Chapt. 15 Eukaryotic mRNA Processing II: Capping and Polyadenylation

Student learning outcomes:
• Explain structure and function of caps
• Explain structure and function of poly(A)
• Describe function of cleavage/polyadenylation complex
• Appreciate complexity of termination by pol II
• Describe relationship of capping, splicing and cleavage/poly(A) to CTD of Rpb1 of pol II
• Figs: 1, 2, 3*, 4*, 8, 9, 10, 12*, 14, 15, 16, 19*, 25, 36*, 40
  problems: 1, 2, 3, 4, 5, 7, 8, 10, 11, 12, 13, 16, 19, 21, 23, 27, 30

15.1 Capping
• 1974, mRNAs from eukaryotes and viruses were found to be methylated at 5'-end = CAP
• Early studies used viral mRNA –
  – reovirus (ds RNA), vaccinia virus (ds DNA)
  – Both replicate in cytoplasm,
  – Easier to purify, investigate than cell mRNA
  Later Adenovirus
    (ds DNA, replicates nucleus)

Cap Structure
• Purified cap by DEAE chromatography; digested with enzymes to figure structure
  β-phosphate of NTP remains only in first nucleotide in RNA
  – Cap is 5'-terminus of RNA
  – Cap is m^7G, 7'-methylguanosine,
  – Linkage is triphosphate
  – Charge on cap area is ~ -5
  — Fig. 1 vaccinia virus
    3H methyl from S-AdoMet; 32P-GTP label RNA; KOH to digest RNA
  — Fig. 2 shows m^7-G
Reovirus Cap Structure
- m^7G (red) contributes positive charge
- Triphosphate linkage (5’-5’) contributes 3 negative charges
- Phosphodiester bond contributes 1 negative charge
- Terminal phosphate contributes 2 negative charges

Cap Synthesis
RNA triphosphatase removes terminal phosphate from pre-mRNA
Guanyl transferase adds capping GMP from GTP
Two methyl transferases methylate N^7 of capping G and 2’-O-methyl group of penultimate nucleotide
Occurs early in transcription, before chain is 30 nt long

4 Functions of Caps
- Protect mRNAs from degradation
- Enhance translatability of mRNAs
- Transport of mRNAs out of nucleus
- Efficiency of splicing mRNAs

Fig. 3 15-4
Fig. 6 15-4
Evidence of synergism between poly (A) and CAP: 
stabilize mRNA for translation

Luciferase (firefly enzyme) as reporter gene; prepare RNA in vitro; inject into tobacco cells; measure light units with luciferin.

<table>
<thead>
<tr>
<th>mRNA</th>
<th>Luciferase mRNA (nM, 30°, 15 min, A540)</th>
<th>Luciferase Activity (light units/µg protein)</th>
<th>Relative Effect of Poly(A) on Activity</th>
<th>Relative Effect of CAP on Activity</th>
</tr>
</thead>
<tbody>
<tr>
<td>Uncapped</td>
<td>Poly(A)−</td>
<td>31</td>
<td>2941</td>
<td>1</td>
</tr>
<tr>
<td></td>
<td>Poly(A)−</td>
<td>44</td>
<td>4400</td>
<td>1.5</td>
</tr>
<tr>
<td>Capped</td>
<td>Poly(A)−</td>
<td>53</td>
<td>62,955</td>
<td>7</td>
</tr>
<tr>
<td></td>
<td>Poly(A)−</td>
<td>100</td>
<td>1,331,917</td>
<td>7</td>
</tr>
</tbody>
</table>


15.2 Polyadenylation

- Addition of series of A residues to 3' end of RNA
- Heterogeneous nuclear mRNA - precursor to mRNA
- Most eukaryotic mRNAs and precursors have chain of AMP residues about 250 nt long at 3'-ends
- Poly(A) polymerase adds Poly(A) post-transcriptionally by (not encoded in genome as TTTTT...)

Fig. 7: electrophoresis of poly(A) from nuclear mRNA digested with T1 (after G) and A (after C, U) is ~200 nt long

Functions of Poly(A)

- Poly(A) enhances stability (half-life) and translatability of mRNA [yet histone mRNAs lack poly(A)]
- Polyadenylation also required for efficient transport of mRNAs from point of origin to cytoplasm
- Relative importance of these effects varies from one system to another

Fig. 9. poly(A) enhances stability. Globin mRNAs injected into Xenopus oocytes
In rabbit reticulocyte extracts, poly(A) enhances translatability by helping recruit mRNA to polysomes.

**Fig. 10.** VSV mRNAs incubated in rabbit reticulocyte extracts with $^{35}$S Met (protein); Fig. 11; VSV mRNAs with $^{32}$P-poly(A+) or $^3H$ (polyA-) in sucrose gradient; polysomes are big.

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**Basic Mechanism of Polyadenylation**

- Transcription extends beyond poly(A) site
- Pol II does not recognize terminators
- Transcript signals recognized cause:
  - Cleavage
  - Polyadenylation at 3’-end created by cleavage
  - 3’ RNA degraded

**Fig. 12 overview of Polyadenylation**

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**Globin transcription can extend at least 500 nt past poly(A) region**

**Fig. 14** Globin synthesis in cells $^{32}$P labeled; hybridized to different restriction fragments and measure molarities: see coding and downstream

Red = exon; yellow = introns
Polyadenylation Signals

- Pol II just keeps synthesizing mRNA
- Cleavage/poly(A) factors bind
- Mammalian polyadenylation signal has:
  - AAUAAA motif about 20 nt upstream of polyadenylation site in pre-mRNA;
  - Followed 23 or 24 bp later by GU-rich motif
  - Followed immediately by a U-rich motif

Cleavage of Pre-mRNA

- Polyadenylation involves:
  - Pre-mRNA cleavage
  - Polyadenylation at cleavage site
- Cleavage in mammals requires several proteins:
  - CPSF – cleavage and polyadenylation specificity factor
  - CstF – cleavage stimulation factor
  - CF I
  - CF II
  - Poly (A) polymerase
  - RNA Pol II CTD

Initiation of Polyadenylation

- Short RNAs that mimic newly created mRNA 3'-end can be polyadenylated (inc. size on gels)
- 2 proteins participate in initiation process
  - Separated by chromatography; functional tests
  - Poly(A) polymerase (DE-100 fraction)
  - CPSF binds to AAUAAA motif (DE-600 fraction)
**Two phases of polyadenylation**

- AAUAAA is needed for first phase, best if followed by 8 nt
- If have template >10 nt, as A40, can be extended even if the AAUAAA is mutated
- Template with X40 will not be extended

**Elongation of mammalian Poly(A) requires PAB II (poly(A)-binding protein II)**

- PAB II (49-kD)
  - Binds preinitiated oligo(A)
  - Aids poly(A) polymerase in elongating poly(A) to 250 nt or more
- PAB II acts independently of AAUAAA motif
  - Depends only on poly(A)
  - Activity enhanced by CPSF

**Polyadenylation Model**

- Factors assemble on pre-mRNA guided by AAUAAA and GU/U motifs
- Cleavage occurs
- Polymerase initiates poly(A) synthesis
- PAB II allows rapid extension of oligo(A) to full-length
Poly(A) Polymerase

- Cloning and sequencing cDNAs encoding calf thymus poly(A) polymerase reveal mixture of 5 cDNAs derived from alternative splicing and alternative polyadenylation
- Structures of enzymes predicted from longest sequence (82-kD = PAPII) includes:
  - RNA-binding domain
  - Polymerase module
  - 2 nuclear localization signals
  - Ser/Thr-rich region – dispensable for activity in vitro

Turnover of Poly(A)

- Poly(A) turns over in cytoplasm; gets shorter
- RNases tear it down
- Cytoplasmic poly(A) polymerase builds it back up
- When poly(A) is gone, mRNA is slated for destruction

Fig. 26 Cytoplasmic poly(A) is shorter; gel electrophoresis of poly(A) of HeLa cells; 3H-Ade

Many proteins are required for 3’-cleavage and polyadenylation in mammals

<table>
<thead>
<tr>
<th>Factor</th>
<th>Polypeptide</th>
<th>Polarity</th>
<th>Properties</th>
</tr>
</thead>
<tbody>
<tr>
<td>Poly(A) polymerase (PAP)</td>
<td>82</td>
<td>Required for 3’-cleavage and polyadenylation, catalyzes poly(A) synthesis</td>
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<tr>
<td>Cleavage and polyadenylation specificity factor (CPSF)</td>
<td>73</td>
<td>Required for cleavage and polyadenylation, binds to AAUAA and interacts with PAP and CFII</td>
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<tr>
<td>Cleavage stimulation factor (CSTF)</td>
<td>77</td>
<td>Required for cleavage and polyadenylation, binds to downstream element and interacts with CPSF</td>
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</tr>
<tr>
<td>Cleavage factor I (CFI)</td>
<td>69</td>
<td>Required only for cleavage, binds RNA</td>
<td></td>
</tr>
<tr>
<td>Cleavage factor II (CFII)</td>
<td>59</td>
<td>Required only for cleavage, binds RNA</td>
<td></td>
</tr>
<tr>
<td>Cleavage factor II (CFII)</td>
<td>59</td>
<td>Required only for cleavage, interacts with poly(A) polymerase</td>
<td></td>
</tr>
<tr>
<td>RNA polymerase II mediator (CTD)</td>
<td>Many</td>
<td>Required only for polyadenylation</td>
<td></td>
</tr>
<tr>
<td>Poly(A) binding protein I (PAP-I)</td>
<td>49</td>
<td>Stimulates poly(A) polymerase, binds growing poly(A) tail, essential for poly(A) tail length control</td>
<td></td>
</tr>
</tbody>
</table>

15.3 Coordination of mRNA Processing Events

- Capping, polyadenylation and splicing are related
- Cap can be essential for splicing, only for first intron
- Poly(A) can be essential, only for splicing last intron

- All 3 processing events occur during transcription
  - Splicing begins when transcription is underway
  - Capping
    - When nascent mRNA is about 30 nt long
    - When 5’-end of RNA first emerges from pol II
  - Polyadenylation occurs when still-growing mRNA is cut at polyadenylation site

Cap preferentially increases excision of first intron

Fig. 29 cloned exons from chicken d-crystallin gene in plasmid; transcribe in vitro to prepare splicing substrates

Poly(A) affects splicing of last intron

Fig. 31 ADML substrates; in vitro reactions; SB2 has no AAUAAA
CTD of Rpb1 binds to mRNA-Processing Proteins

- CTD of Rpb1 subunit of RNA pol II involved in all 3 types of processing
- Capping, poly(A), splicing enzymes bind directly to CTD, which serves as platform for all activities
- Chromatin immunoprecipitation (ChIP) assays show where proteins are bound: antibodies against different factors precipitate cross-linked complex, then PCR with primers for different regions of genes.

ChIP assays show location of yeast proteins binding CAP, poly(A), or pol II

Proteins associated with CTD change:
- Ceg1, Capping guanylyl transferase present when complex close to promoter,
- Polyadenylation factor Hrp1 present in complexes near and remote from promoter

Fig. 34: Ceg1 is CAP enzyme; Hrp1 is polyA factor; Rpb3 is pol II
Pol II all along coding regions; CAP more localized
Buratowski expts.

Model: RNA Processing Organized by pol II CTD

Phosphorylation state of CTD changes during transcription:
- Transcription complexes close to promoter contain P-Ser5 (of 7 as in heptad repeat)
- Complexes farther from promoter contain P-Ser2

Fig. 36 Model of CTD involved in organizing capping, splicing factors; phosphorylation state of CTD organized
Mechanism of Termination; coupled to cleavage, less so to poly(A) addition

- Termination of transcription by pol II:
  - Transcript experiences cotranscriptional cleavage (CoTC) within termination region downstream of poly(A) site – CoTC is ribozyme
    - before cleavage and polyadenylation
    - independent of that process
    - entry site for Xrn2, 5'→3' exonuclease that loads onto RNA and chases pol II by degrading RNA
  - Cleavage & polyadenylation occur at poly(A) site
    - Signals polymerase to dissociate from template

Torpedo Model for Transcription Termination

Fig. 40. After cleavage/poly(A) of mRNA, CoTC self-cleaves, Xrn2 gets on and degrades RNA until contacts pol II > termination, release

Review questions

- Draw diagram of polyadenylation process, beginning with RNA being elongated past poly(A) site.

- Describe experiment that shows size of poly(A)

- Describe experiment that shows importance of AAUAAA polyadenylation motif.

- Outline steps in capping; what are major roles of Cap?