Creatine Phosphate, Not ATP, Is Required for 3' End Cleavage of Mammalian Pre-mRNA * in Vitro

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The poly(A) tail of a mammalian mRNA is generated by endonucleolytic cleavage and poly(A) addition. Previous studies conducted with nuclear extracts suggested an ATP requirement for the cleavage step. We have reexamined the cofactor requirement, initially with the SV40 late pre-mRNA, which requires for cleavage four protein factors, cleavage and polyadenylation specificity factor, cleavage stimulation factor, cleavage factor I, and cleavage factor II. Using highly purified preparations of these factors, which lacked detectable creatine phosphokinase and ATPase activities, creatine phosphate (CP) was, surprisingly, found to be sufficient to promote efficient cleavage.

Although other phosphate compounds substituted poorly or not at all for CP, another phosphoguanidine, arginine phosphate, was fully functional. Notably, ATP was neither necessary nor sufficient, and could in fact inhibit the reaction. Treatment of the purified factors with hexokinase plus glucose (to deplete any contaminating ATP) was without effect, as was addition of EDTA. Using 32P-labeled CP, we found that neither hydrolysis of CP nor phosphate transfer from CP occurred during the cleavage reaction. CP also allowed cleavage of the adenovirus 2 L3 pre-mRNA. However, in this case, ATP both enhanced the reaction and influenced the precise site of cleavage, perhaps reflecting the requirement of poly(A) polymerase for cleavage of this RNA. These results indicate that ATP is not essential for 3' pre-mRNA cleavage and that CP or a related compound can function as a necessary cofactor.

All eukaryotic mRNAs, with the exception of major histone mRNAs, have a poly(A) tail at their 3' end. The process generating the poly(A) tail, referred to as 3' end formation, involves endonucleolytic cleavage of the precursor RNA followed by the sequential addition of adenylate residues. 3' end formation is an essential step in the maturation of pre-mRNAs, and it appears to be coupled with other events in the nucleus, including transcription termination (1–3) and RNA splicing (4–6). The resulting poly(A) tail has been suggested to play important roles in mRNA stability (reviewed in Ref. 7) and translation (reviewed in Ref. 8). Alternate usage of polyadenylation sites can regulate gene expression through changing the capacity of coding or noncoding sequences in the 3' region of mRNA (9–10) (reviewed in Ref. 11).

Although the cleavage and poly(A) addition reactions are tightly coupled in vivo, development of in vitro systems made it possible to study these reactions separately and to purify factors required for each step (for reviews, see Refs. 12 and 13). Cleavage can be assayed by blocking poly(A) polymerase (PAP) activity with either EDTA or chain-terminating ATP analogs, such as 3'dATP, whereas poly(A) addition independent of cleavage can be studied by using “precleaved” RNA substrates. In mammalian cells, two RNA sequence elements are essential for efficient 3' end formation. One is the highly conserved hexanucleotide AAUAAA located 10–30 nucleotides upstream of the cleavage site, and the other is a less conserved GU- or U-rich sequence that lies just downstream of the cleavage site (for review, see Ref. 14). Accurate and efficient cleavage requires five protein factors: cleavage and polyadenylation specificity factor (CPSF), cleavage stimulation factor (CstF), two cleavage factors (CFI and CFII), and, in many cases, PAP (15–18). CPSF consists of at least three subunits (160, 100, and 73) and likely a fourth (30 kDa) that is nonessential in vitro (19–22). CstF is composed of three subunits (77, 64, and 50 kDa) (23–26). CPSF and CstF can specifically and cooperatively interact with AAUAAA and the downstream GU-rich sequence in the precursor RNA, respectively (21, 22, 27–30), and thus they likely can specify the poly(A) site. CFI (16) has been purified from HeLa cells and appears also to be a multimeric subunit factor (31), although the roles of both CFI and CFII in cleavage have not been well defined. PAP, a single polypeptide of ~85 kDa (32, 33), is dispensable for cleavage of SV40 late pre-mRNA in vitro but is required for efficient cleavage of other substrates (Ref. 15). Following cleavage, CPSF, PAP, and poly(A) binding protein II participate in poly(A) addition (34–36). CstF, CFI, and CFII are not necessary for this reaction in vitro, although whether these factors dissociate from the complex at this stage is not clear.

Although the RNA sequences and protein factors responsible for 3' end formation have been well studied, cofactor requirements for the reaction have been examined only with relatively crude extracts (for review, see Refs. 37 and 38). Early experiments conducted with unfraccionated nuclear extracts suggested that ATP is a necessary cofactor for the cleavage reaction (39, 40) and for formation of polyadenylation-specific complexes (40–43). However, several observations have confused the issue. First, the cleavage reaction was found to proceed in the absence of divalent cation and the presence of EDTA (39, 44), which would not be expected for an ATP-requiring reaction. Second, the addition of creatine phosphate (CP), commonly used to regenerate ATP by phosphate transfer from CP to ADP (catalyzed by creatine phosphokinase (CPK)), was shown to be sufficient for cleavage in

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The abbreviations used are: PAP, poly(A) polymerase; CPK, creatine phosphokinase; FPLC, fast protein liquid chromatography; AP, arginine phosphate; CPSF, cleavage and polyadenylation specificity factor; CstF, cleavage stimulation factor; CFI, cleavage factor I; CFII, cleavage factor II.
the absence of exogenously added ATP and divalent cation (15). These observations could reflect an unusual mechanism of ATP regeneration from endogenous ATP/ADP in the crude protein fractions or indicate that ATP is not in fact necessary for cleavage. We therefore reexamined the cofactor requirement for in vitro cleavage using highly purified factors. Here we present evidence that CP can in fact function as a necessary and sufficient cofactor for efficient in vitro cleavage. Although ATP is not required, it can function as either an inhibitor or an activator of the reaction, depending on the specific substrate, and can also affect the precise cleavage site in PAP-dependent cleavage. Possible mechanisms of CP-activated cleavage are discussed.

EXPERIMENTAL PROCEDURES

Materials—Creatine phosphate was purchased from Sigma, Calbiochem, and Boehringer Mannheim. Arginine phosphate was from Sigma. 32P-Labeled creatine phosphate (2.3 × 104 cpmm/μg) was enzymatically synthesized from [γ-32P]ATP (4500 Ci/mmol; ICN) and creatine (Sigma) by creatine phosphokinase (Boehringer Mannheim) and then purified by MonoQ FPLC.

Proteins—Partial purification of CPSF, CFI, and CFII from HeLa cell nuclear extracts was performed as described (16). CPSF (0.12 μg/μl) was further purified by heparin-5PW FPLC. CFI (0.4 μg/μl) and CFII (0.15 μg/μl) were MonoS fractions (16). CstF (0.15 μg/μl) was also a MonoS fraction (purity greater than 90%; Ref. 23). CFI was further purified by phenyl-Superose FPLC (PS-CFI; 0.12 μg/μl). Alternatively, the MonoQ low salt fraction (mixture of CFI and CFII; Ref. 16) was fractionated by heparin-5PW FPLC, MonoS FPLC, and heparin-5PW FPLC (HP-CFI; 0.15 μg/μl). CFII was further purified by MonoS (MSS-CFI; 50 ng/μl), or the MonoQ low salt fraction was fractionated by heparin-5PW FPLC (HP-CFII; 40 ng/μl). Histidine-tagged recombinant bovine PAP (50 ng/μl) was purified from Escherichia coli (gift of K. G. Murthy). All protein fractions were stored in Buffer D (20 mM HEPES-NaOH, pH 7.9; 20% glycerol, 50 mM (NH4)2SO4, 0.2 mM EDTA, 0.5 mM dithiothreitol, and 0.5 mM phenylmethylsulfonyl fluoride). Details of the CFI and CFII purification procedures will be described elsewhere. 2

RNA Substrates and in Vitro Cleavage Assay—32P-labeled pre-mRNAs were prepared by SP6 RNA polymerase (Promega) from linearized plasmids pG3SVL-A and pG3L3-A, which contain the SV40 late site and adenovirus 2 L3 poly(A) site, respectively (15). A standard linearized plasmids pG3SVL-A and pG3L3-A, which contain the SV40 late site and adenovirus 2 L3 poly(A) site, respectively (15). A standard

FIG. 1. Creatine phosphate (CP) can be a sufficient cofactor for efficient 3′ end cleavage. 32P-Labeled SV40 pre-mRNA substrate was incubated in a standard cleavage reaction mixture containing CPSF, CstF, CFI, and CFII, and 2 mM EDTA for 90 min at 30 °C in the presence of (A) no phosphate compound (lane 1), 20 mM CP (lane 3), 1 mM ATP (lane 4), or both (lane 2) or (B) in the presence of the indicated amounts of CP. Processed RNAs were isolated and fractionated on 5% polyacrylamide-8.3 M urea gels. Lanes M and Pre indicate size marker and precursor RNA, respectively. The positions of size markers are indicated (in nucleotides) on the left, and the positions of 5′ and 3′ cleavage products are indicated by arrows on the right.

2Y. Hirose, unpublished data.
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FIG. 2. Certain other phosphate compounds can substitute for CP. Cleavage of SV40 pre-mRNA was assayed under standard reaction conditions, as described in the legend to Fig. 1, in the presence of no addition (−) or the indicated phosphate compounds (concentration was 20 mM for CP, 1 mM for others) (A); increasing amounts of CP (lanes 2–4), KHPO₄ + K₂HPO₄ (lanes 5–7), or Na₂H₂PO₄ + Na₃H₂PO₄ (lanes 8–10) (B); or increasing amounts of CP or AP (C). Concentration of each compound is indicated above each lane. The position of cleavage products are indicated as in Fig. 1.

 compounds were required to induce cleavage as was observed with CP. This is significant because Na₃H₂PO₄ can be a trace contaminant of CP, but since such high amounts were necessary, it cannot be the active component in CP (concentrations lower than those shown in Fig. 2B were inactive; data not shown).

Given the high concentration of CP required for cleavage, we were concerned about the possibility that a contaminant in the CP preparation other than inorganic phosphate might be the actual cofactor (see also below). To address this, we tested four different preparations of CP obtained from three different sources (one was prepared synthetically, whereas the others were prepared enzymatically; see “Experimental Procedures”) and found that all four preparations of CP behaved identically (data not shown). The Tris and sodium salts of CP also gave indistinguishable results (data not shown). We also tested whether another phosphoguanidine compound, arginine phosphate (AP), which exists only in invertebrates (reviewed in Ref. 46), could substitute for CP in the cleavage assay. Strikingly, AP also activated cleavage, and it did so as efficiently as CP (Fig. 2C). This implies that the phosphoguanido group may be an important structural motif of the cofactor. Importantly, the fact that AP can substitute for CP further argues against the possibility that the CP requirement in fact reflects an ATP requirement, through the activity of undetectable contaminating CPK in the purified factors, because AP cannot be used as a substrate by CPK (47).

In the above experiments, we utilized highly purified preparations of CPSF and CstF, whereas the CFI and CFII fractions were less pure (although, as mentioned above, they were free of detectable CPK and ATPase). To provide additional evidence that contaminants in the protein fractions were not in some way responsible for the CP effect, both CFI and CFII were subjected to additional purification steps (see “Experimental Procedures”), and two different preparations of each were used in cleavage assays, as above. Under standard reaction conditions (a solution containing 2 mM EDTA and no ATP), CP was again found to be essential for cleavage, activating the reaction in a concentration-dependent manner, nearly identical to the results observed with the less pure cleavage factors (Fig. 3A) A.

ATP Is Not Required for Cleavage—The above results suggest that CP, not ATP, can be a necessary and sufficient cofactor for efficient 3′ cleavage in vitro. However, given the unexpected nature of this finding, we investigated more extensively the possible role of ATP in 3′ cleavage of the SV40 pre-mRNA. As shown above (Fig. 1A), cleavage in the presence of both ATP and CP appeared less efficient than cleavage in the presence of CP alone. We therefore examined the effect of increasing ATP concentrations on cleavage activated by 40 mM CP. Fig. 4A shows that ATP indeed inhibited cleavage effected by CP in a concentration-dependent manner in the presence of either 0.5 mM MgCl₂ (lanes 2–4) or 2 mM EDTA (lanes 7–9), whereas CP alone allowed efficient cleavage under both reaction conditions (lanes 5 and 10). As shown above (Fig. 1A), cleavage in the presence of both ATP and CP appeared less efficient than cleavage in the presence of CP alone. Therefore, we examined the possibility that a bound form of ATP, perhaps tightly associated with a purified factor(s), might participate in cleavage. To this end, cleavage was assayed using factors treated with hexokinase and glucose to remove any endogenous ATP. Depletion of endogenous ATP by conversion
to glucose 6 phosphate was monitored via addition of trace amounts of [γ-32P]ATP and was analyzed by TLC. After incubation of purified factors with hexokinase and glucose for 10 min in the presence of MgCl₂, the exogenous ATP was entirely depleted (Fig. 5A, lanes 1 and 5). EDTA and substrate pre-mRNA were then added, and reaction mixtures were incubated for the times indicated in Fig. 5, after which RNA products and ATP were both analyzed. Although no labeled ATP was generated during the cleavage reaction.32P-Labeled CP was prepared by enzymatic reaction from [γ-32P]ATP and creatine and purified as described under "Experimental Procedures." Hydrolysis of CP during the cleavage reaction was monitored by removing small aliquots from reaction mixtures containing trace amounts of 32P-labeled CP and 40 mM unlabeled CP at the times indicated in Fig. 6 and then analyzing the fate of the CP by TLC. Whereas efficient cleavage proceeded during the incubation (Fig. 6A), neither hydrolyzed inorganic phosphate (Fig. 6B) nor ATP, conceivably generated by transferring the phosphate group from CP to possible endogenous ADP (Fig. 6C), was detected. These results suggest that CP is neither an energy donor for cleavage nor a phosphate source to produce ATP. This latter finding further excludes the possibility that the observed CP requirement reflects ATP regeneration by CPK contamination in the purified factors. Also, SDS gel electrophoresis of reaction mixtures following incubation with labeled CP failed to provide any evidence of protein phosphorylation (results not shown).

**FIG. 4.** ATP but not EDTA inhibits cleavage. Cleavage of SV40 pre-mRNA was assayed under standard reaction conditions in the presence of 0.5 mM MgCl₂ (lanes 1–5), 2 mM EDTA (lanes 6–10), increasing amounts of ATP (lanes 2–4 and 7–9), 40 mM CP (lanes 5 and 10), or H₂O (lanes 1 and 6); (A) 40 mM CP (all lanes), 0.5 mM MgCl₂ (lanes 1–4), 2 mM EDTA (lanes 5–8), or increasing amounts of ATP (lanes 2–4 and 6–8); (B) 40 mM CP (lanes 1–4), increasing amounts of EDTA (lanes 2–4), or H₂O (lanes 1) (C). Concentrations of ATP (A and B) or EDTA (C) are indicated above each lane. The position of cleavage products are indicated as in Fig. 1.

**FIG. 5.** Depletion of possible endogenous ATP does not affect cleavage. Standard reaction mixtures containing 4 mM [γ-32P]ATP (as a tracer to monitor ATP depletion), cleavage factors, hexokinase, 2 mM glucose, and 2.5 mM MgCl₂ were incubated at 30 °C. After 10 min, reaction mixtures were brought to standard cleavage conditions by adding RNA substrate plus or minus 4 mM EDTA and either 40 mM CP (lanes 1–4) or 1 mM ATP (lanes 5–8) and was further incubated at 30 °C. Reactions were stopped at the indicated times, and aliquots were analyzed either directly by chromatography on polyethyleneimine-cellulose using 1 M formic acid and 0.5 M LiCl as solvent (A) or by fractionation on 5% polyacrylamide-8.3 M urea gels after extraction of RNAs (B). The position of each phosphate compound (A) or 5’ and 3’ RNA cleavage products (B) are indicated on the left.

absence of CP (lanes 1 and 5), but processing was observed as the concentration of CP was increased (lanes 2–4 and 6–8). However, in this case, cleavage was inefficient, and addition of 1 mM ATP with CP greatly enhanced cleavage efficiency (compare lanes 2–4 and lanes 6–8). Note, however, that reaction mixtures contained no divalent cation, suggesting that the role of ATP is likely unusual. It is also noteworthy that assays with ATP yielded two different size upstream cleavage products (Fig. 7, lanes 6–8), whereas assays without ATP yielded only the longer one (Fig. 7, lanes 2–4). These results suggest that ATP can influence the precise site of endonucleolytic cleavage. Such heterogeneity in cleavage site specification with L3 pre-mRNA has also been observed by other groups in the presence of 3’ dATP (49, 50). We also observed similar heterogeneity in the presence of 3’ dATP, but in this case, the shorter upstream cleavage product was less prominent than in the presence of ATP (data not shown). Taken together, our data suggest that CP also functions as a necessary cofactor for cleavage of L3 pre-mRNA, but ATP enhances cleavage efficiency and affects the local choice of cleavage sites.

**DISCUSSION**

ATP has until now been thought to be an essential cofactor for endonucleolytic cleavage of pre-mRNAs containing 3’ end
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CP is a member of the phosphagen family, which consists of high energy phosphorylated guanidine compounds that are believed to provide temporary energy buffers in tissues with high energy demands (reviewed in Ref. 53). CPK catalyzes the reversible transfer of phosphate groups between CP and ADP (46). Several lines of evidence exclude the possibility that the observed CP requirement in the 3' cleavage reaction in fact reflects an ATP requirement through generation of ATP by undetected CPK activity in the purified factors. First, we were not able to detect any CPK activity in any of the purified factors. Second, AP could substitute for CP, even though AP exists only in invertebrates and cannot be a substrate of CPK (47). Third, cleavage proceeds normally even in the presence of 8 mM EDTA, although CPK requires a divalent cation for catalytic activity. Fourth, during cleavage, phosphate transfer from 32P-labeled CP to possible endogenous ADP was not detected. Finally, ATP depletion (by glucose and hexokinase) was without effect on cleavage. Since four different preparations of commercially available CP and one preparation of AP gave identical results, we believe that CP or a related guanidino-phosphate compound is required for efficient cleavage. However, our findings that high concentrations of CP (20–60 mM) are necessary for optimal cleavage and that similar concentrations of sodium phosphate allow a lower level of cleavage leave some question as to the identity of the physiologically relevant cofactor. Our data have shown conclusively, however, that the factor is not ATP.

Is it even reasonable to consider the possibility that CP, at concentrations of ~40 mM, is the physiologically relevant cofactor? Although the answer to this question is probably no (the only tissue where this high a level of CP has been documented is skeletal muscle (54, 55)), many other tissues (e.g., brain, smooth muscle, and kidney), have been shown to have CP concentrations of 5–10 mM (56). Thus, if CP can perform its essential function in polyadenylation (see below) at this level in vivo, then it would appear that the concentration of CP could be adequate. But another requirement is that CP be in the nucleus. Although the bulk of CPK is found in the cytoplasm, a number of studies have found significant levels of the enzyme in the nucleus (57–59). In addition, although to our knowledge, the intracellular localization of CP has not been measured, diffusion through the nuclear pores would likely allow accumulation in the nucleus (60).

Given that our data have established that the role of CP in polyadenylation is not to function as an energy source, what could it be doing to facilitate 3' cleavage? The earliest steps in the reaction have been well studied; they involve specification of the poly(A) site by the cooperative interaction of CstF and CPSF with the pre-mRNA (20, 27, 28). A variety of experiments suggest that this interaction, which is frequently measured by
the UV-cross-linking of the CstF-64 polypeptide to the pre-mRNA, is independent of CP (or ATP) (20, 23, 61–64). Therefore, it is likely that an interaction involving CFI and/or CFII requires CP. These factors are not well characterized, although CFI appears to be a heterotrimer, and all three subunits can be UV-cross-linked to RNA (31). We propose that CP is able to facilitate a conformational change important for the function of one or both of these factors. Interestingly, our early studies are consistent with the idea that CPSF, CstF, CFI, and CFII all coexist in a single large complex (15), and it is thus possible that a conformational change within this complex is facilitated by CP or a related compound. Also supporting the involvement of CFI and/or CFII, when a cruder fraction containing both these factors (MonoQ; see Ref. 16) was employed with CstF and CPSF, a low but significant level of cleavage could be detected in the absence of CP (and ATP). Although we do not have a clear explanation for this phenomenon, it was not observed when cruder fractions of CstF or CPSF were used, and thus it again implicates CFI and/or II as the target of CP.

Another possibility is that CP (or AP) does not in fact play any direct role in 3′ end formation in vivo and functions in vitro by mimicking another component. An intriguing possibility is that this might be an unknown phosphoprotein, perhaps containing phosphoarginine. Although rare, phosphoarginine is known to exist in eukaryotic proteins (65). By this model, the high concentrations of CP required allow it to be recognized by a component of the polyadenylation machinery that naturally recognizes a phosphoprotein. Alternatively, CP (or AP) might bind directly to the RNA, influencing its structure and thereby facilitating cleavage. However, attempts to detect an interaction between 32P[CP] and unlabeled substrate RNA (SV40 late and AdL3) were unsuccessful. Furthermore, neither Mg2+ nor spermidine, which are known to influence RNA structure, had any affect on cleavage of the SV40 late pre-mRNA.

Our data have ruled out a requirement for ATP in 3′ cleavage. However, they also show that ATP can influence the reaction, either positively or negatively, dependent on the pre-mRNA substrate. How does this occur? We suggest the possibility that the inhibitory effect is due to competition between CP and ATP. Given our lack of understanding of how CP functions, it is not possible to comment on the details of this putative competition, or on the possible physiological significance of the inhibition. Indeed, given that the SV40 late pre-mRNA is unusual in its lack of a requirement of PAP for cleavage, it could be that the enhancement of cleavage, observed with the AdL3 pre-mRNA, is most relevant. The sequences within the SV40 RNA that confer PAP independence are limited to nine bases surrounding the cleavage site (48); it may be that CP facilitates interaction of cleavage factors with this region, whereas for most pre-mRNAs, PAP and ATP are also required for optimal cleavage. It is also notable that ATP, in addition, affects the precise site of cleavage in the AdL3 RNA. Interestingly, a possibly related phenomenon was observed over a decade ago by Sperry and Berget (66). These authors observed that in nuclear extract, an SV40 early pre-mRNA (which requires PAP) was cleaved 18–20 bases downstream of the authentic cleavage site when ATP was omitted from reaction mixtures. This apparently aberrant cleavage appeared to be catalyzed by the normal polyadenylation machinery, as judged by its poly(A) signal dependence, and was suppressed by ATP in a concentration-dependent manner. We believe that both the enhancement of cleavage and the switch in site selection resulting from ATP are mediated by PAP. Perhaps the processing complex that assembles when PAP contains bound ATP is different from the complex that assembles in the absence of ATP. We do not know whether ATP hydrolysis is required for these effects, but we suspect that it is not, as our experiments were performed in the absence of divalent cation. It is intriguing to suggest that other treatments that affect PAP activity (e.g. phosphorylation; Ref. 67) could also influence cleavage efficiency or site selection. Future work may elucidate this type of regulation.

To summarize, our studies have shown that contrary to expectation, endonucleolytic 3′ cleavage of mammalian premRNAs does not require energy. Creatine phosphate can function as a necessary and sufficient cofactor, but CP hydrolysis does not occur. Finally, ATP can influence the reaction quantitatively and qualitatively but is not essential.


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