- **NGS, Repeat primed-PCR**

- **Nonsense mediated mRNA decay**

- NMD narušují funkci svalu a to buď přímo v důsledku patologie svalu nebo nepřímo v důsledku patologie nervů a neuromuskulárních spojení.

- **780 typů NMD**
- **16 skupin NMD**
- **417 genů**

http://www.musclegenetable.fr

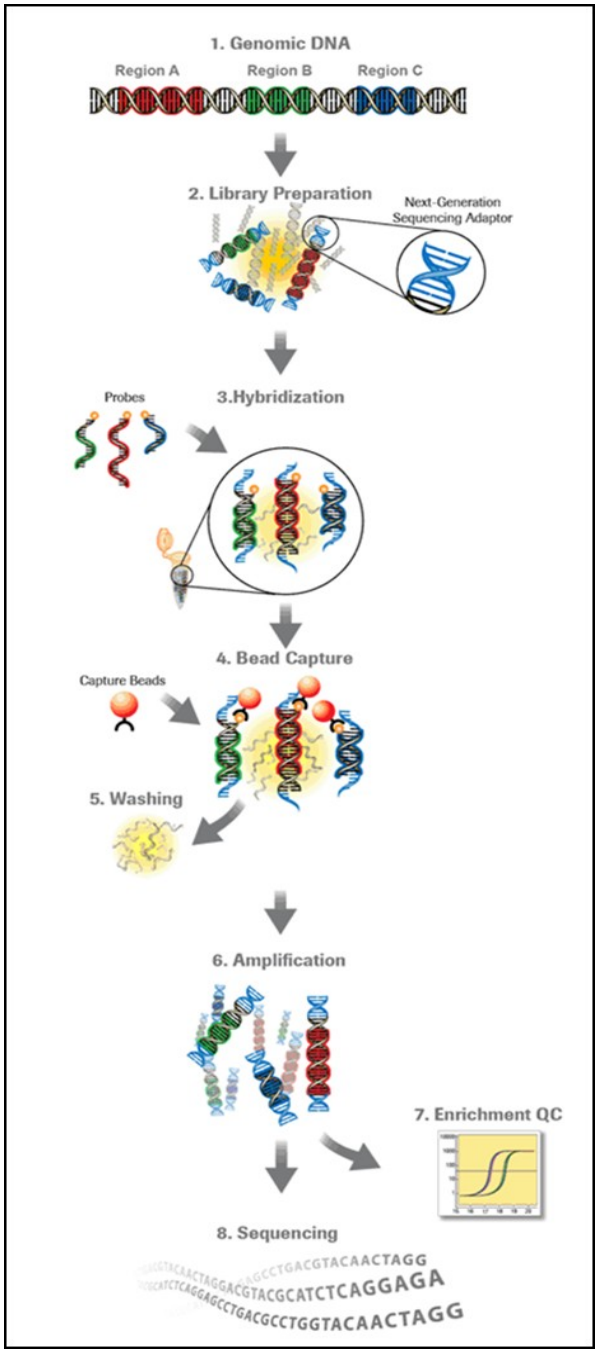
1. Muscular dystrophies	5. Other myopathies	9. Metabolic myopathies	13. Hereditary ataxias
2. Congenital muscular dystrophies	6. Myotonic syndromes	10. Hereditary cardiomyopathies	14. Hereditary motor and sensory neuropathies
3. Congenital myopathies	7. Ion channel muscle diseases	11. Congenital myasthenic syndromes	15. Hereditary paraplegias
4. Distal myopathies	8. Malignant hyperthermia	12. Motor neuron diseases	16. Other neuromuscular disorders

NMD – klinická a genetická heterogenita → většinou obtížné vytypovat určitý gen pro analýzu → vyžití technik sekvenování nové generace

Sekvenace genů spojených s následujícími skupinami NMD

- ❑ Svalové dystrofie
- ❑ Kongenitální svalové dystrofie
- ❑ Kongenitální myopatie
- ❑ Distální myopatie
- ❑ Další myopatie
- ❑ Myotonické syndromy
- ❑ Svalové nemoci spojené s iontovými kanály
- ❑ Maligní hypertermie
- ❑ Hereditární kardiomyopatie
- ❑ Kongenitální myastenické syndromy
- ❑ Nemoci motorického neuronu
- ❑ Další neuromuskulární nemoci

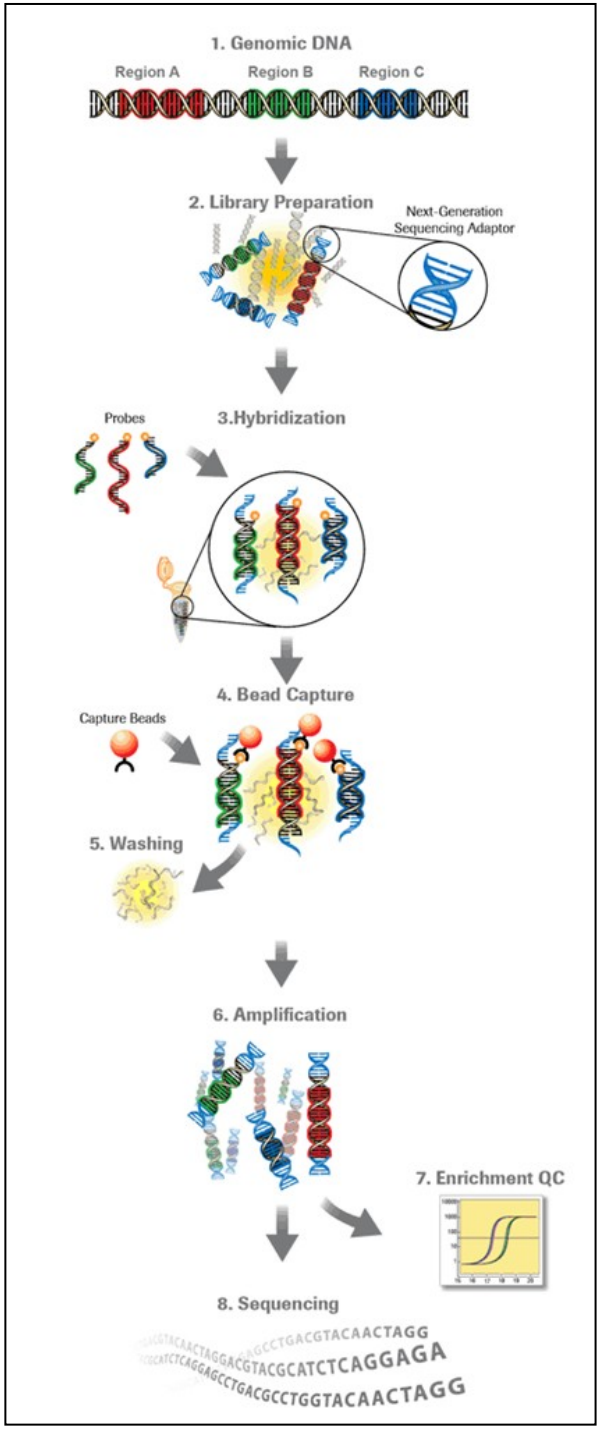
Celkem 250 genů



„Sequence Capture“ (SeqCap EZ Choice Library, NimbleGen) a cílená sekvenace (MiSeq, NextSeq, Illumina)

Postup:

- Zadání cílových úseků, design sond
- Příprava knihovny – fragmentace DNA pacienta, …..
- Hybridizace DNA pacienta se sondami s navázaným biotinem
- Vychytání komplexu sonda-fragment DNA pacienta pomocí magnetických kuliček s navázaným streptavidinem
- Amplifikace „vychytané“ DNA pacienta
- Sekvenace

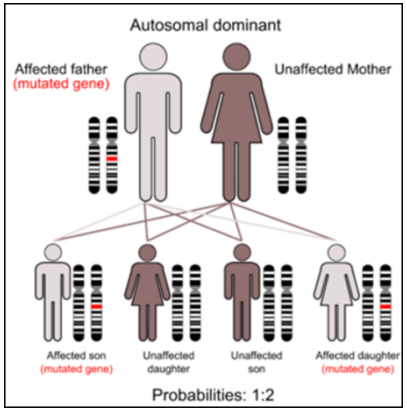


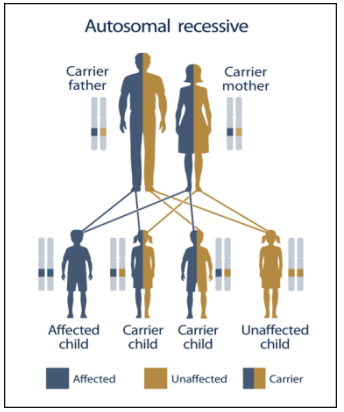
- Nelze detekovat rozsáhlejší delece/duplikace a velké přestavby v genech
- Není vhodnou metodou pro nemoci spojené s expanzí/delecí repetitivních sekvencí
- Sekvenujeme cca 95% vybraných oblastí

- Identifikace nových sekvenčních variant s neznámou kauzalitou

RYANODINE RECEPTOR 1; RYR1

Location	Phenotype	Phenotype MIM number	Inheritance
19q13.2	Central core disease	117000	AD, AR
	King-Denborough syndrome	145600	AD
	Minicore myopathy with external ophthalmoplegia	255320	AR
	Neuromuscular disease, congenital, with uniform type 1 fiber	117000	AD, AR
	{Malignant hyperthermia susceptibility 1}	145600	AD





Syn (NMD)

SeqCap-TR

***LAMA2* (AR, kongenitální svalová dystrofie):**

p.(Asp267Asn)/p.(Glu1231*)

Otec (NMD):

***LAMA2*: p.(Asp267Asn)**

SeqCap-TR

p.(Asp267Asn)/c.9095dupA

Matka (zdravá):

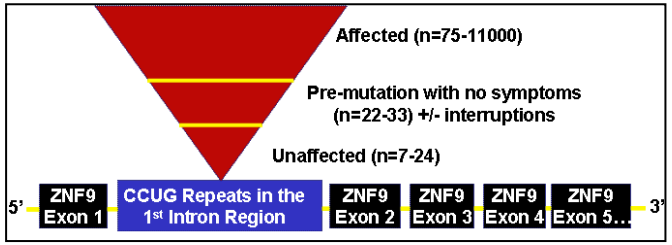
***LAMA2*:**

p.(Glu1231*)

Autosomálně recesivní NMD u syna i otce

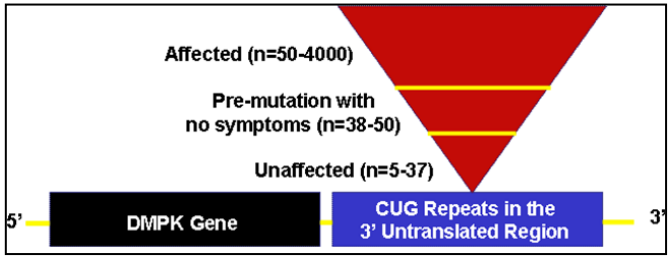
MD1

- Expanze CTG repetice v 3'UTR genu *DMPK* (19q13, *dystrophia myotonica protein kinase*)
- AD dědičnost



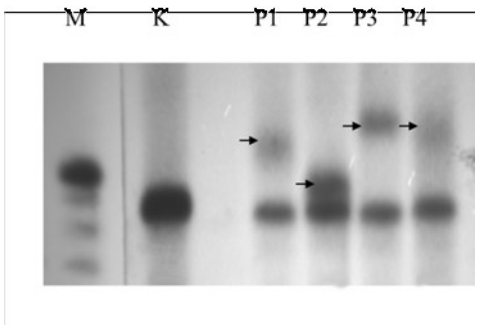
MD2

- Expanze CCTG repetice v 1. intronu genu *ZNF9* (3q21, *zinc finger 9 protein*)
- AD dědičnost

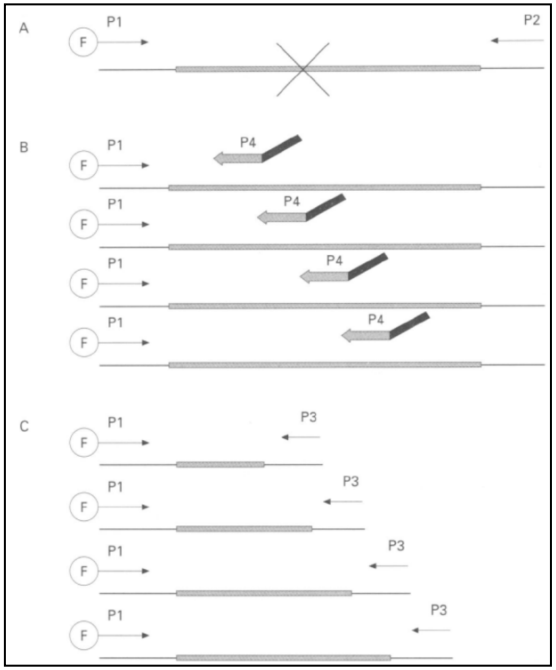


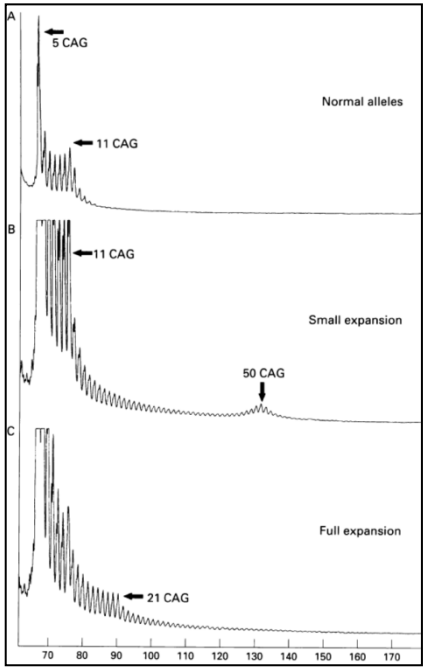
DNA diagnostika, metody:

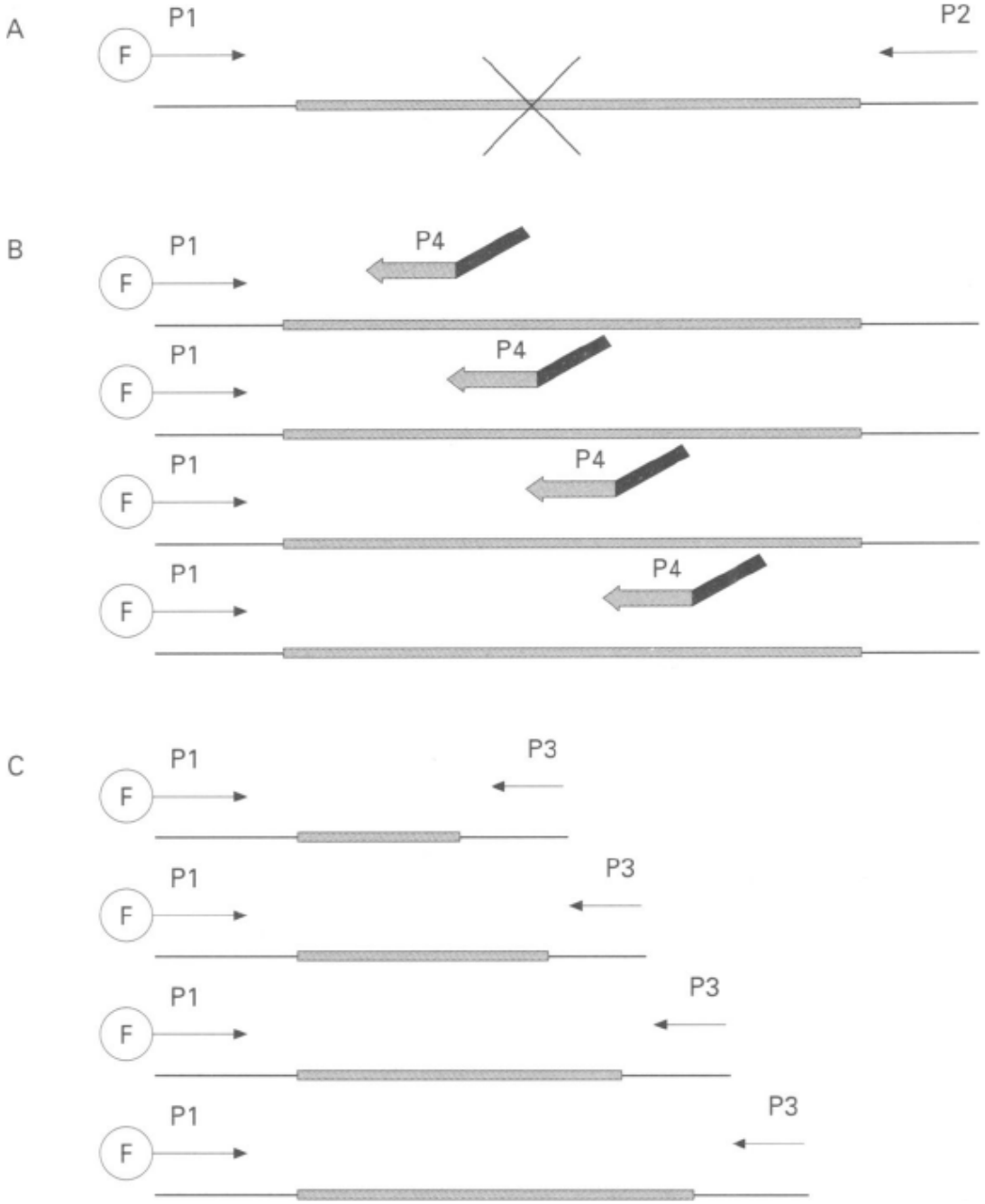
1) **Repeat-primed PCR**
2) **Souther blot a hybridizace**



Warner JP, J Med Genet 1996



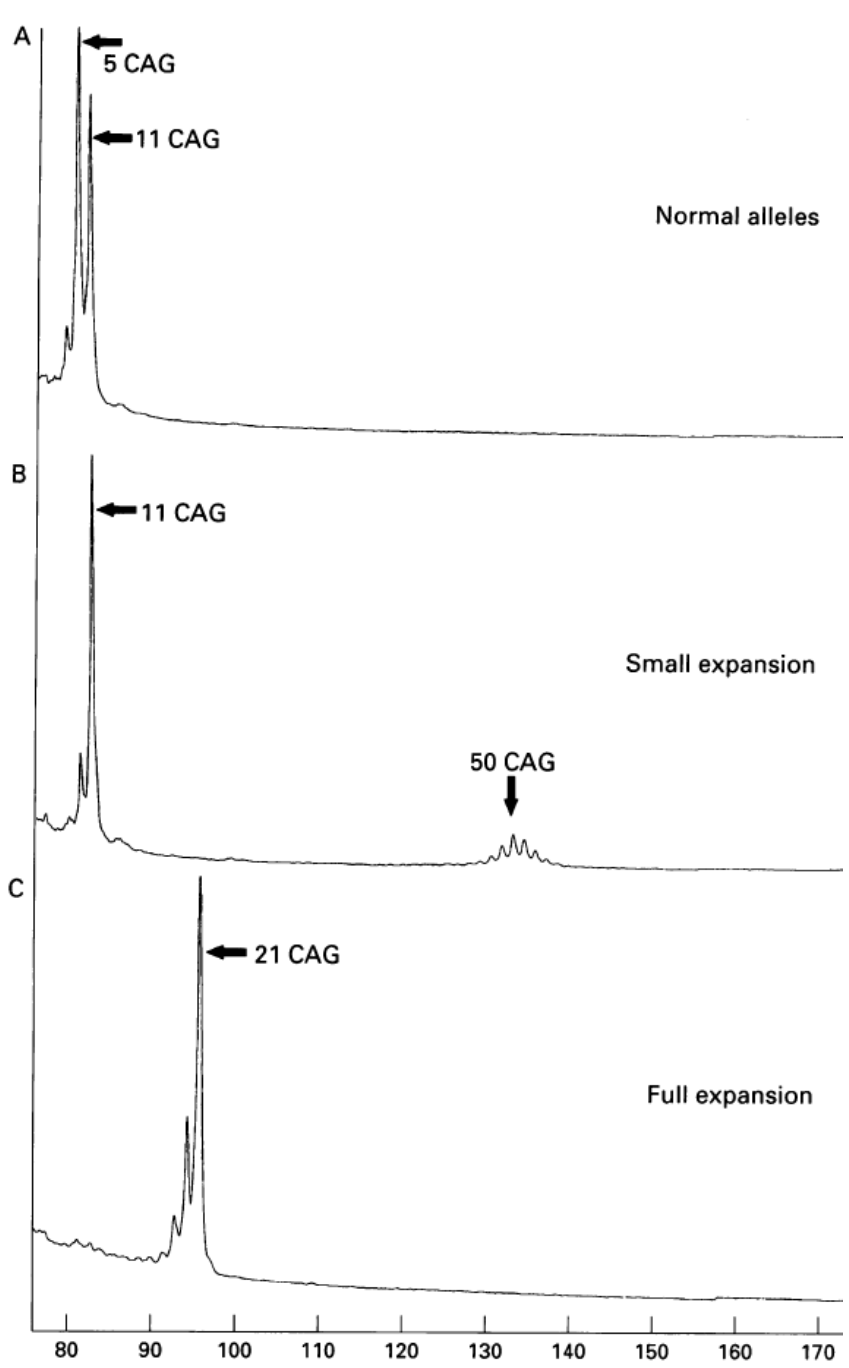




Repeat primed PCR (RP-PCR)

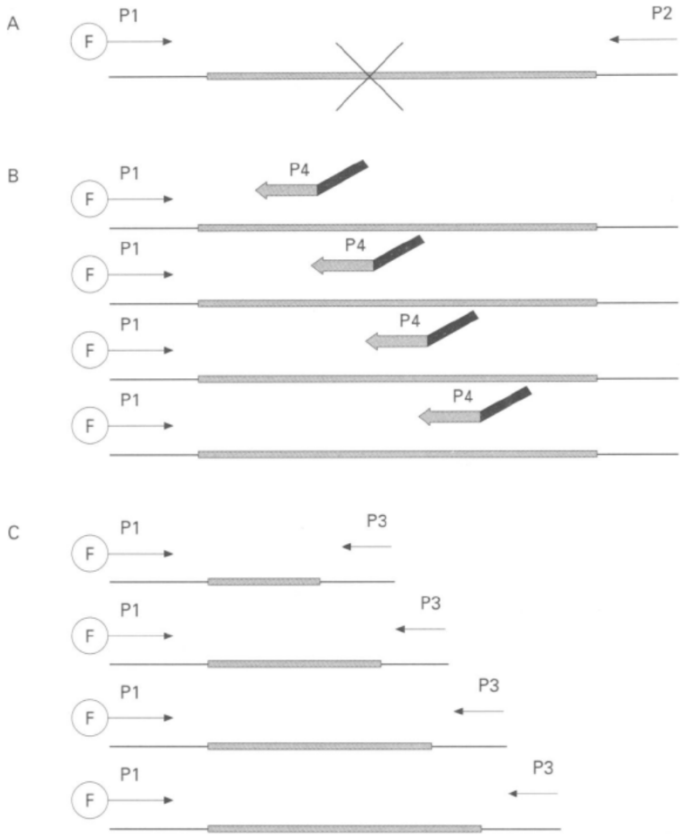
Stippled box represents (CAG)n repeat. F shows 5' fluoresceinated primer.
(A) For large alleles exceeding 100 CAG the PCR using flanking primers P1 and P2 fails to give a product.
(B) RP-PCR: primers P1, P4, P3: in early amplification cycles primer P4 (the repeat specific 3' terminus) binds at multiple sites within CAG alleles giving rise to a mixture of products. Specificity is dictated by P1.
(C) The primer P3 amplifies from the end of products from previous amplification rounds. A long extension time is used to allow complete extension of the larger sized products within the PCR product mixture.
A 10:1 molar ratio of P3 to P4 ensures that primer P4 is exhausted in the early amplification cycles.

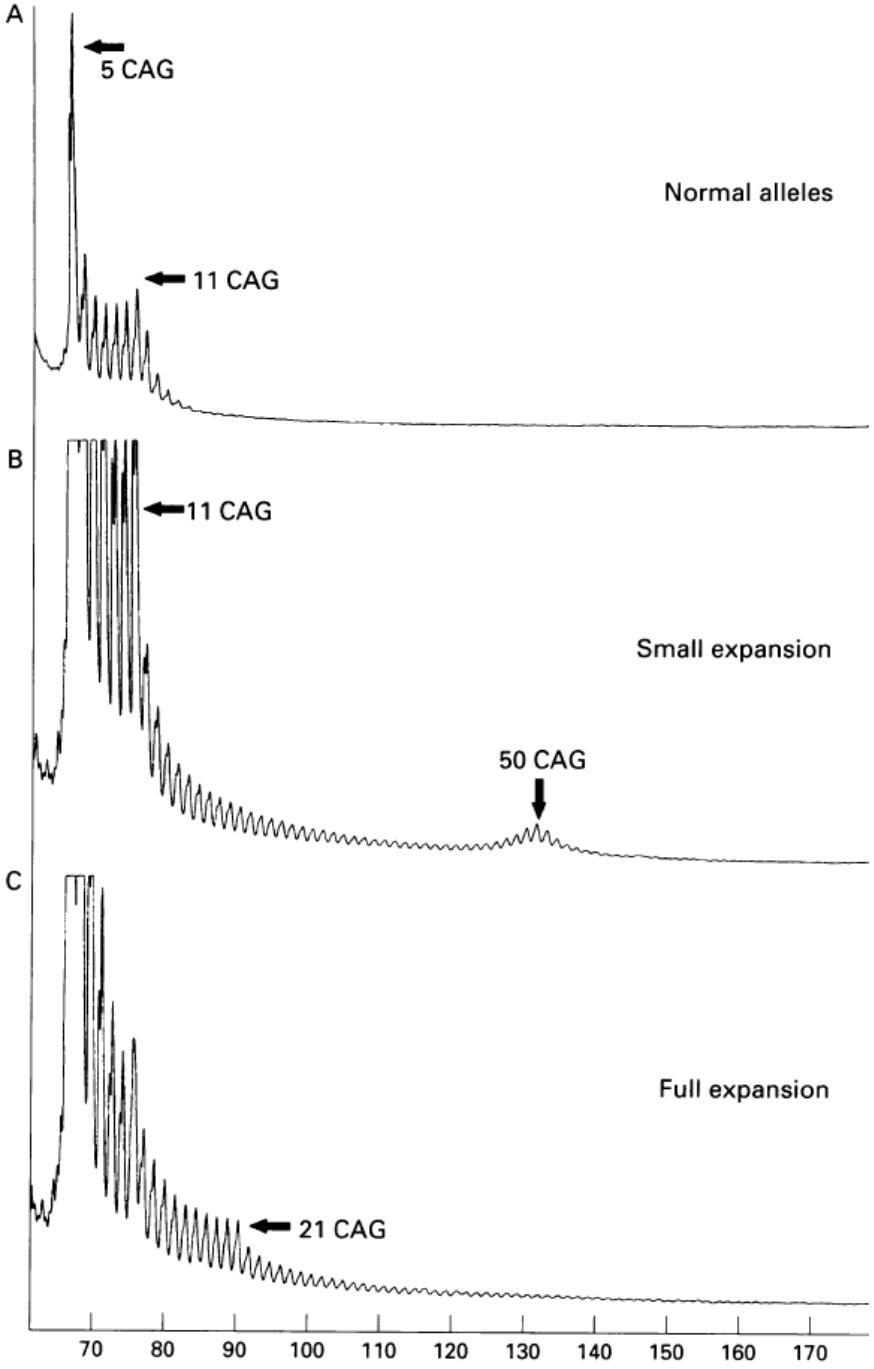
Warner JP, J Med Genet 1996



Warner JP, J Med Genet 1996

Electrophoreogram of PCR (primers P1 + P2)
The axis shows migration time in minutes. CAG allele sizes shown with the arrows.
(A) Trace obtained from a heterozygous normal subject. (B) Trace obtained from a heterozygous subject with a small expansion. (C) Trace obtained from a patient with myotonic dystrophy and an expanded allele size of >4 kb as determined by Southern blot analysis. The larger allele fails to amplify.





Warner JP, J Med Genet 1996

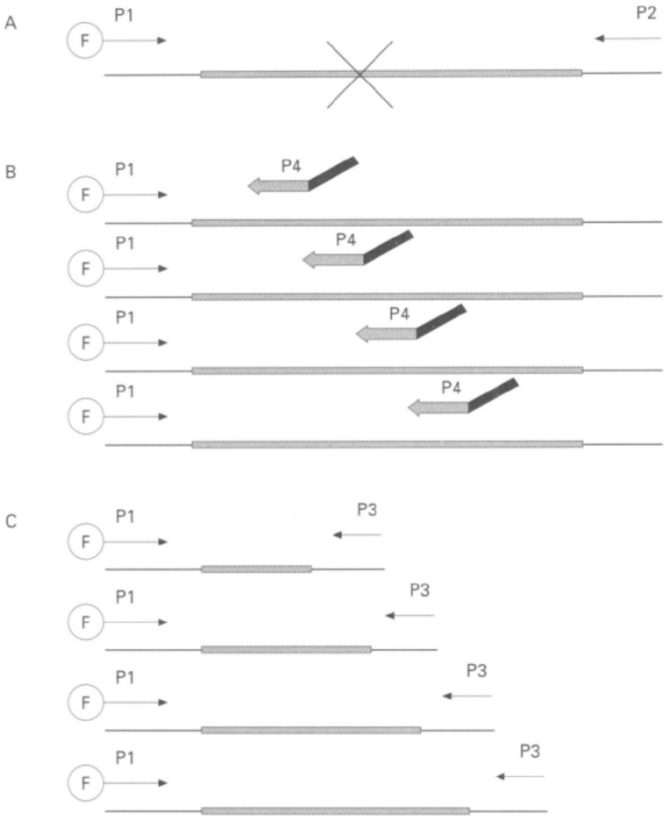
Electrophoreogram of repeat-primed PCR (primers P1+P3+P4)

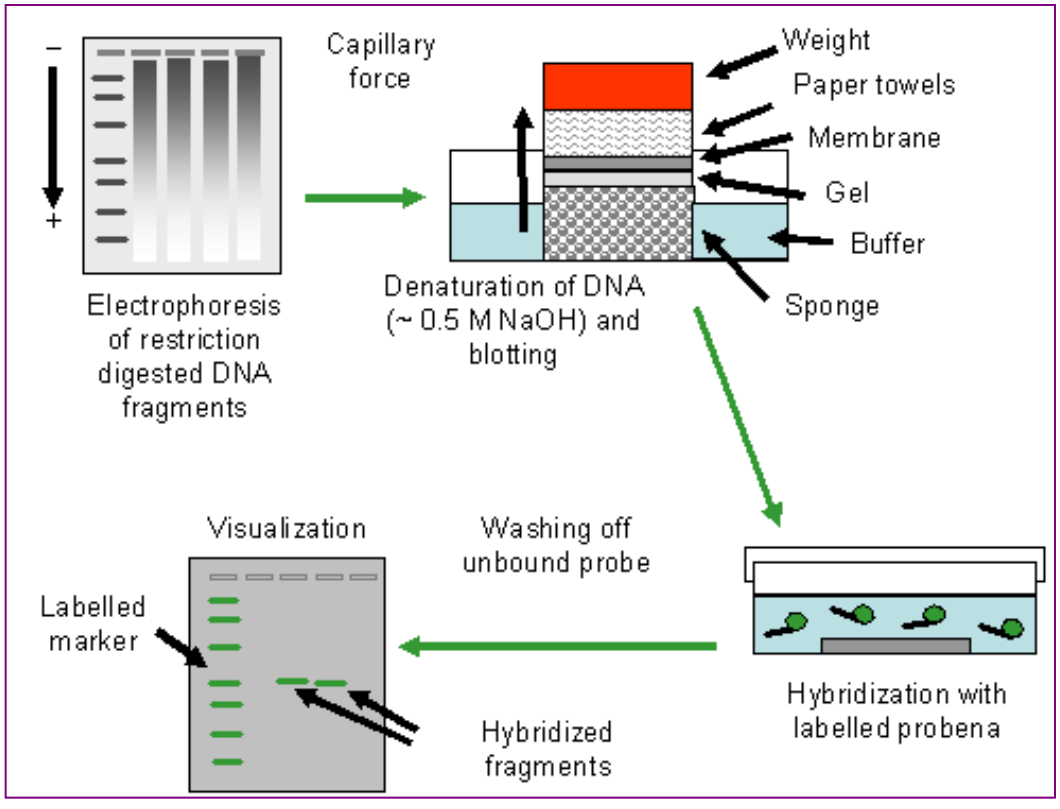
The axis shows migration time in minutes. CAG allele sizes shown with the arrows. Note the characteristic ladder with a 3 bp periodicity.

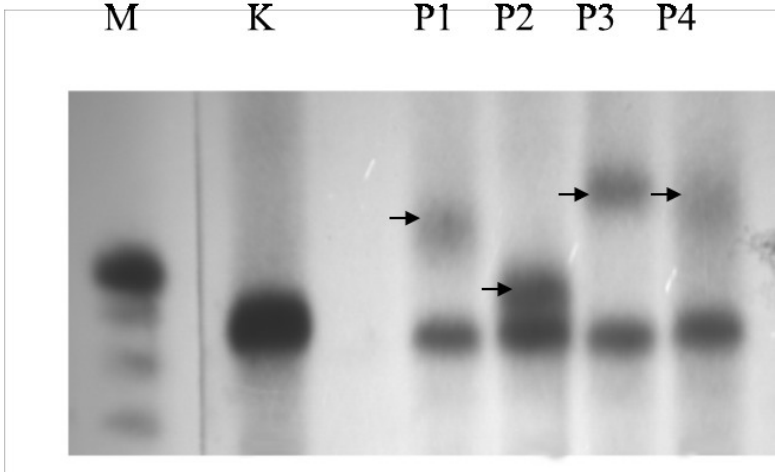
(A) Both alleles give peaks and all the intermediate priming sites give peaks.

(B) Both alleles give peaks as in (A).

(C) The ladder shows the presence of a large CAG allele undetectable using flanking primers.







- Restrikční štěpení DNA.
- Elektroforetické rozdělení naštěpené DNA v agarózovém gelu

Southern blot:

- 0,25 M HCl - depurinace DNA
- 0,5M NaOH - denaturace DNA, rozštěpení cukr-fosfátové vazby v místě depurinace (účinnější přenos DNA z gelu na membránu).
- Alkalický přenos DNA na membránu v 0.5 M NaOH (vazba negativně nabité DNA k pozitivně nabité membráně), *různé možnosti*.

Hybridizace s radioaktivně značenou sondou

Detekce mutací v genu *CAPN3* (LGMD2A)

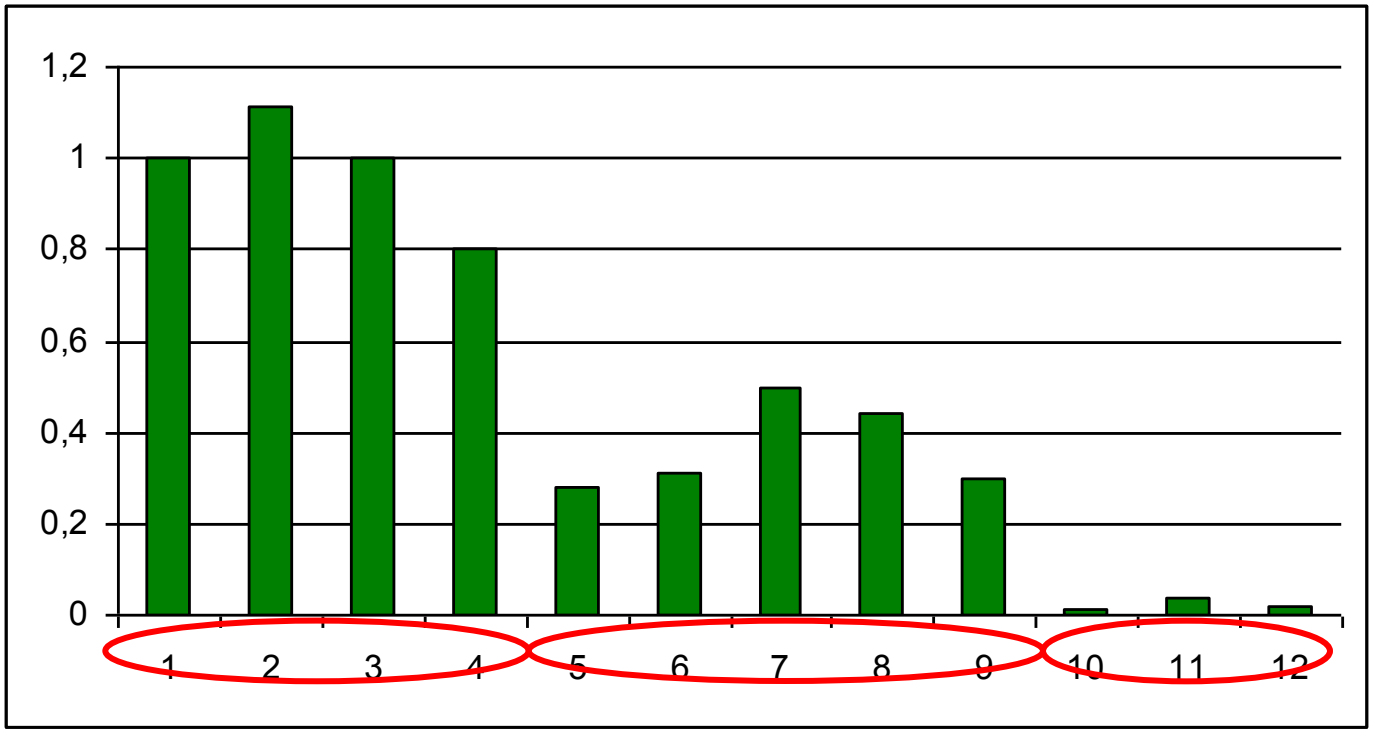
PACIENT	MUTACE DETEKOVANÁ NA ALELE 1	MUTACE DETEKOVANÁ NA ALELE 2
1	245C>T, P82L	*550delA, T184RfsX36*
2	245C>T, P82L	*2314-2317del, D772delK773NfsX3*
3	*550delA, T184RfsX36*	598-612del, F200_L204del
4	*550delA, T184RfsX36*	1468C>T, R490W

- mRNA: homozygotní výskyt *missense* mutace nebo *in-frame* delece

- DNA: heterozygotní výskyt *missense* mutace nebo *in-frame* delece + detekce mutace vytvářející předčasný terminační kodon (PTC)

→mRNA nesoucí PTC byla degradována mechanismem *nonsense mediated mRNA decay*

Stanovení relativního množství mRNA genu *CAPN3*



Pacient 1-4: ***non*-PTC/*non*-PTC**

Pacient 5-9: ***non*-PTC/PTC**

Pacient 10-12: **PTC/PTC**

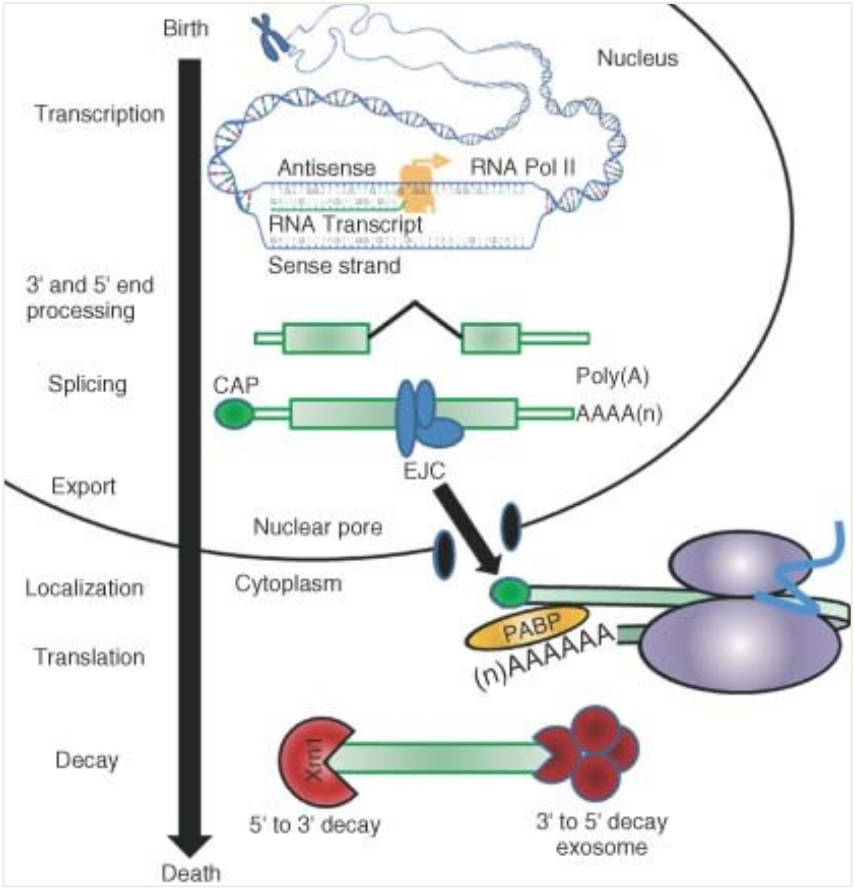
Relativní množství mRNA v závislosti na typu mutace:

***non*-PTC/*non*-PTC: 0,97**

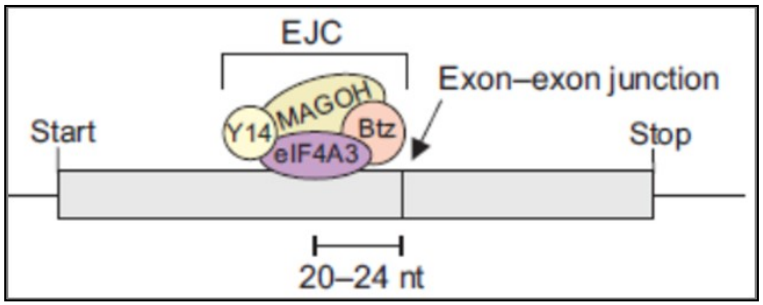
***non*-PTC/PTC: 0,37**

PTC/PTC: 0,02

Nonsense mediated mRNA decay (NMD)



Wiley Interdisciplinary Reviews – RNA, Vol. 1, Is. 1, 2010

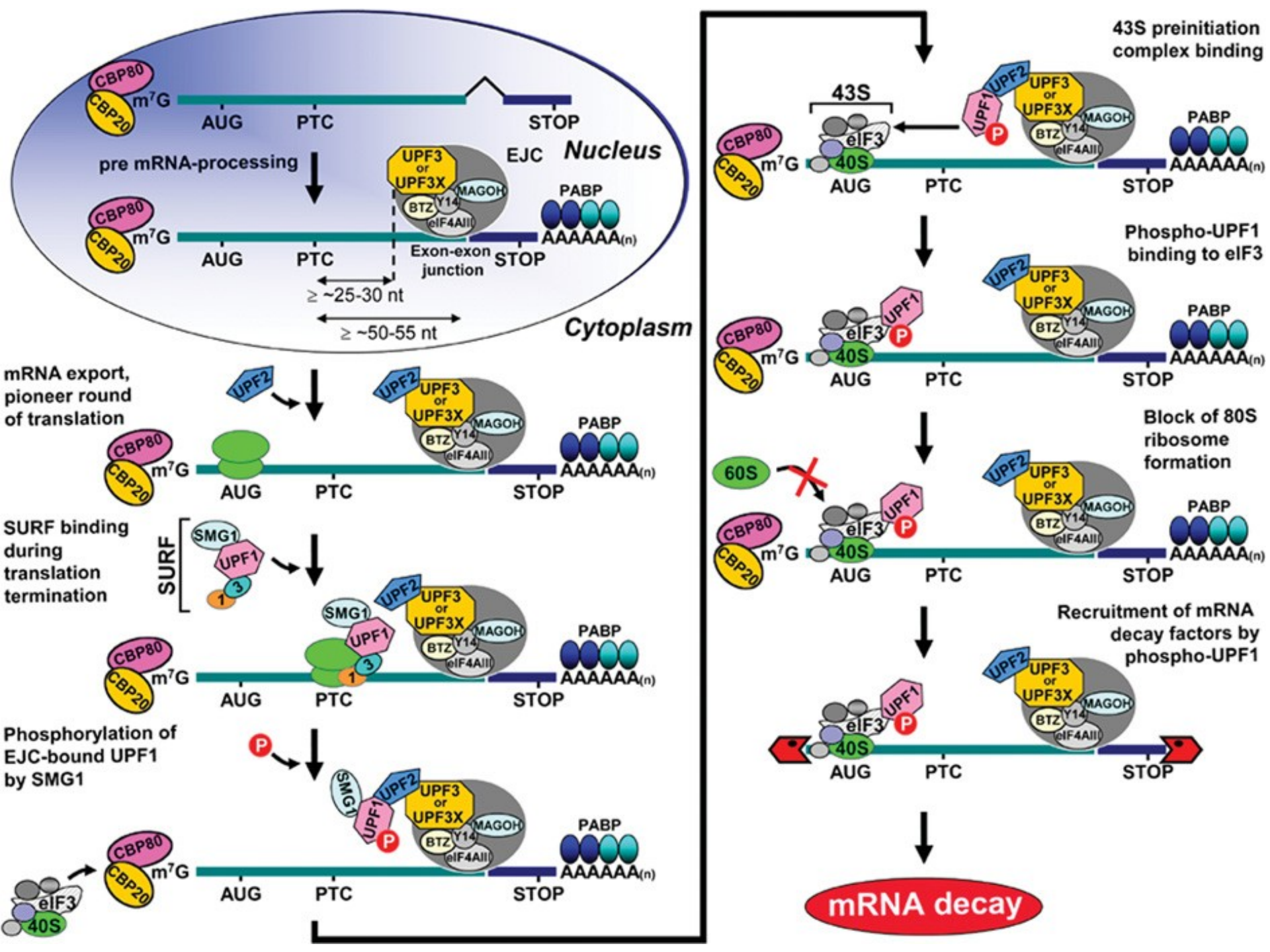


The process of eukaryotic gene expression involves a number of interlinked steps - transcription, splicing, polyadenylation, capping, translation, and mRNA degradation.

Intron splicing of *pre*-mRNA results in the deposition of **the exon-junction complex** (EJC) onto mRNA 20–24 nucleotides upstream of exon–exon junctions during splicing.

In cytoplasm, mRNA undergoes a pioneer round of translation which removes many of the proteins bound to the mRNA in the nucleus.

In the event that a ribosome terminates translation prematurely due to the presence of a PTC, EJCs downstream of the PTC will remain and recruit NMD effectors, forming functional NMD complexes.


CBP80
CBP20
m7G
AUG
PTC
STOP
EJC
Nucleus
pre mRNA-processing
UPF3 or UPF3X
MAGOH
Y14
BTZ
eIF4AIII
PABP
AAAAAA(n)
Exon-exon junction
≥ ~25-30 nt
≥ ~50-55 nt
Cytoplasm
mRNA export, pioneer round of translation
UPF2
SURF binding during translation termination
SURF
SMG1
UPF1
Phosphorylation of EJC-bound UPF1 by SMG1
P
eIF3
40S
43S preinitiation complex binding
43S
Phospho-UPF1 binding to eIF3
Block of 80S ribosome formation
60S
Recruitment of mRNA decay factors by phospho-UPF1
mRNA decay

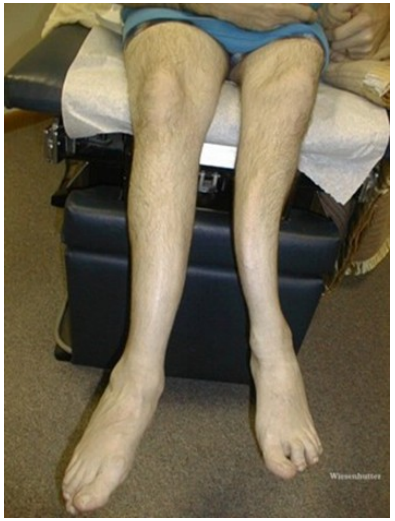
In mammals, newly synthesized CPB80–CBP20-bound mRNA is targeted for NMD once mRNA has been generated by pre-mRNA processing and exported from the nucleus to the cytoplasm. During pre-mRNA processing, splicing results in the deposition of an EJC of proteins upstream of mRNA exon–exon junctions. EJC components include eIF4AIII, Y14, MAGOH, BTZ and many other proteins. The UPF3 (UPF3a) or UPF3X (UPF3b) join EJCs in the nucleus so as to be exported with mRNA to the cytoplasm. In the cytoplasm, UPF3 or UPF3X recruits UPF2. The translation of CBP80–CBP20-bound mRNA constitutes the pioneer round. Translation termination during the pioneer round at a PTC that is situated 50–55 nt upstream of an exon–exon junction (i.e. 25–30 nt upstream of an EJC) involves the SURF complex, which consists of the PI3K-related protein kinase that phosphorylates UPF1, SMG1, together with UPF1, eRF1 and eRF3. As a consequence, NMD generally occurs. During the process, UPF1 together with SMG1 is thought to bind EJC-associated UPF2
in a way that is promoted by CBP80. UPF1 binding to the EJC results in UPF1 phosphorylation. Phospho-UPF1 triggers NMD by promoting translational repression of the NMD target. Translational repression involves the binding of phospho-UPF1 to eIF3 within the 43S pre-initiation complex that is poised at the AUG translation initiation codon so as to prevent 60S ribosomal subunit joining. Phospho-UPF1 also promotes NMD by recruiting mRNA degradative activities. Not shown are SMG5, SMG6 and SMG7, which activate UPF1 dephosphorylation and thus recycling. SMG6 appears to additionally function as an endonuclease. Very recently, roles for SMG8 and SMG9 as SMG1-interacting proteins have been defined. Nucleolytic activities are indicated by the red irregular hexagons. PABP, poly(A)-binding protein, where darker shapes specify the largely nuclear PABPN1 and lighter shapes denote the largely cytoplasmic PABPC1; AUG, translation initiation codon; STOP, normal termination codon; 1, eRF1; 3, eRF3.

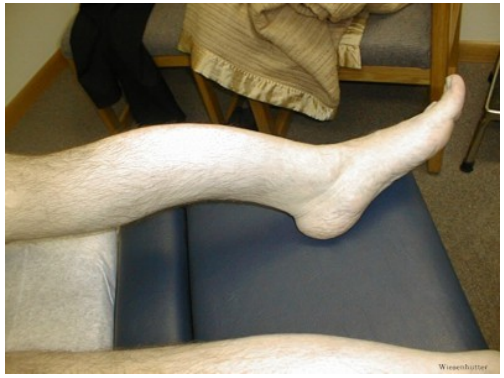
Maquat LE, 2010

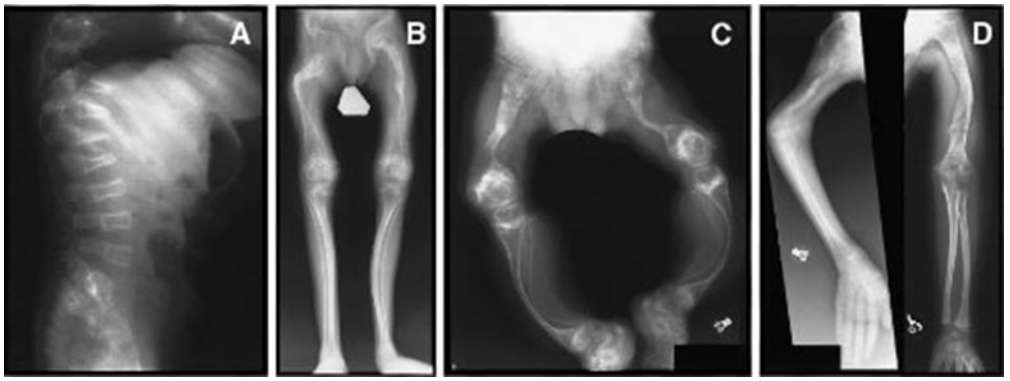
- **NMD zhoršuje klinické projevy nemocí –** Duchennova svalová dystrofie
- **NMD zmírňuje klinické projevy nemocí –** Osteogenesis imperfecta

Osteogenesis imperfecta:

Mutace kolagenu typu I (geny *COL1A1* a *COL1A2*); kolagen typu I - hlavní strukturní protein kostí, mutace mají za následek náchylnost k lomivosti kostí a deformitám skeletu.



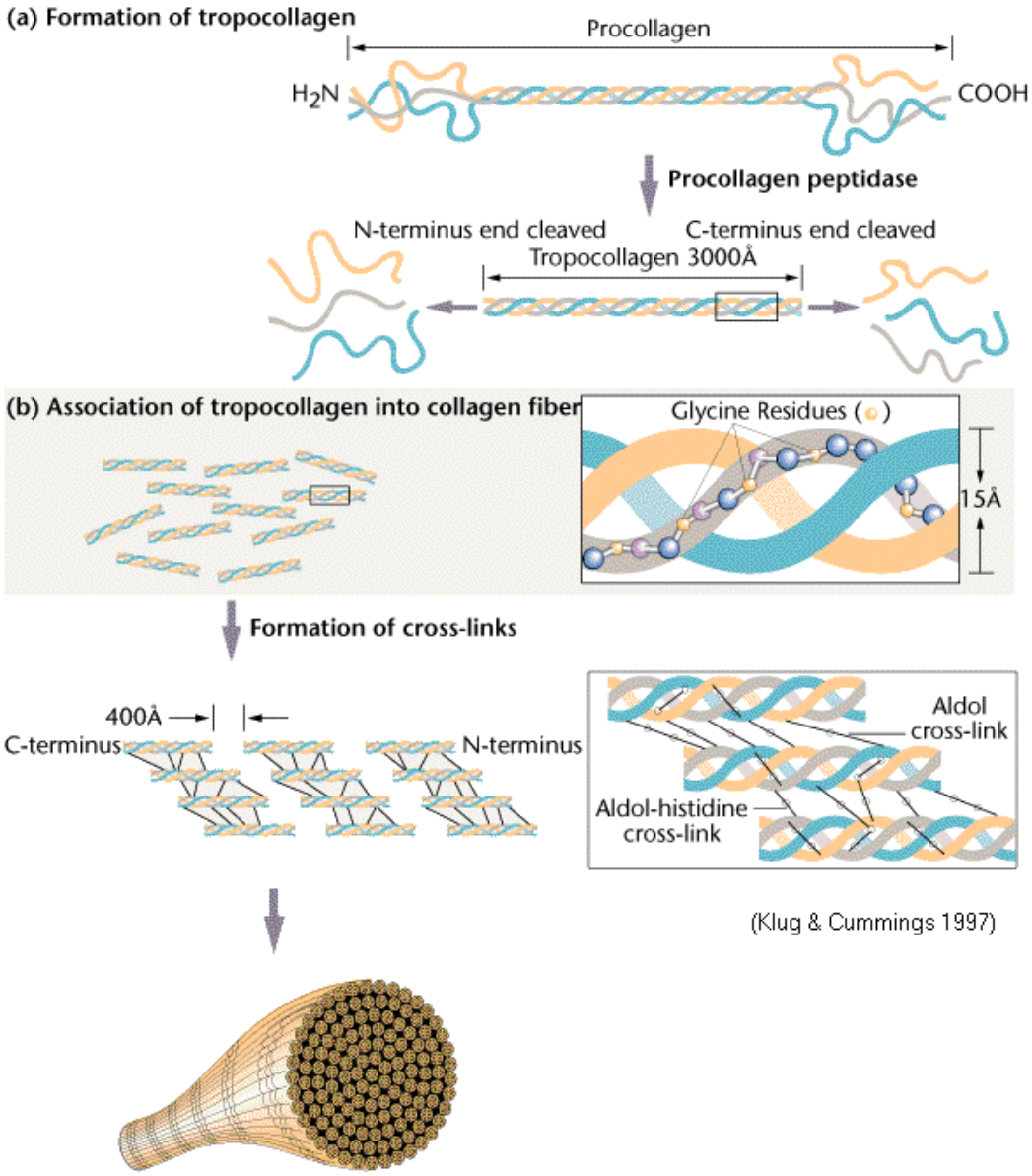




• Kolagen typu I – tvořen ze dvou řetězců prokolagenu α1 (*COL1A1*) a jednoho prokolagenu α2 (*COL1A2*).

• **Missense mutace** asociované s genem ***COL1A*** jsou příklady **dominantně-negativních alel** ..... ruší konformaci kolagenových podjednotek a jsou spojeny s těžkými klinickými fenotypy **osteogenesis imperfecta typu II–IV.**

• **PTC mutace** vyvolávající NMD (nevzniká mutantní protein účastnící se struktury) a jsou spojeny s mírnějšími klinickými fenotypy **osteogenesis imperfecta typu I.**



Therapies based on translational read-through

PTC124 - a new drug in development for mutation-specific treatment of inherited diseases such as DMD, CF, ….
PTC124 is able **to bind the decoding centre of the ribosome and decrease the accuracy of codon–anticodon pairing**. The recognition of PTC is suppressed and, instead of chain termination, an amino acid is incorporated into the polypeptide chain.
PTC124 promotes read-through of PTCs without affecting normal stop codons. PTC124 is being investigated in clinical studies.