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An investigation into the role of ATP in the mammalian pre-mRNA 3' cleavage reaction

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Abstract

RNA Polymerase II transcribes beyond what later becomes the 3' end of a mature messenger RNA (mRNA). The formation of most mRNA 3' ends results from pre-mRNA cleavage followed by polyadenylation. In vitro studies have shown that low concentrations of ATP stimulate the 3' cleavage reaction while high concentrations inhibit it, but the origin of these ATP effects is unknown. ATP might enable a cleavage factor kinase or activate a cleavage factor directly. To distinguish between these possibilities, we tested several ATP structural analogs in a pre-mRNA 3' cleavage reaction reconstituted from DEAE-fractionated cleavage factors. We found that adenosine 5'-(β,γ -methylene)triphosphate (AMP-PCP) is an effective in vitro 3' cleavage inhibitor with an IC_{50} of ~ 300 μ M, but that most other ATP analogs, including adenosine 5'-(β,γ -imido)triphosphate, which cannot serve as a protein kinase substrate, promoted 3' cleavage but less efficiently than ATP. In combination with previous literature data, our results do not support ATP stimulation of 3' cleavage through cleavage factor phosphorylation in vitro. Instead, the more likely mechanism is that ATP stimulates cleavage factor activity through direct cleavage factor binding. The mammalian 3' cleavage factors known to bind ATP include the cleavage factor II (CF II_m) Clp1 subunit, the CF Im25 subunit and poly(A) polymerase alpha (PAP). The yeast homolog of the CF II_m complex also binds ATP through yClp1. To investigate the mammalian complex, we used a cell-line expressing FLAG-tagged Clp1 to co-immunoprecipitate Pcf11 as a function of ATP concentration. FLAG-Clp1 co-precipitated Pcf11 with or without ATP and the complex was not affected by AMP-PCP. Diadenosine tetraphosphate (Ap4A), an ATP analog that binds the Nudix domain of the CF Im25 subunit with higher affinity than ATP, neither stimulated 3' cleavage in place of ATP nor antagonized ATP-stimulated 3' cleavage. The ATP-binding site of PAP was disrupted by site directed mutagenesis but a reconstituted 3' cleavage reaction containing a mutant PAP unable to bind ATP nevertheless underwent ATP-stimulated 3' cleavage. Fluctuating ATP levels might contribute to the regulation of pre-mRNA 3' cleavage, but the three

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Conflict of interest

We confirm that there is no conflict of interest.

subunits investigated here do not appear to be responsible for the ATP-stimulation of pre-mRNA cleavage.

Keywords

Pre-mRNA processing; Cleavage and polyadenylation; 3' End formation; ATP

1. Introduction

Eukaryotic pre-messenger RNAs (pre-mRNAs) undergo endonucleolytic cleavage at one or more specified positions downstream from the stop codon during mRNA biogenesis [1,2]. This 3' cleavage reaction defines the downstream end of the 3'-untranslated region (3'-UTR), and prepares most pre-mRNA for polyadenylation. In yeast and in mammalian cells, several large multi-subunit protein complexes responsible for these two reactions have been identified and characterized (for reviews, see Refs. [1–4]). The mammalian complexes were first fractionated on chromatography media such as DEAE-sepharose, and named according to functions identified through the use of the in vitro reconstituted 3' cleavage reaction [5–8]. These multi-protein complexes include the cleavage polyadenylation specificity factor (CPSF), the cleavage stimulation factor (CstF) and the mammalian cleavage factors I and II (CF I_m and CF II_m, or when not separated from one another, CF_m). Symplekin, thought to fulfill a scaffolding role during 3' cleavage, is present in DEAE-separated CPSF, and poly(A) polymerase (PAP), which is required for all substrates tested in vitro so far except one, co-elutes in the flow-through with CstF during DEAE-sepharose fractionation. The C-terminal domain of the largest RNA polymerase II subunit is also necessary, but its requirement can be replaced in vitro by a high concentration of creatine phosphate [9]. Most of the yeast cleavage factor proteins, or their protein sub-domains, have orthologs among the mammalian cleavage factors [1,4,10,11]. Despite differences in the *cis*-acting pre-mRNA 3' sequences and the primary structures of the various cleavage factors, the basic biochemical reactions of cleavage and polyadenylation are conserved from yeast to mammals. In vivo, the cleavage and polyadenylation reactions are coupled to transcription [12] and to one another, but the two individual reactions – cleavage and polyadenylation – are not mechanistically coupled, and can be studied separately in vitro [13].

Despite many years of study, some fundamental questions pertaining to the 3' cleavage step remain unanswered. One of these is the role that ATP plays in the reaction. While polyadenylation obviously requires ATP, a role for ATP in the 3' cleavage step has not been clear. During the early development of the in vitro 3' cleavage cell-free system, where a substrate containing the adenovirus-2 late 3 (L3) pre-mRNA sequence was used, ATP was found to be required for the cleavage reaction in HeLa nuclear extract [13]. The ATP concentration-dependence of the reaction was complex: stimulation was evident from 0 to 1 mM, but between 1 and 5 mM a maximum was reached, after which dose-dependent inhibition by ATP was found, with complete inhibition observed at 10 mM. Since cellular ATP ranges from about 1 to 10 mM [14,15], this behavior suggests the possibility of both positive and negative regulation of 3' cleavage within the normal range of cellular ATP concentrations.

Other 3' pre-mRNA substrates were subsequently found to require ATP for correct cleavage in HeLa nuclear extract [16–18] and in yeast whole cell extract [19]. The in vitro substrates requiring ATP included those from the herpes simplex virus 1 thymidine kinase pre-mRNA [18] and the simian virus 40 early (SV40E) pre-RNA [16,17]. However, employing another commonly used substrate from the SV40 late (SV40L) pre-mRNA [20], ATP was found to be unnecessary for in vitro 3' cleavage [7,21]. In one study, which in contrast to most ATP studies used partially purified HeLa cleavage factors rather than nuclear extract, the effect of ATP on cleavage of the SV40L and L3 substrates was starkly different [21]. While ATP was not required for SV40L cleavage, it strongly enhanced the in vitro cleavage of the L3 substrate. Without added ATP, only trace levels of L3 cleavage were observed. This was taken as evidence that the 3' cleavage reaction was an ATP-independent process [21]. These two viral substrates, SV40L and L3, have been commonly used in place of mammalian substrates because they work well in vitro, but it is not known which one might be more representative of the RNA sequence surrounding the typical mammalian polyadenylation site. It has been suggested that L3 is the more representative [21], implying that ATP is generally required for efficient 3' cleavage, at least in vitro. The biological significance of why ATP is needed for all substrates except SV40L is odd, but this inconsistency may pertain more to SV40L than to mammalian gene expression.

We previously found that dephosphorylating HeLa cell nuclear extract, and more specifically, a mammalian Cleavage Factor I and II fraction (CF_m) derived from it by DEAE chromatography, led to the loss of in vitro cleavage activity [22]. We interpreted this result to mean that one of the mammalian CF_m subunits must be phosphorylated in order to carry out its function in the cleavage reaction. The phosphorylation state of any such cleavage factor might be set during cell growth, prior to extract preparation, but since protein kinases use ATP as a phosphate donor, it is at least formally possible that ATP stimulates L3 cleavage in vitro by serving as a phosphate donor for a cleavage factor kinase. An argument against this possibility is that protein Ser/Thr kinases require magnesium for phospho transfer from ATP [23,24], while the in vitro 3' cleavage reaction is typically carried out at concentrations between 2 and 8 mM of EDTA, a divalent cation chelator [21]. EDTA binds Mg²⁺ more avidly (K_d 1.2 μM) [25] than ATP binds Mg²⁺ (K_d 50 μM) [26] such that, under millimolar EDTA equilibrium conditions, no significant amount of Mg-ATP can be available to a kinase for catalysis. Alternatively, it was long ago suggested that ATP might enhance in vitro cleavage allosterically [17], while not undergoing hydrolysis or other chemical modification during the cleavage reaction. This possibility is also suggested by cleavage studies in yeast, where ATP is required [27] and the ortholog of human Clp1, a CF II_m subunit, has been found by X-ray crystallography in a complex with ATP [28,29]. Though mammalian Clp1 is an ATP-dependent RNA kinase [30], the yeast ortholog is not [31,32]. Thus, ATP might stabilize the fold of mammalian Clp1, or stabilize its complexation with the CF II_m Pcf11 subunit, or play such a role for one of the other cleavage factors. In the present study, we test hypotheses seeking to explain how, in the range of 1 mM, ATP enhances the 3' cleavage of the L3 substrate in vitro. Our results lead us to favor the hypothesis that ATP enhances in vitro cleavage through a binding-only role. However, we present evidence that ATP is not needed to maintain the integrity of the mammalian Clp1-Pcf11 CF II_m complex, and that ATP does not exert its effect through the PAP II ATP-binding site.

2. Materials and methods

2.1. Buffer composition and protein determination

The following buffers were used: Buffer D: 20 mM Na-HEPES, pH 7.9, 20% glycerol, 0.2 mM PMSF, 0.5 mM DTT, 0.2 mM EDTA, 50 mM $(\text{NH}_4)_2\text{SO}_4$; Buffer D_{10Glyc}: Buffer D with only 10% glycerol; Buffer D_{40AS}, Buffer D with only 40 mM $(\text{NH}_4)_2\text{SO}_4$; His₆-Tag Native Lysis Buffer: 50 mM NaH_2PO_4 pH 8.0, 300 mM NaCl, 10 mM imidazole; His₆-Tag Native Wash Buffer: lysis buffer but with 20 mM imidazole; His₆-Tag Elution Buffer: lysis buffer but with 250 mM imidazole; Sodium-Chloride-Tris-EDTA (STE) Buffer: 10 mM Tris pH 8.0, 50 mM NaCl, 1 mM EDTA; Phosphate Buffer Saline: 137 mM NaCl, 2.7 mM KCl, 10 mM Na_2HPO_4 , 1.8 mM KH_2PO_4 pH 7.4. Total protein concentration of nuclear extract, cleavage factor fractions, wild-type and mutant PAP protein purifications was determined using the Bio-Rad Bradford protein assay with BSA standards.

2.2. Preparation of HeLa cell nuclear extract and cleavage factors fractionation

HeLa cell pellets were purchased from the National Cell Culture Center (Biovest International). Nuclear extract was prepared as described [7]. Cleavage factor activities (CPSF, CstF, CF_m) were fractionated from Buffer D_{40AS}-dialyzed HeLa nuclear extract on a DEAE-sepharose column, followed by $(\text{NH}_4)_2\text{SO}_4$ precipitation and dialysis into Buffer D, as previously described [22,33]. The CSF fraction was prepared from the 20–40 $(\text{NH}_4)_2\text{SO}_4$ fraction as described [8] with minor modifications: one ml of the 20–40 $(\text{NH}_4)_2\text{SO}_4$ fraction (≈ 10 mg protein) was passed through a Superose 6 column (10/300 GL, GE Healthcare) equilibrated with Buffer D containing 200 mM $(\text{NH}_4)_2\text{SO}_4$, and 0.5 ml fractions were collected. Adjacent fractions were pooled and 0.37 g of solid $(\text{NH}_4)_2\text{SO}_4$ was added to each tube, gently agitated for 1 h at 4 °C followed by centrifugation (13,200 rpm, 20 min, at 4 °C). The precipitate was resuspended in 100 μl of Buffer D and dialyzed against the same buffer. Active CSF fractions were identified using in vitro cleavage of the SV40L substrate (see below). PAP fractions, eluting after CSF, were identified using a non-specific in vitro polyadenylation assay [34].

2.3. The 3' pre-mRNA cleavage reactions

Capped, uniformly ^{32}P -labeled pre-mRNA SV40L and L3 substrates were made by in vitro transcription from linearized plasmids pG3SVL-A and pG3L3-A [7]. In vitro pre-mRNA 3' cleavage reactions were carried out as previously described [22,33,35]. The total reaction volume was 12.5 μl and in place of nuclear extract the following DEAE-fractionated cleavage factors (~ 2 –4 mg/ml total protein each) were included: 0.9 μl of CPSF, 0.45 μl of CstF, 1.8 μl of CF_m. HeLa PAP elutes from DEAE-sepharose to some extent with CstF in the flow-through but to be sure there was sufficient PAP present when the DEAE cleavage factors were used with the L3 substrate, ~ 5 ng of recombinant PAP (see below) was included. Relative Cleavage (R.C.) was defined as $(5' \text{ fragment/uncleaved}) \times 100$, where gel band values were taken from a Molecular Dynamics Phosphorimager volume measurements. Nucleotides were included at the indicated final concentration. In reactions testing PAP mutants, 5 μl CSF were used per 12.5 μl reaction.

2.4. ATP and analogs

Stock solutions were made in MilliQ (EMD Millipore) de-ionized water, with or without buffer as indicated below, and stored at -80°C . ATP was dissolved in water and the pH was adjusted to ~ 7 using concentrated NaOH and pH paper. The final stock concentration for ATP (and all analogs) was estimated by OD_{260} using an extinction coefficient of 15,400. A 100 mM, pH 7.4 stock solution of 2'-dATP was purchased from Fermentas/ThermoFisher (99%). Sources for other analogs were as follows: AMP-CPP (Sigma-Aldrich cat. M6517, 94% according to manufacturer, no information on residual ATP given, dissolved in 2 mM Tris-HCl, pH7). AMP-PCP (Sigma-Aldrich cat. M7510, >95% pure according to the manufacturer, no information on residual ATP given, dissolved in water). γ -S-ATP (MP Biomedical cat. 159551, 85% pure, contains 0.3% ATP according to the manufacturer, dissolved in 2 mM Tris-HCl, pH7). Cordycepin triphosphate (Sigma-Aldrich cat. C9137, 95%, no information on residual ATP given, dissolved in water and then extracted with phenol/ CHCl_3 followed by pure CHCl_3). Ap4A (Sigma-Aldrich D1262, 98% pure, dissolved in water). AMP-PNP (Sigma-Aldrich cat. A2647; tetralithium salt, 93% pure according to the manufacturer, no information on residual ATP given, dissolved in water). Since AMP-PNP is both non-hydrolyzable and gave a positive cleavage result in place of ATP, we used ^{31}P NMR to check for residual ATP after the in vitro cleavage was completed. The stock solution was stored at -80°C for several months between the cleavage reactions and when the NMR spectrum could be taken. The sample was found to contain approximately 22% AMPPNH_2 , a hydrolysis breakdown product and possible synthetic by-product [36] of AMP-PNP, and a commensurate amount of inorganic phosphate, but no ATP was detected. Similarly, the ATP sample after the same amount of time under the same storage conditions (but buffered) showed no hydrolysis or impurities. At the time AMP-PNP was used in 3' cleavage, it therefore contained between 78% and 86% AMP-PNP and no ATP. Likewise, the AMP-PCP showed no trace of ATP by ^{31}P NMR.

2.5. pIRESneo3-Clp1-3XFlag plasmid construction

The expression vector pIRESneo3 (Clontech) was used to establish a Clp1-expressing stable cell line. To enable co-immunoprecipitation experiments, a 3X-FLAG tag was inserted into the C-terminus of human Clp1 cDNA. The expression plasmid was constructed as follows: The pIRESneo3 plasmid was first modified by ligating two complementary 85-nt DNA oligonucleotides encoding BamHI-XhoI-3XFLAG-Stop-NotI into the BamHI and NotI sites of pIRESneo3, to form plasmid pIRESneo3-3X-FLAG. The ligated adaptor was: Sense: 5' phosphoGATCCGCCTCGAGTGACTACAAAGACCATGACGGTGATTATAAAGATCATGATATCGATTACAAG GATGACGATGACAAGTAGGC3'; Antisense: 5' phosphoGGCCGCCTACTTGTCATCGTCATCCTTGTAATCGATATCATGATCTTTATAATCACC GTCATGGTCTTTGTAGTCACTCGAGGCG3'. The ligation junctions of this plasmid were verified by Sanger sequencing. Separately, the cDNA of the hClp1 open reading frame was amplified from the full length cDNA (NCBI NM_006831, Open Biosystems, clone IHS1380-97432823, a.k.a. LIFESEQ258153) using PCR primers containing EcoRI and XhoI restriction sites at the 5' and 3' ends, respectively, and high fidelity Pfu taq (Invitrogen). The sequences for Clp1 PCR primers were: Forward: 5'-TTAGAATTCGCCACCATGGGAGAAGAGGCTAATGATGAC3'; Reverse: 5'-TTACTCGAGGCCTTCAGATCCATGAACCGGATATCC3'. The resulting PCR product

was digested with EcoRI and XhoI, cloned into the pcDNA3.1.myc-His plasmid (Invitrogen), and then subcloned into the pIRES-3X-FLAG EcoRI and XhoI sites. A clone was selected and its entire hClp1 ORF and ligation junctions were verified by sequencing. This plasmid, pIR-ESneo3-Clp1-3XFLAG, was used to generate stably-transfected HeLa cells.

2.6. Stable cell line establishment (Clp1-FLAG cell line)

A HeLa cell line stably expressing hClp1-3XFLAG was established as follows. HeLa cells (JW36, ATCC) were grown in DMEM containing 10% cosmic calf serum (CCS), 10 I.U./ml penicillin and 10 µg/ml streptomycin (PS) at 5% CO₂ and 37 °C. A 4 cm² tissue culture plate well was seeded with 2 × 10⁵ HeLa cells and grown overnight without PS. The next day cells were transfected for 5 h with Lipofectamine 2000 (Life Technologies) and pIRESneo3-Clp1-3XFlag at 1:1 ratio (µl/µg). The cell medium was then exchanged for fresh medium containing PS, and the cells were grown overnight. The cells were re-plated into a 10 cm diameter plate and incubated for 7 h. A solution of G418 disulfate antibiotic (RPI Corp.) (0.6 mg/ml medium concentration) was added and incubated with the cells for 2 days. The medium was then exchanged for fresh medium containing 1 mg/ml of G418 and thereafter replaced every five days. Single colonies became visible after two weeks of incubation at 37 °C. Each colony was isolated using a cloning cylinder (Corning), expanded, and tested for the presence of FLAG-tagged Clp1 by western blotting with anti-FLAG antibody (Sigma-Aldrich).

2.7. Co-immunoprecipitation

Lysates were made from plated (10 cm) HeLa cells grown to ~80% confluency and the Clp1-FLAG cell line. The cells were detached with trypsin for 5 min, washed in 1 ml of PBS, pelleted, snap-frozen in liquid nitrogen and thawed to lyse cells, then gently rocked in 50 µl of Buffer D_{10Glyc} per 10 cm plate for 10 min at 4 °C. The cells were centrifuged at 4 °C for 10 min at 10,000 rpm and the supernatant was taken as the lysate for co-immunoprecipitation (co-IP) experiments on the same day.

Four co-IP experiments were done simultaneously, one using HeLa cell lysate and three using Clp1-Flag cell lysate. Each IP solution contained 200 µg of lysate protein and (for the ATP-depletion step) glucose (2 mM), MgCl₂ (2.5 mM) and hexokinase (0.6 u) (Hirose 1997). The lysates were ATP-depleted at 30 °C for 10 min. EDTA was added to a final concentration of 4.5 mM and the lysates were placed at 30 °C for 10 min and then iced. 10 µl (drained beads volume) of anti-FLAG affinity beads (Sigma-Aldrich) were mixed with each sample. ATP or AMP-PCP (final concentration at final IP volume, 4 mM) was added each to one of the Clp1-FLAG lysates while the third Clp1-FLAG and the HeLa lysate received no nucleotide. To simulate in vitro cleavage conditions in the lysates that contained ATP or AMP-PCP, creatine phosphate (50 mM) and L3 RNA substrate (~3 nM, with 40 u RNase inhibitor) were included. Buffer D_{10Glyc} was added as necessary to produce in each tube a final volume of 60 µl. IPs were done for 2 h at 4 °C with gentle rocking. The supernatants were collected from each tube and the beads were washed three times with 200 µl of Buffer D_{10Glyc} and eluted with 13 µl of 1.2 µg/µl 3X-FLAG peptide (Sigma-Aldrich). Lysates (10% input, i.e. 20 µg), supernatants (10% input, 6 µl), and the entire eluate were

resolved on 10% SDS-PAGE and probed by western blotting with anti-Pcf11 (Proteintech Cat. No. 23540-I-AP) and anti-Clp1 (Abcam Cat. No. ab128939) antibodies. In some experiments (Fig. 3B), the cell lysates were dialyzed 2×30 min at 4 °C against 1 L of Buffer D_{10Glyc} and the co-IP was done as described above beginning with the ATP-depletion step.

2.8. Generating and purifying wild-type and mutant PAP proteins

Primers for making the mutants of wild-type full length PAP (bovine PAP α , isoform II) from plasmid pUK-wt His-PAP plasmid [37](gift of E. Wahle and U. Kühn) were generated using the QuikChange Primer Design Program (Agilent Technologies). K228A primers were: Forward 5'-TAACCCTGAGAGCTATCGCACTGTGGGCCAAACGCC-3'; Reverse 3'-ATTGGGACTCTCGATAGCG-TGACACCCGGTTTGCGG-5'. Primers for Y237A were: Forward 5'-GGGCCAAACGCCACAACATCG-CTTCCAATATATTAGGTTTCCT-3'; Reverse 3'-CCCGGTTTGCGGTGTTGTAGCGAAGGTTAT-ATAATCCAAAGGA-5'. The double mutant was generated by using Y237A primers on the K228A mutant plasmid. The PCR reactions and transformations were done as recommended by the manufacturer. The plasmid DNA was purified using a MiniPrep Kit (GenScript). Plasmid DNA regions with introduced mutations were verified by sequencing.

About 50 μ l of competent *E. coli* BL21 (DE3) cells were transformed with 5 ng of either wild-type or one of the mutant PAPs. Each was then inoculated into 50 ml LB (100 μ g/ml ampicillin) culture and grown overnight. The overnight culture was inoculated into 4 L of LB/ampicillin culture and grown at 37 °C to 0.15 OD₆₀₀, the temperature was then changed to 18 °C and cells were grown to 0.5 OD₆₀₀, at which point cells were induced with 1 mM IPTG and grown at 18 °C for 18 h. Cells were harvested and stored at -80 °C. The pellet from 1L of cell culture was resuspended in 32 ml of His-Tag Native Lysis Buffer, lysed two times in a French Press, centrifuged at 10,000 rpm for 20 min at 4 °C. The lysate was incubated under gentle agitation with 0.8 ml of drained volume Ni-NTA Agarose (Qiagen) beads for 2 h at 4 °C, followed by transfer to a column where beads were washed 2 times with 4 ml of His-Tag Native Wash Buffer, and then eluted with 4 consecutive 0.8 ml aliquots of His-Tag Elution Buffer. Elutions were dialyzed twice against 1 L of Buffer D for 2 h at 4 °C.

2.9. Nonspecific polyadenylation reaction

Carried out at 30 °C for 30 min in 12.5 μ l with: Buffer D, 2.5% of PVA, 0.1 μ g of BSA, 20 mM CP, 1 mM MnCl₂ (except 10 mM in Fig. 5D), 3 nM uniformly ³²P-labeled L3 RNA, and the amount of ATP and recombinant bPAP (wt or mutant) as indicated in figures.

2.10. The 3' pre-mRNA cleavage reactions using mutant PAPs and CSF

In vitro cleavage was carried out in 12.5 μ l at 30 °C for 2 h as described [22,33,35]. The final reaction component concentrations were: 1.25 μ g of tRNA, 1.6 mM DTT, 50 mM CP, 6 u of recombinant RNase Inhibitor (Promega), 2.5% PVA, 2 mM EDTA, 3 nM ³²P-labeled L3 substrate, 5 μ l of CSF, and PAP and ATP as indicated in Fig. 5.

3. Results and discussion

3.1. ATP and 3' cleavage stimulation: a role as phosphate donor?

ATP has been investigated as a possible energy-supplying cofactor necessary for the 3' cleavage step [13,16–18,38]. These studies used a number of ATP structural analogs, including the non-hydrolyzable analogs adenosine 5'-(α,β -methylene)triphosphate (AMP-CPP) and adenosine 5'-(β,γ -methylene)triphosphate (AMP-PCP). A consensus was found regarding the effect of AMP-CPP on 3' cleavage using HeLa cell nuclear extracts as the source of the cleavage factors. This analog was able to replace ATP's cleavage stimulation property for three different mammalian pre-mRNA substrates: HSV-1 TK [18], L3 [13] and the Simian Virus 40 early (SV40E) poly(A) site [16,17]. It also promoted the formation of what appeared to be ATP-dependent protein-RNA complexes on the path to AAUAAA-dependent cleavage and polyadenylation [18,38,39].

Results from the use of AMP-PCP during *in vitro* cleavage, where the β - and γ -phosphates are joined by a methylene group, were less clear. This analog can in principle be incorporated into a nascent RNA chain, but cannot serve as a kinase substrate or yield energy by hydrolysis of the γ -phosphate by ATPases. AMP-PCP could not replace ATP in pre-mRNA cleavage of the HSV-1 TK substrate [18], nor did it allow cleavage of the CYC1 yeast substrate in yeast whole cell extract [19], but it did appear to support both cleavage and polyadenylation of L3 in HeLa nuclear extract [13]. Another report mentioned that AMP-PCP inhibited both cleavage and polyadenylation of an unspecified substrate, but no data was shown [38]. Gel mobility shift experiments appeared to show that AMP-PCP was able to substitute for ATP in the formation of pre-cleavage complexes [18,38].

Our previous evidence that Ser/Thr phosphorylation of a cleavage factor protein in the CF_m DEAE fraction is necessary for efficient 3' cleavage *in vitro* [22] led us to revisit the effect of these and other ATP analogs on the L3 cleavage reaction, here reconstituted from DEAE-sepharose fractionated CPSF, CstF and CF_m. As structurally similar substitutes for ATP in 3' cleavage, we initially used 2'-dATP, 3'-dATP, AMP-PCP, AMP-CPP, γ -S-ATP (which when used as a kinase substrate produces phosphorylated proteins resistant to phosphatase activity [40]) and, as a negative control, GTP. Our *in vitro* cleavage conditions included 50 mM creatine phosphate (CP), shown with the more highly fractionated factors to be required for SV40L and L3 pre-mRNA cleavage, independent of any ATP requirement [21], and 2 mM EDTA to prevent Mg-ATP formation. We included EDTA in our standard cleavage reaction to prevent polyadenylation of the cleavage product, which would obscure the outcome of the 3' cleavage step, and because ATP has been repeatedly shown to stimulate L3 cleavage in the presence of EDTA. Using these ATP analogs, we sought to re-evaluate the role of ATP in L3 cleavage in view of the possibility that ATP might stimulate *in vitro* cleavage by serving as a phosphate donor substrate for a cleavage factor protein kinase, and to resolve the effect AMP-PCP has on reconstituted *in vitro* 3' cleavage.

Lanes 1–3 in Fig. 1A show that our DEAE-fractionated factors require the addition of ATP to cleave a significant amount of the L3 substrate. With one exception, the ATP structural analogs we tested produced less L3 cleavage than did ATP. The exception, 2'-dATP,

enhanced in vitro cleavage by ~50% (compared to ATP) (lane 4). In addition, AMP-PCP stood out for its inability to stimulate even a trace of L3 cleavage above background (lane 6).

In contrast to the L3 substrate ATP requirement, when cleavage factors that are more highly purified than our DEAE-fractionated factors were used to reconstitute in vitro cleavage of the SV40L substrate, ATP was not required and in fact slightly inhibited cleavage at 1 mM [21]. To see whether this contrasting ATP effect was the same for the ATP analogs, we repeated the L3 experiment on the SV40L substrate using the DEAE-fractionated factors. As expected, ATP was not required for substantial cleavage (Fig. 1B, lane 11). However, in contrast to what was found using the more highly fractionated cleavage factors, ATP led to a small increase of cleavage above the no-ATP cleavage level (Fig. 1B, lanes 11 and 12). The other analogs tested followed a relative pattern similar to their effect on L3 cleavage: 2'-dATP gave the greatest enhancement (lane 13), AMP-PCP failed to enhance cleavage (lane 15) and the other analogs gave intermediate levels of cleavage. AMP-PCP not only failed to enhance SV40L cleavage but suppressed it below the no-ATP background, reducing the background by about half. This suggested that AMP-PCP was not merely failing to stimulate cleavage of SV40L and L3, but that it was functioning as a cleavage reaction inhibitor. This result also supported the idea that for both substrates, L3 and SV40L, ATP can influence the cleavage reaction through an as yet unidentified ATP-binding protein whose role in cleavage is general but more important for L3 than it is for SV40L.

We tested a second ATP analog with a non-transferable γ -phosphate, AMP-PNP (Fig. 1C). Hydrolysis of this analog to AMP-PN and phosphate [36], or transfer of its γ -phosphate by a kinase [41] cannot occur on the time scale and under the conditions of in vitro cleavage, and it is generally considered to be a non-hydrolyzable analog of ATP. Additionally, this analog is known to bind to the CF II_m Clp1 subunit's ATP binding site [42]. ATP is not a by-product contaminant of AMP-PNP synthesis or hydrolysis [36,43] but can form over a period of weeks under abnormal storage conditions [44], which we took care to avoid. Unlike AMP-PCP, when substituted for ATP, AMP-PNP stimulated a reproducibly detectable amount of L3 cleavage (Fig. 1D), though about 5-fold less than that produced by ATP. This result provided additional indirect evidence against the possibility that ATP works via cleavage factor phosphorylation.

The effect of AMP-PCP on SV40L cleavage (Fig. 1B, lane 15) raised the question whether it is a 3' cleavage inhibitor or just fails to do whatever ATP does to stimulate in vitro cleavage. To address this question, we titrated AMP-PCP into L3 substrate cleavage reactions in the presence of 1 mM ATP (Fig. 2). Over the concentration range of 0.1–2 mM, AMP-PCP inhibited the stimulation of cleavage by ATP in a concentration-dependent manner. The reaction produced half of the normal cleavage product at an AMP-PCP concentration of 300 μ M, which we take to be an approximate IC₅₀ under our in vitro conditions. Given their similarity in structure, this result suggests that AMP-PCP competes with ATP for a binding site on one of the cleavage factors but, unlike ATP, fails to stimulate cleavage. An alternative explanation that we cannot rule out is that two different cleavage factors, one stimulatory and one inhibitory, bind ATP with different affinities, and AMP-PCP has a dominant effect through the inhibitory factor at these concentrations. The simpler explanation is that AMP-PCP competes for the site through which ATP stimulates 3' cleavage.

The results of these indirect experiments support a role in which ATP stimulates cleavage without serving as a protein kinase substrate. The stimulation of cleavage by ATP in the presence of EDTA (absence of Mg-ATP), and the detectable stimulation of cleavage by the non-hydrolyzable ATP analog AMP-PNP, are inconsistent with the transfer of the γ -phosphate to a cleavage factor during in vitro cleavage. We also tried the more direct experiment where [γ - ^{32}P]-ATP was included during an in vitro cleavage reaction, but no clearly labeled cleavage factor proteins could be identified by SDS-PAGE mobility (not shown).

3.2. ATP and 3' cleavage stimulation: a binding-only role for ATP in 3' cleavage?

A more likely alternative mechanism for cleavage stimulation by ATP entails mere binding of ATP to one or more cleavage factors in such a way as to stabilize its folded structure or its complex formation with other factors [17]. Among the core cleavage factors, there are three proteins known to bind ATP in the concentration range where it stimulates in vitro L3 cleavage: PAP (ATP K_M = 0.23–0.3 mM) [45,46], the 25-kDa subunit of the CF I_m cleavage factor, CF $\text{Im}25$ (K_d = 1.5 mM for the isolated subunit) [47] and Clp1 (kinase reaction $K_M \approx$ 0.26–1 mM) [30,42].

3.3. ATP and the cleavage factor II_m Clp1–Pcf11 interaction

Mammalian CF II_m has been extensively fractionated from HeLa nuclear extract [48]. A chromatographic fraction co-eluting with CF II_m in vitro cleavage activity was found to contain at least fifteen polypeptides, only some of which likely contribute to CF II_m cleavage activity. Two of the proteins, Clp1 and Pcf11, drew attention because they eluted precisely with CF II_m activity and have yeast cleavage factor orthologs. These two remain the only validated CF II_m subunits. Full length yeast Clp1 has been crystallized in a ternary complex with a fragment of yeast Pcf11 spanning the predicted Clp1-Pcf11 binding interface, and ATP [29]. That study revealed that the ATP binding site, centered on a conserved Walker A motif, or P-loop, is remote from the Clp1-Pcf11 interface, and that the ATP appears not to undergo any phosphate transfer as a consequence of Clp1 binding. Disruptive mutations made in the yeast Clp1 ATP-binding site were found to influence Clp1-Pcf11 binding [31,49], and mutant yeast Clp1 strains having impaired 3' processing were found to contain synergizing mutations in the Clp1-Pcf11 interface and ATP-binding site [50]. Although the identity between yeast and human Clp1 is only 24%, these yeast studies pointed to a possible link between ATP-binding and Clp1-Pcf11 association. In yeast, Clp1-Pcf11 binding occurs within the CF IA complex, which also contains the CstF homologs Rna14 and Rna15. Yeast Clp1-Pcf11 binding can be disrupted without loss of viability, perhaps because CF IA is held together by additional protein-protein interactions [49]. However, in mammals, where there may be no other interactions to hold CF II_m together, the Clp1-Pcf11 association might be important for CF II_m activity and, we speculated, ATP binding might be involved and influence Clp1-Pcf11 binding stability. Mammalian Clp1 has other cellular roles outside of pre-mRNA cleavage that involve ATP-binding: Clp1 is an oligonucleotide kinase in vitro [30], and its ATP-binding is involved in an important tRNA splicing role demonstrated in vivo [51,52]. During the mammalian Clp1 experiments, the question had arisen whether Clp1 ATP-binding and kinase activity were needed for both its tRNA and pre-mRNA processing roles [51]. Accordingly, nuclear extracts from mouse embryonic

fibroblast cells expressing a mutant Clp1 with weakened ATP-binding in place of wt Clp1 were tested for in vitro 3' cleavage activity. The extract showed normal in vitro 3' cleavage, but only the SV40L pre-mRNA substrate, which is ATP-independent in vitro, was tested, leaving the question of Clp1's ATP role in 3' cleavage unanswered. The contribution of ATP-binding to Clp1's pre-mRNA 3' cleavage function is sufficiently unclear that we considered the possibility that ATP contributes to 3' cleavage by stabilizing the mammalian Clp1-Pcf11 complex, and that AMP-PCP might inhibit in vitro cleavage by replacing ATP in this complex.

To test whether ATP contributes to Clp1-Pcf11 binding, we made a HeLa cell line stably expressing FLAG-tagged Clp1 (Clp1-FL). Whole cell lysates were made from plated Clp1-FL cells and from regular HeLa cells. To prevent competition from endogenous ATP, the lysate was depleted of ATP (using hexokinase and glucose) or, in some experiments, dialyzed and then depleted. FLAG-Clp1 was then immunoprecipitated from the lysate using anti-FLAG-beads in the presence of ATP, AMP-PCP or neither nucleotide. Endogenous Clp1, FLAG-Clp1 and Pcf11 were all visible on a Western blot of the IP supernatants in each case (Fig. 3A, lanes 4–7). FLAG-Clp1 co-immunoprecipitated endogenous Pcf11 in the absence of ATP (lane 9), indicating that excess ATP is not needed to maintain Clp1-Pcf11 binding in vitro. Neither ATP nor AMP-PCP had an effect on the amount of Pcf11 co-immunoprecipitated with FLAG-Clp1 (Fig. 3A, lanes 10, 11 and Fig. 3B). We cannot know from this experiment if ATP is present in Clp1-Pcf11 complex. We can however conclude that either excess ATP is not needed for the mammalian Clp1-Pcf11 association or, if it is required, the ATP is bound with such high affinity that it does not dissociate during immunoprecipitation in the absence of excess ATP. Since AMP-PCP had no effect on the Clp1-Pcf11 immunoprecipitation, we conclude that AMP-PCP does not inhibit in vitro cleavage by disrupting the Clp1-Pcf11 complex through the Clp1 ATP-binding site.

3.4. ATP and the cleavage factor I_m 25 kDa subunit

We next considered the ATP-binding capability of mammalian cleavage factor I 25 kDa protein (CF I_m25). CF I_m is a tetrameric protein composed of two heterodimers, each containing one CF I_m25 subunit, also known as CPSF5 or NUDT25, and one of two possible larger homologous subunits, CF I_m59 (CPSF7) or CF I_m68 (CPSF6). The CF I_m25/68 complexes dimerize through CF I_m25 [53–55]. CF I_m25 contains a Nudix domain, whose occurrence is typically associated with divalent metal coordination, nucleotide binding and release of nucleotide diphosphate through hydrolysis of various nucleotide polyphosphates [56]. The CF I_m25 Nudix domain is missing two key glutamates, preventing metal binding and nucleotide hydrolysis [47]. Instead of its typical function, the Nudix domain in CF I_m25 makes an important contribution to the sequence-dependent binding of the UGUA RNA substrate motif [55] found upstream of many polyadenylation sites [57,58], including the SV40L and L3 poly(A) substrates used here. The location where the RNA binds to CF I_m25 is also the binding site where the common Nudix substrate, diadenosine tetraphosphate, Ap₄A, was found by X-ray crystallography to bind (K_d , 2.4 μ M) and where, presumably, ATP also binds, though with lower affinity (K_d = 1.5 mM) [47]. The ATP concentration range over which the CF I_m25 RNA binding site becomes saturated with ATP, albeit measured in the context of the isolated CF I_m25 homodimer, is close to the range

where ATP inhibits in vitro cleavage [13,21]. The mutually exclusive nature of ATP and UGUA RNA binding makes it unlikely that ATP stimulates in vitro cleavage through CF Im25, but at high concentrations ATP might interfere with cleavage by displacing the UGUA RNA substrate from the Nudix site. Since Ap4A binds to the site with over 600-fold greater affinity than ATP, we tested the effect of Ap4A on in vitro cleavage in the presence of a concentration of ATP that stimulates 3' cleavage. At concentrations where Ap4A binds the isolated CF Im25 subunit, it did not inhibit ATP-stimulated cleavage (Fig. 4A). We repeated the experiment in the absence of ATP and confirmed that Ap4A did not stimulate 3' cleavage (Fig. 4B). Thus, despite its ability to bind the isolated CF Im25 subunit with micromolar affinity and to occupy the CF Im25 RNA binding site, Ap4A had no effect on in vitro cleavage. The RNA recognition motif (RRM) of the CF Im68 subunit increases the affinity of CF Im25 for UGUA-containing RNA [55,57] and it is reasonable to expect that in the structural context of the functional CF Im25/68 heterodimer, CF Im25 strongly prefers the UGUA sequence over small Nudix substrates like ATP or Ap4A. In summary, recent structural studies into the way that CF Im25 binds to UGUA make it unlikely that ATP stimulates 3' cleavage through this cleavage factor in the 0.5–1 mM range, and we found indirect evidence supporting this. It remains possible that high ATP concentrations inhibit 3' cleavage through this site, but we have not yet been able to definitively test this.

3.5. ATP and the Poly(A) polymerase alpha 3' cleavage factor

The nuclear ATP-binding protein poly(A) polymerase α (PAP) [46,59,60] is perhaps the most logical candidate for the cleavage factor that mediates cleavage stimulation by ATP because efficient cleavage of the L3 substrate requires both PAP and ATP [7]. To test whether ATP stimulates in vitro cleavage through PAP, we disrupted PAP's ATP-binding ability using site-directed mutagenesis, and then tested the effect of the mutations on ATP-stimulated L3 cleavage in concert with the other HeLa cleavage factors. To carry out this experiment, we could not use the HeLa DEAE-fractionated factors because endogenous PAP co-elutes with CstF. Instead, we used the cleavage specificity factor fraction (CSF) [8]. This size-exclusion chromatography fraction contains all of the cleavage factors except PAP. Using CSF as the source of the cleavage factors allowed us to supplement them with recombinant PAP proteins during in vitro L3 cleavage.

The mammalian PAP (isoform II) residues required for binding ATP have been identified through mutagenesis [61] and X-ray crystallography in the presence of Mn^{2+} [62] and Mg^{2+} [45]. We chose two active site bovine PAP (bPAP) residues whose mutation to alanine caused a large increase in the ATP Michaelis constant (K_M), and which made important contacts with bound ATP in both crystal structures. When changed to alanine, lysine 228 (K228A) and tyrosine 237 (Y237A) weakened ATP affinity in the context of the full length bPAP and the C-terminally truncated form (bPAP1-513) containing the intact polymerase domain. In one report using bPAP1-513, the K228A ATP K_M increased 10-fold compared to the wild type from 0.23 mM to 2.2 mM, while the Y237A mutant increased the ATP K_M to 1.4 mM [45]. An earlier study using the full length bPAP reported smaller increases [63]. Because the affinity appeared to be weakened less in the context of the full length bPAP, and because we do not yet know which part of the PAP primary structure is needed for its role in the 3' cleavage reaction, aside from any question of ATP dependence, we made a new

double-mutant that included both of these mutations, K228A/Y237A. The bovine wild type and mutants have a N-terminal His₆-tag for affinity purification. An SDS-PAGE characterization of the bacterially expressed, His₆-tag-purified proteins is shown in Fig. 5A. To verify that ATP binding was compromised in the mutants, we used the non-specific polyadenylation assay [34], reasoning that the efficiency of the reaction would indicate how well the mutants bind ATP. The non-specific polyadenylation of a uniformly labeled substrate RNA by the wild type and mutant PAPs at 1 mM ATP and ~50 nM polymerase is shown in Fig. 5B. These conditions led to a level of polyadenylation expected for the mutants based on their K_M values. The Y237A mutant, which is predicted to be about 50% bound at 1 mM (K_M 0.88 mM) [63] gave the same level of polyadenylation as the wild type (lanes 8 and 10). The K228A mutant gave detectably shorter tails, but the result still showed that under these high concentration conditions, a significant fraction can bind to ATP. As expected, the ability of the K228A/Y237A double mutant to carry out non-specific polyadenylation was greatly impaired (lane 11). Close inspection however revealed a weak smear of polyadenylated substrate RNA with a comparatively short poly(A) tail. This result indicated that the mutant is intact, and the small fraction binding ATP under these conditions is functional.

We carried out *in vitro* 3' cleavage using the same high concentrations of ATP and bPAP proteins. Under these conditions (1 mM ATP, 50 nM PAP, 2 mM EDTA, RNA < 5 nM), both wt and mutant bPAPs stimulated similar levels of L3 cleavage (Fig. 5C). Here, at a bPAP concentration that is much higher than that of the RNA and likely those of the CSF cleavage factors, neither bPAP nor ATP is limiting, and the small fraction of the mutant bPAP able to bind ATP may suffice to stimulate normal 3' cleavage. We therefore lowered the bPAP concentration to 10 nM, and lowered the ATP concentration to the wt K_M (0.23 mM). An assessment of ATP-binding by nonspecific polyadenylation under these conditions is shown in Fig. 5D. The wt bPAP shows clear concentration dependence under these conditions over the range of 10–50 nM (lanes 22, 26, 27). The K228A mutant showed slightly reduced polyadenylation, while the Y237A appeared slightly more active than the wt bPAP (lanes 23 and 24). While unexpected, we cannot rule out that the Y237A difference results from uncertainty in the estimate of the protein concentration. More importantly, for our purposes, the double mutant showed no detectable polyadenylation, even at long exposures, and hence appears unable to bind ATP at this lower concentration. The same concentrations of bPAP (10 nM) and ATP (0.23 mM) were next used to stimulate *in vitro* L3 cleavage (Fig. 5E). The dose dependent response to the wt bPAP (lanes 32, 36, 37) shows that at the 10 nM concentration, the cleavage stimulation property of bPAP is not saturating, but rather in a range where cleavage stimulation is sensitive to the amount of bPAP, and where differences, if any, between the wild type and the mutants should be detectable. Under these conditions, all of the mutants stimulated significant cleavage despite their mutations. Since the K228A/Y237A double mutant appears unable to bind ATP, and small differences in the amount of the bPAP proteins added might confound direct comparisons among the mutants, we decided to lower the bPAP and K228A/Y237A double mutant concentration further, to 5 nM, and to ask simply whether ATP would stimulate L3 cleavage (Fig. 5F). The result showed that in the presence of bPAP Y237A/K228A, which appears unable to bind ATP, there was unequivocal ATP-dependent 3' cleavage stimulation (lanes 43 and 44). The most likely

explanation for this result is that bPAP is not the cleavage factor through which ATP stimulates in vitro 3' cleavage. Rather, one of the wild type cleavage factors present in the CSF fraction allowed the ensemble of cleavage factors to respond to the addition of ATP.

4. Conclusions

In conclusion, although most pre-mRNA substrates require about 0.5–1 mM ATP for efficient 3' cleavage in vitro, and L3 is strongly inhibited by 5–10 mM ATP, nothing is known about how ATP brings about its effects or whether these in vitro observations are physiologically relevant. Here we have found additional indirect evidence supporting a binding-only, rather than a kinase substrate, role for ATP in cleavage stimulation. We identified AMP-PCP as an effective inhibitor of the cleavage reaction with an IC₅₀ of 300 μM. We found that AMP-PCP does not work by disrupting the Clp1-Pcf11 complex in CF II_m, and that either this complex does not need ATP for stability, or that it binds ATP so tightly as to be irreversible when excess ATP is removed. We also rule out PAP as a candidate for the positive regulation of 3' cleavage by ATP. Although we have not identified the cleavage factor that uses ATP to promote 3' cleavage, we suggest the CF Im25 subunit as a possible candidate for the cleavage factor that inhibits 3' cleavage of L3 at high ATP concentrations. Lastly, we speculate that since cellular ATP increases during mitosis [15], the inhibition of 3' cleavage by ATP at high concentrations may be part of the shutdown of key gene expression and RNA processing activities that takes place during M-phase [64].

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Abbreviations

ATP	Adenosine 5'-triphosphate
AMP-PNP	Adenosine 5'-(β,γ-imido)triphosphate
AMP-CPP	Adenosine 5'-(α,β-methylene)triphosphate
AMP-PCP	Adenosine 5'-(β,γ-methylene)triphosphate
ATP-γ-S	Adenosine 5'-[γ-thio] triphosphate
3'-dATP	cordycepin triphosphate
Ap4A	diadenosine tetraphosphate
L3	adenovirus-2 late 3 poly(A) sequence
SV40L	Simian Virus 40 Late poly(A) sequence
HSV-1 TK	herpes simplex virus type I thymidine kinase poly(A) sequence

PAP	poly(A) polymerase
CPSF	cleavage polyadenylation specificity factor
CstF	cleavage stimulation factor
CF I_m	mammalian cleavage factor 1
CF II_m	mammalian cleavage factor 2
bPAP	bovine poly(A) polymerase α isoform II
L3	adenovirus-2 late 3 pre-mRNA poly(A) signal

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Fig. 1. The effect of ATP and ATP structural analogs on the pre-mRNA 3' cleavage reaction reconstituted from HeLa DEAE-fractionated cleavage factors

A. In vitro reconstituted cleavage of the uniformly labeled, 5' capped L3 pre-mRNA substrate. Nucleotide concentration, 0.5 mM H₂O, water added in place of nucleotide. Input, unprocessed RNA. 5', upstream cleavage product; 3', downstream cleavage fragments; R.C. Relative cleavage, normalized to ATP lane. Bar charts were made using the average of three independent identical experiments, $\pm$ one S.D. See text for nucleotide abbreviations. **B.** Same as in panel A except the SV40L pre-mRNA substrate was used. **C.** Structural differences of three of the nucleotides used. **D.** Same as in panel A, except 1 mM AMP-PNP was used. Spaces between gel lanes indicate rearrangement of lanes from a single representative experiment.

Fig. 2. The effect of AMP-PCP on ATP-stimulated L3 pre-mRNA cleavage
ATP (1 mM) and AMP-PCP at the indicated concentrations were included in a reconstituted in vitro cleavage reaction as in Fig. 1. The bar chart was made using the average of three independent identical experiments, $\pm$ one S.D.

Fig. 3. Excess ATP is not needed for CF II_m Clp1-Pcf11 complex stability

A. FLAG-tagged Clp1 was expressed in a stable HeLa cell line (Clp1-FLAG) and immunoprecipitated using FLAG-beads from whole cell extract with no added nucleotide or with 4 mM ATP or AMP-PCP. Normal HeLa cells were used as a control. The indicated fractions were resolved on SDS-PAGE and blotted. The top portion of the blot was probed with an anti-Pcf11 antibody. The lower portion was probed with an anti-Clp1 antibody. W.B., Western blot. **B.** The experiment shown in panel A was repeated (n) times with either ATP-depletion (as shown in A) or dialysis of the whole cell extract followed by ATP depletion. Bars indicate the Pcf11-Clp1 ratio normalized to the control where no nucleotide (nt) was included in the precipitation.

Fig. 4. Ap4A, a CF Im25 Nudix domain ligand, does not affect in vitro pre-mRNA 3' cleavage
A. Increasing concentrations of Ap4A do not inhibit ATP-stimulated cleavage of L3 pre-mRNA. **B.** Ap4A alone does not stimulate pre-mRNA cleavage in place of ATP. Cleavage reactions were done as described in Fig. 1.

Fig. 5. ATP does not stimulate pre-mRNA 3' cleavage through the canonical PAP ATP-binding site

A. Bacterially expressed, N-term-His₆-tag affinity purified wt and mutant bovine PAP proteins (bPAP, ~3 µg) used in this study. SDS-PAGE with Coomassie staining. Asterisk (*) denotes bands arising through adventitious C-terminal proteolysis during expression or purification. BSA, bovine serum albumin standards. **B.** Evaluation of ATP binding using 1 mM Mn²⁺-mediated non-specific polyadenylation at 50 nM PAP concentration. wt(2×) denotes 100 nM PAP. RNA substrate/primer was the uniformly labeled L3 pre-mRNA. **C.** In vitro L3 RNA cleavage reconstituted from the HeLa nuclear extract CSF fraction supplemented with the indicated PAP protein and ATP. **D.** Evaluation of ATP binding as in panel B but with lower PAP (10 nM) and ATP concentrations. wt(2×), 20 nM PAP. wt(5×), 50 nM. **E.** Reconstituted in vitro cleavage using CSF and recombinant PAP as in panel C but using 10 nM PAP and 0.23 mM ATP. **F.** Reconstituted in vitro cleavage using CSF and recombinant PAP as in panels C and E but here only 5 nM PAP, with or without 0.23 mM ATP.