Reverse transcription (RT);

Analysis of RNA ends;

Techniques for studying transcription

Michał Koper, IGiB UW

RNA steady-state level = equilibrium between transcription, processing, and degradation

Methods of studying transcripts

Steady state transcript level *(Presence in the cel):*

- Northern-blot technique
- RT-PCR
- RT-qPCR
- SAGE (*Serial Analysis of Gene Expression*)
- Microarrays
- *In situ* hybridization (microscopic technique)
- RNAseq: High-throughput sequencing of transcriptomes

Nascent transcripts (RNA *„in statu nascendi”*):

- TRO (*Transcription Run-On*)
- FRAP (*Fluorescence Recovery After Photobleaching*; microscopic technique).
- GRO-Seq (*Global Run-On Sequencing*).

***In vivo* Protein-DNA/RNA interactions:**

- ChIP (*Chroamtin Immuno-precipitation*), e.g., presence of RNA Pol on gene; TFs-DNA interactions; "ChIP on CHIP"; ChIP-seq
- RIP (*RNA Immuno-precipitation*) – interactions of RRM proteins with RNA
- DIP (DNA Immuno-precipitation)
- CLIP (*in vivo Cross-Linking and Immunoprecipitation; RNA-protein complexes*).

Reverse transcription (RT) reaction

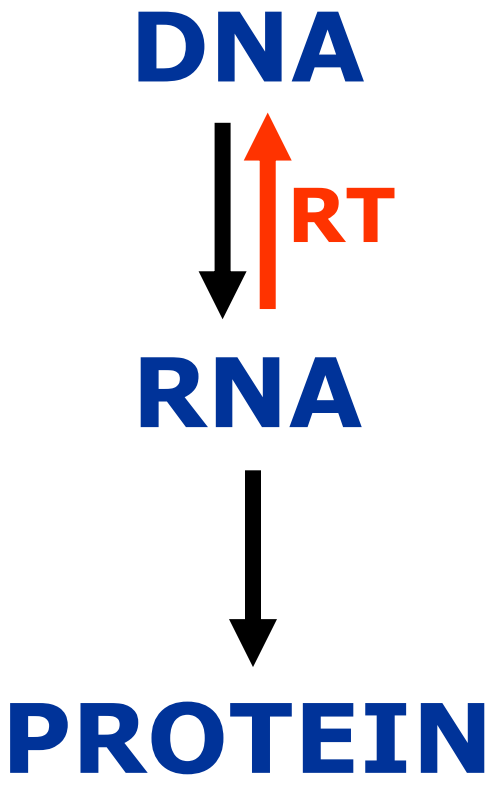
Discovery of reverse transcriptase

Nobel Prize in Medicine and Physiology 1975.

Howard M. Temin

David Baltimore

Renato Dulbecco



„For their discoveries concerning the interaction between tumour viruses and the genetic material of the cell"

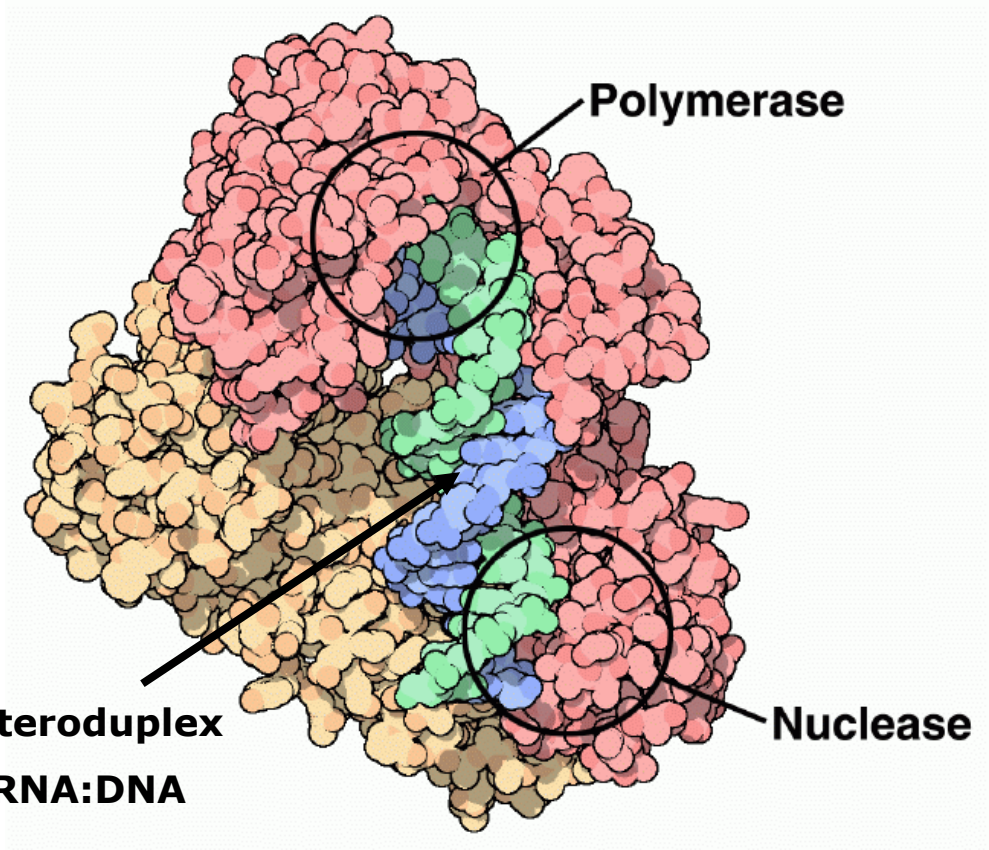
RNA-DEPENDENT DNA POLYMERASE

Temin, H. M., and S. Mizutani. 1970. **RNA-dependent DNA polymerase in virions of Rous sarcoma virus**. Nature 226: 12 1 l-l 2 13.

Reverse transcriptase: 2 enzymes in 1

HIV-1 RT

(heterodimer)



***Ding J**. et al, 1998, J. Mol. Biol. 284; 1095-1111*

RNA-dependent DNA polymerase activity

(and DNA dependent DNA Pol)

Ribonuclease H

(RNase H) activity

RT requires a primer to start DNA synthesis

gene-specific primers

Oligo-dT primers,
Can be "anchored", i.e.
TTTTTTTTTTT(A/C/G)

short random primers,
e.g. hexamers, octamers

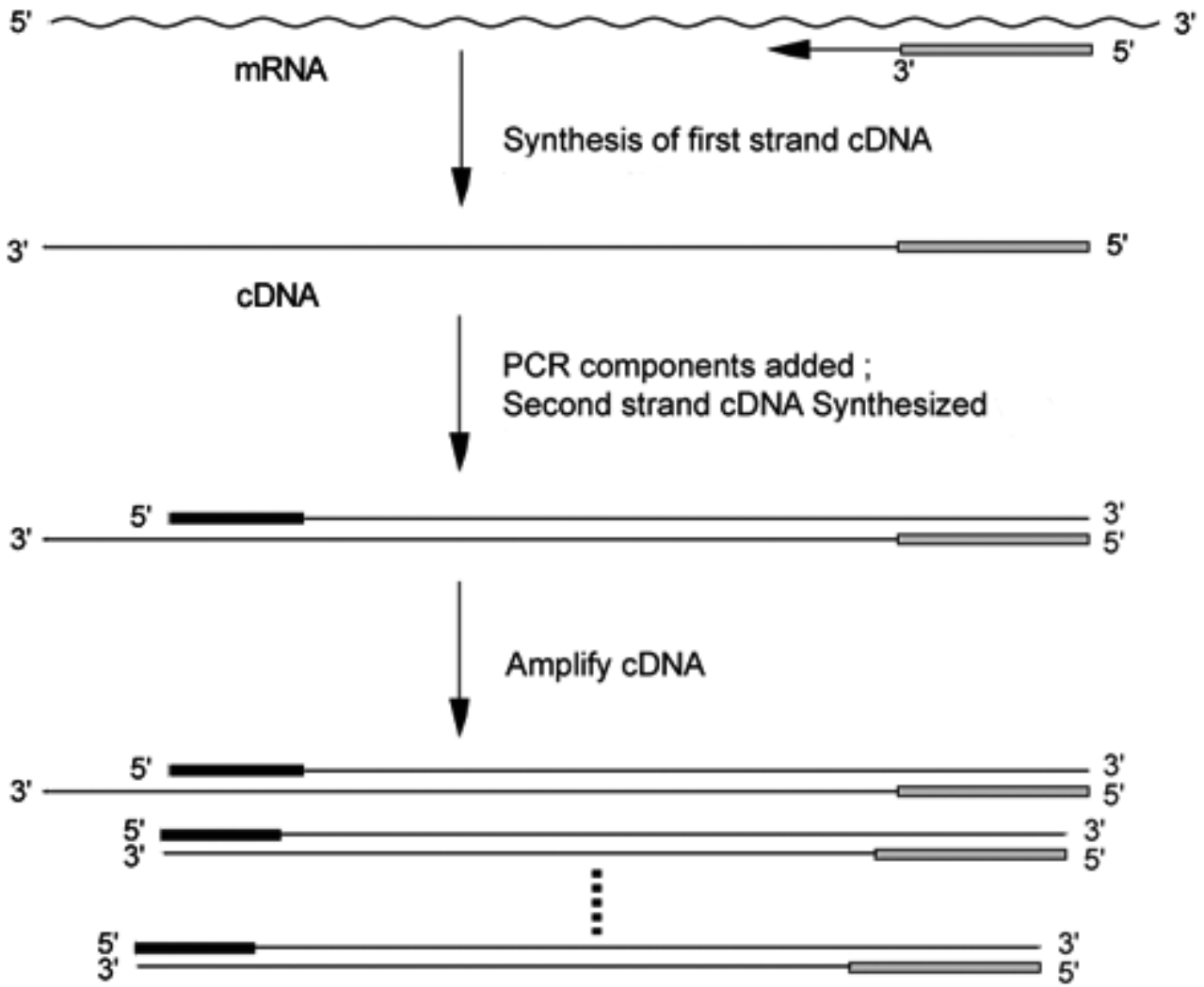
Applications of reverse transcription

QUALITATIVE techniques („presence”):
- **structure of the genes (introns)**
- **determination of the RNA 5’ ends**
- **structure of the RNA 3’ ends**
- **detecting the expression of constructs**

QUANTITATIVE techniques („levels”):
- **semiq RT-PCR**
- **RT-qPCR**

BOTH – QUALITATIVE and QUANTITATIVE techniques
- **RNAseq - transcriptome sequencing**

Principle of RT-PCR



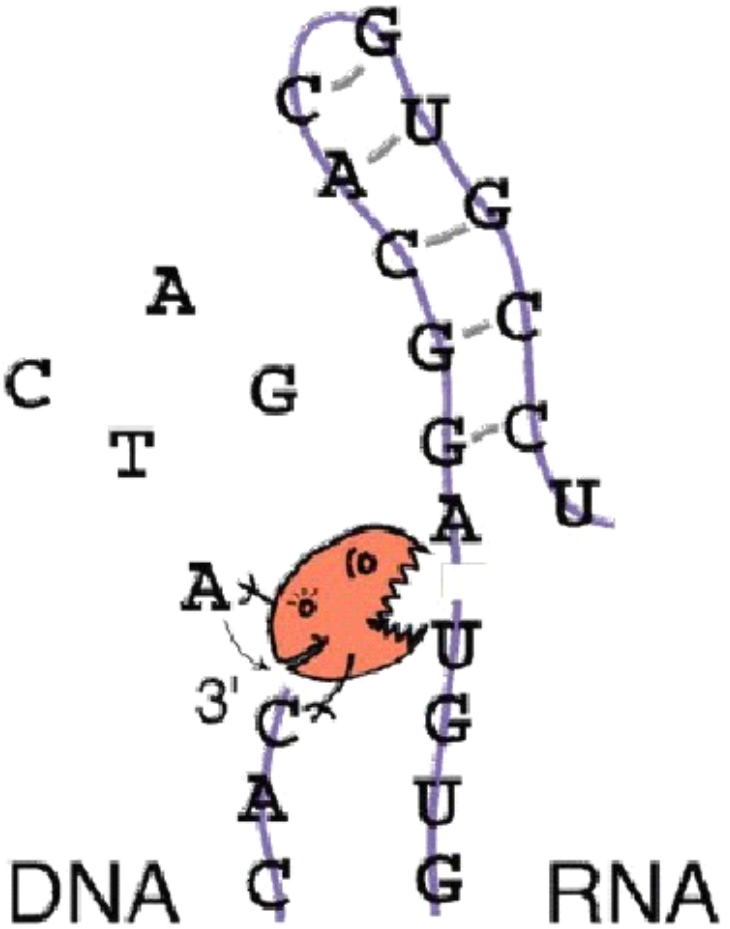
RNase H activity of wild type RT

Responsible for:

- ***removal of DNA:RNA hybrids***
- ***generation of PPT primers***
- ***removal of tRNA and PPT primers***

Particularly active at sites with RNA secondary structure where RT slows down.

In some commercially available RT Rnase H activity is lowered or removed.



Courtesy of the TATA Biocenter

Properties of various RTs

	Temp
MMLV RNase H- Minus (Promega, Germany)	37
M-MLV (Promega)	45
Avian Myeloblastosis Virus (AMV) (Promega)	37
Improm-II (Promega)	45
Omniscript (Qiagen, Germany)	37
cloned AMV (cAMV) (Invitrogen, Germany)	45
ThermoScript RNase H- (Invitrogen)	50
SuperScript III RNase H- (Invitrogen)	50

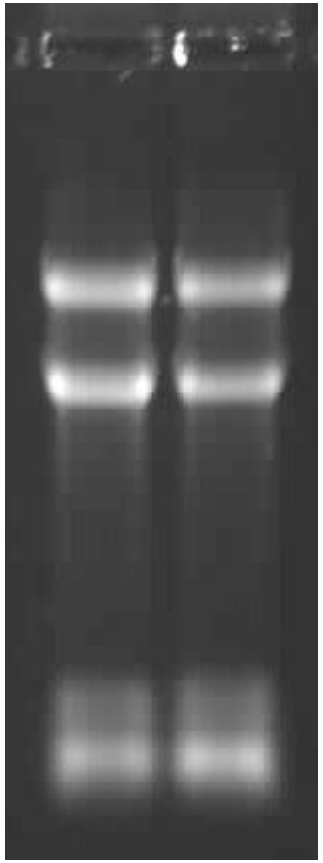
Sthalberg et al, 2004, Comparison of reverse transcriptases In gene expression analysis, Clin Chem. 50(9):1678-80.

AMV: Avian Leukemia Virus
MMLV: Moloney Mouse Leukemia Virus

GOOD LABORATORY PRACTICE AT EVERY STAGE OF WORK WITH RNA!

DNase treatment
1h, 37° C

- +



RNA quality is a key factor for RT!

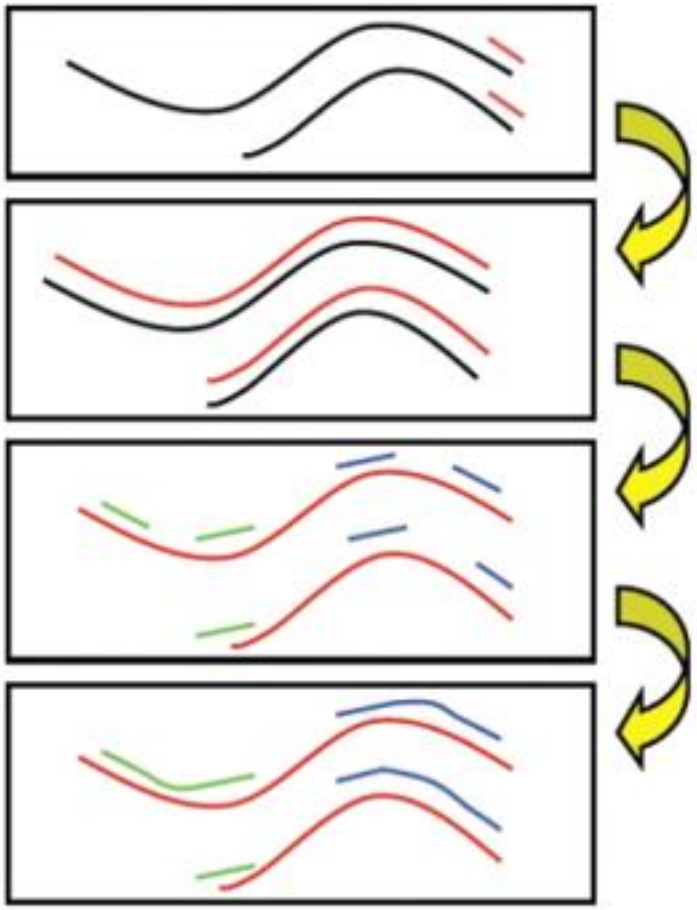
RNA quality control after isolation is always necessary!

DNase treatment is always necessary!

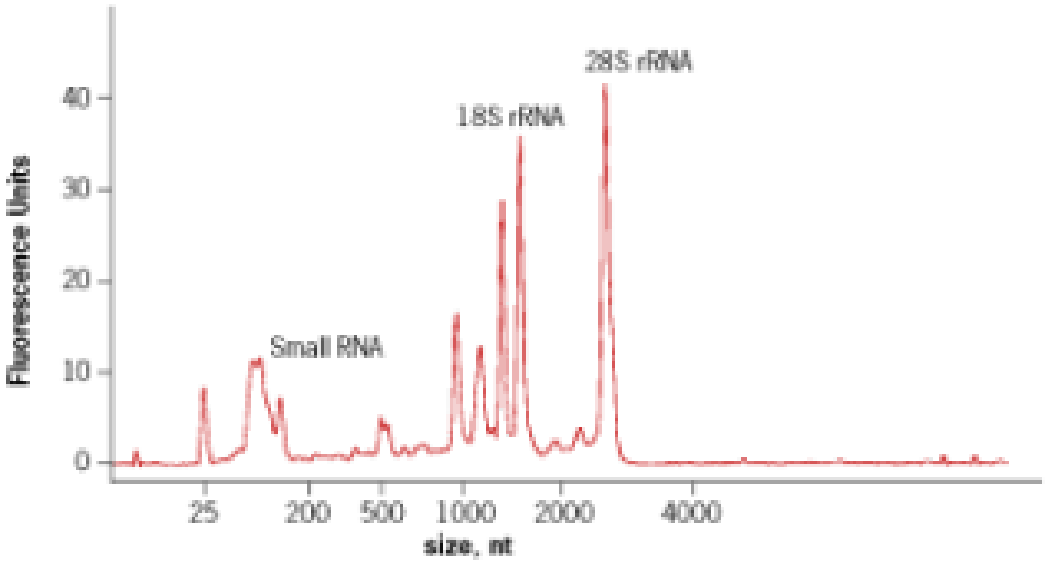
Recommended use of RNase inhibitors!

RNA quality assessment

5'-3' RNA ends integrity test



Microfluidic Analyzers



- **Agilent: Bioanalyzer, Tapestation**
- **Biorad: Experion**
- **Capillary electrophoresis**

Efficiency and specificity of RT

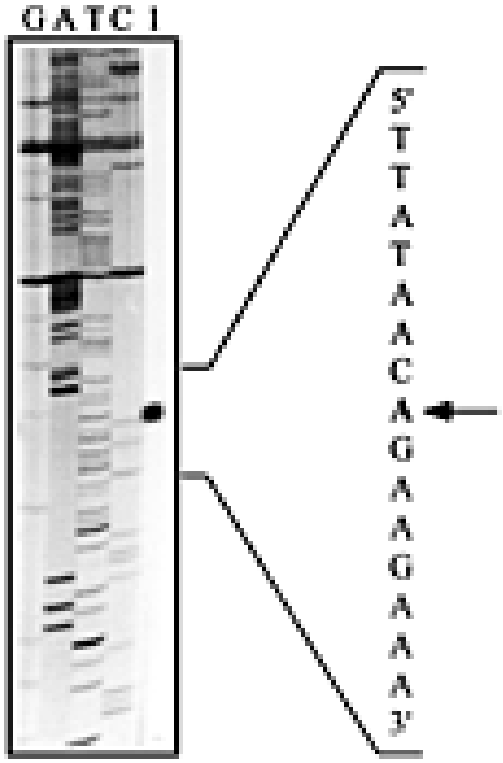
Efficiency and specificity of reverse transcription reaction STRONGLY depends on:

- **Methods of priming**
- **Type of reverse transcriptase (features of the enzyme)**
- **The sequence of a particular RNA molecule – it's tendency to form complex secondary structures**
- **Performance differences can be as high as 100X!!!**
- **The optimal RT conditions should always be determined experimentally**

Analysis of 5' and 3' RNA ends

Primer extension

Determination of the transcription start site



T.-R. Lee, H.-P. Hsu, and G.-C. Shaw, 2001, J. Biochem. Vol. 130, pp. 569-574

Annealing of radioactively labeled GS primer to the RNA 5' end

↓

Reverse transcription

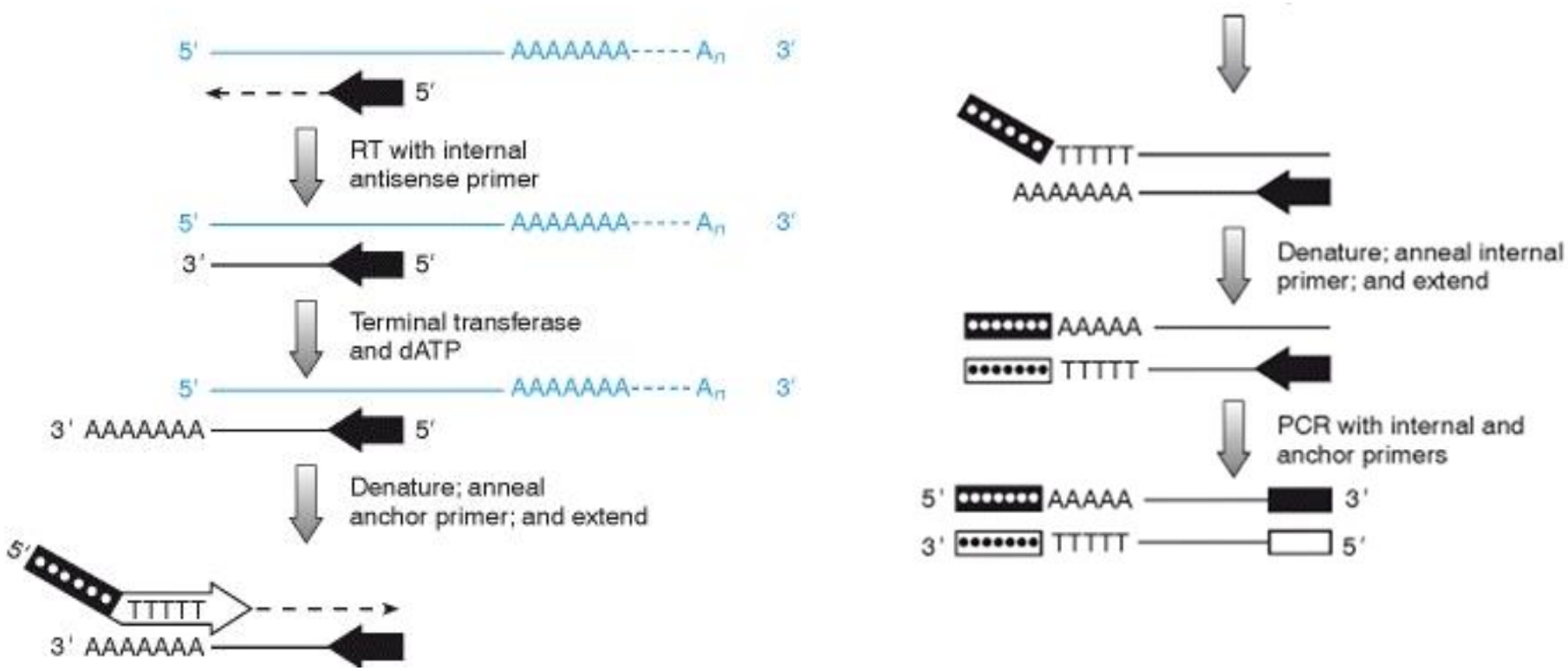
↓

Separation in polyacrylamide gel together with sequencing reactions carried out from the same primer

Analysis of 5' RNA ends - 5' RACE

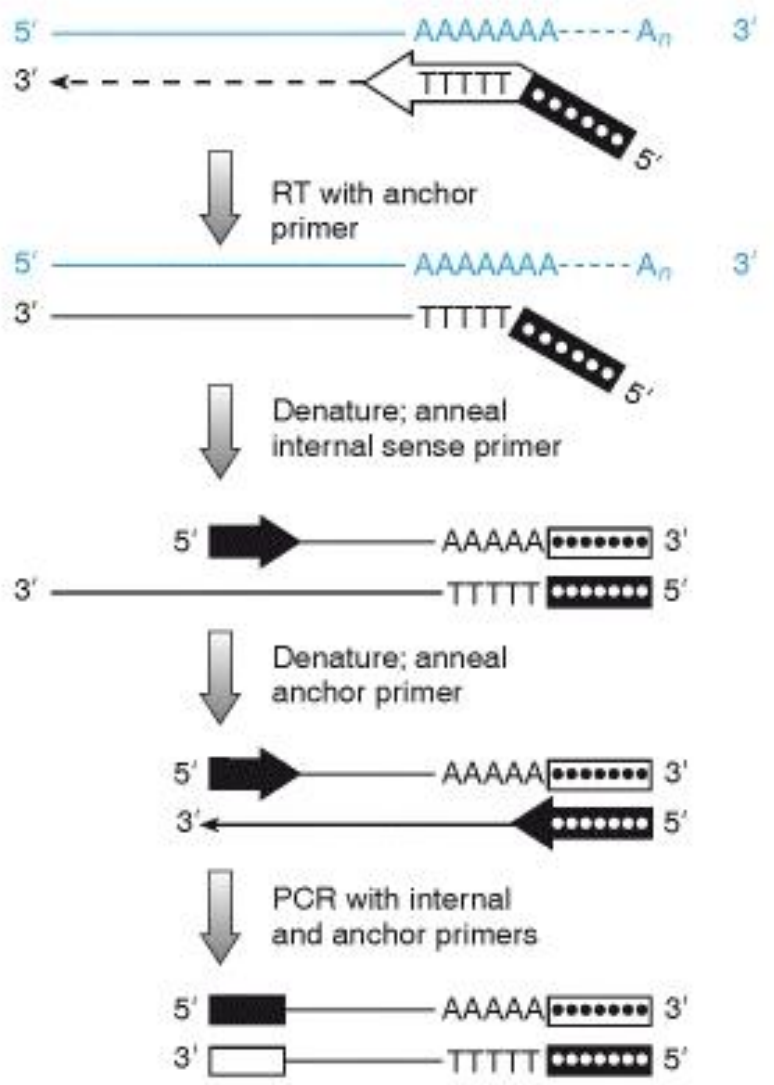
Rapid Amplification of 5'cDNA Ends

anchored PCR

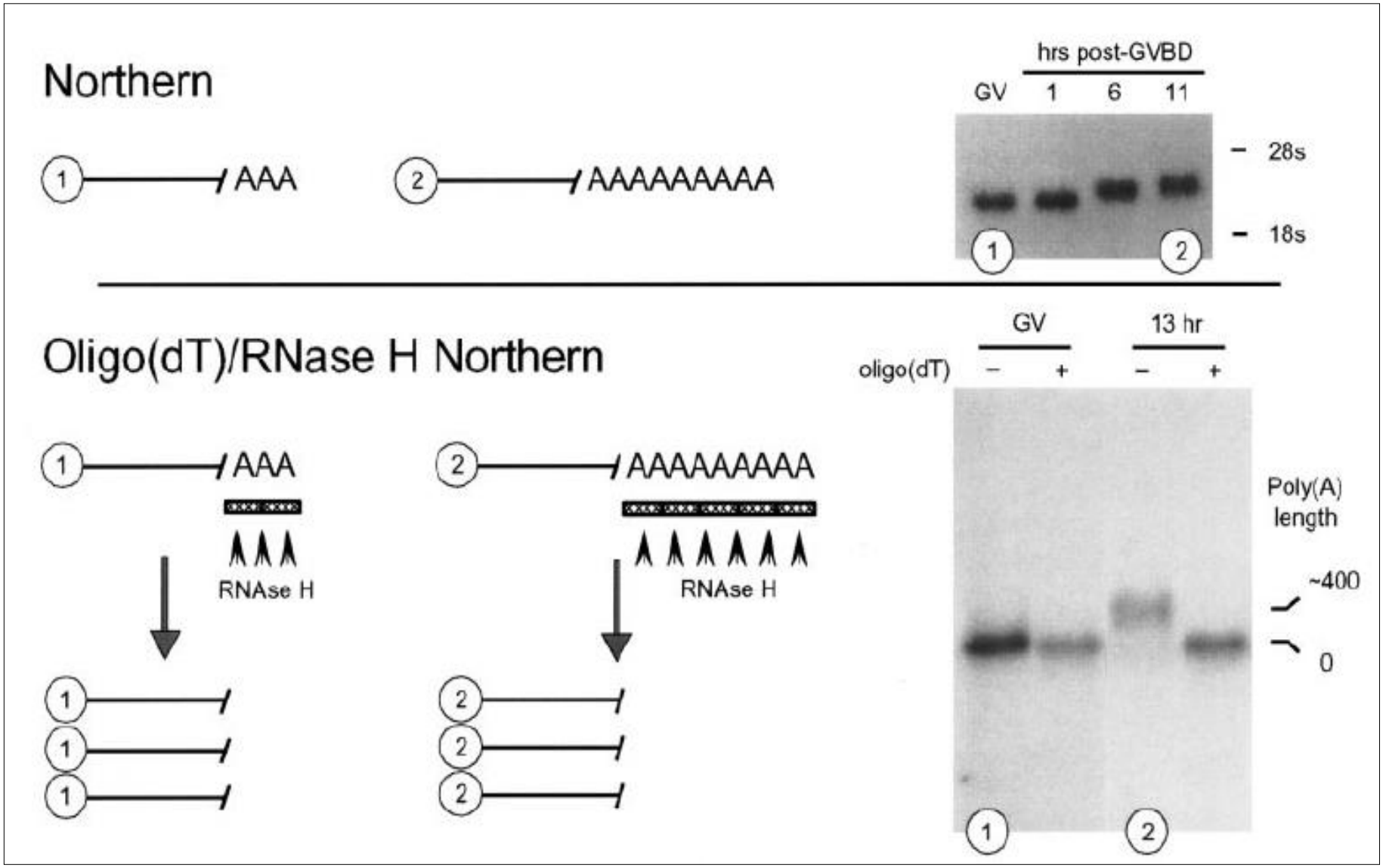


Analysis of 3' ends of polyadelinated RNAs - 3' RACE

Rapid Amplification of 3'cDNA Ends

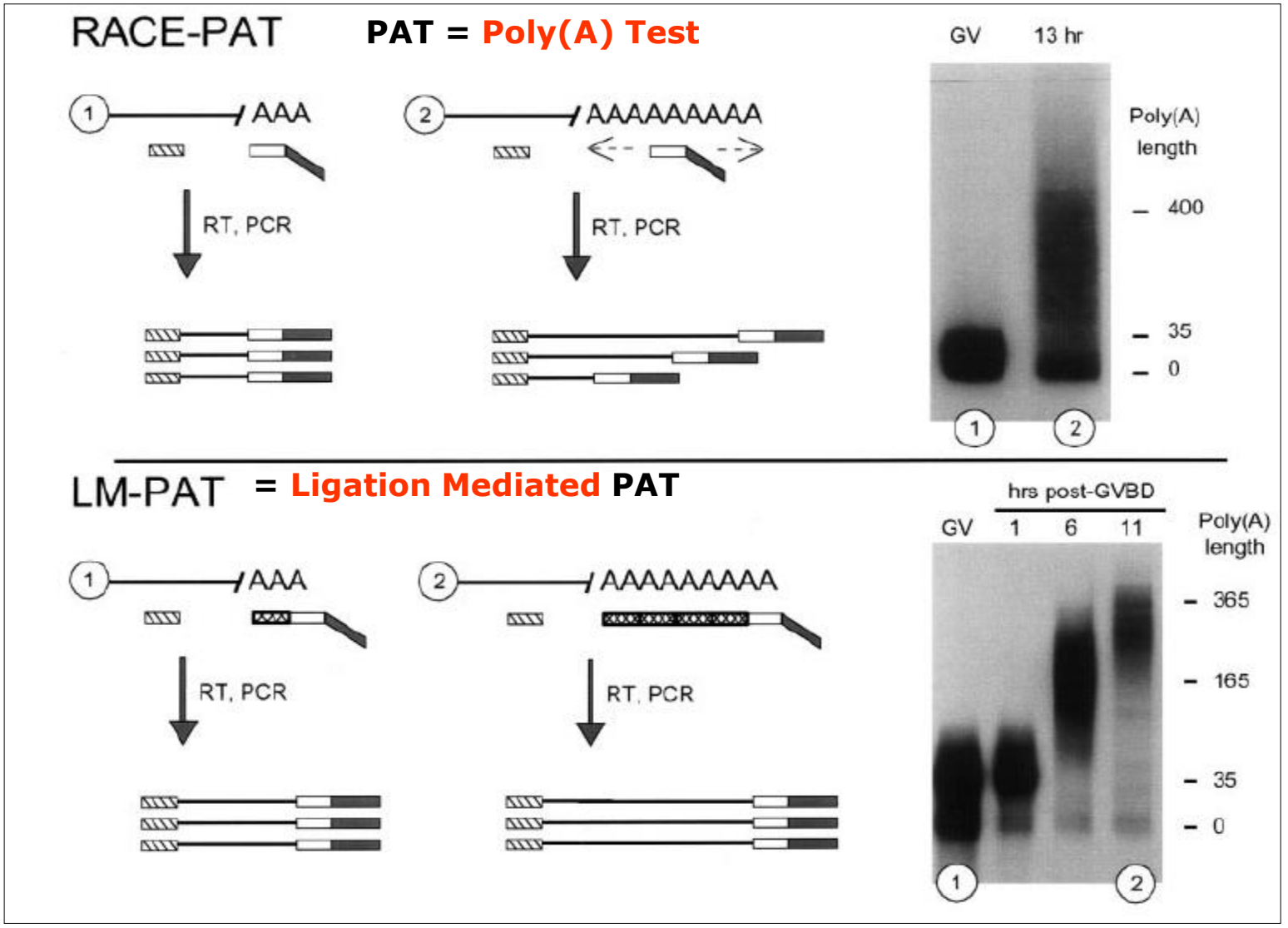


Analysis of polyA$^+$ 3′ ends

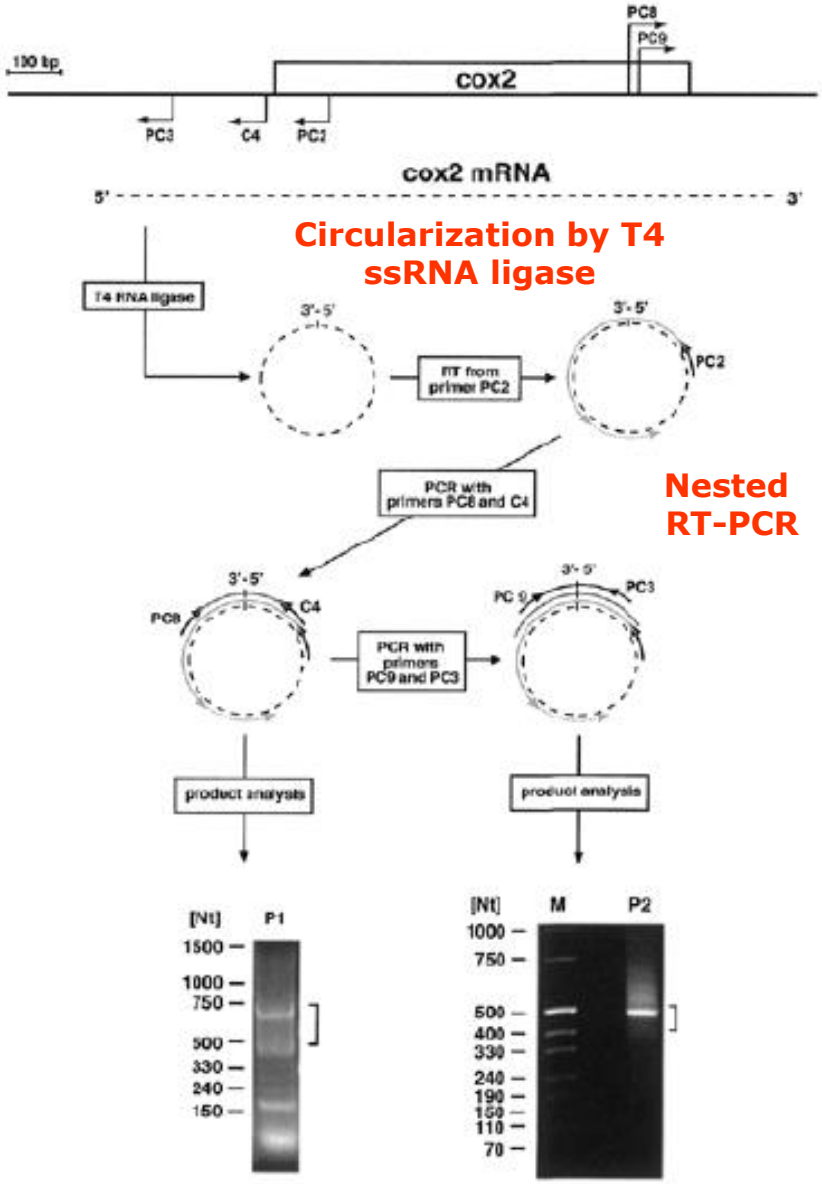


Sallés et al, Methods, 1999

Analysis of polyA$^+$ 3′ ends



Circular RT-PCR (cRT-PCR)



Analysis of 5' and 3' ends of the mitochondrial COX2 transcript in plants

Khun and Binder, NAR, 2002.

1	TCGCAATAGCGAGCCTACTTATCGCTTTTTTT – AGAAAGAA....	+300 -277
3	TCGCAATAGCGAGCCTACTTATCGCTTTTTT – GATAGAAA....	+299 -280
1	TCGCAATAGCGAGCCTACTTATCGCTTTTTT – TAGAAAGA....	+299 -278
1	TCGCAATAGCGAGCCTACTTATCGCTTTTTT – AGAAAGAA....	+299 -277
1	TCGCAATAGCGAGCCTACTTATCGCTTTTTT – AGAATCGT....	+299 -273
1	TCGCAATAGCGAGCCTACTTATCGCTTTTTT – ATCGTTCA....	+299 -270
1	TCGCAATAGCGAGCCTACTTATCGCTTTTTT – TCGTTCAG....	+299 -269
1	TCGCAATAGCGAGCCTACTTATCGCTTTTTT – ATCACGTA....	+298 -291
2	TCGCAATAGCGAGCCTACTTATCGCTTTTT – TCGTTCAG....	+298 -269
2	TCGCAATAGCGAGCCTACTTATCGCTTTTT – TAGGCTAA....	+298 -291
1	TCGCAATAGCGAGCCTACTTATCGCTTTT – GTAGGCTA....	+297 -282
Σ 15		

Circular RT-PCR (cRT-PCR) cont.

- **RNA ligase ligates RNA molecules lacking a cap at the 5' end**
- **When testing molecules with a cap, the cap must be removed to detect RNA**
- **The cap can be removed by enzymes that hydrolyze P-P bonds, e.g. bacterial RNA 5´-pyrophosphohydrolase RppH (NEB) or plant acid pyrophosphatase Cap-Clip (CellScript) (formerly acid pyrophosphatase from tobacco TAP, now not produced) or by digesting RNA-DNA duplexes with RNase H (makes sense when the RNA is highly expressed, exact 5' end of the transcript is known and we are not interested in alternative 5' ends)**

https://www.neb.com/en/tools-and-resources/selection-charts/mrna-decapping-selection-chart

Techniques for studying transcription

Transcription Run ON technique

Transcription Run-On (TRO)

early log-phase yeast culture

↓

sarkosyl treatment
- permeabilize cells
- inhibit transcription initiation

↓

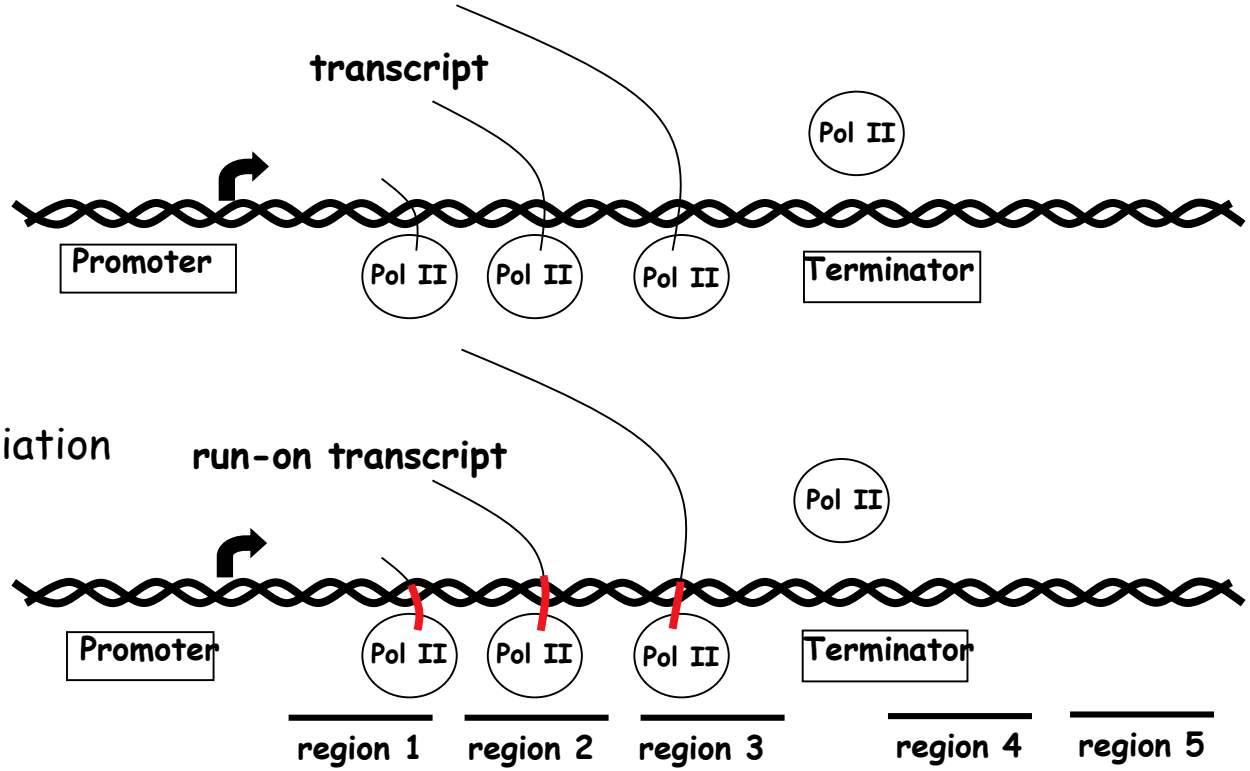
transcription reaction with P-UTP^{32}

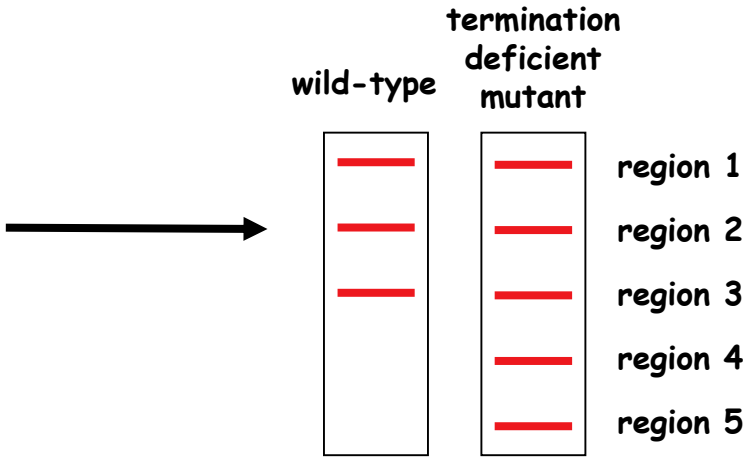
↓

RNA extraction

↓

Hybridize RNA to single strand DNA probes relative to different regions on the gene of interest





Courtesy of B. Dichtl

Molecular probes used in TRO

Antisense probes that are approx. 70 to 400 nt in length:

- **ssDNA obtained from M13-derived phagmids**
- **Oligonucleotides usually 70-85 nt**
- ***in vitro* transcribed RNA (e.g., using T7 promoter)**
- **PCR products**
- **dsDNA plasmids**

Blotting TRO probes on filters

Standard nylon membranes for nucleic acids, e.g. *Amersham Hybond N.*

Vacuum application, e.g. *Bio-Rad slot/dot blotter*.

The amounts of probes applied depends on the expression levels of the transcripts under study, e.g. for ACT1, 25s or 18s rRNA - 5 µg oligonucleotides 75 nt. or 5 µg ssDNA M13 probes (inserts about 250 nt).

Performing TRO in yeast - *in vivo* transcription

Modified from Birse et al, Science 280 (1998):

Culture cells to OD_{600} ~ 0.2-0.3 (0.1-0.4).

Permeabilization with sarcosyl - sarcosyl breaks down complexes - "rips off" proteins that interact with the polymerase - any induction before cell harvesting!

Transcription with a mixture of AGC and ^{32}P-UTP for 5 min - longer makes no sense because the polymerase "falls off" after a few tens/hundreds of sec.

Performing TRO in yeasts - isolation of labeled RNA and hybridization

Isolation of RNA by any method, e.g. "hot phenol", GTC/phenol/zirconia beads.

Hydrolysis of RNA in NaOH solution before application to filters. Depending on the duration of hydrolysis, different sizes of fragments obtained.

Hybridization, washes and exposure similar to the **northern-blot** technique.

TRO applications and variants

Quantitative applications:

- Testing the transcriptional activity (power) of promoters
- Determination of transcription rates for individual genes
- Differences in transcriptional activity between mutants or upon external conditions.

Qualitative applications:

- Study of transcription termination defects (*read-through*)
- Distinguishing between RNA Pol presence and RNA synthesis

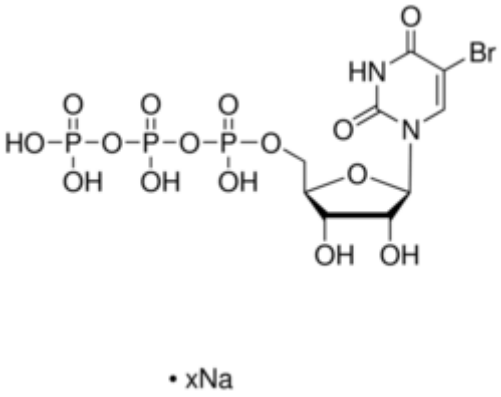
TRO Variants:

- Nuclear Run-On, on isolated cell nuclei: Gariglio (1981) and Brown (1984)
- Mitochondrial Run-On, on isolated mitochondria

GRO-seq: Global (Genomic) Run-On

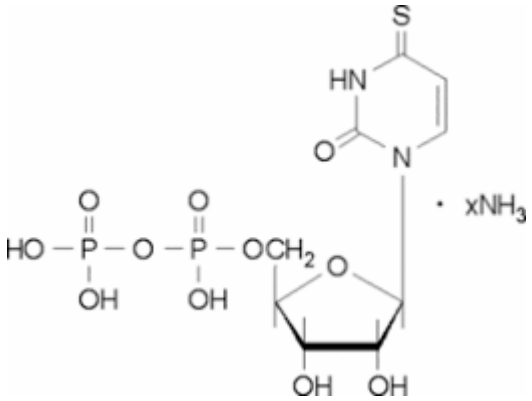
RNA labeling: Run-On in the presence of BrU or 4tU

5-Bromouridine (BrU)



RNA fragmentation and immunoprecipitation on agarose-anti BrU resin

4-thiouridine (4tU)

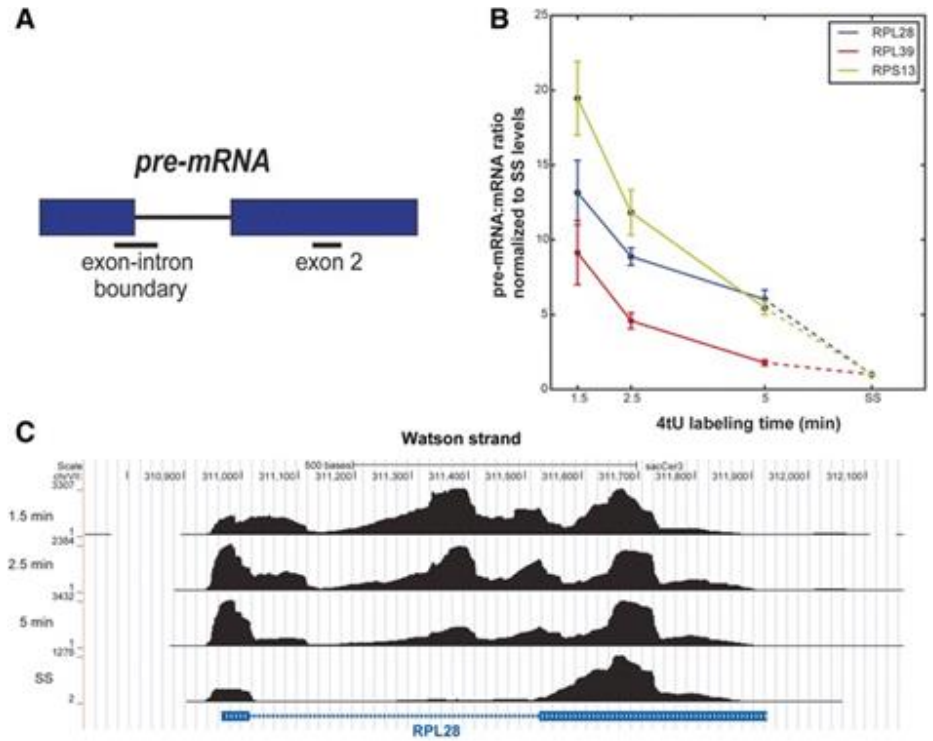


Biotinylation of the thiol group and binding to the streptoavidin complex

Reverse transcription -> RNAseq

4tU-seq

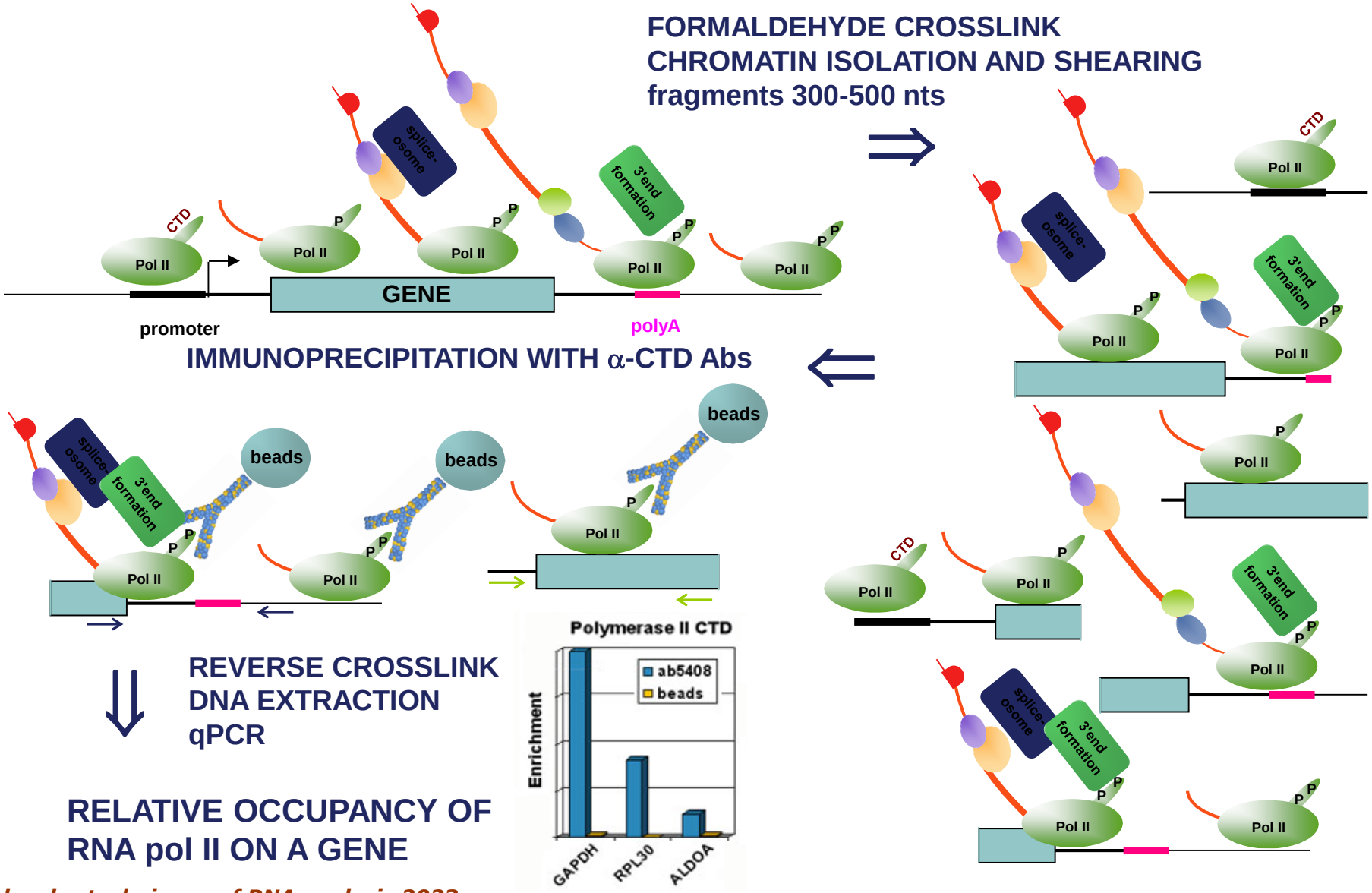
Transcriptome-wide RNA processing kinetics revealed using extremely short 4tU labeling



(Barras et al, Genome Biol 2015)

ChIP – Chromatin Immunoprecipitation

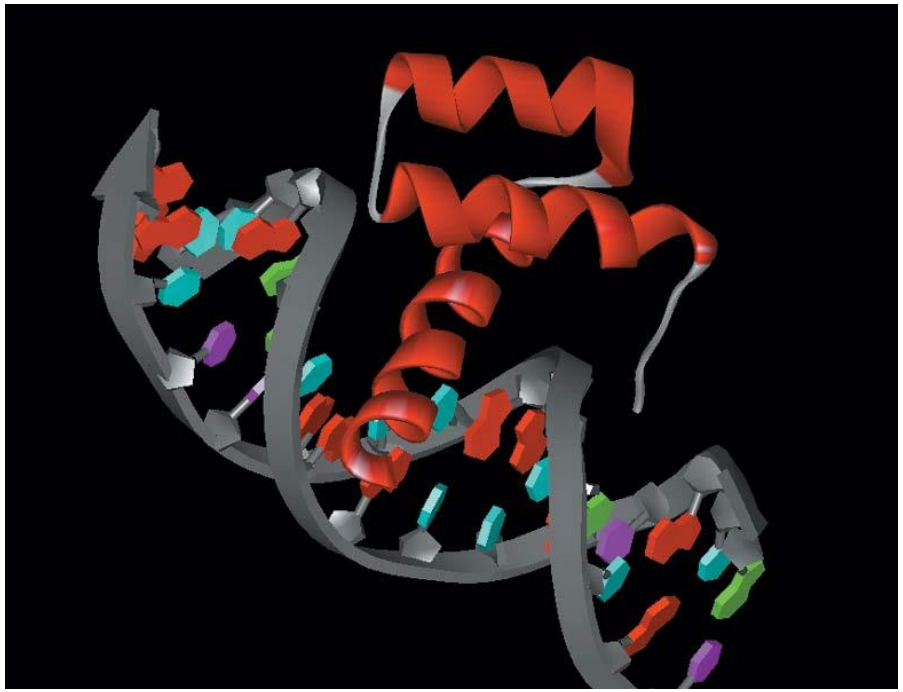
Chromatin immunoprecipitation - ChIP



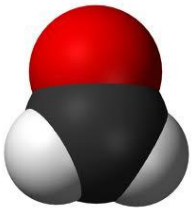
ChIP applications

Studying protein-DNA interactions *in vivo*!

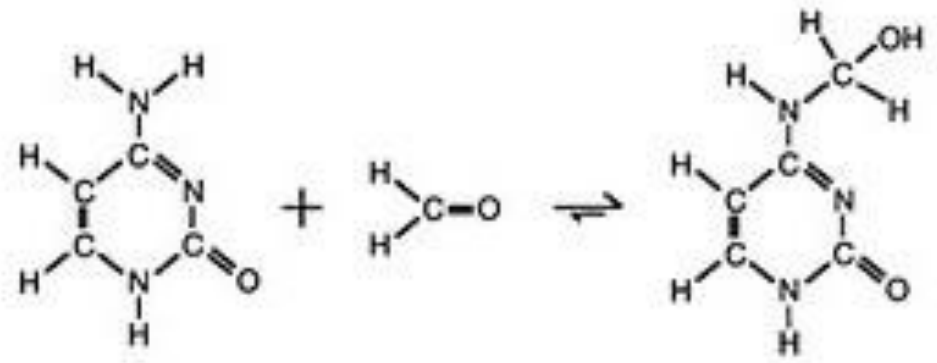
- **Transcription factor binding sites**
- **Presence (distribution) of RNA Pols along chromatin (genes)**
- **Histone modifications**
- **Chromatin remodeling factors**



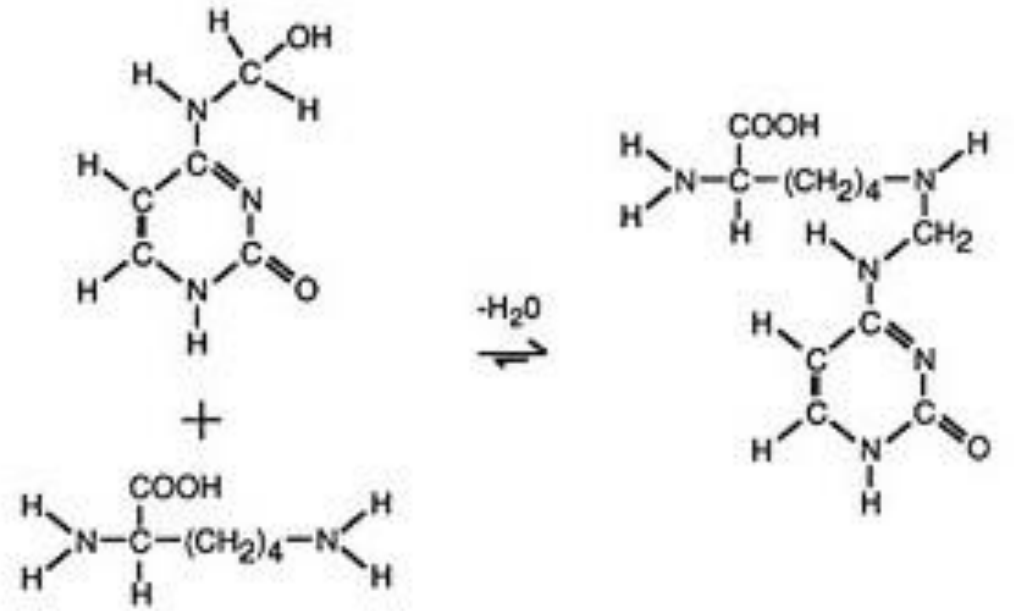
Crosslinking agent: formaldehyde



Reaction 1 (Shiff base is formed)



Reaction 2

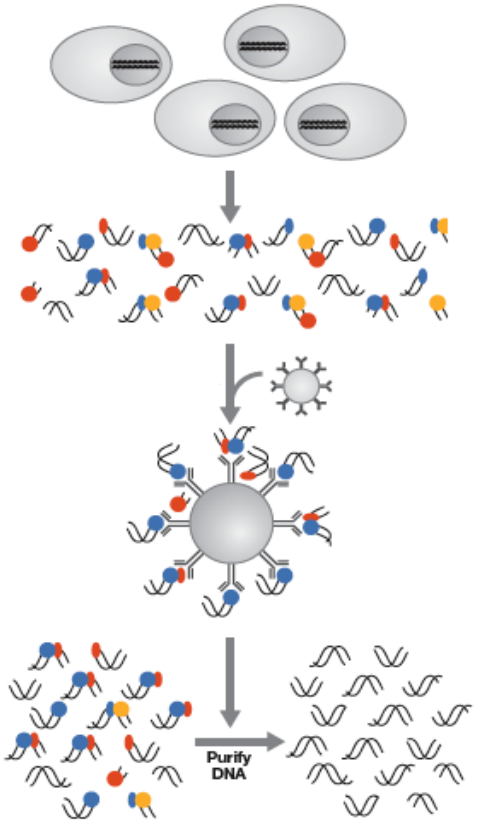


Resolution vs. specificity of crosslinking

- ***In vivo* crosslinking, high reactivity of formaldehyde**
- **High resolution: 2Å**
- **Problem with crosslinking of weaker interacting proteins with DNA (greater distance between atoms in residues)**
- **Other cross-linking agents (e.g., DTBP)**

ChIP procedure

Chromatin Immunoprecipitation (ChIP)

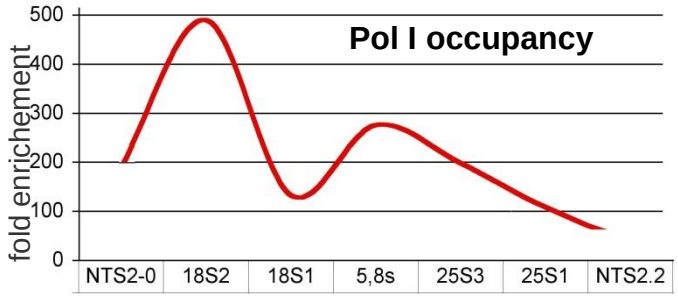


DNA-protein crosslink *in vivo*

Chromatin fragmentation

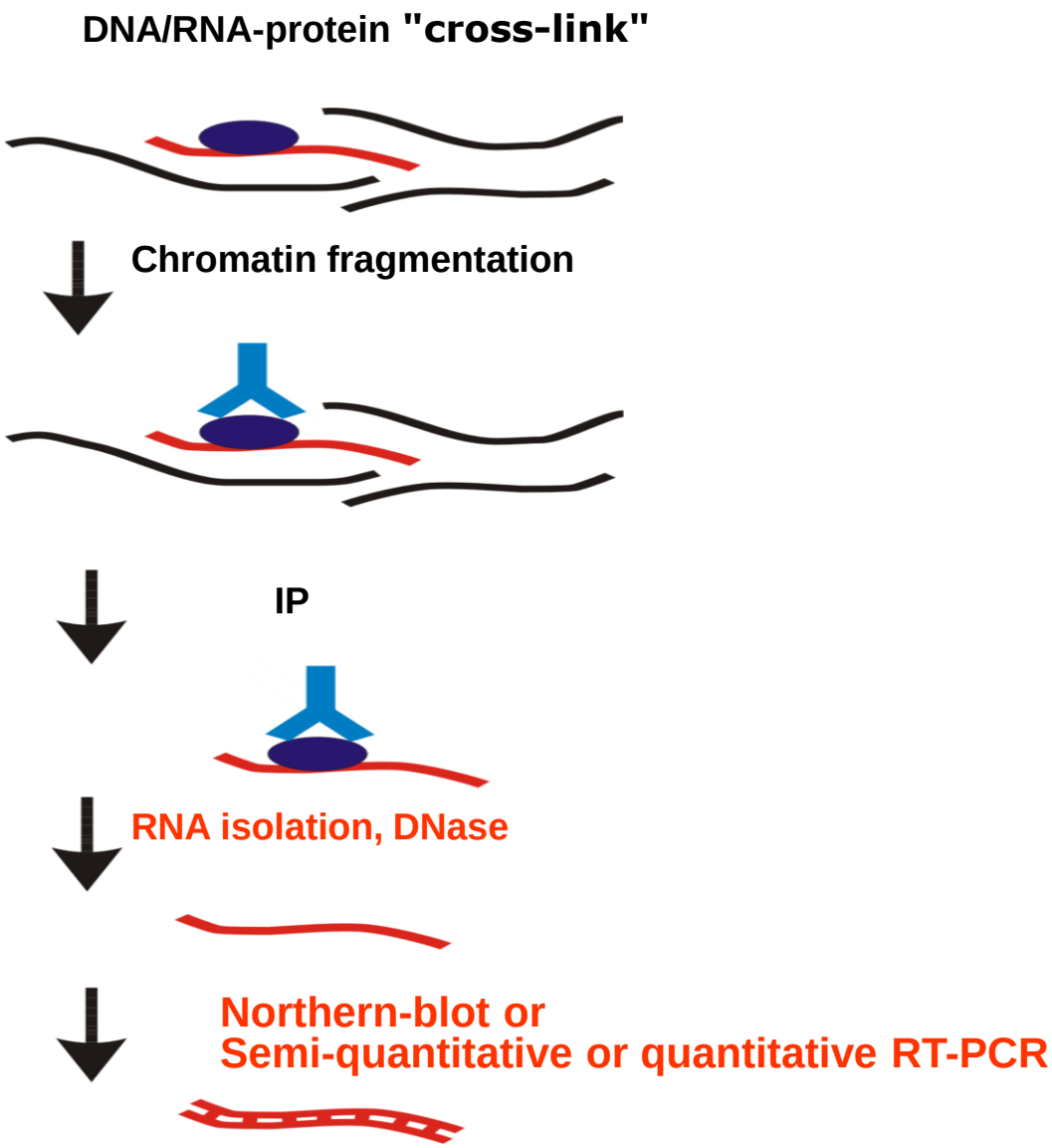
IP using Pol Abs or tagged Pol subunit

Southern-blot or semi-quantitative or quantitative PCR NGS sequencing (ChIP-seq)



RNA Immunoprecipitation - RIP

RNA immunoprecipitation is similar to ChIP



Transcription termination

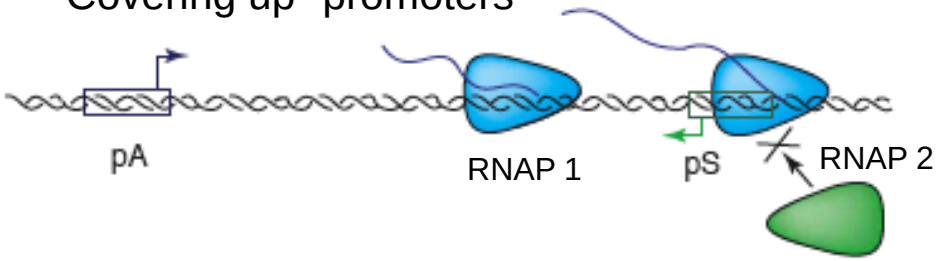
Why to terminate transcription?

In eukaryotes, few genes are organized in polycistrons (exceptions: ncRNA, mtRNA)

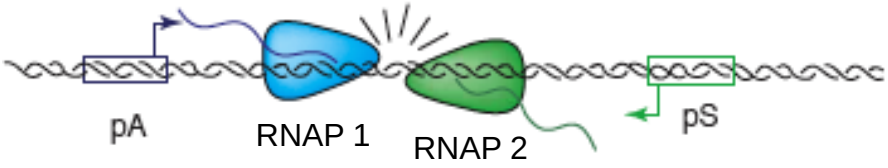
Incorrect transcription termination affects gene expression

1. co-transcriptionally

"Covering up" promoters

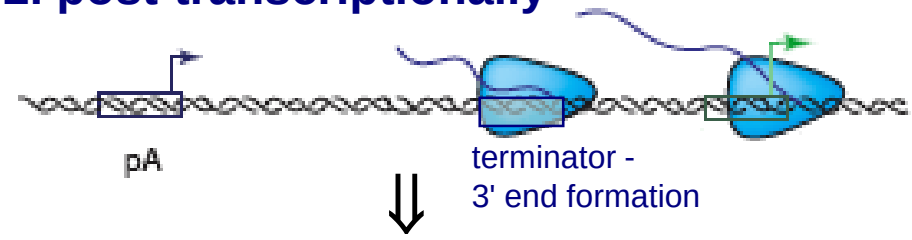


Polymerase collisions

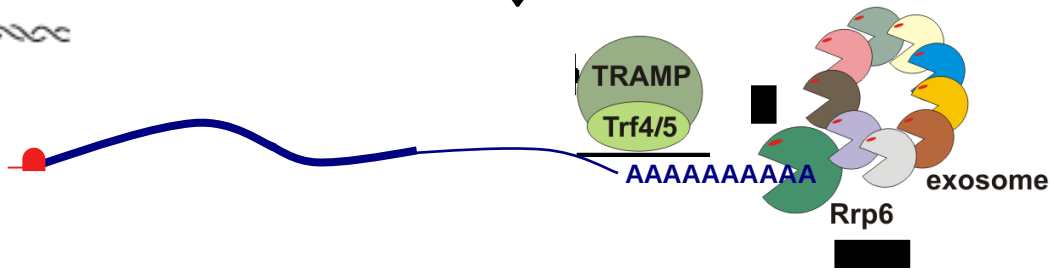


Shearwin et al, TiG., 2005

2. post-transcriptionally



"Readthrough" - Transcription Readthrough (**RT**).

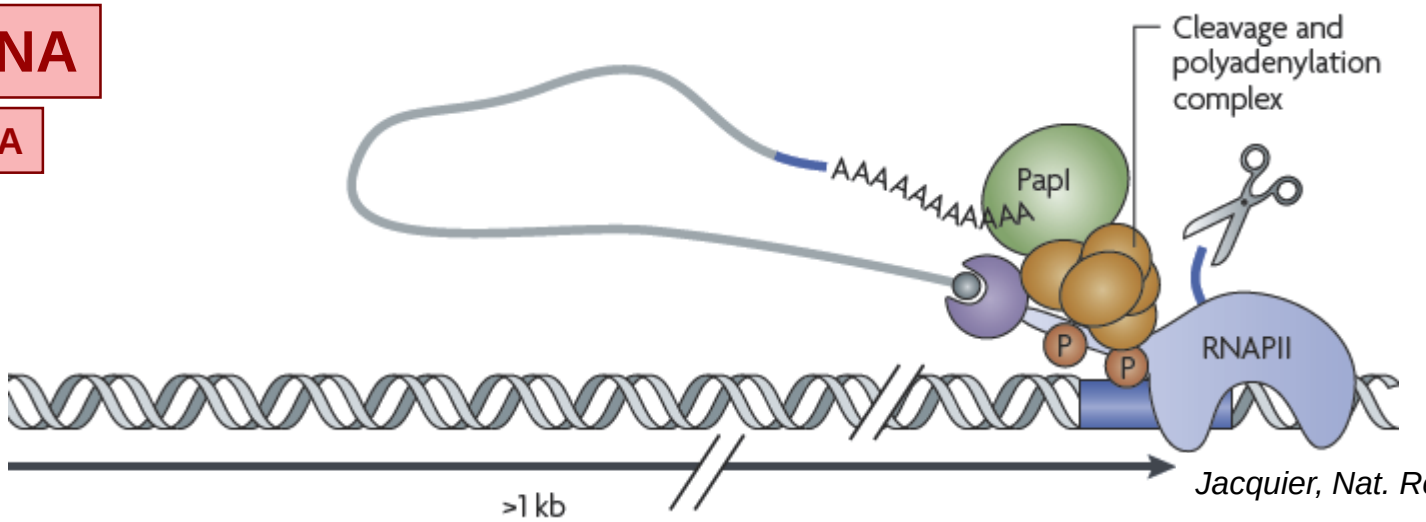


Removal of defectively elongated transcripts (nuclear RNA surveillance)

RNA polymerase II - cleavage and polyadenylation

mRNA

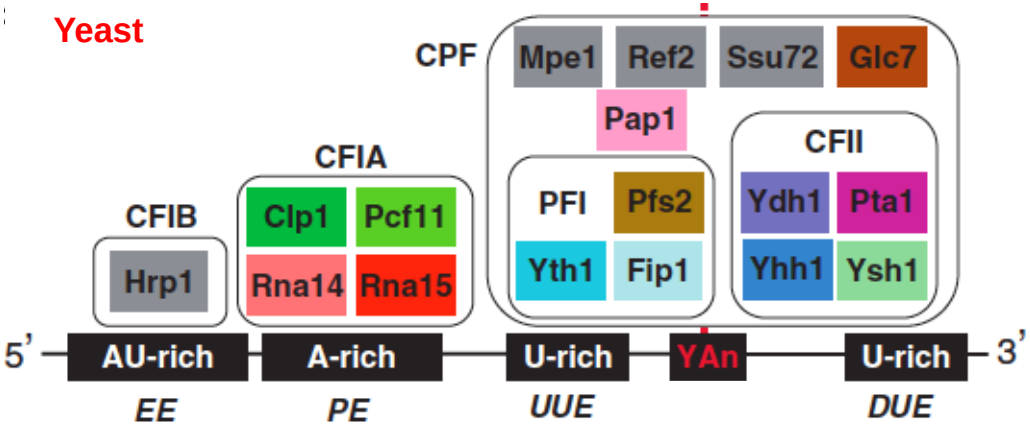
ncRNA



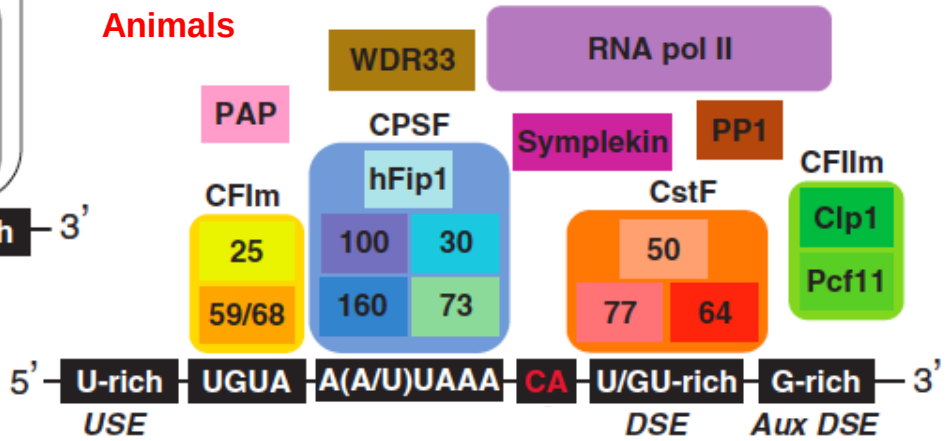
Jacquier, Nat. Rev. Genet, 2009

Cleavage and polyadenylation complex (CP) (recruitment via Ser2-P CTD)

Yeast



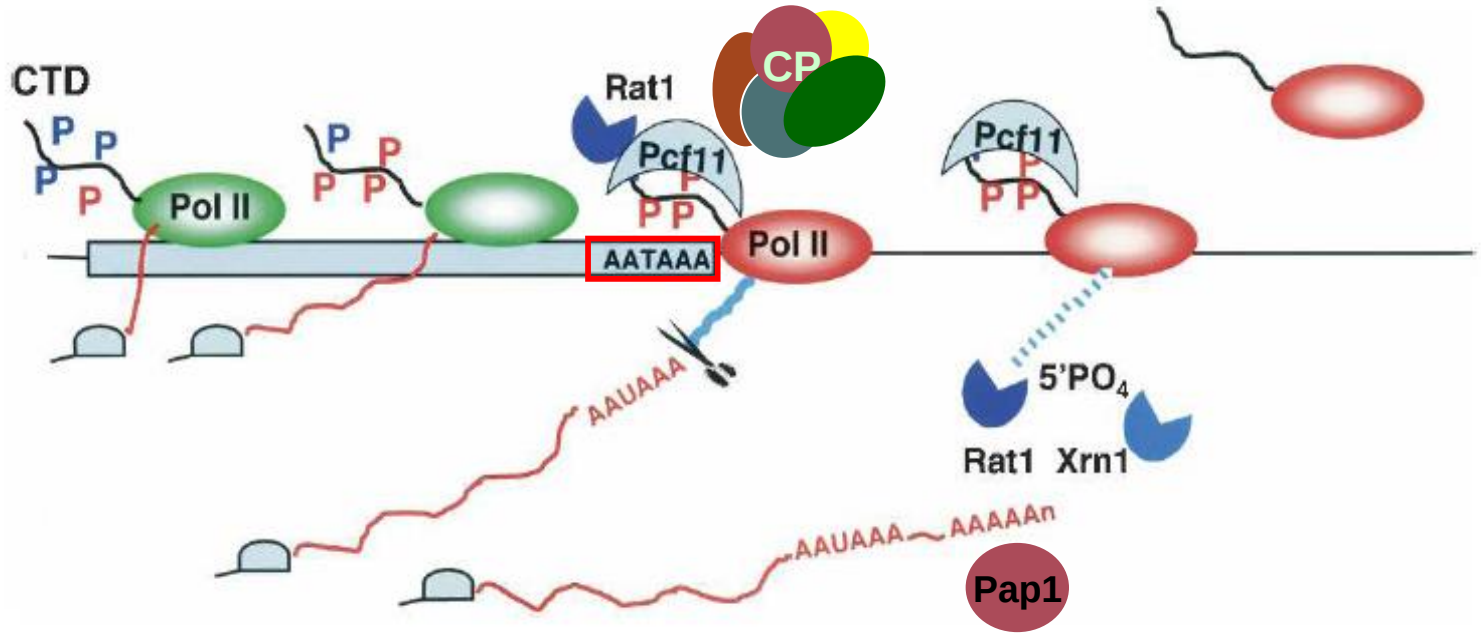
Animals



In yeast, cleavage by Brr5/Ysh1 and in humans by CPSF-73

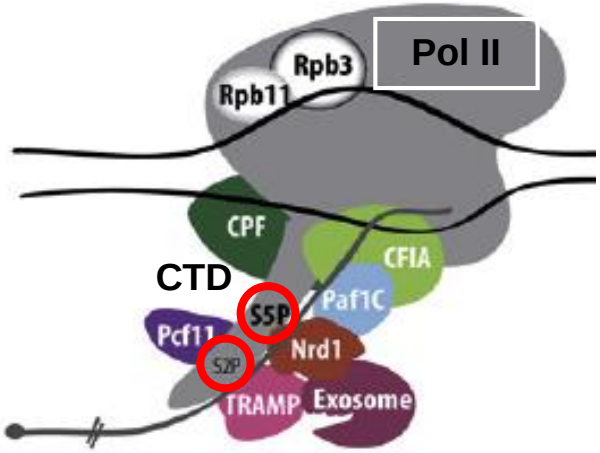
Millevoi and Vagner, NAR, 2008

Hybrid - allosteric-torpedo - model of RNA polymerase II transcription termination



Luo and Bentley, Gene Dev, 2006

Nrd1/Nab3/Sen1 dependent termination of pol II



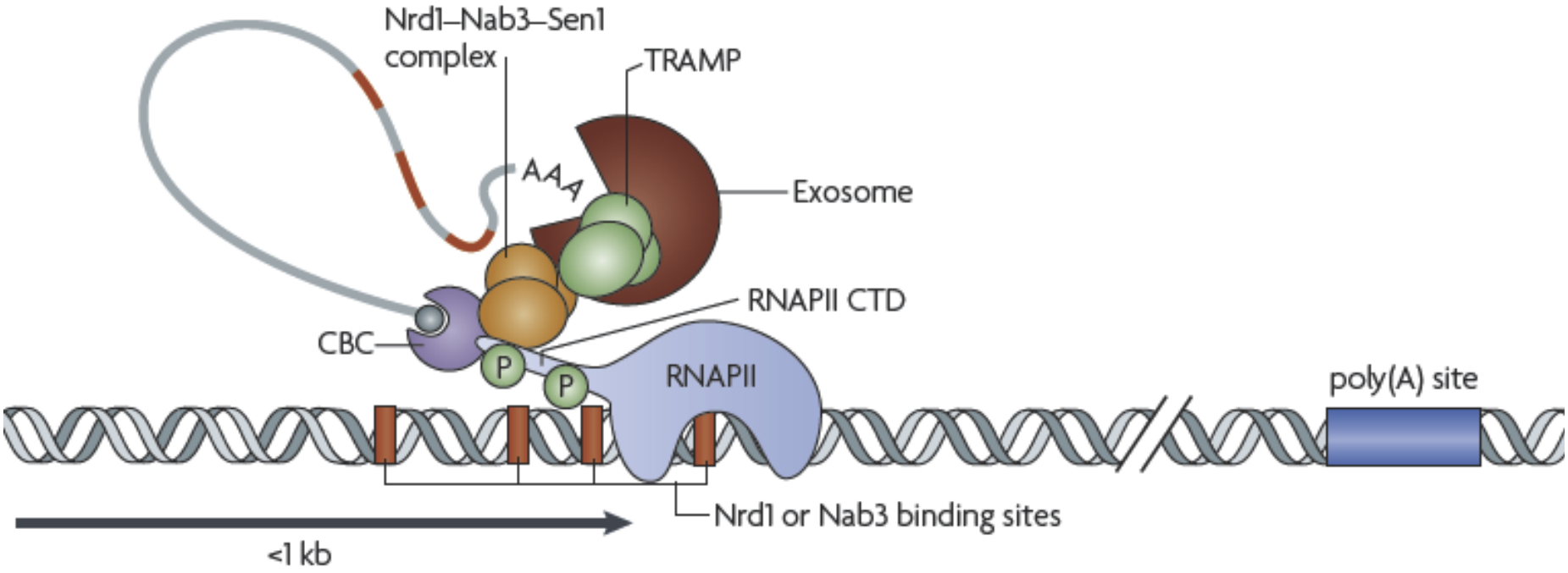
Nrd1/Nab3/Sen1 - NNS complex

- **sn/snoRNAs**
- **CUTs**
- **short mRNAs (< 600 nt)**

ncRNA

mRNA

(Recruitment through Ser5-P CTD)

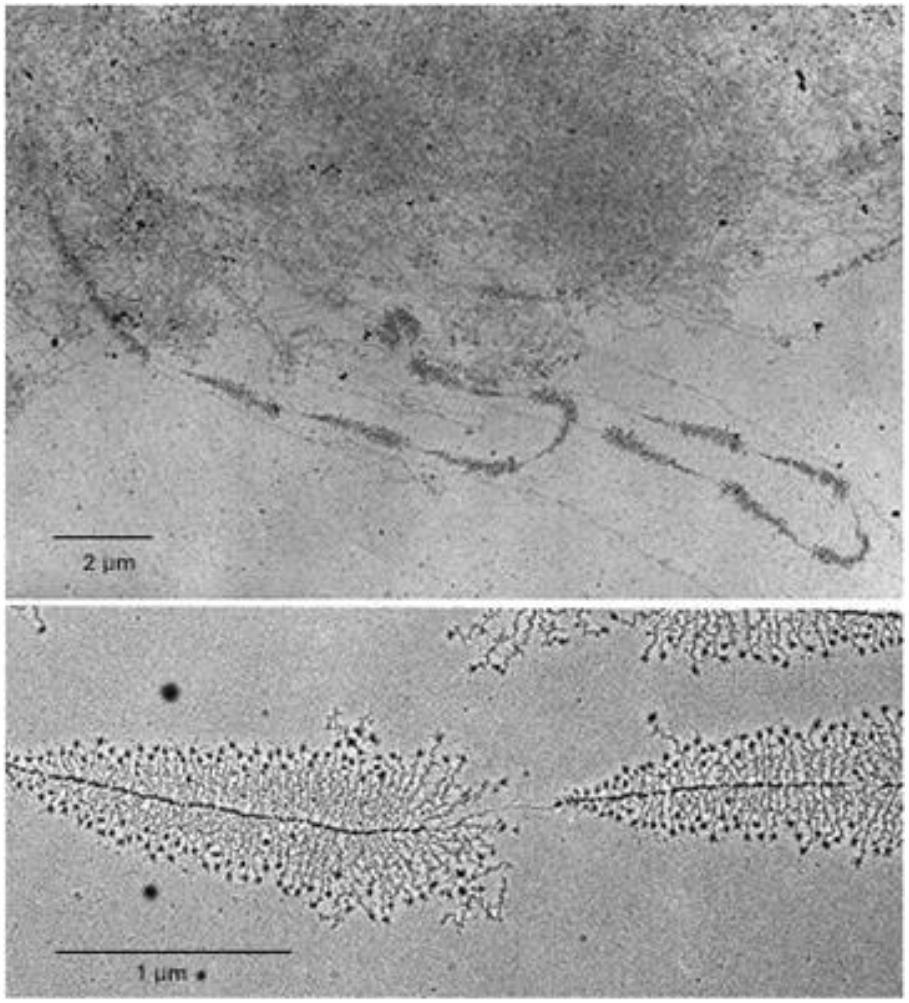


Jacquier, Nat. Rev. Genet 2009

Transcription termination of RNA polymerase I

rDNA genes in the nucleous

Miller's spreads - "Christmans tree".

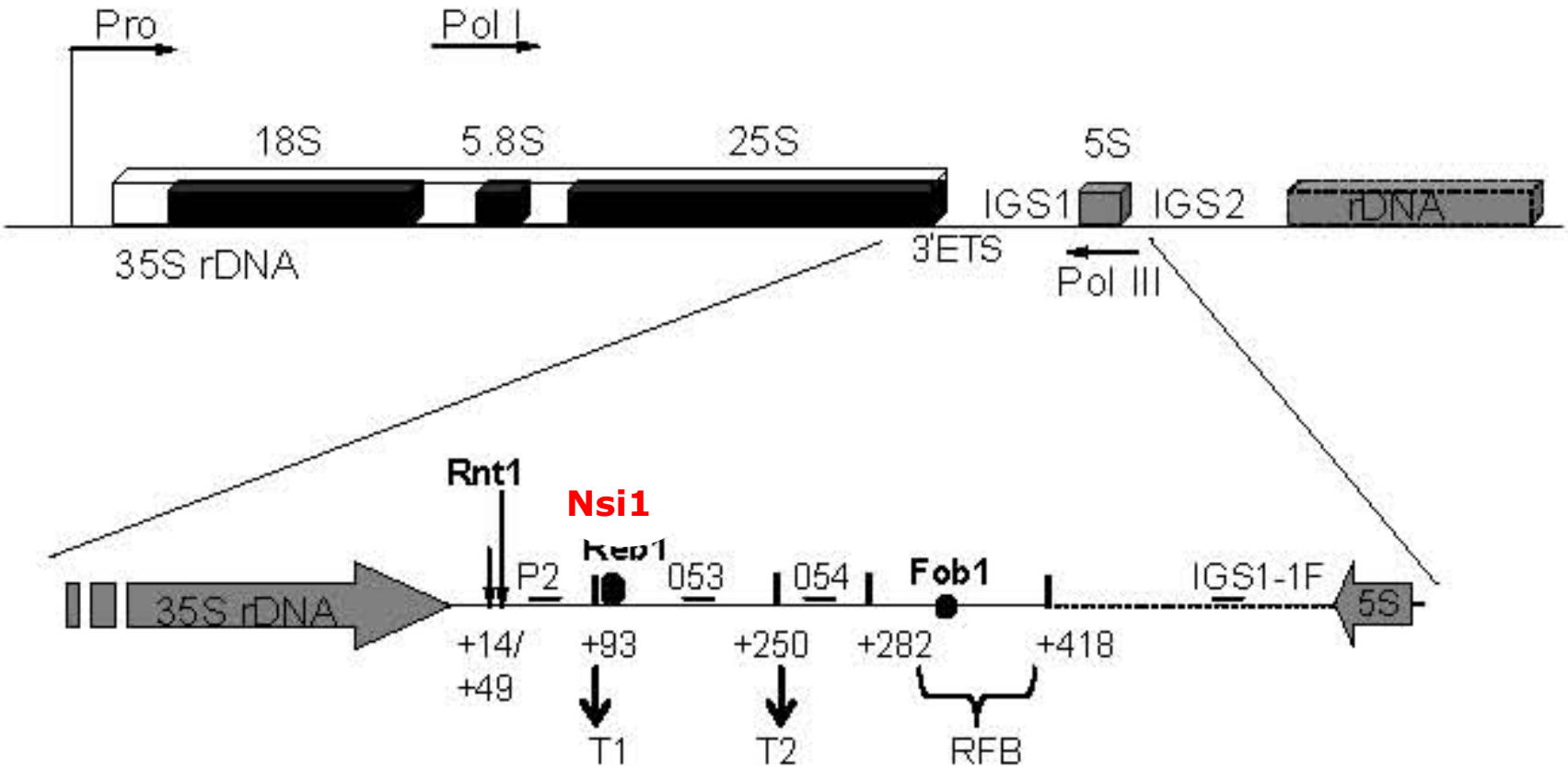


Top: V.E. Foe, Cold Spring Harbor Symp. Quant. Biol. 42:723-740, 1978; bottom: Ulrich Scheer, MBC on NCBI.

- **Pol I transcribes ribosomal RNA (35S rRNA)**
- **Pol I RNA – up to 80% of all transcriptional activity of the cell nucleus!!!**
- **In yeast, rDNA genes are organized in 150-200 tandem repeats**

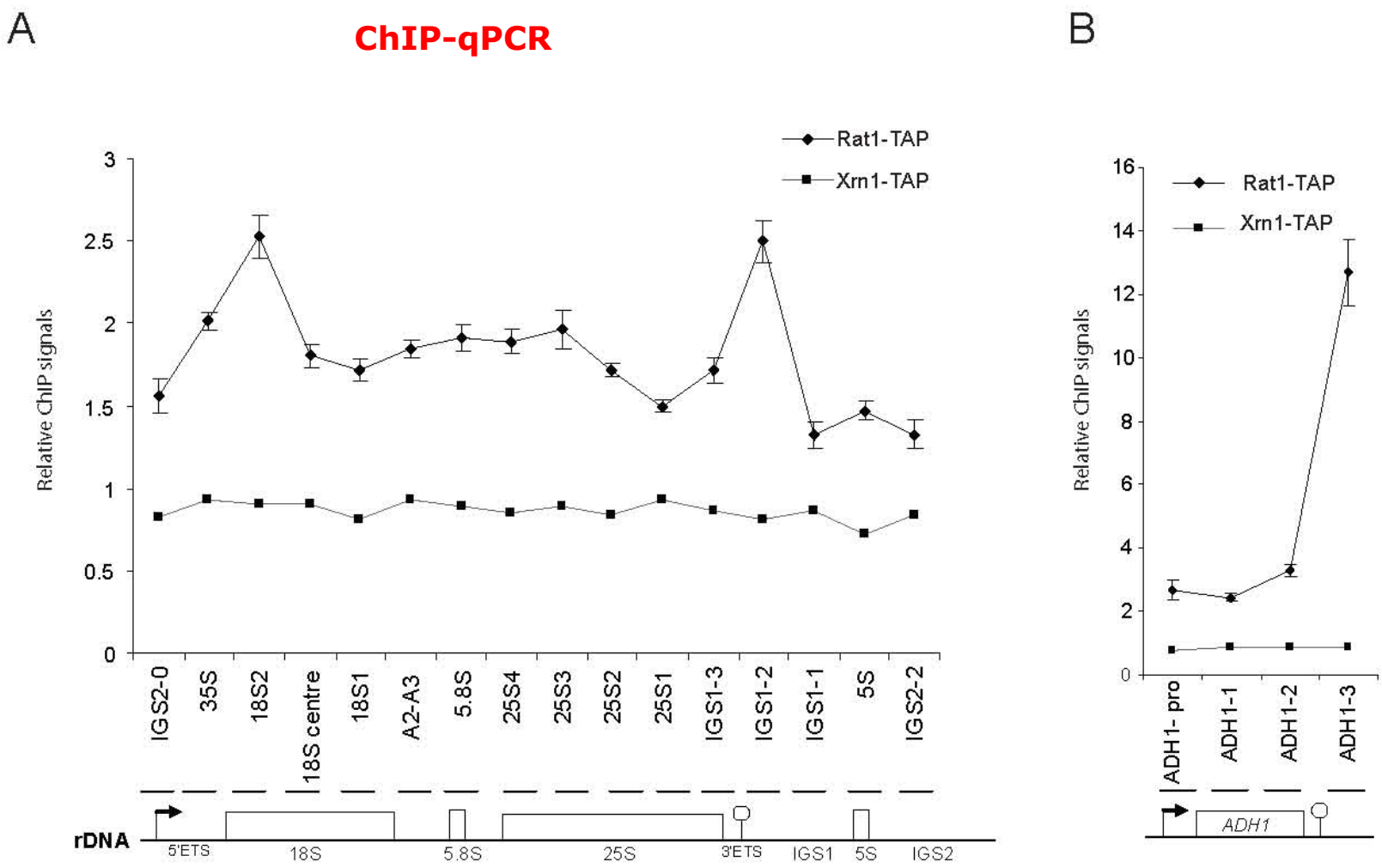
Schematic of an rDNA repeat in *S. cerevisiae*

with detailed transcription termination region



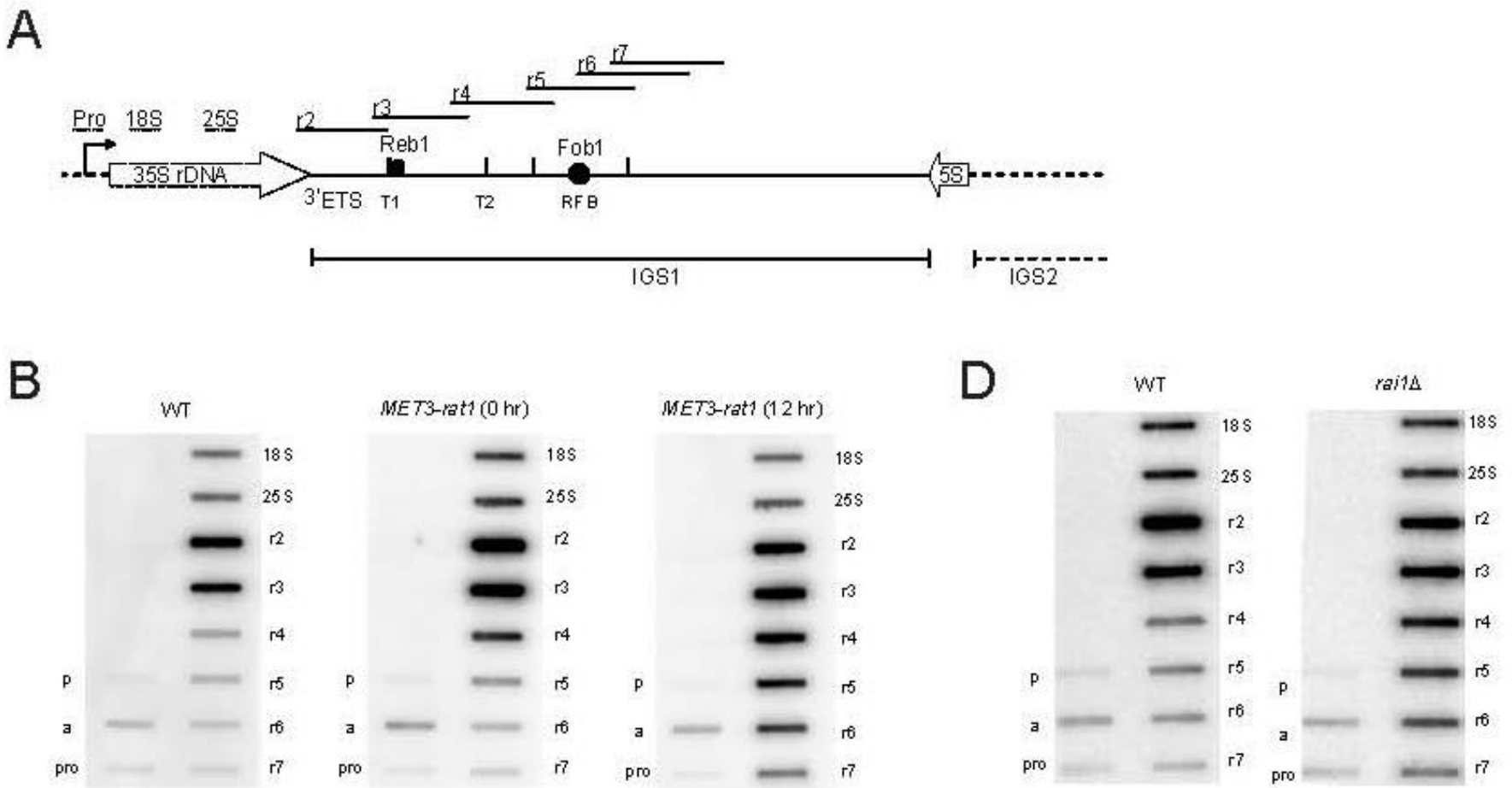
El Hage et al, Genes Dev., 2008,15;22(8)

Rat1 associates with the 3'-ETS region on the rDNA



El Hage et al, Genes Dev., 2008,15;22(8)

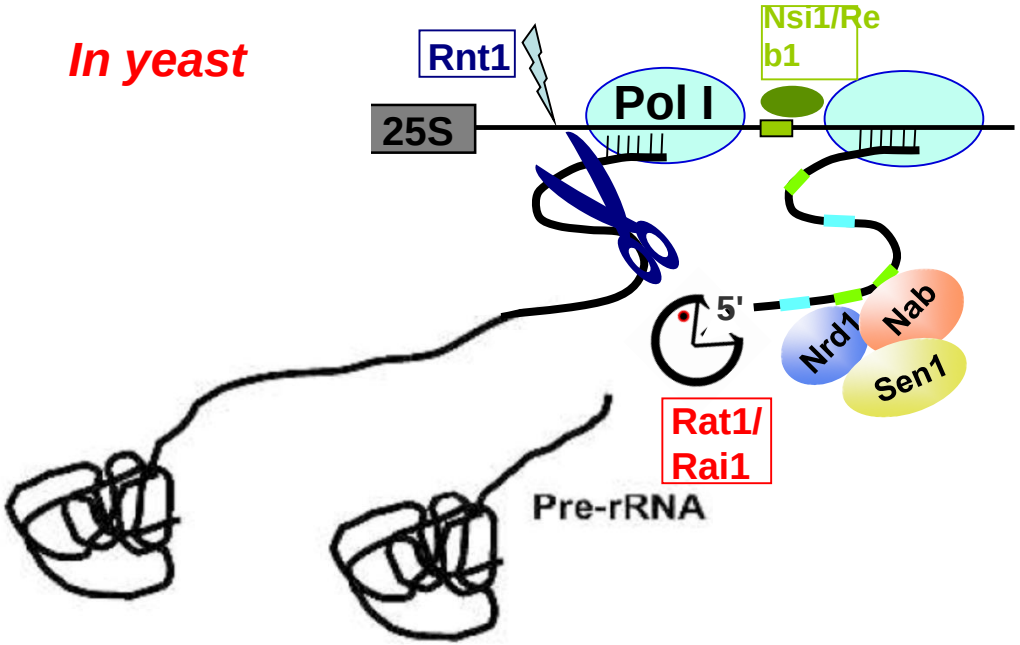
Transcription Run-On shows read-through of the major Pol I terminator in strains lacking Rat1 or Rai1



El Hage et al, Genes Dev., 2008,15;22(8)

RNA polymerase I transcription termination

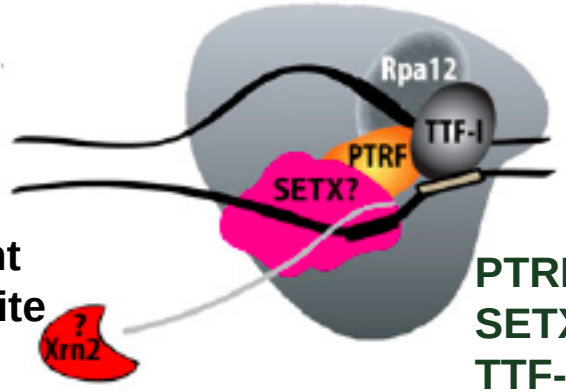
In yeast



Pol I termination factors:

- DNA-binding protein Nsi1
- Pol I subunit Rpa12
- endonuclease Rnt1
- RFB binding protein Fob1
- 5'-3' exonuclease Rat1/Rai1 *(torpedo mechanism)*
- RNA helicase Sen1
- Nrd1/Nab3 complex (??)

In mammalian cells

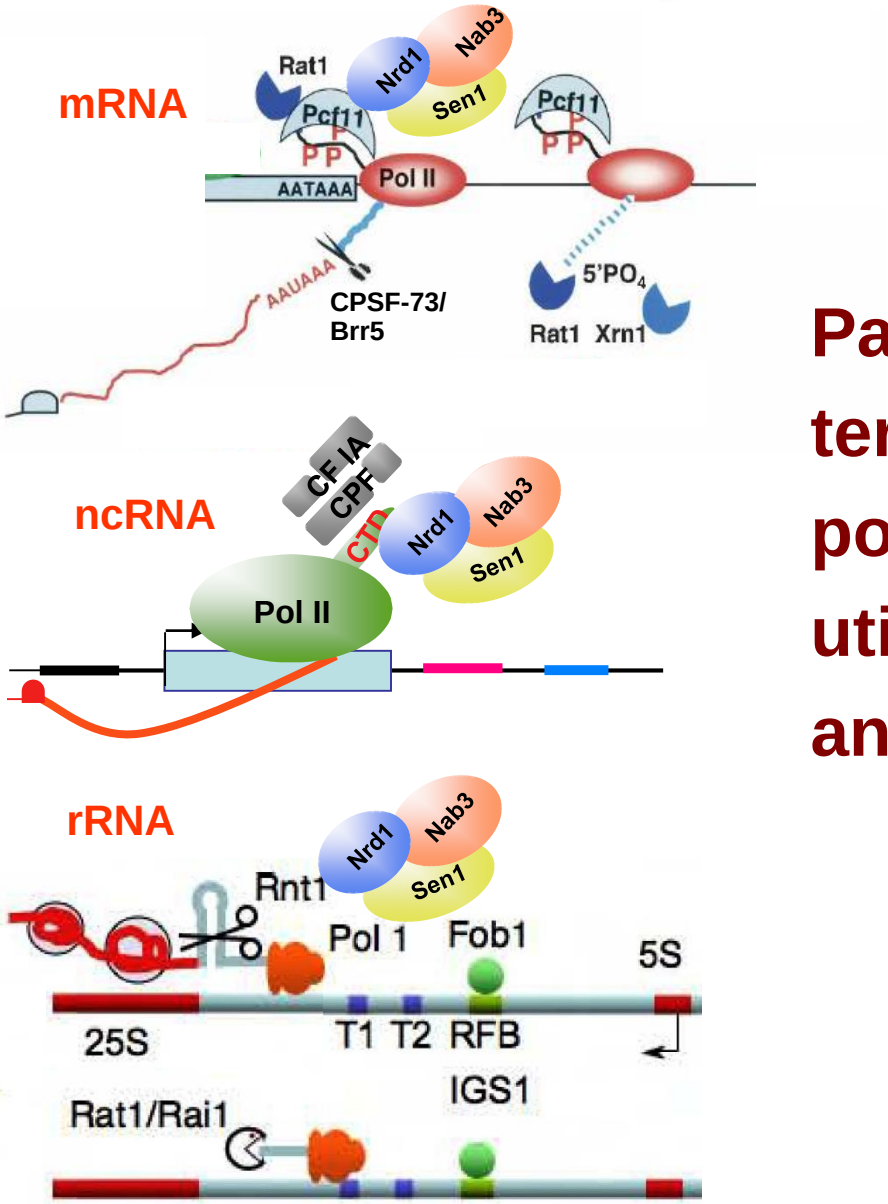


transcript release element
T-stretch + TTF-I pause site

PTRF - release factor
SETX - helicase, Sen1 homolog
TTF-I - transcription termination factor I

Richard and Manley, GeneDev., 2009

Conclusion - RNA pol I and RNA pol II „share" termination pathways



Pathways for transcription termination by different polymerases, Pol I and II, utilize overlapping factors and mechanisms

SUMMARY OF TECHNIQUES

The level of RNA in a cell is the result of its synthesis and degradation.

The fact that RNA polymerase is bound to DNA does not mean that it transcribes RNA.

To study a transcript at the time of its formation, various molecular techniques should be used:

TRO allows to study qualitatively and quantitatively the „ongoing" transcription (to detect nascent transcripts).

ChIP makes it possible to study *in vivo* the interaction of proteins with DNA.

RIP allows to study *in vivo* the interaction of proteins with RNA.

THANK YOU
FOR
YOUR ATTENTION