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Evidence of two forms of poly(A) polymerase in germinated wheat embryos and their regulation by a novel protein kinase

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Abstract

Two forms of poly(A) polymerase (PAPI and PAPII) from germinated wheat embryos have been resolved on DEAE–cellulose ion-exchange chromatography by a linear gradient of 0–500 mM $(NH_4)_2SO_4$. Further purification shows that both forms are monomeric in nature with an identical molecular weight, approximately 65 kDa. The phosphoprotein nature of PAPI and PAPII has been established by in vivo labelling with ^{32}P-orthophosphate. Acid hydrolysis of both ^{32}P-labelled purified PAPI and PAPII has revealed that phosphorylations generally take place in serine and threonine residues. PAPI and PAPII have also been characterised with respect to V_{max} and K_m for poly(A). The V_{max} and K_m values of PAPI are 28.57 and 11.37 μg, respectively, whereas 34.48 and 7.04 μg of PAPII. In vitro dephosphorylation of the purified enzyme by alkaline phosphatase leads to a significant loss of the enzyme activity, which is regained upon phosphorylation by a 65 kDa protein kinase (PK) purified from wheat embryos. The extent of phosphorylation by protein kinase shows that PK has similar affinity towards both PAPI and PAPII, whereas the phosphate incorporation in PAPII is twofold higher than PAPI suggesting their distinct chemical nature.

Keywords: PAPI, poly(A) polymerase I; PAPII, poly(A) polymerase II; PK, protein kinase; *Triticum aestivum*

Poly(A) polymerase (EC.2.7.7.19) catalyses the covalent sequential addition of 50–200 AMP residues at the 3-terminus of hnRNA [1,2]. This polyadenylation of mRNA is an important part of the RNA processing in eukaryotes and virtually all mRNAs end in a poly(A) tail post transcriptionally [3]. Multiple forms of poly(A) polymerase have been reported in rat liver [4], HeLa cells [5,6], and Ehrlich ascites tumour cells [7,8]. Two isoforms were purified from different origins of HeLa cells, one from cytoplasmic fraction and other from nuclear fraction. In addition, Ryner et al. [6] reported three forms of poly(A) polymerase in HeLa cells with an identical molecular weight and immunological properties.

The regulation of enzyme activity is mediated through its phosphorylating state. For example, poly(A) polymerase from rat liver and a rapidly growing rat Hepatoma (Morris hepatoma 3924A) could be phosphorylated in isolated nuclei by the endogenous kinase [9]. The poly(A) polymerase activity was found to be activated due to phosphorylation as reported by Jacob and Rose [10] and Rose and Jacob [11]. Infection of MOLT-3 cells with HIV-I virus also stimulated the activity of poly(A) polymerase [12] and the stimulation was ascribed to enzyme phosphorylation by NI kinase or protein kinase C.

In plants, poly(A) polymerase had been detected in tobacco [13], and was purified from wheat [14], maize [15], and mung [16]. Nevertheless, there were no reports of multiple forms of the enzyme. In the present communication we show the existence of two physically separable forms in germinated wheat embryos, which have been purified to their electrophoretic homogeneity. These two forms vary from their substrate affinity and polymerase activity. In vivo labelling of the enzyme with ^{32}P-orthophosphate showed that the phosphate

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incorporation was associated with serine and threonine residues of the purified preparation of the enzyme. In vitro dephosphorylation of both forms led to a significant loss of their activities, which was restored by in vitro phosphorylating the enzyme by a novel protein kinase purified from wheat embryo per se. Also the varied level of phosphorylation of two forms indicated their distinct chemical nature.

Materials and methods

Plant material. Wheat grains (*Triticum aestivum* L. var. Sonalika) obtained from Indian Agricultural Research Institute, New Delhi, were surface sterilised with $HgCl_2$ solution (0.02%) for 10 min, thoroughly rinsed in sterile distilled water, and germinated in dark for 48 h at 25 °C. The germinated embryos were manually excised, frozen in liquid nitrogen, and stored at −80 °C.

Purification of two forms of poly(A) polymerase. All steps of enzyme preparation were carried out at 4 °C. Germinated wheat embryos were homogenised in buffer A containing 50 mM Tris–acetate, pH 7.6, 5 mM β-mercaptoethanol (β-ME), 100 mM NaF, and 2% Polyvinylpolypyrrolidone. The crude extract was brought to 30% saturation with solid $(NH_4)_2SO_4$ and centrifuged for 15 min at 15,000*g*. The supernatant was brought to 50% saturation with $(NH_4)_2SO_4$ and centrifuged at 15,000*g* for 15 min. The resulting pellet was suspended in buffer B (20 mM Tris–acetate, pH 7.6, 5 mM β-ME and 5% glycerol) and dialysed overnight against the same buffer. After dialysis the extract was loaded on DEAE–cellulose column that had been pre-equilibrated with buffer B. The unbound protein was eluted with the same buffer and the bound protein was eluted with a linear gradient of 0–500 mM $(NH_4)_2SO_4$. Twenty fractions (5 ml each) were collected, concentrated against PEG 20,000 overnight, dialysed for 12 h against buffer B and assayed for poly(A) polymerase activity. The active fractions were pooled and loaded onto ATP–Sepharose column (10 × 3 cm) that had been pre-equilibrated with 10 mM Tris–acetate, pH 7.6 containing 2 mM β-ME, 2 mM $MnCl_2$, 0.25 mM EDTA, 50 mM NH_4Cl, 20% glycerol, and BSA (2 mg/ml) and subsequently with the same buffer without BSA (equilibration buffer). The column was washed thoroughly with equilibration buffer followed by the same buffer containing 2 mM ATP. Poly(A) polymerase was eluted with two bed volumes of equilibration buffer containing 4 mM ATP. The ATP–Sepharose fraction was concentrated against PEG 20,000 overnight, dialysed for 12 h for removing the excess ATP against buffer containing 10 mM Tris–HCl, pH 7.6, and 2 mM β-ME. The purified enzyme was analysed on native and SDS–PAGE according to the method of Weber and Osbern [17]. Protein band was stained according to the method of Davis et al. [18]. For molecular mass determination the purified protein was applied to a Sephacryl S-200 (Pharmacia) column (2.6 × 100 cm) equilibrated with 10 mM Tris–acetate, pH 7.6 containing 5 mM β-ME, 1 μM $ZnCl_2$, and 0.5 mM $MgCl_2$. The fractions of 2 ml were collected and each fraction was assayed for the poly(A) polymerase activity. The molecular weight of the poly(A) polymerase was determined by calibrating the column with standard protein markers: fructose 6-phosphate kinase (84 kDa), rinderserum albumin (68 kDa), huhnerai albumin (45 kDa), and chymotrypsinogen A (25 kDa).

Purification of a protein kinase. The crude enzyme extract was subjected to $(NH_4)_2SO_4$ precipitation as described, and the desalted ammonium sulphate fraction was loaded on DEAE–cellulose column that had been pre-equilibrated with buffer C (10 mM Tris–acetate, pH 7.6, 2 mM β-ME, and 5% glycerol). The bound protein was eluted with the buffer C containing 450 mM $(NH_4)_2SO_4$ and subjected to 50% saturation with solid $(NH_4)_2SO_4$. The precipitated protein was collected by centrifugation at 10,000*g* for 10 min, dissolved in buffer C, and dialysed against the same buffer for 12 h. After dialysis, the protein was loaded onto ATP–Sepharose column (15 × 3 cm) that had been pre-equilibrated with equilibrium buffer as described. The protein kinase activity was recovered by elution with the same buffer containing 2 mM ATP. The eluted protein was concentrated by PEG 20,000 and dialysed against 10 mM Tris–acetate, pH 7.6, and 2 mM β-ME for 12 h. The ATP–Sepharose fraction was loaded onto casein–Sepharose affinity matrix pre-equilibrated with 25 mM Tris–acetate buffer, pH 7.6 containing 100 mM KCl. The column was thoroughly washed with 25 mM Tris–acetate, pH 7.6 containing 250 mM KCl. The protein kinase activity was recovered by the same buffer containing 500 mM KCl, concentrated against PEG 20,000 and dialysed with same buffer without KCl.

In vivo labelling of poly(A) polymerases. Excised germinated wheat embryos were incubated in sterile 2% sucrose solution and carrier-free ^{32}P-orthophosphoric acid (25 mCi) for 12 h in dark. PAPI and PAPII were purified to electrophoretic homogeneity from these radiolabelled tissues according to the method described earlier. The incorporation of the ^{32}P-label into the purified preparation of both forms was determined by soaking 40 μl sample on a Whatman 3 MM disc. The radioactivity was determined as described earlier [19].

Assay of poly(A) polymerase. Poly(A) polymerase was assayed according to the procedure of Lakhani et al. [20] with modification. The assay components are 100 mM Tris–HCl, pH 8.0, 4 mM DTT, 2 mM $MnCl_2$, 50 μg poly(A) primer, and [^{3}H]ATP (4 μCi, 0.4 μmol, 10 μCi/μmol) in 200 μl reaction mixture. The reaction was carried out for 30 min at 30 °C, terminated by chilling and the radioactivity was determined by soaking 30 μl of aliquot on Whatman 3 MM disc as described [19]. The one unit of enzyme (IU) is defined as pmol of AMP incorporated per mg protein in 30 min. The amount of protein was estimated by Bradford methods [21].

Assay of protein kinase. Protein kinase was assayed according to the method of Ansari and Sachar [22]. The reaction mixture (250 μl) contains 20 mM Tris–acetate, pH 8.0, 15 mM Mg^{2+} acetate, 50 μg dephosphorylated casein, 1 μg purified protein kinase, and [γ-^{32}P]ATP (50 μM, 420 dpm/picomol). The assay was initiated by addition of [γ-^{32}P]ATP and carried out at 25 °C for 30 min. An aliquot (40 μl) of the assay mixture was spotted on Whatman 3 MM disc. The incorporation of radioactivity was determined as described earlier [19]. The activity of protein kinase is expressed as pmols of phosphate incorporated per mg protein by 30 min.

Chemical characterisation of ^{32}P-labelled poly(A) polymerases. The ^{32}P-labelled poly(A) polymerases (100 μg each, PAPI and PAPII) were hydrolysed separately with 6 N HCl for 1 h at 110 °C under nitrogen in a sealed ampule. The acid hydrolysate was lyophilised, and solubilised in 100 μl H_2O. The acid hydrolysate and authentic phosphoserine, phosphothreonine, and phosphotyrosine (20 μg each) were applied on Whatman Chromatograph paper, and were separated as described by Saluja et al. [16]. After the run the paper was dried and sprayed with ninhydrin to develop the spots of authentic phosphoamino acids. The lane of acid hydrolysate was cut into 2 mm pieces across the run and radioactivity was measured as described.

Dephosphorylation of poly(A) polymerases by alkaline phosphatase. The purified poly(A) polymerases were incubated with calf intestine alkaline phosphatase in 10 mM Tris–acetate buffer, pH 8.0 for 30 min at 25 °C. The reaction was terminated by adding sodium orthovanadate (10 μM), an inhibitor of alkaline phosphatase [23,24]. Alkaline phosphatase was then removed completely by passing the reaction mixture through ATP–Sepharose affinity column that had been equilibrated with a buffer containing 10 mM Tris–acetate, pH 7.6, 2 mM $MnCl_2$, and 50 mM NH_4Cl_2. Poly(A) polymerase was eluted from the column with above buffer containing ATP (4 mM). Enzyme was then concentrated against PEG 20,000 overnight and dialysed against 25 mM Tris–HCl, pH 7.6.

In vitro phosphorylation of poly(A) polymerase by a protein kinase. Poly(A) polymerase (500 μg) was incubated with 100 μg of purified wheat protein kinase activity along with cold ATP (100 μM) in 20 mM Tris–acetate, pH 7.6. The above mixture was incubated for 30 min at

25 °C. Immediately after the incubation, the reaction mixture was diluted two times and was passed through the casein–Sepharose matrix for the removal of protein kinase. Poly(A) polymerase was collected in the unbound fraction from the casein–Sepharose matrix, concentrated against PEG 20,000, and dialysed against 20 mM Tris–acetate, pH 7.6 buffer with 20% glycerol (v/v).

Data analysis. The experiments of protein purifications were repeated at least thrice and representative data are given. The enzyme assays were done in triplicate. The kinetic analysis was repeated thrice and representation data are given.

Results

Purification of two forms of poly(A) polymerases

Two forms of poly(A) polymerase were purified to homogeneity from wheat embryos. The total protein was extracted from dark grown embryos and subjected to 30–50% fractionation of $(NH_4)_2SO_4$. Ammonium sulphate-fractionated protein was resolved into two activity peaks of poly(A) polymerase (PAPI at 150 mM and PAPII at 450 mM) on DEAE–cellulose ion-exchange chromatograph by a linear gradient of 0–500 mM $(NH_4)_2SO_4$ (Fig. 1A). The active fractions of two peaks were loaded individually onto ATP–Sepharose affinity chromatography and activity was recovered in between 2 and 4 mM ATP elution. The total protein and enzyme activities at each step are listed in Tables 1 and 2. PAPI and PAPII were purified about 1666-fold and 2688-fold, respectively. The SDS–PAGE analysis of the purified enzyme preparations (PAPI and PAPII) showed a single protein band at an identical position with a subunit of approximately 65 kDa (Fig. 1B). Molecular sieve chromatography of PAPI and PAPII on Sephacryl S-200 separately also revealed their similar molecular weights of approximately 65 kDa (Fig. 1C).

Poly(A) polymerases are phosphoproteins

The regulation of poly(A) polymerase through enzyme phosphorylation was well established in animal

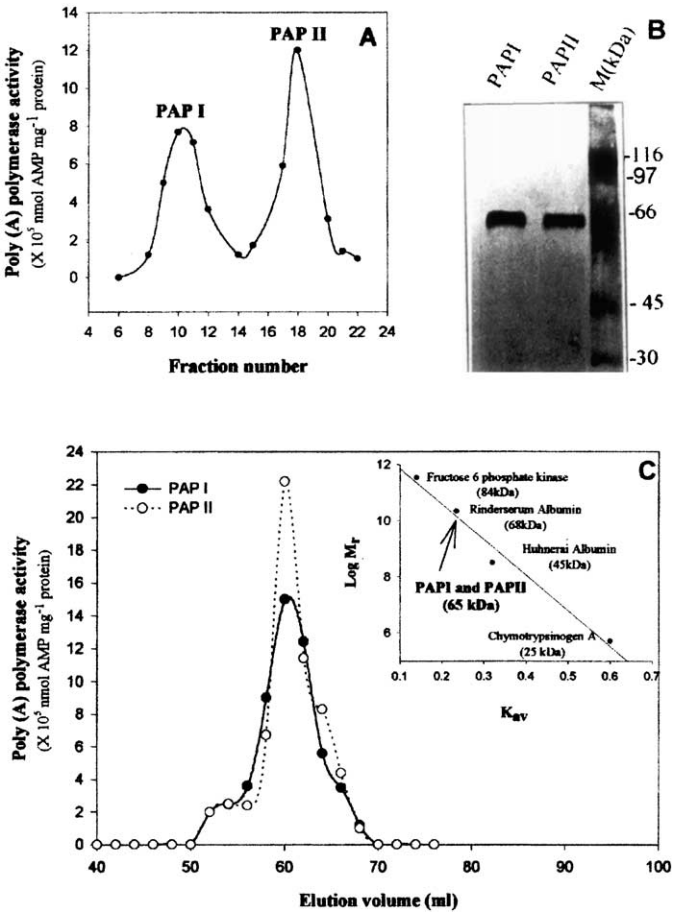

Fig. 1. Two forms of poly(A) polymerase. (A) The DEAE–cellulose bound protein was eluted by a linear gradient of $(NH_4)_2SO_4$, 25 fractions (5 ml each) were collected and assayed for poly(A) polymerase activity. (B) The SDS–PAGE analysis of PAPI and PAPII. The purified PAPI and PAPII were run on 10% polyacrylamide gel and protein band was visualised by silver staining. (C) Molecular weight of PAPI and PAPII by gel exclusion chromatography. Sephacryl S-200 was calibrated with protein markers of known molecular weight. The molecular weights of PAPI and PAPII have been determined by K_{av} value.

Table 1
Protocol for the purification of poly(A) polymerase I (PAPI) from dark grown germinated wheat embryos

Steps	Total protein (mg)	Specific activity IU/mg protein	Fold purification	Yield (%)
Crude	4550	6.0×10^2	1	100
30–50% (sat) $(NH_4)_2SO_4$	1500	1.5×10^3	2.5	83.3
DEAE–cellulose (0–500 mM $(NH_4)_2SO_4$)	200	4.4×10^3	33	33.00
ATP–Sepharose (4 mM ATP)	0.4	1.0×10^7	1666	17.2

Total protein was estimated at every step of purification and specific activity was calculated as described in Materials and methods. The yield was calculated from the crude preparation.

Table 2
Protocol for the purification of poly(A) polymerase II (PAPII) from dark grown germinated wheat embryos

Steps	Total protein (mg)	Specific activity IU/mg protein	Fold purification	Yield (%)
Crude	4550	6.0×10^2	1	100
30–50% (sat) $(NH_4)_2SO_4$	1500	1.5×10^3	2.5	83.3
DEAE–cellulose (0–500 mM $(NH_4)_2SO_4$)	100	9.3×10^3	15.5	34.50
ATP–Sepharose (4 mM ATP)	0.2	1.6×10^7	2688	11.0

Total protein was estimated at every step of purification and specific activity was calculated as described in Materials and methods. The yield was calculated from the crude preparation.

cells as reported by Jacob and Rose [9] and Rose and Jacob [11]. Verma and Sachar [25] demonstrated the phosphoprotein nature of poly(A) polymerase in wheat embryos, and in vitro dephosphorylation by alkaline phosphatase resulted in significant loss of enzyme activity. Therefore, in order to examine whether PAPI and PAPII are phosphoprotein excised wheat embryos were incubated with ^{32}P-orthophosphate as described by Verma and Sachar [25] and both forms were purified from in vivo labelled tissue as described. Purified enzyme preparation was fractionated in two parallel lanes on SDS–PAGE. One lane was sliced (2 mm) across the run and the radioactivity was measured. The other lane was silver stained for the detection of the protein band. The enzyme preparation showed a single ^{32}P-labelled peak that matched with the protein stained band of PAPI and PAPII on SDS–PAGE (Fig. 2). Therefore, it appears that both PAPI and PAPII are phosphoproteins. Also acid hydrolysis of both PAPI and PAPII by 6 N HCl for 1 h and subsequent paper chromatography revealed that phosphorylation generally occurred at serine and threonine residues (Fig. 3).

Kinetics of PAPI and PAPII

In order to examine the nature of poymerase activities, PAPI and PAPII were assayed (Fig. 4A) individually over a broad range of poly(A) concentration (0–50 μg). The V_{max} and K_m values are calculated from Lineweaver Burk plot (Fig. 4B), using least squares fits generated with computer software. The V_{max} values for PAPI and PAPII are 0.95 and 1.14 pmol/min, respectively, whereas K_m values are 11.37 and 7.04 μg/ml.

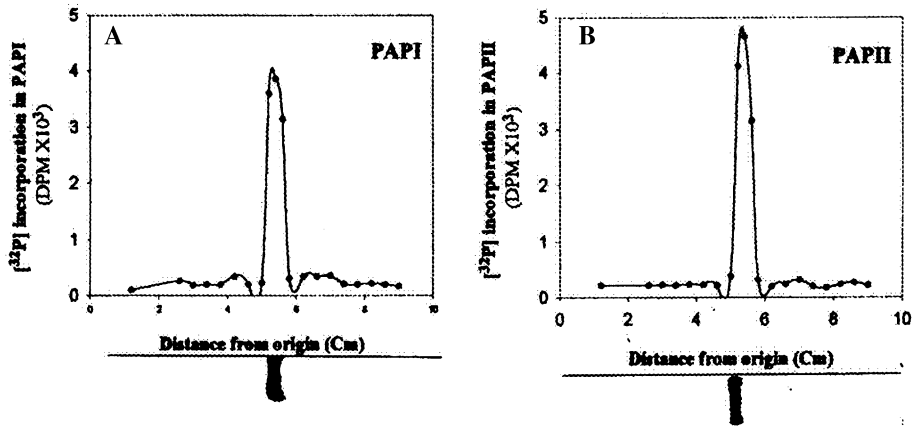

Fig. 2. Phosphoprotein nature of PAPI and PAPII. In vivo ^{32}P-labelled purified PAPI (A) and PAPII (B) were fractionated on SDS–PAGE (10%). One lane was stained for detecting the protein (lower panel) and other lane was serially sliced (2 mm) across the run. The radioactivity was measured in each gel slice. A single radioactivity peak coincided with the silver stained protein band.

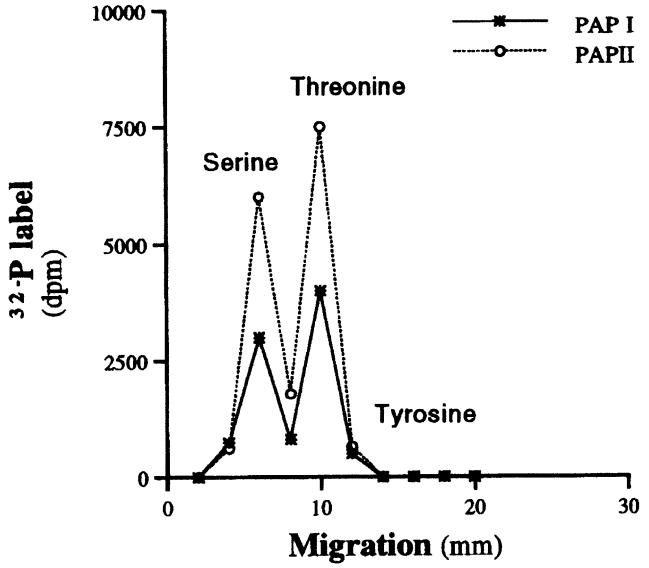

Fig. 3. Incorporation of ^{32}P-orthophosphate in purified PAPI and PAPII. Poly(A) polymerases were in vivo labelled by [^{32}P]-orthophosphate, and PAPI and PAPII were purified to homogeneity. The purified enzymes were acid hydrolysed by HCl and hydrolysates were paper chromatographed along with standard amino acids as described in Materials and methods. The paper was sprayed with ninhydrin to detect the position of phosphoamino acid standards. The lane loaded with acid hydrolysate was cut into pieces (2 mm) across the run and radioactivity was measured.

Therefore we suggest that PAPII has higher substrate affinity as well as polymerase activity.

Purification of a protein kinase

A novel protein kinase has been purified to electrophoretic homogeneity from wheat embryo. Ammonium sulphate-fractionated (30–50% saturation) protein from dark grown wheat embryo was loaded on DEAE–cellulose column and the activity was recovered by 450 mM $(NH_4)_2SO_4$. Protein kinase activity was then loaded on ATP–Sepharose column and eluted by 2 mM ATP. Protein kinase activity was finally loaded on casein–Sepharose column and activity was recovered in between 0.25 and 0.5 M KCl elute. The total protein and enzyme activities at each step are listed in Table 3. The SDS–PAGE-analysis of purified protein kinase showed that the molecular weight of protein was approximately 65 kDa (Fig. 5A). The molecular sieve chromatography revealed that molecular weight of the native enzyme was approximately 70 kDa (Fig. 5B).

Regulation of poly(A) polymerases by phosphatase and protein kinase

Alkaline phosphatases are known to remove the phosphate moiety from macromolecules [26]. Therefore, poly(A) polymerase was dephosphorylated by alkaline phosphatase and the dephosphorylated enzyme showed a significant loss of enzyme activities (70% and 76% for PAPI and PAPII, respectively, Fig. 6). The appearance of two peaks on ion-exchange chromatography due to partial dephosphorylation was ruled out as partially dephosphorylated peak II appeared at 450 mM of $(NH_4)_2SO_4$ (Fig. 7). In addition, phosphorylation of dephosphorylated enzyme by protein kinase restores the polymerase activity. The extent of phosphorylation was further studied by incubating protein kinase with various concentrations of dephosphorylated PAPI (Fig. 8A) and PAPII (Fig. 8C). It was observed that the phosphorylation of PAPII was approximately twofold higher than PAPI. These data are analysed by Eadie–Hofstee plot (Figs. 8B and D) using least square fits generated with computer software. The slopes of straight lines for PAPI and PAPII are 14.22 and 13.58 μg/ml, respectively, indicating the similar binding pattern of PK and polymerase, whereas the intersections of PAPII-curve on both axes are approx-

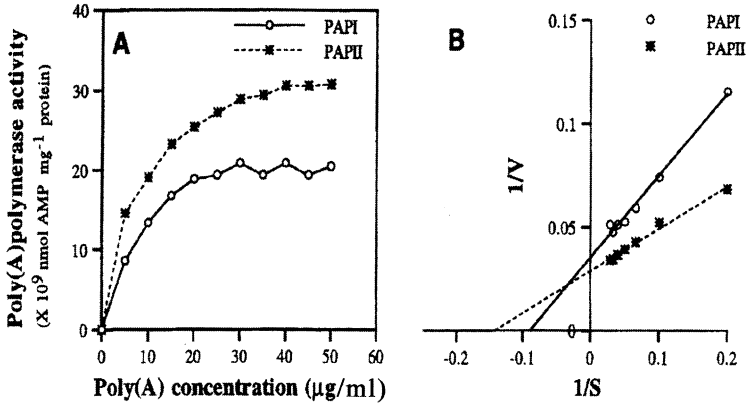

Fig. 4. Kinetics of PAPI and PAPII. (A) PAPI and PAPII were assayed with various concentration of poly(A). The polymerase activities were plotted against poly(A) concentration. The data were analysed by Lineweaver Burk plot (B), using least squares fits generated with computer software.

Table 3
Protocol for the purification of protein kinase: the protein kinase was purified from dark grown wheat embryos

Steps	Total potein (mg)	Specific activity IU/mg protein	Fold purification	Yield (%)
Crude	2355	0.8×10^2	1.00	100
30–50% (sat) $(NH_4)_2SO_4$	800	2.4×10^2	2.7	92.6
DEAE–cellulose (0–500 mM $(NH_4)_2SO_4$)	158	5.1×10^2	5.8	39.0
ATP–Sepharose (2 mM ATP)	10.0	2.7×10^3	31.0	13.2
Casein–Sepharose (0.25–0.5 M KCl)	0.153	1.6×10^5	1853	12.1

The enzyme activity was assayed at each step of the purification taking purified poly(A) polymerase as a substrate. Specific activity was calculated as described in the materials and methods. The percent yield was calculated taking crude as the 100%.

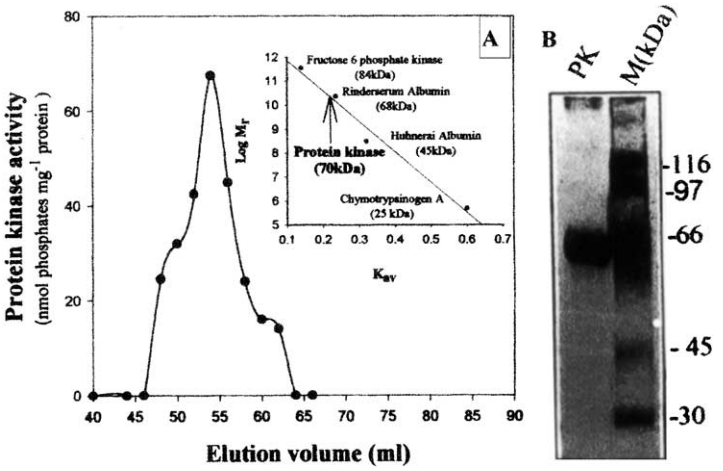

Fig. 5. Analysis of purified protein kinase. (A) Molecular weight of protein kinase (PK) by gel exclusion chromatography. Sephacryl S-200 was calibrated with protein markers of known molecular weight. The molecular weight of PK has been determined by K_{av} value. (B) Purified PK was fractionated on 10% polyacrylamide gel along with molecular weight marker (M) and protein was visualised by silver staining.

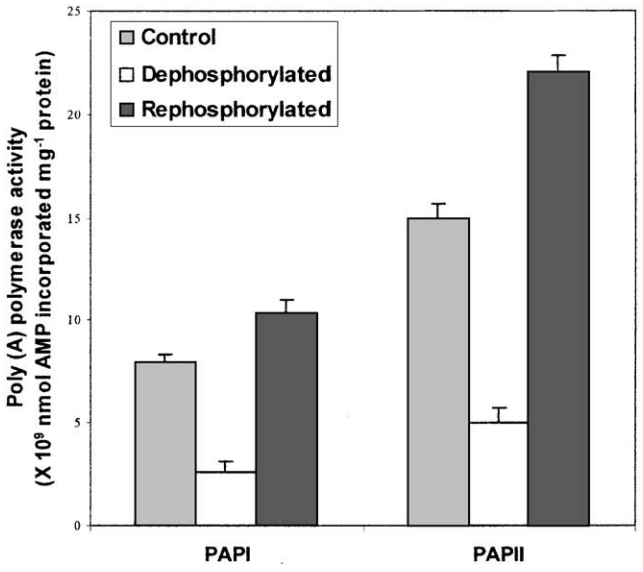

Fig. 6. The activities of PAPI and PAPII upon dephosphorylation by alkaline phosphatases and rephosphorylation by protein kinase. The purified poly(A) polymerases (PAPI and PAPII) were in vitro dephosphorylated by alkaline phosphatase, and dephosphorylated ones were again rephosphorylated by purified wheat protein kinase. The histogram shows the enzyme activities of phosphorylated and dephosphorylated forms.

imately double of PAPI, suggesting the two different isozymes of poly(A) polymerase with respect to their chemical nature.

Discussion

Various forms of poly(A) polymerase on the basis of their chromatographic properties, different ion requirements and subcellular localisation were reported from animal systems. For instance, three poly (A) polymerases were isolated from HeLa cells infected with vaccinia virus. One was located in the nucleus, comprised of a single polypeptide with a Mr 60 kDa and stimulated by Mn^{2+} and also by Mg^{2+}. The second one was located in the cytoplasm, consisting of a single polypeptide with Mr 75 kDa and absolutely dependent on Mn^{2+}. The third enzyme was present only in the virus-infected cells and composed of two polypeptides with Mr 57 and 37 kDa [5]. Avramova et al. [7] reported the two distinct poly(A) polymerases from the cytoplasm of Ehrlich ascites tumour cells. The first one had an absolute requirement for Mn^{2+} and used ATP as substrate. The second enzyme required either Mn^{2+} or Mg^{2+} and used ATP or UTP as substrate. In plants, single poly(A) polymerase had been purified by Verma and Sachhar

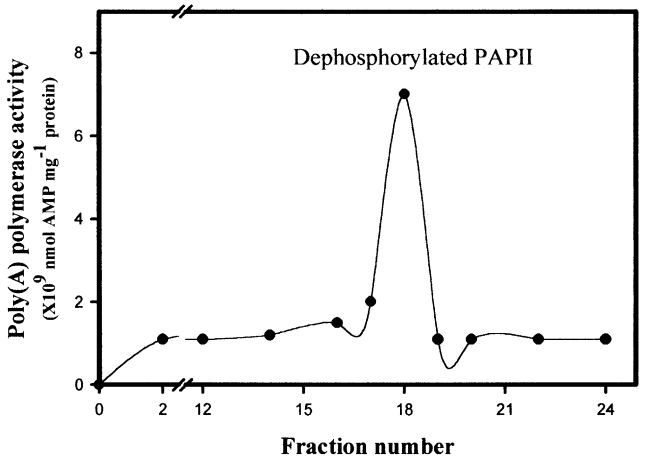

Fig. 7. Analysis of dephosphorylated PAPII on ion-exchange chromatography. The partially dephosphorylated PAPII was fractionated on DEAE–cellulose ion-exchange chromatography and the bound protein was eluted with 0–500 mM $(NH_4)_2SO_4$ linear gradient.

[25]. But, there were no reports regarding the different forms of poly(A) polymerase in plant system. In the present paper we established a protocol to purify two forms of poly(A) polymerase from wheat embryos. Both forms are absolutely dependent on Mn^{2+} for their activities (data not shown). Non-denaturing polyacrylamide gel electrophoresis and molecular sieve chromatography confirmed their identical molecular weight of approximately 65 kDa. The SDS–PAGE analysis revealed a single polypeptide with Mr 65 kDa, demonstrating the monomeric nature of the protein. This is in accordance with the molecular weight of poly(A) polymerase purified to homogeneity from wheat 65 kDa [14] and from maize 70 kDa [15]. However, mung poly(A) polymerase was shown to be a tetramer with a holoenzyme of 120 kDa and composed of four equal subunits of 30 kDa [27].

Chemical characterisation of in vivo labelled enzyme showed that both PAPI and PAPII were phosphoprotein in nature and the incorporation of radioactivity was on the serine and threonine residues. Also, it appeared that two peaks resolved on ion exchange chromatography was due to varied levels of their phosphorylation. But this possibility had been ruled out as partial dephosphorylated PAPII eluted as a single peak at 450 mM of $(NH_4)_2SO_4$. The kinetics analysis has shown that PAPII has higher affinity to poly(A) and polymerase activity as well.

The regulation of poly(A) polymerase through phosphorylation is well established in animals. Incubation of isolated nuclei with [γ-^{32}P]ATP, followed by purification of poly(A) polymerase yielded enzyme containing radioactive phosphate. Also phosphoprotein nature had been confirmed by incubating purified protein with exogenous protein kinase [9]. Rose and Jacob [10] showed that phosphorylation of poly(A) polymerase by a protein kinase NI (a cyclic-nucleotide independent nuclear kinase) resulted in sevenfold activation of enzyme. Verma and Sachar [25] demonstrated the phosphoprotein nature of poly(A) polymerase in wheat. They also reported that phosphorylated form was more active than dephosphorylated one in vitro.

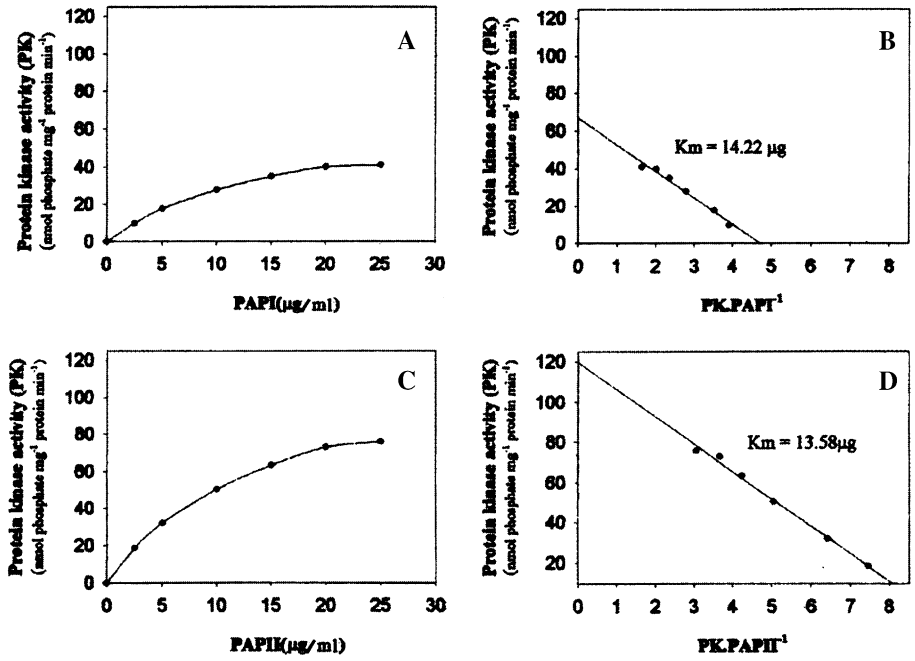

Fig. 8. Phosphorylation of PAPI and PAPII by protein kinase.The protein kinase was assayed taking different concentrations of substrates (PAPI and PAPII). The protein kinase activity was plotted against poly(A) polymerase concentration (A, C). The data were analysed by Edie–Hofstee plot (B, D), using least square fits generated by computer software ($r^2 = 0.98$, 0.99, respectively).

Therefore, to prove the physiological significance of phosphorylation in regulation of both forms of poly(A) polymerase, purified enzyme preparations were dephosphorylated by alkaline phosphatase. The comparison of the enzyme activities of dephosphorylated poly(A) polymerases with the purified one showed that they lost almost 70% activity. In principle phosphorylation or dephosphorylation is required to change the conformation of target protein by the cognate protein kinase, protein phosphatase, or both [28,29]. A protein kinase was purified from pea in our laboratory [22] and was used to phosphorylate wheat poly(A) polymerase. But no phosphorylation was observed. Therefore, our next attempt was to purify a protein kinase from the same system, and it was observed that in vitro phosphorylation by this protein kinase (Mr 65 kDa) from wheat embryo was found to regain its activity. The molecular sieve chromatography showed that the protein kinase was of approximately 70 kDa. This small difference in their size is not clear and needs further characterisation. The phosphorylation by PK showed that PK had similar affinity towards both PAPI and PAPII. But phosphate incorporation in PAPII is twofold higher than PAPI, suggesting their distinct nature with respect to the chemical properties.

Poly(A) polymerase was regulated by hormones, progesterone, and esterogen in rabbit uterus [30], by corticosteroids in rat liver [31] and in kidney cortex [32] and by testerosterone in rat prostate gland [33]. In plant cells phytohormonal regulation of poly(A) polymerase by GA_3 was first reported in wheat aleurones by Berry and Sachar [34,35]. In wheat embryos, the induction of poly(A) polymerase by GA_3 was paralleled by an increase in the relative abundance of poly$(A)^+$ RNA [20]. The GA_3 mediated stimulation of poly(A) polymerase was strongly inhibited by cycloheximide in wheat aleurones and excised wheat embryos. This was accompanied by a significant decrease in the levels of total poly $(A)^+$ RNA [19,20]. The origin and physiological importance of two different isozymes of poly(A) polymerase in wheat embryo remain to be elucidated.

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