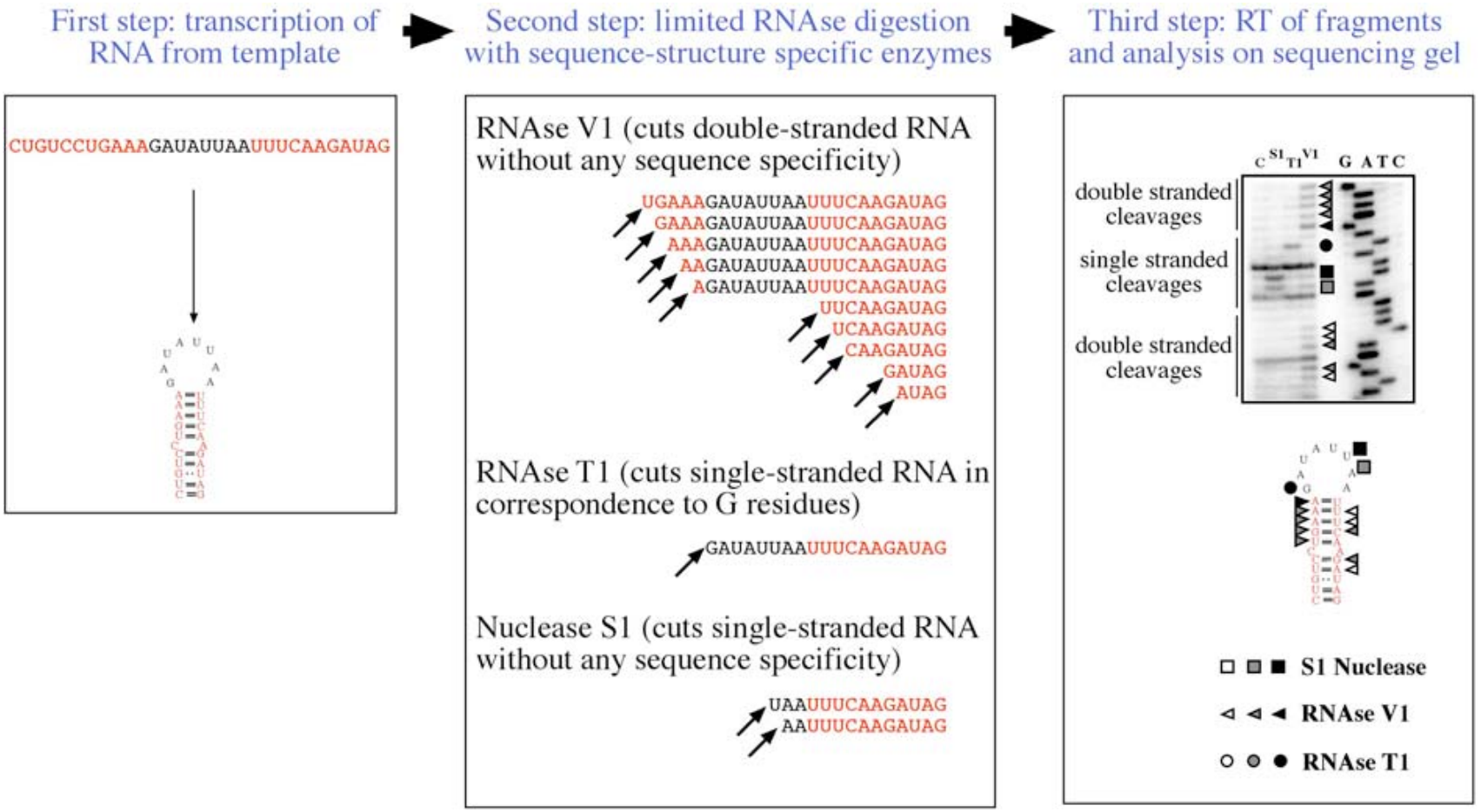
Buratti and Baralle MCB 2004; 24: 10505-10514

**Surface representation with electrostatic potential map of the human tau exon 10 splicing regulatory element (left) and of its complex with neomycin B (right). Binding causes a marked increase in the stability of the regulatory wild type and mutant elements. This kind or results represent a starting point for the development of small compounds aimed at developing new splicing-based therapies.**



Varani, L. et al. NAR 2000 28:710-719

## A practical approach to obtain structural information on RNA folding:




## The observed cleavages can then be used to discriminate between competing predicted structures or to detect structural changes following the introduction of mutations/deletions/insertions.

to Austria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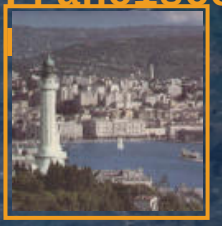

Tries te

to Venice

Piran (Slo)

to Pula (Croatia)