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Inhibition of poly(A) polymerase requires p34^{cdc2}/cyclin B phosphorylation of multiple consensus and non-consensus sites

D.F.Colgan, K.G.K.Murthy, W.Zhao, C.Prives and J.L.Manley[1]

Department of Biological Sciences, Columbia University, New York, NY 10027, USA

[1]Corresponding author

We showed previously that p34^{cdc2}/cyclin B (MPF) hyperphosphorylates poly(A) polymerase (PAP) during M-phase of the cell cycle, causing repression of its enzymatic activity. Mutation of three cyclin-dependent kinase (cdk) consensus sites in the PAP C-terminal regulatory domain prevented complete phosphorylation and MPF-mediated repression. Here we show that PAP also contains four nearby non-consensus cdk sites that are phosphorylated by MPF. Remarkably, full phosphorylation of all these cdk sites was required for repression of PAP activity, and partial phosphorylation had no detectable effect. The consensus sites were phosphorylated *in vitro* at a 10-fold lower concentration of MPF than the non-consensus sites. Consistent with this, during meiotic maturation of *Xenopus* oocytes, consensus sites were phosphorylated prior to the non-consensus sites at metaphase of meiosis I, and remained so throughout maturation, while the non-consensus sites did not become fully phosphorylated until after 12 h of metaphase II arrest. We propose that PAP's multiple cdk sites, and their differential sensitivity to MPF, provide a mechanism to link repression specifically to late M-phase. We discuss the possibility that this reflects a general means to control the timing of cdk-dependent regulatory events during the cell cycle.
Keywords: cyclin-dependent kinase/p34^{cdc2}–cyclin B/phosphorylation/poly(A) polymerase

Introduction

During mitosis and meiosis (M-phase), a series of strictly regulated, major structural rearrangements are temporally controlled, culminating in cell division. Extensive studies have shown that a burst of phosphorylation events drives the cell through M-phase of the cell cycle (King *et al.*, 1994). Numerous proteins become phosphorylated, resulting in important changes in their structure or activity (reviewed by Nigg, 1991). During this time of dynamic change, major metabolic activities such as mRNA and protein synthesis are transiently repressed (e.g. Prescott and Bender, 1962; Steward *et al.*, 1968; Fan and Penman, 1970; Sagata *et al.*, 1980; Kanki and Newport, 1991; Shermoen and O'Farrell, 1991; reviewed by Gottesfeld, 1997), most likely in order to accommodate events such as chromosome condensation and nuclear envelope breakdown. It is becoming evident that the silencing of gene expression that accompanies M-phase is not a passive effect of cell division but rather an active and specific shut-down of key cellular processes. This view has been strengthened recently by studies showing the phosphorylation and resulting M-phase-specific transient repression of several essential proteins, such as TFIIIB (Gottesfeld *et al.*, 1994), TFIID (Segil *et al.*, 1996) and poly(A) polymerase (PAP) (Colgan *et al.*, 1996).

Mitosis/meiosis-promoting factor (MPF) is a heterodimeric cyclin-dependent kinase (cdk) consisting of p34^{cdc2} and cyclin B (for a recent review, see Nigg, 1995). M-phase is characterized by the appearance and increase of MPF kinase activity; late in M-phase the abrupt ubiquitinization of the cyclin B subunit triggers cytokinesis and allows progression to G_1 (reviewed in King *et al.*, 1994). MPF phosphorylates a wide range of targets, acting both as a 'master regulator' of mitosis (triggering cascades of reactions by activating downstream protein kinases) and as a 'work horse' (phosphorylating directly some of the proteins that bring about certain metabolic and structural changes) (Nigg, 1991). The optimal phosphorylation site for cdc2 kinase, as well as other cdks, fits the consensus T/SPXK/R (Nigg, 1991; Songyang, 1994). However, phosphorylation of sites where the requirement for a basic residue at the +3 position has been relaxed is not uncommon (Nigg, 1991). It is noteworthy that several substrates of MPF contain multiple phosphorylation sites, e.g. lamins (Peter *et al.*, 1990), non-muscle caldesmon (Yamashiro *et al.*, 1995) and p60^{src} (Morgan *et al.*, 1989; Shenoy *et al.*, 1989). Often such proteins contain a combination of consensus and non-consensus sites. However, the function of the multiplicity and degeneracy of a protein's target sites remains unknown.

Our studies focus on the M-phase-specific regulation of PAP activity. It has been shown previously that PAP is hyperphosphorylated in *Xenopus laevis* M-phase oocytes and unfertilized eggs and in mitotic HeLa cells (Ballantyne and Wickens, 1995; Colgan *et al.*, 1996). Our earlier studies showed that PAP preparations, from either mitotic HeLa cells or Sf9 insect cells co-infected with PAP, p34^{cdc2} and cyclin B baculoviruses, showed a significant reduction in activity and that this repression could be reversed by treatment with phosphatase. Direct phosphorylation of PAP by MPF was demonstrated in *in vitro* assays, and a PAP derivative with mutations in all three of its clustered cdk consensus sites was observed to be resistant to repression by MPF. However, mutation of the consensus cdk sites did not completely prevent the phosphorylation observed during oocyte maturation, suggesting either that PAP is phosphorylated by non-cdks during M-phase or that PAP is among the targets that contain non-consensus, as well as consensus, cdk sites (Colgan *et al.*, 1996).

PAP is a single subunit enzyme, the catalytic core of which is conserved from yeast to man (Ligner *et al.*, 1991;

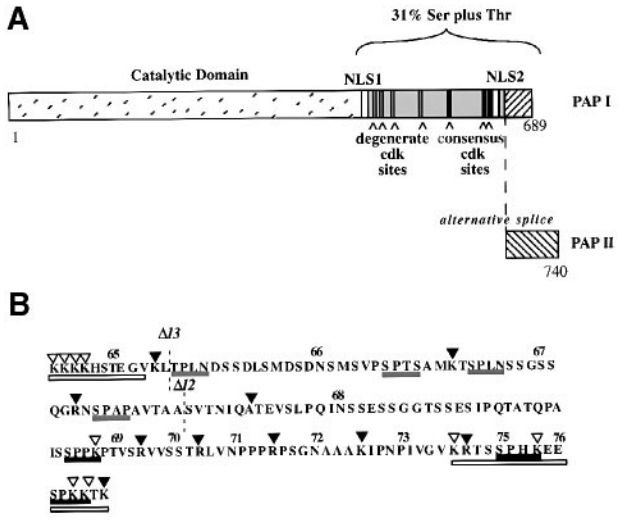

Fig. 1. Poly(A) polymerase. (**A**) Schematic diagram showing significant features of PAP including the catalytic domain and the C-terminal S/T-rich region (which contains two nuclear localization signal sequences, three consensus cdk sites and four potential non-consensus cdk sites), followed by an alternative splice site used to produce PAP II. (**B**) Amino acid sequence of the S/T-rich region of PAP I and II showing NLSs I and II (striped underline), consensus (black underline) and non-consensus (gray underline) cdk sites, as well as sites of tryptic digestion. Filled triangles denote sites where complete trypsin digestion is expected, while open triangles denote sites where partial trypsin digestion is expected. Potential tryptic peptides in the S/T-rich region are numbered 65–76 and are labeled as such. Also shown are the sites of truncation used to construct the Δ12 and Δ13 PAP deletion mutants.

Raabe *et al.*, 1991; Wahle *et al.*, 1991). Primary transcripts encoding mammalian PAP are processed alternatively to give rise to multiple isoforms in mammals (Zhao and Manley, 1996). The longer forms, typified by PAP I and II (Raabe *et al.*, 1991), all display equivalent activities in *in vitro* assays, while several shorter forms are catalytically inactive and their function is unknown (Zhao and Manley, 1996). The long forms all contain a C-terminal S/T-rich region that is dispensable for activity *in vitro* (Raabe *et al.*, 1991; Raabe and Murthy, 1994) and is lacking in the yeast enzyme. This region spans ~180 amino acid residues, with a nuclear localization signal (NLS) at either end. Immediately after the second NLS, the primary sequence of PAP I diverges from the other long forms, due to alternative splicing. Numerous potential phosphorylation sites exist in this S/T rich-region, including the aforementioned three cdk consensus sites (all in exon 19; Zhao and Manley, 1996) as well as four potential non-consensus cdk sites located between the first NLS and the consensus sites (see Figure 1).

The function of PAP in the nucleus is to add a poly(A) tail to nearly every newly transcribed pre-mRNA. PAP is part of a multisubunit complex that tightly couples precise pre-mRNA cleavage to subsequent poly(A) addition (for recent reviews, see Wahle and Keller, 1996; Colgan and Manley, 1997). In general, the site of 3′ end formation is defined by two sequence elements in the pre-mRNA; upstream of the cleavage site lies the nearly invariant polyadenylation signal sequence AAUAAA, and downstream of the cleavage site lies the less well conserved GU-rich element. AAUAAA is recognized by the multisubunit cleavage–polyadenylation specificity factor (CPSF) and the GU-rich element is recognized by another multisubunit protein, cleavage stimulation factor (CstF). Following site definition, two factors, cleavage factors I and II, participate in the cleavage reaction and PAP then polymerizes a tail of ~250 adenosines to the 3′ end of the 5′ cleavage product.

Poly(A) addition to pre-existing mRNAs can also occur in the cytoplasm, and has been observed in a variety of organisms during early development. Such cytoplasmic poly(A) addition has been shown to control gene expression until activation of zygotic transcription (reviewed in Richter, 1996; Wickens *et al.*, 1997). Here, temporally regulated translation of cytoplasmic maternal mRNAs is controlled by dynamic changes in poly(A) tail length, with messages possessing a long poly(A) tail being recruited to polysomes for active translation, and those with short tails being translationally inactive. In both frog and mouse, the polyadenylated state of a mRNA has been found to be controlled by sequence elements in the 3′-untranslated region (UTR) known as cytoplasmic polyadenylation elements (CPEs). While interactions of such elements with developmentally specific factors probably control the timing of poly(A) addition (Paris *et al.*, 1991; Hake and Richter, 1994), 3′ end recognition and the elongation of the tail itself appear to rely on the same proteins that participate in nuclear polyadenylation, CPSF and PAP (Fox *et al.*, 1992; Bilger *et al.*, 1994). Thus PAP plays an important role during both 3′ end formation of newly transcribed pre-mRNAs in the nucleus and translational activation of maternal mRNAs in the cytoplasm during early development. Taken together, it can be concluded that the ubiquitous presence and obvious importance of the poly(A) tail make it likely that repression of PAP activity would affect the synthesis, stability and/or translational efficiency of all polyadenylated mRNAs.

Here we describe more precisely the complex pattern of MPF-induced phosphorylation of PAP and requirements for inhibition. We present evidence that in addition to the three consensus cdk sites, PAP is phosphorylated by MPF on four non-consensus cdk sites, and that complete phosphorylation of all these sites is required for MPF-induced repression. Furthermore, we show that while the consensus sites are phosphorylated at a low concentration of MPF, phosphorylation of the non-consensus sites requires a much higher concentration. We propose that progressive hyperphosphorylation of PAP, first on consensus cdk sites and subsequently on non-consensus sites, may be a sensing mechanism that specifically links PAP repression to late M-phase. This suggests the possibility that a transient shut-down in the polyadenylation machinery late in M-phase, in combination with the repression of transcriptional activity, may be an important component of M-phase cells.

Results

PAP is phosphorylated by MPF on non-consensus cdk sites in vitro

Previously we described the construction of PAP cdk$^-$, in which the serine residues of the three cdk consensus sites in bovine PAP I were mutated to alanine or glycine (Colgan *et al.*, 1996). When Sf9 cells were co-infected with a PAP cdk$^-$ recombinant baculovirus and viruses

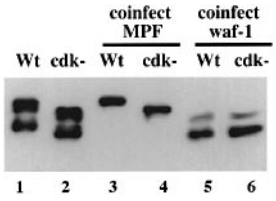

Fig. 2. The phosphorylation pattern of recombinant baculovirus PAPs can be altered by co-infection with MPF or p21/waf-1 viruses. Recombinant bovine PAPs were purified from Sf9 cells infected with viruses expressing wild-type or cdk⁻ PAP alone (lanes 1 and 2) or in combination with $p34^{cdc2}$ plus cyclin B viruses (lanes 3 and 4) or a p21/waf-1 virus (lanes 5 and 6). Proteins were resolved by SDS–PAGE and visualized by Western blotting and chemiluminescence.

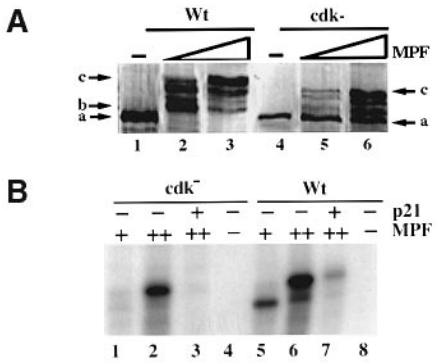

Fig. 3. PAP is differentially phosphorylated by MPF on consensus and non-consensus cdk sites. (**A**) Consensus cdk sites are phosphorylated at a 10-fold lower concentration of MPF than the non-consensus sites. *In vitro* phosphorylation of 400 ng of wild-type (lanes 1–3) and cdk⁻ (lanes 4–6) PAP with 0, 20 and 200 ng of MPF. Proteins were analyzed by SDS–PAGE followed by silver staining.
(**B**) Phosphorylation of both consensus and non-consensus cdk sites is inhibited by p21/waf-1. Four hundred ng of cdk⁻ (lanes 1–4) and wild-type (lanes 5–8) PAP were treated with 20 ng (lanes 1 and 5), 200 ng (lanes 2, 3, 6 and 7) or 0 ng (lanes 4 and 8) of MPF, plus p21/waf-1 (lanes 3 and 7) in the presence of [γ-^{32}P]ATP. Proteins were analyzed by SDS–PAGE followed by autoradiography.

expressing the two subunits of MPF ($p34^{cdc2}$ and cyclin B), no inhibition of PAP activity was detected, contrasting with the strong inhibition observed when wild-type PAP was used in such an experiment (Colgan *et al.*, 1996). These results suggested that cdk⁻ PAP, unlike wild-type PAP, was not hyperphosphorylated in Sf9 cells when co-expressed with MPF. To test this directly, wild-type and cdk⁻ PAP were expressed in Sf9 cells in the presence or absence of MPF, and the exogenously expressed PAP (isolated via an N-terminal His tag) was analyzed by Western blotting (Figure 2). Unexpectedly, the patterns displayed by the two enzymes were remarkably similar: in the absence of MPF (compare lanes 1 and 2), both gave rise to multiple, apparently related species, while in the presence of MPF (compare lanes 3 and 4) each was detected as a single, slower migrating hyperphosphorylated species. However, each cdk⁻ PAP species displayed slightly greater gel mobilities, consistent with some degree of underphosphorylation. Note that much of the PAP phosphorylation, even in the absence of co-infected MPF, was due directly or indirectly to a cdk, as co-infection with a virus encoding the cdk inhibitor p21/waf-1 (El-Deiry *et al.*, 1993) greatly reduced phosphorylation and caused wild-type and cdk⁻ PAP to display identical mobilities (compare lanes 5 and 6).

Two significant points emerge from this analysis of MPF-induced hyperphosphorylation. First, because the mobilities of hyperphosphorylated wild-type and cdk⁻ PAP were nearly identical, significant phosphorylation must occur on sites other than the three cdk consensus sites. Moreover, since this additional phosphorylation was inhibited by p21/waf-1, and promoted by MPF, these additional sites are almost certainly non-consensus cdk sites. Second, despite their similar mobilities, hyperphosphorylated cdk⁻ PAP is fully active, while wild-type hyperphosphorylated PAP is nearly inactive (Colgan *et al.*, 1996). Together, these results suggest that considerable cdk phosphorylation can occur without influencing PAP activity, and the enzyme is inhibited only when it is fully phosphorylated.

To examine more directly the relationship between cdk phosphorylation and PAP inhibition, recombinant wild-type and cdk⁻ PAP were produced as unphosphorylated forms in *Escherichia coli* and the purified proteins were treated *in vitro* with increasing amounts of purified MPF plus ATP. When analyzed by silver staining (Figure 3A), it is evident that at a low kinase concentration the unphosphorylated form of wild-type PAP was completely converted to several lower mobility forms (lane 2); lower amounts of kinase resulted in conversion to form b alone (data not shown). In contrast, after treatment with low concentrations of MPF, cdk⁻ PAP remained largely unphosphorylated (lane 5). However, several additional forms with decreasing mobilities were observed in both wild-type and cdk⁻ PAP treated with a 10-fold higher concentration of MPF (Figure 3A, lanes 3 and 6). Similar results were observed when [γ-^{32}P]ATP was included in the kinase reaction and products were detected by autoradiography (Figure 3B). At the low MPF concentration, only wild-type PAP was significantly phosphorylated (lane 5), while at the higher concentration both forms were extensively modified (lanes 2 and 6). Significantly, all of these phosphorylations were inhibited by purified p21/waf-1 (Figure 3B, lanes 3 and 7), supporting the conclusion that MPF directly phosphorylates not only the cdk consensus sites, but also additional sites present in both wild-type and cdk⁻ PAP.

The sequence of PAP reveals four potential non-consensus cdk sites (S/TPXX) located in a 45 residue region just N-terminal to the consensus sites (see Figure 1). To determine whether these sites correspond to the additional sites phosphorylated by MPF, we first examined the ability of two C-terminal truncation mutants to be phosphorylated by MPF *in vitro*. One, Δ12, lacks 93 residues including the three consensus cdk sites, and the other, Δ13, lacks an additional 51 residues, which also removes all the non-consensus sites (see Figure 1). In the experiment shown in Figure 4, wild-type PAP and the two truncation mutants were produced (in essentially unphosphorylated form) by *in vitro* translation in a rabbit reticulocyte lysate, phosphorylated with purified MPF and analyzed by SDS electrophoresis. The pattern of phosphorylated wild-type PAP was similar to that seen in Figure 3A (Figure 4, compare lanes 1 and 2), and all of this mobility shift was inhibited by the cdk inhibitor olomoucine (lane 3). When Δ12 was treated with MPF, it too was shifted significantly

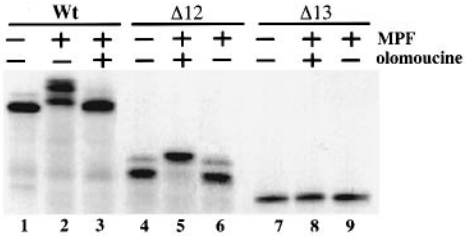

Fig. 4. Localization of non-consensus cdk sites in the S/T-rich region. Full-length wild-type PAP (lanes 1–3) and C-terminal truncation mutants, deleting either the consensus cdk sites alone (Δ12; lanes 4–6) or the consensus and non-consensus cdk sites (Δ13; lanes 7–9), were produced by *in vitro* translation in the presence of [^{35}S]methionine, treated with MPF in the absence (lanes 2, 5 and 8) or presence (lanes 3, 6, and 9) of olomoucine.

in mobility, though somewhat less than wild-type, consistent with the absence of the cdk consensus sites but the presence of non-consensus sites (Figure 4, lanes 5 and 6). Despite the lack of consensus cdk sites, and consistent with the data obtained with cdk$^-$ PAP, Δ12's mobility shift was also prevented by olomoucine (lane 6). In contrast, no mobility change was detected when Δ13 was incubated with MPF (Figure 4, lanes 7–9), strongly suggesting that one or more non-consensus cdk sites present in Δ12 but lacking in Δ13 was phosphorylated by MPF.

The data in Figure 3A imply that the consensus and non-consensus cdk sites are sensitive to different concentrations of MPF, with the consensus sites being phosphorylated at a low concentration of kinase (inducing a minor mobility shift) and the non-consensus sites modified significantly only at higher levels of kinase (inducing a more dramatic mobility shift). To provide additional support for this idea, and also to identify the sites of phosphorylation more precisely, wild-type and cdk$^-$ PAPs were expressed in *E.coli*, phosphorylated with MPF and [γ-^{32}P]ATP, resolved by SDS–PAGE, and different forms were isolated and subjected to two-dimensional tryptic phosphopeptide analysis. Full digestion of PAP by trypsin should result in 112 different peptides, with peptides 65–75 derived from the region containing the S/T-rich domain. Figure 1 shows the predicted sites of trypsin digestion and the corresponding numbered peptides in the S/T-rich region of wild-type PAP I, which includes all potential cdk sites. Triangles denote the predicted sites of trypsin digestion (K and R residues), with filled triangles showing sites of complete digestion and open triangles indicating sites where complete or partial inhibition of digestion is expected due to local amino acid composition. Since trypsin cannot cleave between K and P, peptides 68 and 69 will be a single peptide, designated 68+69. Peptide 68+69 and peptide 66 are relatively large, have a low charge and so should be retained in the electrophoretic dimension (*x* axis). They are also relatively hydrophobic and therefore more mobile in the chromatographic dimension (*y* axis). Peptide 68+69 also has the unique property of having both a consensus and non-consensus site. The other two consensus sites are in peptides 74 and 75, which are relatively small (mobile in the electrophoretic dimension) and, due to partial cleavage by trypsin, predicted to appear as several spots.

Figure 5A shows tryptic phosphopeptide analysis of

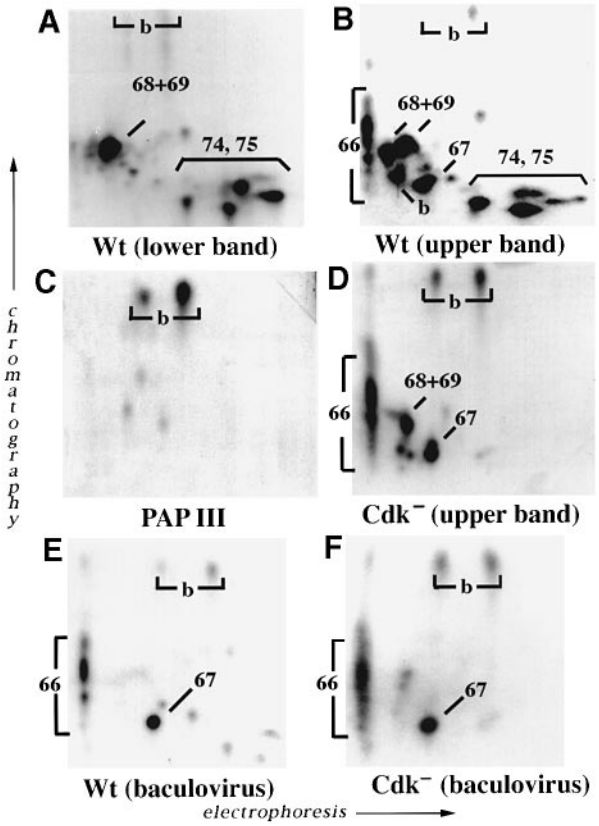

Fig. 5. PAP can be phosphorylated by MPF on three consensus and four non-consensus cdk sites. (**A**) Wild-type PAP is phosphorylated on three consensus cdk sites at low concentrations of MPF. *E.coli*-expressed wild-type PAP (500 ng) was phosphorylated with 25 ng of MPF, isoforms were resolved by SDS–PAGE, eluted from the gel, and form b (see Figure 3A) subjected to tryptic phosphopeptide analysis (see Materials and methods). (**B**) Wild-type PAP is phosphorylated on three consensus and four non-consensus cdk sites at high concentrations of MPF. *E.coli*-expressed wild-type PAP (500 ng) was phosphorylated with 250 ng of MPF *in vitro*, followed by tryptic phosphopeptide analysis of form c (see Figure 3a). (**C**) PAP lacking the S/T-rich region (PAP III) shows only background phosphorylation. PAP III (500 ng) was treated with 250 ng of MPF and the single phosphorylated form (not shown) was isolated by SDS–PAGE and analyzed by tryptic phosphopeptide analysis. (**D**) Cdk$^-$ PAP is phosphorylated on four non-consensus cdk sites at high concentrations of MPF. *E.coli*-expressed cdk$^-$ PAP (1 mg) was treated with 400 ng of MPF, followed by tryptic phosphopeptide analysis of form c (see Figure 3A). (**E**) Baculovirus-expressed wild-type PAP I is phosphorylated *in vivo* on three consensus and one non-consensus cdk site. Baculovirus-expressed wild-type PAP I (500 ng) was phosphorylated with 250 ng of MPF, followed by tryptic phosphopeptide analysis of form c (see Figure 3A). (**F**) Phosphotryptic peptide maps of cdk$^-$ and wild-type PAP produced in Sf9 cells and phosphorylated *in vitro* are identical. Baculovirus-expressed cdk$^-$ PAP (500 ng) was phosphorylated with 250 ng of MPF, followed by tryptic phosphopeptide analysis of form c (see Figure 3A).

wild-type PAP phosphorylated by a low concentration of MPF *in vitro*. (The band isolated from the gel for analysis was analogous to form b, lane 2 of Figure 3A.) As mentioned above, it is predicted that this form is phosphorylated only on cdk consensus sites, since it was not observed in phosphorylated preparations of cdk$^-$ PAP. The map obtained from this form of wild-type PAP supports this prediction. A prominent spot that displays low mobility in the electrophoretic dimension and high mobility in the chromatographic dimension is likely to be peptide 68+69

(see below). In addition, several small peptides were detected that are consistent with phosphorylation of partially digested peptides 74 and 75. When the lowest mobility form of wild-type PAP was analyzed (analogous to form c in lane 3 of Figure 3A), labeled peptides consistent with phosphorylation not only on the three cdk consensus sites but also on additional sites were observed (Figure 5B). The migration pattern of the additional spots is consistent with phosphorylation of the non-consensus cdk sites and identification as phosphorylated peptides 66, 67 and 68+69. Peptide 68+69 appeared as two spots, with the position of one the same as in a and the other spot slightly displaced in a way consistent with a change in mobility resulting from a second phosphorylation, on the non-consensus cdk site (see below). Thus the non-consensus site on peptide 68+69 (SPAP) was incompletely phosphorylated by MPF. If peptide 66 is phosphorylated on both of its cdk sites, then it would have no charge and would not migrate in the electrophoretic dimension. Confirming phosphorylation of both sites, peptide 66 (which appeared as a smear) did not migrate in the electrophoretic dimension, and no additional spots were observed in positions that would indicate partial phosphorylation. Finally, a spot also appeared in the position where peptide 67 was predicted to be, confirming at least partial phosphorylation of this non-consensus site. To provide additional support for the above assignments, PAP III, an isoform produced by alternative splicing (Zhao and Manley, 1996) that lacks the S/T-rich region, was produced in *E.coli*, purified, phosphorylated by MPF and subjected to tryptic phosphopeptide mapping (Figure 5C). Only a low level of phosphorylation was detected, the spots observed were identical to those indicated as background (b) at the top of the maps in Figure 5A and B, and none of the spots described above were detected.

The tryptic phosphopeptide analysis described above is consistent with phosphorylation by MPF of all seven predicted cdk sites of wild-type PAP. The phosphopeptide map obtained from hyperphosphorylated PAP cdk$^-$ (Figure 5D) strongly supports this interpretation. The spots identified as partially digested peptides 74 and 75 were completely absent, consistent with mutation of the consensus sites. Similarly, the spot assigned as peptide 68+69 migrated as phosphorylated on a single site, reflecting a single phosphorylation on its non-consensus site. Furthermore, non-consensus site-containing peptides 66 and 67 were again detected. Together, these data support the conclusion that phosphorylation of the consensus cdk sites causes the minor mobility shift observed when wild-type but not cdk$^-$ PAP was treated with low levels of MPF (Figure 3A, compare lanes 1, form a with lane 2, form b). In addition, the phosphopeptide maps indicate that the non-consensus cdk sites, present in both wild-type and cdk$^-$ PAP, are phosphorylated only at high levels of MPF. The data also indicate that it is phosphorylation of these non-consensus sites that induces the major mobility change.

As mentioned above, wild-type and cdk$^-$ PAP produced by recombinant baculoviruses exhibited similar migration patterns during SDS–PAGE, with the wild-type PAP isoforms migrating slightly more slowly than the corresponding cdk$^-$ isoforms (Figure 2, compare lanes 1 and 2). The difference in mobility between wild-type and cdk$^-$ is

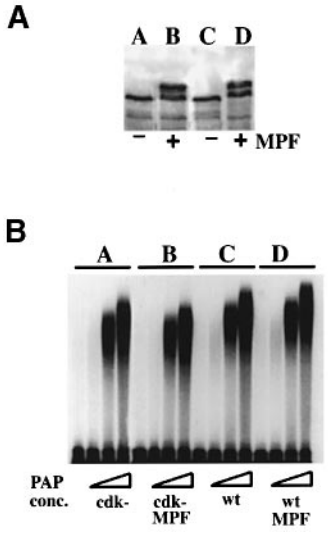

Fig. 6. Partial phosphorylation of PAP's cdk sites does not affect its activity. (**A**) One mg of *E.coli*-produced cdk$^-$ (lanes A and B) and wild-type (lanes C and D) PAPs were incubated without (lanes A and C) and with (lanes B and D) 200 ng of MPF. Proteins were equilibrated on a desalting column (Smart System, Pharmacia), resolved by 7.5% PAGE and visualized by silver staining. (**B**) Increasing concentrations of the PAP preparations shown in (a) (5, 10 and 20 ng) were used in a non-specific polyadenylation assay (see Materials and methods).

consistent with consensus cdk site phosphorylation of wild-type PAP in baculovirus-infected Sf9 cells. To test this, wild-type and cdk$^-$ PAP purified from Sf9 cells were phosphorylated by MPF *in vitro* and subjected to two-dimensional phosphopeptide analysis (Figure 5, maps e and f). In this analysis, sites phosphorylated *in vivo* will not be detected following *in vitro* labeling, and only sites unphosphorylated in Sf9 cells will be labeled. These results in both cases showed phosphorylation *in vitro* primarily of peptides 66 and 67, with little phosphorylation of the three consensus sites. This confirmed that wild-type PAP purified from insect cells was nearly fully phosphorylated on the three cdk consensus sites, and possibly on the non-consensus site on peptide 68+69. Alternatively, the non-consensus site on peptide 68+69 may be more difficult to phosphorylate *in vitro*.

The above results are consistent with the idea that phosphorylation of all cdk sites (consensus and non-consensus) is necessary for repression of PAP activity. To provide additional support for this hypothesis, we compared the activities of unphosphorylated wild-type and cdk$^-$ PAP purified from *E.coli* and treated with a concentration of MPF that resulted in full phosphorylation of the consensus sites, but only limited phosphorylation of the non-consensus sites. Figure 6A shows the PAPs after SDS–PAGE and detection by silver staining, while Figure 6B shows the activity of increasing amounts of the PAPs shown in Figure 6A. Strikingly, no difference in activity was detected amongst any of the PAPs. As shown by the tryptic phosphopeptide mapping above (Figure 5), wild-type PAP was completely phosphorylated on the consensus cdk sites (as judged in Figure 6A by the slightly reduced mobility of the lower band relative to unphosphorylated PAP; compare lanes C and D), yet in the absence of full phosphorylation of the non-consensus sites, enzymatic

activity was unaffected. Together with our previous results comparing wild-type and cdk$^-$ PAP hyperphosphorylated by co-infection with MPF baculoviruses, which indicated that only wild-type PAP was inhibited (Colgan *et al.*, 1996), these findings indicate that full phosphorylation is necessary for inhibition of activity. Strengthening this conclusion, hyperphosphorylated, inactive PAP isolated from HeLa cells can be completely reactivated by phosphatase treatment (Colgan *et al.*, 1996).

PAP is differentially phosphorylated on non-consensus cdk sites during meiotic maturation of Xenopus oocytes

We have shown two examples in which the non-consensus cdk sites of PAP are phosphorylated when the concentration of MPF is high: *in vitro* phosphorylation assays with purified proteins and in baculovirus co-infections of PAP with p34^{cdc2} and cyclin B viruses. This raises the question of whether these non-consensus sites are differentially phosphorylated *in vivo* at physiological levels of MPF. Our previous analysis of PAP isolated from cycling and M-phase HeLa cells (Colgan *et al.*, 1996) is consistent with complete cdk site phosphorylation specifically during M-phase. Meiotic maturation of *Xenopus* oocytes provides an ideal environment to examine possible changes in PAP's phosphorylation pattern during M-phase because of MPF's well-characterized cycling as the oocyte traverses the two phases of meiosis. Furthermore, oocytes can be examined at two naturally arrested states, one in which MPF is inactive, and another when MPF is maximally active (for a review, see Stern and Nurse, 1996). Fully grown (stage VI) *Xenopus* ooctyes are arrested in prophase of meiosis I and contain inactive MPF. Exposure to progesterone triggers MPF activation, and allows meiosis to proceed. MPF activity increases until metaphase, at which point a white spot is clearly visible at the oocytes' animal pole, a sign of germinal vesicle breakdown (GVBD). Then ubiquitination of the cyclin B subunit of MPF causes turnover of MPF and completion of meiosis I. Meiosis II immediately begins, accompanied by the characteristic rise in MPF activity, and progresses to metaphase when the oocyte is again arrested. An oocyte arrested at metaphase of meiosis II retains high MPF activity, and is in many ways equivalent to an unfertilized egg. It has been established that while the time from progesterone treatment to GVBD is variable among oocytes, the time between GVBD and metaphase of meiosis II is a fixed 3 h, and MPF activity reaches a minimum between 45 and 60 min after GVBD (Oshumi *et al.*, 1994).

It has been shown that PAP translated from mRNA injected into *Xenopus* oocytes is phosphorylated during meiotic maturation (Ballantyne and Wickens, 1995; Colgan *et al.*, 1996). M-phase-specific phosphorylation induced a major shift in PAP's mobility, and mutation of the three cdk consensus sites only inhibited a small fraction of this shift (Colgan *et al.*, 1996), consistent with the minor difference that phosphorylation of the consensus sites causes *in vitro* and in baculovirus-infected Sf9 cells (see above). To provide evidence that the additional phosphorylation observed during meiotic maturation occurs on non-consensus sites, the C-terminal truncation mutant deleting the three consensus sites, but not the four non-consensus sites (Δ12), was examined following injection of its mRNA into oocytes and induction of maturation with progesterone. Figure 7A (lanes 3 and 4) shows that, consistent with the *in vitro* results described above, Δ12 exhibited a significant mobility shift during oocyte meiotic maturation, characteristic of phosphorylation of the non-consensus cdk sites. In contrast, wild-type PAP (lanes 1 and 2) gave rise to two predominant forms, one displaying only a minor shift reflecting phosphorylation of consensus sites, and the other a significant shift as expected from modification of non-consensus sites as well.

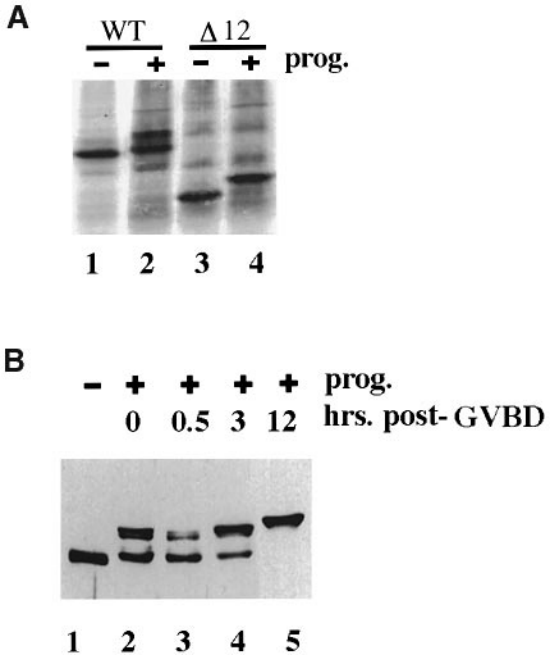

Fig. 7. PAP I is differentially phosphorylated on its consensus and non-consensus cdk sites during *Xenopus* oocyte maturation. (**A**) A C-terminal deletion mutant lacking the consensus but retaining the non-consensus cdk sites is phosphorylated during meiotic maturation in *Xenopus* oocytes. Groups of 10 oocytes were injected with 50 ng of wild-type (lanes 1 and 2) or C-terminal deletion mutant Δ12 (lanes 3 and 4) bovine PAP I mRNA. Oocytes were incubated overnight in medium containing [^{35}S]methionine, and groups analyzed in lanes 2 and 4 were exposed to progesterone to induce maturation. Following maturation, oocytes were lysed, PAP was immunoprecipitated using rabbit polyclonal antibodies, and proteins were resolved by SDS–PAGE. The gel was dried and exposed to X-ray film. (**B**) Consensus and non-consensus cdks are differentially phosphorylated during oocyte maturation. Oocytes were injected with 50 ng of bovine PAP I mRNA, incubated overnight to allow for expression and specified oocytes were treated with progesterone. Single oocytes were extracted at the indicated time points, resolved by SDS–PAGE and PAP isoforms visualized by Western blot using a rabbit polyclonal antibody and chemiluminescence.

To extend this analysis, we wished to assay the phosphorylation state of wild-type PAP during the course of oocyte maturation to observe how phosphorylation of the PAP cdk sites responds to the rise and fall of MPF activity that drives meiosis. To this end, oocytes were injected with wild-type PAP mRNA, incubated overnight to allow for protein expression and, as opposed to the above experiments where groups of 10 oocytes were examined, single oocytes were analyzed at varying times following progesterone addition. Western blot analysis showed that, as reported previously (Ballantyne and Wickens, 1995), PAP remained essentially unphosphorylated until GVBD

(Figure 7B, lane 1). However, oocytes harvested at the first sign of GVBD (when changes at the animal pole were barely detectable) showed apparently complete phosphorylation of PAP's consensus sites and significant phosphorylation of non-consensus sites (Figure 7B, lane 2). Oocytes harvested 60 min after GVBD, during prophase of meiosis II when MPF activity is low (Ohsumi *et al.*, 1994), still showed full phosphorylation of the consensus sites, although the non-consensus sites became largely dephosphorylated (Figure 7B, lane 3). At 180 min after GVBD, the non-consensus sites again became heavily phosphorylated (Figure 7B, lane 4), but it was not until after 12 h of arrest in metaphase of meiosis II that hyperphosphorylation of PAP was observed (lane 5). This time course is consistent with our *in vitro* observations, in which the non-consensus sites were only phosphorylated when the concentration of MPF was high. Furthermore, the data suggest that once phosphorylated, the consensus sites remain phosphorylated throughout maturation, while the non-consensus sites are dephosphorylated. We have analyzed PAP in hundreds of maturing oocytes, individually and in groups, and have never detected dephosphorylation of the consensus sites after GVBD. We conclude from these studies that the non-consensus and consensus cdk sites display a differential response to changing levels of MPF activity, which may link repression of PAP activity to a critical concentration of MPF.

Fig. 8. Clustered cdk sites are conserved in vertebrate PAPs. Amino acid sequences of frog, cow, human, chicken and mouse PAP C-terminal S/T-rich domains are shown aligned. The two conserved bipartite nuclear localization signal sequences are boxed, and potential cdk phosphorylation sites are shaded. Consensus cdk sites are shaded in dark gray and non-consensus sites in light gray. Sources of sequence data are: cow, Raabe *et al.* (1991) and Wahle *et al.* (1991); human, Thuresson *et al.* (1994); frog, Ballantyne and Wickens (1995); mouse, Zhao and Manley (1996); and chicken, W.Zhao and J.L.Manley, unpublished data.

Discussion

We have shown that PAP is phosphorylated by MPF on multiple consensus and non-consensus cdk sites, with higher levels of MPF required to phosphorylate PAP on the non-consensus sites. Complete phosphorylation of the consensus sites and partial phosphorylation of non-consensus sites was shown not to affect PAP activity. Together with our previous data that phosphorylation of all non-consensus sites also does not detectably affect activity (Colgan *et al.*, 1996), these findings provide strong evidence that complete phosphorylation of all cdk sites is required to inhibit PAP. This complex on/off switch provides a mechanism whereby PAP will be repressed only in the presence of high or prolonged levels of MPF. Our data showing that PAP becomes hyperphosphorylated only late during meiotic maturation of oocytes not only supports this idea, but also provides an explanation for why such a complex switch may have evolved.

Bovine PAP I was a good substrate for studying the responsiveness of consensus and non-consensus cdk sites to increasing levels of MPF. Phosphorylation of non-consensus versus consensus sites resulted in characteristic changes in the mobility of PAP on SDS–polyacrylamide gels, providing a relatively simple way to assess the phosphorylated state of the different types of sites. However, PAP II appears to be the most abundant form of full-length PAP in most cells (e.g. Zhao *et al.*, 1996), and it is therefore important to know whether it responds to MPF in the same way as PAP I. We observed that PAP II is hyperphosphorylated, and its activity repressed, in Sf9 cells co-infected with MPF (Colgan *et al.*, 1996), suggesting that PAP II undergoes the same changes in its phosphorylated state as does PAP I. Supporting this, phosphopeptide mapping of PAP II phosphorylated *in vitro* with MPF revealed the same patterns of consensus and non-consensus phosphorylation as did PAP I (unpublished data). Thus both forms appear to respond identically to MPF, despite the presence of alternative C-terminal exons. It is also important to note that the presence of clustered consensus and non-consensus cdk sites is conserved in all vertebrate PAPs analyzed to date (see Figure 8). In contrast, it is notable that *Saccharomyces cerevisiae* PAP entirely lacks a C-terminal S/T-rich region (Ligner *et al.*, 1991), suggesting that cell cycle regulation of PAP may be restricted to metazoa.

The on/off switch in PAP activity is a dramatic one, and in the absence of more data it is difficult to envisage how complete hyperphosphorylation, probably of all seven cdk sites, causes a nearly total repression of activity, while partial phosphorylation has no detectable effect. Our current model is that repression involves an inhibiting interaction between the hyperphosphorylated C-terminus and the catalytic N-terminus, and perhaps this requires high negative charge. It will be of interest to determine whether partial PAP phosphorylation in fact affects some aspect of PAP function, despite our current evidence to the contrary. It is conceivable that this phosphorylation only provides a buffer to ensure that PAP inhibition does not occur at inappropriate times during the cell cycle (see below).

In order to make general conclusions about MPF activity from our observations of exogenously expressed PAP in *Xenopus* oocytes, it is important that the observed changes in PAP's phosphorylated state reflect those of endogenous proteins, which we believe it does. It has been demonstrated that MPF is reactivated prematurely at prophase

of meiosis II, probably as a mechanism to allow metaphase to occur twice without an intervening S-phase (Furuno *et al.*, 1994). This premature reactivation results in only a dip, rather than a prolonged decline in MPF levels. This dip is reflected in the observed underphosphorylation of PAP non-consensus sites after metaphase of meiosis I. Thus the observed changes in the phosphorylated state of the exogenously supplied PAP correlate well with the endogenous MPF activity.

Our results support a model in which progressive phosphorylation of PAP, first on consensus cdk sites and later on non-consensus sites, provides a mechanism for linking repression of PAP activity exclusively to late M-phase. In this way, repression of PAP can be controlled via phosphorylation by MPF, but only during a prolonged period of MPF activity. It would be especially important that PAP retain activity in the presence of moderate levels of MPF during oocyte maturation, since maturation-specific gene expression relies on the oocyte's capacity to polyadenylate maternal mRNAs. Therefore, it is significant that we have not observed hyperphosphorylated PAP at or shortly after GVBD, since at this time PAP must be active, playing an important role in cytoplasmic polyadenylation. The requirement for full phosphorylation of non-consensus sites for PAP inhibition allows for a significant period during which MPF is active, performing other essential functions, but PAP nonetheless retains full activity, thus being able to polyadenylate the appropriate maternal mRNAs.

The study of the phosphorylation pattern of PAP during M-phase has revealed not only information about PAP regulation during the cell cycle, but also general properties applicable to other cdk targets. For example, several substrates have been shown to have multiple phosphorylation sites, and some of these contain both consensus and non-consensus sites, similar to PAP (see Nigg, 1993, and references therein). One, non-muscle caldesmon, is known to have seven cdk sites in its C-terminal region, three of which are consensus and four non-consensus, very similar to PAP. Mitosis-specific phosphorylation of non-muscle caldesmon by cdc2 causes it to dissociate from microfilaments, which is believed to play a role in cell rounding during mitosis (for a review, see Yamashiro and Matsumura, 1991). It is possible that multiple cdk sites in the non-muscle caldesmon C-terminus may allow it to respond to increasing concentrations of MPF in a manner similar to PAP, linking its activity to the course of M-phase progression. Another MPF substrate, the NIMA kinase, which plays a role in coordinating chromosome condensation with MPF activity during M-phase (for a review, see Fry and Nigg, 1995), has 10 potential cdk sites in a C-terminal S/T-rich regulatory domain, only five of which match the consensus. Like PAP, NIMA's enzymatic activity has been shown to be regulated by MPF during M-phase (for a review, see Fry and Nigg, 1995), although its activity is up-regulated by MPF phosphorylation. Again, perhaps the multiple consensus and non-consensus sites allow NIMA to respond differently to either high or low levels of MPF activity. In contrast, other substrates, such as histone H1, contain only consensus sites (Nigg, 1991) and are readily phosphorylated at relatively low concentrations of MPF. Taken together, it is possible that the existence of different types of substrates, responding to different levels of MPF, allows MPF to regulate directly both early and late events of M-phase simply through its rise in activity or concentration during the course of M-phase.

Our results support a model in which phosphorylation of MPF substrates such as PAP is, in part, dependent upon quantitative differences in MPF activity during M-phase. It has been suggested previously that a similar mechanism functions to modulate the cell cycle in *Schizosaccharomyces pombe* (Stern and Nurse, 1996). *Schizosaccharomyces pombe* expresses three B-type cyclins, cig1, cig2 and cdc13, and all pair with a single catalytic subunit, cdc2. In wild-type strains, cig1 is the main partner of cdc2 during S-phase and cdc13 is the main partner of cdc2 during G_2/M and M. *cig1* and *cig2* can be deleted without affecting cell viability, and such mutants can traverse the cell cycle with a single oscillation of the cdc13 protein (for a review, see Fisher and Nurse, 1995). In contrast, conditional mutants of *cdc13* cannot enter M-phase at the restrictive temperature, and they continually re-replicate their DNA (Enoch and Nurse, 1990). This suggests that the M-phase-containing complexes can perform the functions of the S-phase cyclins, but the reverse is not the case. A model to explain these results was proposed in which the cell cycle in *S.pombe* is driven by a general cdk activity that is lower during S-phase than M-phase. While the M-phase cdk (containing cdc13) can reach the level of general activity of the S-phase cdk (containing cig2), and therefore can substitute for it, S-phase cdk activity never reaches a level that can push the cell through M-phase if cdc13 is not functioning. This model suggests that simply increasing the level of cdk activity can be sufficient to coordinate cell cycle events. Analogously, our data provide evidence that in higher organisms quantitative changes in MPF activity can temporally control the response of a target protein during M-phase. Furthermore, our data provide evidence for a hierarchy among cdk sites, and a rationale for its existence.

Materials and methods

Preparation of PAPs

A total of 10^9 Sf9 cells were infected with 1 p.f.u./cell of PAP I or cdk recombinant baculoviruses alone, or in combination with 3 p.f.u./cell of $p34^{cdc2}$ plus 3 p.f.u./cell of cyclin B or 3 p.f.u./cell of Waf-1 viruses. After 40 h, cells were lysed in 10 ml of 50 mM Tris pH 8.0, 150 mM NaCl, 40 mM imidazole, 0.1% aprotinin, 10 mM benzamidine, 30 μg/ml leupeptin, 1 mg/ml bacitracin, 10 mg/ml α-2-macroglobulin and 0.35 mM phenylmethylsulfonyl flouride (PMSF). Lysates were spun for 15 min at 37 000 *g*, supernatants were collected and rocked for 1 h with 0.25 ml Ni^{2+}-NTA agarose (Quiagen). Beads were washed five times with RIPA buffer (150 mM NaCl, 50 mM Tris, pH 7.2, 1.0% NP-40, 2% sodium deoxycholate, 0.1% SDS) and eluted with 0.2 ml of 40 mM HEPES, pH 7.5, 20% glycerol, 100 mM imidazole, plus protease inhibitors. To produce PAPs in *E.coli*, N-terminally His-tagged PAPs were expressed for 3 h in 250 ml of LB buffer plus 25 mg/ml rifampicin. Cells were pelleted, washed twice in ice-cold TEK (20 mM Tris pH 7.5, 100 mM KCl, 1 mM EDTA), resuspended in 2 ml of ice-cold TEK, sonicated three times for 30 s and PAP was then purified as above. Preparations were equilibrated in buffer D [20 mM HEPES–KOH (pH 7.9), 50 mM $(NH4)_2SO_4$, 0.2 mM EDTA, 0.5 mM PMSF, 0.5 mM dithiothreitol (DTT), 10% glycerol] on a Fast desalting column (Smart System, Pharmacia).

Phosphorylation of PAP

Rabbit reticulocyte lysate-, baculovirus- or *E.coli*-produced PAPs were incubated for 1 h at 33°C in 50 μl of kinase buffer (5 mM $MnCl_2$,

0.1 mM DTT, 25 mM HEPES, pH 7.5, and 100 mM ATP) in the presence of 200 ng of purified MPF (Wang and Prives, 1995) alone or with 10 μg/ml olomoucine.

Two-dimensional phosphotryptic peptide maps

The indicated amounts of PAP purified as above were treated with the indicated amounts of MPF *in vitro* in the presence of [γ-^{32}P]ATP. PAPs were resolved by 7.5% SDS–PAGE, the gel dried, exposed to film and the portion containing the desired band excised. Protein elution and subsequent trypsin digestion were performed precisely as described (Boyle *et al.*, 1991), with three aliquots of trypsin added at 2 h incubation intervals. The electrophoretic dimension was performed in pH 1.9 buffer for 35 min and the chromatographic dimension in isobutyric acid buffer (see Boyle *et al.*, 1991) for 10 h.

Polyadenylation assays

Polyadenylation assays were performed as described (Raabe *et al.*, 1991). The indicated amounts of PAPs were added to reaction mixtures containing ^{32}P-labeled SV40 late pre-RNA and 0.5 mM Mn^{2+} instead of Mg^{2+}. RNA products were analyzed on a 5% polyacrylamide–8 M urea gel.

Oocyte injections

Oocytes were cytoplasmically injected singly, or in groups of 10 with 50 ng of bovine PAP I or Δ12 mRNA [including a poly(A) tail of 40 residues] synthesized *in vitro* and, where indicated, labeled with [^{35}S]methionine. Oocytes were processed as described (Prives and Foukal, 1991) and either immunoprecipitatied from lysates with anti-PAP polyclonal antisera, or loaded as whole-cell extracts and resolved on a 7.5% polyacrylamide gel. PAP was visualized by exposure to X-ray film, or by Western blotting and chemiluminescence, respectively.

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References

Ballantyne,S. and Wickens,M. (1995) Poly(A) polymerase in the nucleus and cytoplasm of frog ooctyes: dynamic changes during oocyte maturation and early development. *RNA*, **1**, 64–78.

Bilger,A., Fox,C.A., Wahle,E. and Wickens,M. (1994) Nuclear polyadenylation factors recognize cytoplasmic polyadenylation elements. *Genes Dev.*, **8**, 1106–1116.

Boyle,W.J., Van der Geer,P. and Hunter,T. (1991) Phosphopeptide mapping and phosphoamino acid analysis by two-dimensional separation on thin-layer cellulose plates. *Methods Enzymol.*, **201**, 110–149.

Colgan,D.F. and Manley,J.L. (1997) Mechanism and regulation of mRNA polyadenylation. *Genes Dev.*, **11**, 2755–2766.

Colgan,D.F., Murthy,K.G.K., Prives,C. and Manley,J.L. (1996) Cell-cycle related regulation of poly(A) polymerase by phosphorylation. *Nature*, **384**, 282–285.

El-Deiry,W.S., Tokino,T., Veculescu,V.E., Levy,D.B., Parsons,R., Trent,J.M., Mercer,W.E., Kinzler,K.W. and Vogelstein,B. (1993) WAF-1, a potential mediator of p53 tumor suppression. *Cell*, **75**, 817–825.

Enoch,T. and Nurse,P. (1990) Mitosis of fission yeast cell cycle control genes abolishes dependence of mitosis on DNA replication. *Cell*, **60**, 665–673.

Fan,H. and Penman,S. (1970) Regulation of protein synthesis in mammalian cells. *J. Mol. Biol.*, **50**, 665–670.

Fisher,D. and Nurse,P. (1990) Cyclins of the fission yeast *Schizosaccharomyces pombe. Seminars Cell Biology*, **6**, 73–78.

Fox,C.A., Sheets,M.D., Whale,E. and Wickens,M. (1992) Polyadenylation of maternal mRNA during oocyte maturation: poly(A) addition *in vitro* requires a regulated RNA-binding activity and a poly(A) polymerase. *EMBO J.*, **11**, 5021–5032.

Fry,A.M. and Nigg,E.A. (1995) The NIMA kinase joins forces with cdc2. *Curr. Biol.*, **5**, 1122–1125.

Furuno,N., Nishizawa,M., Okazaki,K., Tanaka,H., Iwashita,J., Nakajo,N., Ogawa,Y. and Sagata,N. (1994) Suppression of DNA replication via Mos function during meiotic divisions in *Xenopus* oocytes. *EMBO J.*, **13**, 2399–2410.

Gottesfeld,J.M. (1997) Mitotic repression of the transcriptional machinery. *Trends Biochem. Sci.*, **22**, 197–202.

Gottesfeld,J.M., Wolf,V.J., Dang,T., Forbes,D.J. and Hartl,P. (1994) Mitotic repression of RNA polymerase III transcription *in vitro* mediated by phosphorylation of a TFIIIB component. *Science*, **263**, 81–84.

Hake,L.E. and Richter,J.D. (1994) CPEB is a specificity factor that mediates cytoplasmic polyadenylation during *Xenopus* oocyte maturation. *Cell*, **79**, 617–628.

Kanki,J.P. and Newport,J.W. (1991) The cell cycle dependence of protein synthesis during *Xenopus laevis* development. *Dev. Biol.*, **146**, 198–213.

King,R.W., Jackson,P.K. and Kirschner,M.W. (1994) Mitosis in transition. *Cell*, **79**, 563–571.

Ligner,J., Kellermann,J.E. and Keller,W. (1991) Cloning and expression of the essential gene for poly(A) polymerase. *Nature*, **354**, 496–498.

Morgan,D.O., Kaplan,J.M., Bishop,J.M. and Varmus,H.E. (1989) Mitosis-specific phosphorylation of pp60src by p34cdc2 associated protein kinase. *Cell*, **57**, 775–786.

Nigg,E.A. (1991) The substrates of the cdc2 kinase. *Semin. Cell Biol.*, **2**, 261–270.

Nigg,E.A. (1993) Targets of cyclin-dependent protein kinases. *Curr. Opin. Cell Biol.*, **5**, 187–193.

Nigg,E.A. (1995) Cyclin-dependent protein kinases: key regulators of the cell cycle. *BioEssays*, **17**, 471–480.

Ohsumi,K., Sawada,W. and Kishimoto,T. (1994) Meiosis-specific cell cycle regulation in maturing Xenopus oocytes. *J. Cell Sci.*, **107**, 3005–3013.

Paris,J., Swenson,K., Piwnica-Worms,H. and Richter,J.D. (1991) Maturation-specific polyadenylation: *in vitro* activation by p34cdc2 and phosphorylation of a 58-kDa CPE-binding protein. *Genes Dev.*, **5**, 1697–1708.

Peter,M., Nakagawa,J., Doree,M., Labbee,J.C. and Nigg,E.A. (1990) *In vitro* disassembly of the nuclear lamina and M-phase-specific phosphorylation of lamins by cdc2 kinase. *Cell*, **61**, 591–602.

Prescott,D.M. and Bender,M.A. (1962) Synthesis of RNA and protein during mitosis in mammalian tissue culture cells. *Exp. Cell Res.*, **26**, 260–268.

Prives,C. and Foukal,D.F. (1991) Use of oligonucleotides for antisense experiments in *Xenopus laevis* oocytes. *Methods Cell Biol.*, **36**, 185–210.

Raabe,T. and Murthy,K.G.K. (1994) Poly(A) polymerase contains multiple functional domains. *Mol. Cell. Biol.*, **14**, 2946–2957.

Raabe,T., Bollum,F. and Manley,J.L. (1991) Primary structure and expression of bovine poly(A) polymerase. *Nature*, **353**, 229–234.

Richter,J.D. (1996) Dynamics of poly(A) addition and removal during development. In Hershey,J.W.B., Mathews,M.B. and Sonenberg,N. (eds), *Translational Control*. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY, pp. 481–503.

Sagata,N., Shiokawa,K. and Yamana,K. (1980) A study on the steady-state population of poy(A)$^+$ RNA during early development of *Xenopus laevis*. *Dev. Biol.*, **77**, 431–448.

Segil,N., Guermah,M., Hoffmann,A., Roeder,R.G. and Heintz,N. (1996) Mitotic regulation of TFIID: inhibition of activator-dependent transcription and changes in subcellular localization. *Genes Dev.*, **10**, 2389–2400.

Shenoy,S., Choi,J.K., Bagrodia,S., Copeland,T.D., Maller,J.L. and Shalloway,D. (1989) Purified maturation promoting factor phosphorylates pp60src at the sites phosphorylated during fibroblast mitosis. *Cell*, **57**, 763–774.

Shermoen,A.W. and O'Farrell,P.H. (1991) Progression of the cell through mitosis leads to abortion of nascent transcripts. *Cell*, **67**, 303–310.

Songyang,Z., Blechner,S., Hoagland,M.F., Piwnica-Worms,H. and Cantley,L.C. (1994) Use of an oriented peptide library to determine the optimal substrate of protein kinases. *Curr. Biol.*, **4**, 973–982.

Stern,B. and Nurse,P. (1996) A quantitative model for the cdc2 control of S phase and mitosis in fission yeast. *Trends Genet.*, **12**, 345–350.

Steward,D.L., Schaeffer,J.R. and Humphrey,R.M. (1968) Breakdown and assembly of polyribosomes in synchronized Chinese hamster cells. *Science*, **161**, 791–793.

Thuresson,A., Astrom,J., Astrom,A., Grovnik,K. and Virtanen,A. (1994) Multiple forms of poly(A) polymerases in human cells. *Proc. Natl Acad.Sci. USA*, **91**, 979–983.

Wahle,E. and Keller,W. (1996) The biochemistry of polyadenylation. *Trends Biochem. Sci.*, **21**, 247–250.

Wahle,E., Martin,G., Schiltz,E. and Keller,W. (1991) Isolation and expression of cDNA clones encoding mammalian poly(A) polymerase. *EMBO J.*, **10**, 4251–4257.

Wickens,M., Anderson,P. and Jackson,R.J. (1997) Life and death in the cytoplasm: messages from the 3′ end. *Curr. Opin. Genet. Dev.*, **7**, 220–232.

Yamashiro,S. and Matsumura,F. (1991) Mitosis-specific polyadenylation of caldesman: possible molecular mechanisms of cell rounding. *Bioessays*, **13**, 563–568.

Yamashiro,S., Yamakita,Y., Yoshida,K., Takiguchi,K. and Matsumura,F. (1995) Characterization of the COOH terminus of non-muscle caldesmon mutants lacking mitosis-specific phosphorylation sites. *J. Biol. Chem.*, **270**, 4023–4030.

Zhao,W. and Manley,J.L. (1996) Complex alternative RNA processing generates an unexpected diversity of poly(A) polymerase isoforms. *Mol. Cell. Biol.*, **16**, 2378–2386.

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