A 43 kDa DNA binding protein from the pea chloroplast interacts with and stimulates the cognate DNA polymerase

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ABSTRACT

A DNA binding protein with DNA polymerase 'accessory activity' has been identified and purified to apparent homogeneity from pea chloroplasts. This protein consists of a single subunit of 43 kDa and binds to DNA regardless of its base sequence and topology. It increases cognate DNA polymerase–primase activity in a dose dependent manner. Using solid phase protein–protein interaction trapping and co-immuno-precipitation techniques, the purified protein was found to associate with the chloroplast DNA polymerase. The chloroplast DNA polymerase also binds directly to the radioiodinated 43 kDa protein. The specific interaction between 43 kDa protein and chloroplast DNA polymerase results in the synthesis of longer DNA chains. The 43 kDa protein, present abundantly in the pea chloroplast, appears to increase processivity of the chloroplast DNA polymerase and may play an important role in the replication of pea chloroplast DNA.

INTRODUCTION

DNA metabolism, namely replication, repair, and recombination, requires the concerted assembly and activity of multiprotein complexes (1). DNA synthesis, in particular, is the result of an interplay between the core DNA polymerases and associated accessory proteins. Accessory proteins are either subunits of holoenzymes or those that interact with the DNA polymerases for processive movement of the replication fork. For example, the *E.coli* DNA *Pol*III core enzyme requires the so-called γ–β complex (holoenzyme subunits) along with the homologous single stranded DNA binding protein (SSB) to achieve fork movement at a rate of 700 nucleotides (nt) per second at 37°C, a value close to *in vivo* fork movement of 1000 nt per second (2,3). Eukaryotic DNA polymerases α and δ also have their respective holoenzyme-like structures (4,5). Here again, the eukaryotic SSBs, namely Replication Protein A (RPA), interact with the polymerases to increase processivity (6). In addition to SSBs, some general DNA binding proteins such as UL42 protein of Herpes Simplex Virus are also reported to associate with and accentuate the homologous DNA polymerizing activity (7).

The mechanisms of DNA replication are fairly well understood in many prokaryotic and lower eukaryotic systems. On the other hand, DNA replication in plants is poorly studied (8–10). Many studies on plant DNA replication center around those on chloroplast DNA (ctDNA) because of the relative ease of isolating and handling the intact chloroplast genome (11–13). A partially purified *in vitro* system from pea chloroplast that can faithfully replicate plasmids containing replication origin sequences of pea ctDNA has been developed (14). The enzymes, namely DNA polymerase, DNA topoisomerase and a nuclease have been identified from this system and purified to homogeneity (15–17). In our continuing effort to study the *in vitro* replication system, we now report the identification, purification and characterization of an accessory protein of pea ctDNA polymerase.

The purified accessory protein is a monomer of 43 kDa and exhibits non specific binding to DNA. Cognate DNA polymerase/primase activity is stimulated several-fold depending upon the nature of template and amounts of the 43 kDa protein. The protein binds to the pea ctDNA polymerase in a specific manner and causes synthesis of longer DNA chains. This protein is found only in green tissues of the plant and is localized within the chloroplasts.

MATERIALS AND METHODS

Purification of the 43 kDa protein

Triton disrupted chloroplasts (Fraction I) were prepared from 750 g of pea leaves (18) and loaded on to a 50 ml DEAE cellulose column (DE 52, Whatman) equilibrated with buffer A (50 mM Tris, pH 8, 10 mM β-mercaptoethanol, 10% glycerol and five protease inhibitors namely 1 mg/ml benzamidine, 10 mM sodium-metabisulfite, 10 μM pepstatin A, 2 μg/ml leupeptin and 100 μM phenylmethylsulfonylfluoride) and 100 mM NaCl. The column was washed with 100 ml of buffer A. The flow-through and wash fractions were concentrated by ultrafiltration and equilibrated to buffer B (25 mM sodium acetate, pH 5.2, 10% glycerol plus the protease inhibitors) using the Amicon RC 8400 system (10 kDa cut off, Amicon). This was called Fraction II and was applied on a 50 ml CM cellulose column (CM 52, Whatman)

equilibrated with buffer B containing 25 mM NaCl. After thorough washing, bound proteins were eluted with a 500 ml linear gradient of 25–600 mM NaCl in buffer B. Active fractions were identified by southwestern hybridization (19) with radio-labelled denatured DNA (specific activity = 5×10^7 c.p.m./µg) and were found to elute at 400–475 mM NaCl. The active fractions were pooled and dialysed against buffer A containing 50 mM NaCl and 5 mM $MgCl_2$. Dialysed proteins (Fraction III) were then loaded onto a 10 ml calf thymus ssDNA cellulose column (U.S.B.). Following washing with buffer C (buffer A with 5 mM $MgCl_2$) containing 75 mM NaCl, the bound proteins were eluted with a 50 ml linear gradient of 75–750 mM NaCl in buffer C. The active fractions eluting between 250–350 mM NaCl were pooled, concentrated and dialysed against buffer C containing 25 mM NaCl. This concentrate (Fraction IV) was subsequently chromato-graphed on a Sephadex G-100 (Superfine, Sigma) column (50×1 cm). The eluted active fractions were pooled, concentrated by ultrafiltration (Fraction V) using the centricon membrane (30 kDa cut off, Amicon) and either stored at –70°C or used immediately. All the chromatographic steps were performed at 4°C. The 43 kDa protein obtained as fraction V was used for all biochemical studies.

DNA binding

Appropriate amounts of different forms of DNA and 43 kDa protein were incubated in 20 µl of reaction buffer containing 50 mM Tris, pH 7.5 and varying concentrations of NaCl (as mentioned in the text) at 37°C for 30 min. Some of the binding reactions were also performed in presence of 2 mM ATP or other substrates as described in the text. Bound complexes were processed for agarose-gel electrophoresis, filter binding assay or electron microscopy.

For south-western blots, autoradiograms containing 43 kDa protein were used to calculate the DNA binding units, described in Table 1. Blots were exposed for sufficient time intervals to ensure linearity of autoradiographic bands in the densitometric scale (20). Unit activity was taken to correspond to an arbitrarily chosen amount of band intensity. Based on this measurement, the intensity of bands in lanes 1 and 5 of Figure 1B would represent 2 and 10 units respectively.

Nitrocellulose filter binding assay

Ten nanograms of radiolabelled linear pUC19 DNA (3′ end labelled with [α³²P]dCTP; specific activity of DNA = 10^7 c.p.m./ µg) was incubated with 50 ng 43 kDa protein in 50 mM Tris buffer pH 8, containing 50 mM NaCl. Various competitors were added as indicated in the text. Following incubation at 37°C for 30 min, the DNA–protein complexes were trapped in the Millipore nitro-cellulose filters (DAWP, pore size 65 µm). Filters were washed, dried and subjected to scintillation counting to determine retained radioactivity.

Electron microscopy (EM)

DNA–protein complexes were crosslinked with 0.1% glutaralde-hyde and purified by gel filtration chromatography (21). Contour length measurements were performed using a digitizer (Sigma-Scan, Jandel Scientific, USA).

Partial purification of the ctDNA polymerase

DNA polymerases were obtained by a slight modification of the published procedure (15). Briefly, Triton X-100 supernatant of
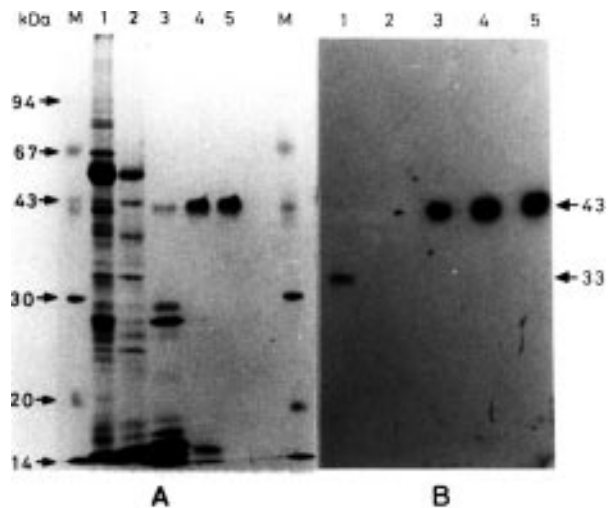
Figure 1. Purification and activity profile of the 43 kDa protein (**A**) SDS–PAGE of the various chromatographic fractions. A quantity of 50 µg of fraction I, 20 µg of fraction II, 10 µg of fraction III, 3 µg of fraction IV and 2 µg of fraction V proteins were electrophoresed on a SDS 10% PAGE in lanes 1 through 5 respectively and stained subsequently with Coomassie blue. Molecular weight of the marker proteins in lane M has been indicated by arrows. (**B**) Autoradiogram of south western blot corresponding to the gel shown in (A). Denatured radiolabelled DNA containing OriA sequence of pea ctDNA was used as the probe.

pea chloroplast derived from 1 kg of leaf tissue was loaded onto a 100 ml DEAE cellulose column equilibrated with Buffer D (10 mM potassium phosphate pH 8, 20% glycerol, 1 mM EDTA, 10 mM β-mercaptoethanol and five protease inhibitors). Following a thorough washing, bound enzymes were step-eluted with 350 mM potassium phosphate in buffer D. Fractions containing DNA polymerase activity were pooled and dialysed against buffer D (Fraction 1). Fraction 1 was either chromatographed on heparin–Sepharose (Pharmacia) or phosphocellulose (Whatman) columns. Fractions containing the active ctDNA polymerase were recovered from heparin–Sepharose with a 100–500 mM KCl gradient in buffer D and from phosphocellulose with a 100–500 mM gradient of potassium phosphate, pH 8.0. The active fractions eluted at about 300 mM KCl and 350 mM potassium phosphate from heparin–Sepharose and phosphocellulose columns, respectively. These active fractions were dialysed against buffer D containing 50 mM KCl to obtain Fraction 2A (heparin–Sepharose) and Fraction 2B (phosphocellulose) which were then separately subjected to single-strand DNA (ssDNA)–cellulose chromatography. DNA polymerases eluted from this column at around 500 mM KCl, following application of a linear gradient of 100–600 mM KCl in buffer D. The eluted enzymes were dialysed and concentrated using the Amicon system (30 kDa cut off) to obtain Fraction 3. This fraction was adjusted to 700 mM KCl and applied on a 1 ml phenyl–Sepharose column. Bound enzyme eluted with a linear gradient of 700–0 mM KCl in buffer D. Active fractions, eluting between 200–150 mM KCl, were pooled, concentrated (Fraction 4) and stored after freezing in liquid nitrogen in presence of 1 mg/ml bovine serum albumin (BSA) (Gibco BRL).

The total number of major polypeptides in Fractions of 2A, 2B, 3 and 4 were about 45, 35, 15 and 4 respectively, as examined by SDS–PAGE and silver staining. No DNA primase activity was detected in Fraction 3 or 4.

Table 1. Purification of the 43 kDa protein

No.	Fraction	Protein (mg)	DNA binding activity (U)	Specific activity (U/mg)	Fold purification
1.	Lysed chloroplasts	2050	13700	6.7	–
2.	DEAE cellulose flowthrough	1058	10587	9.9	1.3
3.	CM cellulose	9.99	5595	560	84
4.	ssDNA cellulose	0.65	2167	3334	498
5.	Sephadex G-100	0.33	1650	5000	746

One unit represents the amount of activity corresponding to an arbitrarily chosen unit scale of intensity in a densitometric scan of the autoradiogram from the southwestern blot. Intensity of the bands in lanes 1 and 5 of Figure 1B is 2 and 10 respectively.

DNA synthesis

DNA synthesis was carried out as described by Reddy *et al.* (14). One unit of DNA polymerase activity was defined as the amount responsible for incorporation of 1×10^5 c.p.m. of [^{3}H]TMP (specific activity of [^{3}H]TTP = 5×10^{13} c.p.m./mmol) into acid insoluble radioactivity. The reactions were usually carried out at 37°C for 30 min using 1 µg of activated calf thymus DNA as template. Under similar conditions, 1 µg of unprimed M13 ssDNA template yielded an insoluble radioactivity of about 20 000 c.p.m. The unprimed ssDNA template was used for monitoring activity of DNA primase coupled DNA polymerase activity. Wherever necessary, synthesis was also carried out using [^{35}S]dATP (specific activity = 10^7 c.p.m./nmol) and appropriate templates.

Solid phase assay for protein–protein interactions

The 43 kDa protein was allowed to bind to the polystyrene surface of the wells of an ELISA plate. The binding was carried out in 50 µl B1 buffer (50 mM Tris pH 8, 10 mM β-mercaptoethanol, 10% glycerol and protease inhibitors) with 150 mM NaCl for 2 h at 37°C. The unbound proteins were removed, followed by two washes with 100 µl B1 buffer containing 150 mM NaCl. The wells were then blocked with 1% BSA for 1 h at 37°C in 200 µl B1 buffer containing 150 mM NaCl. After three washes as above, salt-free Fraction 2A containing 1–2 U of DNA-polymerase activity in 50 µl B1 buffer with 50 mM NaCl was added to the coated wells. Incubation was continued at 25°C for 20 min in the presence or absence of various reagents such as 2 mM ATP or 1 µg of M13ssD or 40 µg polyclonal anti-43 kDa antibodies. Unbound enzymes were removed by washing three times with 200 µl B1 buffer containing 200 mM NaCl. The bound enzymes were eluted with 400 mM NaCl in 50 µl B1 buffer. The eluted enzyme (25 µl) was added to 75 µl of DNA synthesis reaction. Care was taken to keep the salt concentration below 125 mM NaCl, the inhibitory limit for the DNA polymerase activity (15).

Co-immunoprecipitation

Fraction 2A containing 1.5 U DNA-polymerase activity was mixed with 5 µg of 43 kDa protein (or 5 µg of BSA as control) at 37°C in 30 µl of Bl buffer containing 150 mM NaCl. After 30 min, 50 µg of rabbit anti-43 kDa antibodies were added, incubated at 25°C for 15 min, followed by the addition of 15 µl of Protein G-Sepharose (capacity = 19 µg of rabbit IgG/µl, Pharmacia). The mixture was shaken mildly at 37°C for another 30 min. The immunoprecipitate was removed by centrifugation at 3000 *g* for

10 min and the supernatant was assayed for DNA polymerase activity. The presence of DNA polymerase within the immunoprecipitate was also established by a DNA polymerase activity gel (22).

Radioiodination of 43 kDa protein

Approximately 30 µg of 43 kDa protein was conjugated with 0.5 mCi of Na[^{125}I] (specific activity = 1500 Ci/mmol, Amersham) using preloaded iodobeads and published protocol (Stratagene). Radioiodinated protein (6 µg in 150 µl) at a specific activity of 1.8×10^7 c.p.m./µg was obtained after removing unincorporated radioactivity by gel filtration chromatography. This was used as a probe for far western hybridization as described below.

Various fractions of ctDNA polymerase were electrophoresed in a SDS–10% PAGE and transferred onto nitrocellulose membrane. The membrane was incubated with a blocking buffer containing phosphate buffered solution (PBS), 0.3% Tween 20, 1% BSA (Sigma) for 1–2 h at room temperature followed by incubation with [^{125}I]-43 kDa protein in the same buffer (2 µl of radioiodinated protein/ml of blocking buffer) for 16 h. The membrane was then washed 3–4 times with buffer containing $1.5 \times$ PBS and 0.3% Tween 20, dried and exposed for autoradiography.

Size determination of the nascent DNA

Five units of Fraction 2A ctDNA polymerase and 10 µCi of [^{35}S]dATP (specific activity = 10^7 c.p.m./nmol) were used in each DNA synthesis reaction with DNA templates and substrates as described in the text. While 80% of the synthesised DNA was processed to check trichloroacetic acid (TCA) insoluble incorporation, the rest was analysed for size distribution on a urea–8% PAGE at 45°C. The gel was fixed, dried and exposed for autoradiography.

The autoradiogram was scanned by a laser densitometer. The intensity (I) and the Molecular weight (M) of each DNA species was plotted to generate an I versus M curve for each lane of the gel. Molecular weight (M) of nascent DNA species was calculated using the relationship between the electrophoretic mobility (µ) and the molecular weight (M) i.e. $\log M = K_1\mu + K_2$ (K_1 and K_2 are the empirical constants, the values of which were determined from standard markers). The average molecular weight ($\overline{M}$) was found as $\overline{M} = \int IdM/\int dM$ from the plots of I versus M.

Others

Other procedures such as purification of antibodies from rabbit antisera, agarose gel electrophoresis, labelling of probes by nick translation and 3′-end labelling, western blotting using anti 43 kDa rabbit polyclonal IgG or antisera (dilution 1:20 000) etc. were carried out using published procedures (23).

RESULTS

Purification of 43 kDa protein

A 43 kDa DNA binding protein was purified 800-fold to apparent homogeneity from the pea chloroplasts using a combination of ion exchange, affinity and gel filtration chromatography (Table 1). The enrichment of the protein was followed up by south western hybridization with radiolabelled denatured DNA. Due to its basic nature (pI > 9, not shown), the 43 kDa protein did not bind to DEAE cellulose but was retained on a CM cellulose column at pH 5.2. Purity of the protein was enhanced significantly by affinity chromatography over ssDNA cellulose where the only co-eluant was a 16 kDa protein as shown in lane 4 of Figure 1A. The 16 kDa
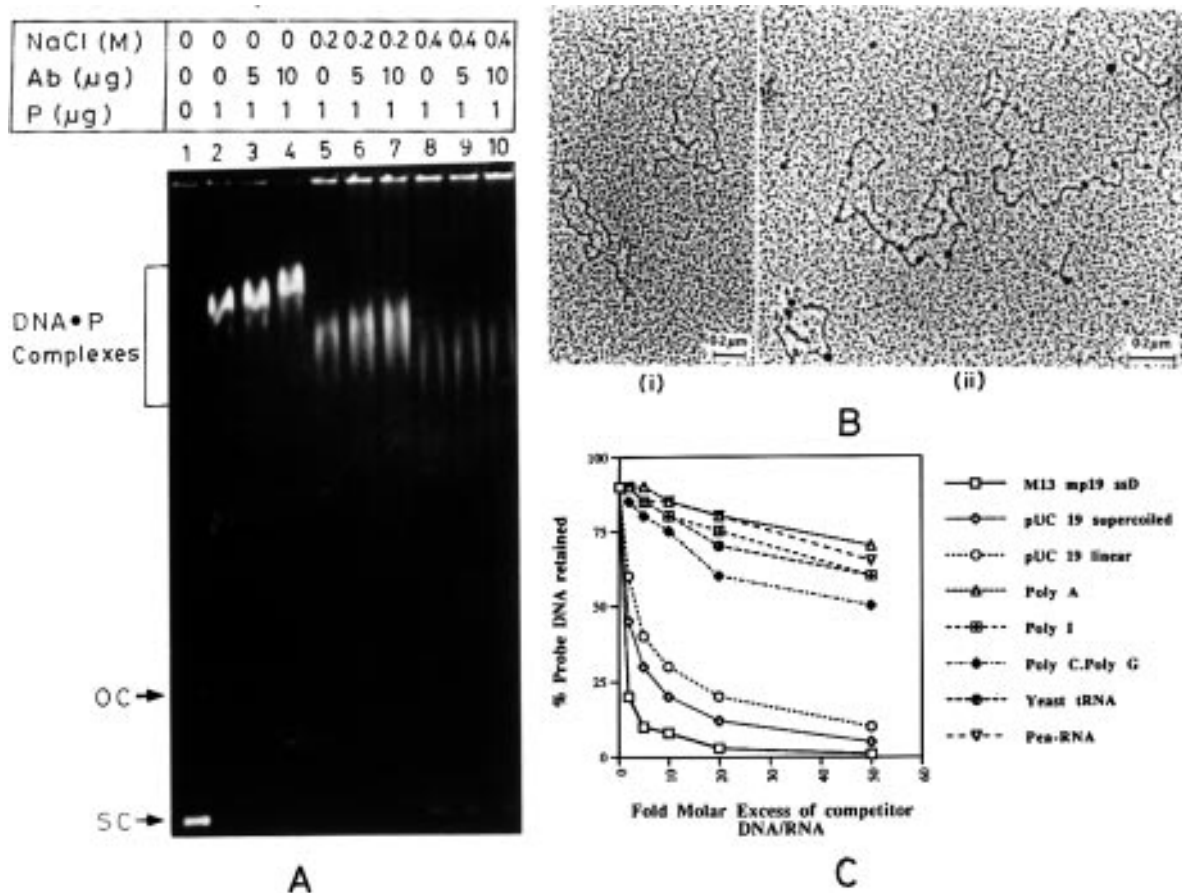
Figure 2. DNA-binding activities of the 43 kDa protein. (**A**) Gel-retardation of the DNA–protein complexes. One microgram of p-bluescipt (pBS) supercoiled DNA complexed with 43 kDa protein was electrophoresed on a 0.7% agarose gel in TBE buffer at 1 V/cm for 16 h. Conditions of complexation reactions have been indicated on the top of the figure. The gel was stained with ethidium bromide and photographed. P, OC and SC represent p43, open circular and the supercoiled form of pBS DNA respectively. (**B**) Electron micrograph of M13ssDNA and M13ssDNA–43 kDa protein complexes. One microgram of M13ssDNA was incubated in absence (i) or presence (ii) of 1 µg of the 43 kDa protein and the reaction products were examined in a Philips EM 410 transmission electron microscope. (**C**) Filter-binding of DNA–protein complexes. Complexes were prepared in the presence of various unlabelled competitors as indicated on the right hand side of the figure. Filter bound radioactivity was determined and values are expressed as percent of the probe DNA.

protein however did not bind DNA on a southwestern blot (Fig. 1B) and could be separated from the 43 kDa protein by a subsequent step of gel filtration chromatography. It is important to note that the proteins of ~33 kDa in size were abundantly present in Triton X 100 lysate (Fig. 1B, lane 1) and have been reported earlier to be the RNA binding proteins (24). These were removed from the 43 kDa protein following DEAE cellulose chromatography.

The molecular mass of the purified protein was similar when analysed either by gel filtration chromatography or SDS–PAGE. Therefore the 43 kDa protein is a monomer in its native state. The DNA binding activity of 43 kDa protein was stable at 4°C for 2–3 weeks and for 6 months at –70°C when stored at a concentration of 1 mg/ml. The activity was lost when treated at 60°C for 15 min. The purified 43 kDa protein did not have any detectable DNA polymerase, DNA-primase, DNA topoisomerase, DNA helicase, ATPase or nuclease activity.

Purified 43 kDa protein binds to any sequence and all structural forms of DNA

DNA binding properties were evaluated by gel retardation, filter-retention and electron microscopic visualization of the DNA–protein complexes. These experiments revealed that the 43 kDa protein could complex with any topological form and sequence of DNA. At low salt concentration (<50 mM NaCl), strong binding was observed with either the supercoiled, topoisomers, linear, nicked, or single stranded form of any plasmid DNA and the complex could easily be detected by agarose gel electrophoresis. Lane 2 of Figure 2A shows the retarded mobility of the complex between the protein and supercoiled DNA. The retardation depended on the amount of 43 kDa protein and salt concentration. Addition of anti-43 kDa polyclonal antibody during the complex formation (or after complex formation) induced a 'supershift' of the complex (lanes 3 and 4). The DNA–protein interaction was, however, sensitive to high salt and was reduced to the extent of 80% in presence of 400 mM NaCl (lane 8). The smear in lanes 5–10 may reflect either the gradual loss of complex-forming ability during electrophoresis or formation of heterogenous species in the presence of high salt.

ssDNA complexed with 43 kDa protein could also be visualized by electron microscopy. Approximately 10 protein-binding sites, distributed randomly over the entire contour of the DNA molecule, could be determined in the micrograph as shown in Figure 2B. A linear increment of binding sites was observed with the increasing
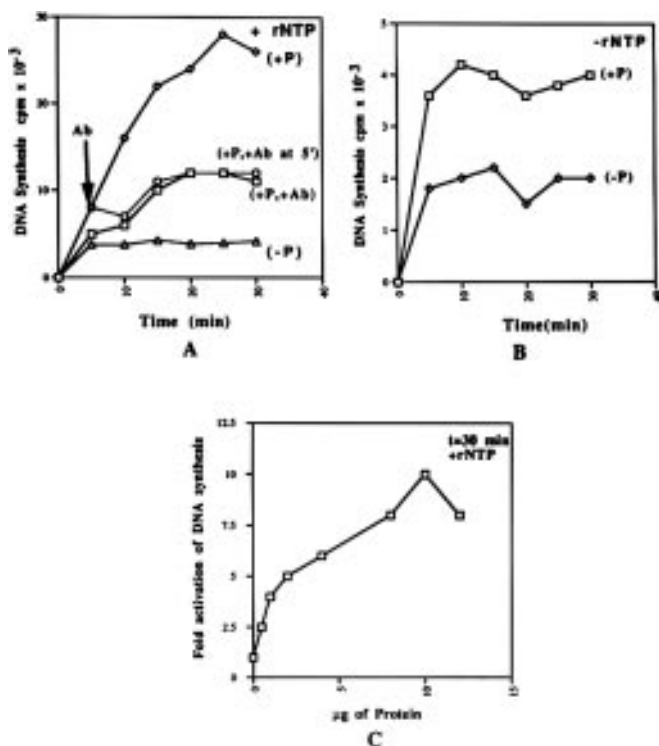
Figure 3. Enhancement of the ctDNA polymerase/primase activity. (**A**) Kinetics of DNA synthesis. One microgram of unprimed M13ssDNA was incubated with 0.2 U Fraction 2A ctDNA polymerase enzyme under various conditions both in absence (–) and presence (+) of 3.5 µg (35 µg/ml) of the 43 kDa protein (P). DNA synthesis was measured in terms of incorporation of [³H]TMP. Thirty-five microgram (350 µg/ml) of anti-43 kDa antibodies (Ab) were either allowed to precomplex with the 43 kDa protein (+P, +Ab) or added 5 min after the onset of DNA synthesis reaction (+P,+Ab at 5 min). (**B**) Kinetics of synthesis. Reactions were carried out similarly as in (A) withholding (–) the ribose triphosphates (rNTPs) as substrates. (**C**) Fold activation is the ratio of DNA synthesis observed in presence and absence of 43 kDa protein. Reactions were performed as in (A) but terminated after 30 min.

concentration of 43 kDa protein (up to 6 µg) as long as the ssDNA concentration remained unchanged.

In order to explore the relative binding affinities of various forms of DNA, loss of the radiolabelled DNA–protein complex in the presence of various unlabelled competitor nucleic acids was monitored by a filter-binding assay. As shown in Figure 2C, ssDNA competed most efficiently for binding of 43 kDa protein to radiolabelled linear DNA whereas RNA was the weakest competitor. The ssDNA binding was, however, marginally preferred over the binding of supercoiled DNA as shown in Figure 2C. Of the various forms of RNA tested, the homopolymer duplex polyC·polyG bound to the 43 kDa protein most efficiently.

The 43 kDa protein stimulates ctDNA polymerase/primase activity

Many DNA binding proteins are known to stimulate their homologous DNA polymerase activities. Hence the role of the 43 kDa protein on DNA synthesis was examined. Since attempts were not made to purify the DNA polymerase to homogeneity, due to its extreme low abundance in pea chloroplasts, the chromatographic fractions specifically enriched for DNA polymerase activity (Materials and Methods) were used. Of the various

fractions tested, stimulation of DNA synthesis by the 43 kDa protein was maximal with the DNA polymerase Fraction 2A.

Fraction 2A contained proteins other than the pea ctDNA polymerase and the primase but was devoid of 43 kDa protein as detected by western blotting. This fraction was capable of synthesising DNA chains on unprimed M13mpl9ssDNA template. A kinetic analysis of DNA synthesis (Fig. 3A) revealed that synthesis was rapid, reaching a plateau within 10 min. Inclusion of 43 kDa protein in the synthesis reaction resulted in a gradual enhancement of DNA synthesis though the plateau was not reached before 25 min. The stimulation of DNA synthesis in the presence of 43 kDa protein depended upon the amount of DNA polymerase. For example, the DNA synthesis increased 6-fold with 0.2 U DNA polymerase in presence of 3 µg of 43 kDa protein. Under similar experimental conditions using 1 U DNA polymerase, the increase was only 2.5-fold (not shown). The increase of DNA synthesis was reversed by anti-43 kDa antibodies as shown in Figure 3A. When the antibodies were added after 5 min of initiation of DNA synthesis, inhibition occurred within 1–2 min. In a control reaction, pre-immune rabbit antibodies did not show any significant effect on DNA-synthesis (not shown).

DNA synthesis by Fraction 2A was reduced by 50% in the absence of rNTPs (Fig. 3B). The presence of endogenous rNTPs within Fraction 2A and/or the ability of the primase to partially utilize dNTPs for formation of primers (25) may account for the residual DNA synthesis. Figure 3B shows that the stimulation of DNA synthesis by 43 kDa protein still occurred in such a situation. Figure 3C demonstrates that the stimulatory effect on ctDNA polymerase/primase activity was optimal at about 10 µg of 43 kDa protein. Higher concentrations were inhibitory for stimulation of DNA synthesis.

To investigate whether the enhancement of DNA polymerase/primase activity was specific for 43 kDa protein, the effect of nonhomologous DNA binding proteins on pea ctDNA polymerase mediated DNA synthesis was also studied. As shown in Table 2, *Escherichia coli* SSB and calf thymus histone Hl proteins were inhibitory rather than stimulatory for ctDNA polymerase/primase activity. Stimulatory activity of other stabilizing proteins like BSA was also marginal. Table 2 also shows that the 43 kDa protein did not have any inherent DNA polymerase activity. Since dideoxynucleotides (ddNTPs) were poor chain terminators for the ctDNA polymerase, a 10-fold molar excess of ddNTPs over dNTPs was required to reduce the stimulatory activity by 60%. This reduction was also consistent with the inference that 43 kDa protein acts as an accessory to the ctDNA polymerase/primase activity.

The 43 kDa protein binds to the ctDNA polymerase

The data of Figure 3 and Table 2 suggested that the purified protein might interact with the ctDNA polymerase. Since homogeneously purified ctDNA polymerase was not available in sufficient amounts, we used three independent approaches to verify this.

The first approach employed a solid phase assay in which 43 kDa protein was coated onto the wells of an ELISA plate. The binding of 43 kDa protein to the wells was strong with no disruption even at a concentration of 500 mM NaCl. After blocking nonspecific sites on the plate, Fraction 2A DNA polymerase was applied to the wells and incubated. Subsequently, proteins bound by 43 kDa protein were eluted and the eluant was assayed for DNA polymerase activity.

Table 2. Chloroplast DNA polymerase/primase activity is enhanced specifically by the 43 kDa protein

No.	Reaction	Addition	Synthesis (c.p.m. × 10⁻³) at *t* = 30 min
(a)	Standard reaction without Fraction 2A DNA polymerase	None	0.35
(b)	Same as (a)	4.0 µg p43	0.34
(c)	Standard reaction with Fraction 2A DNA polymerase	None	4.20
(d)	Same as (c)	ddNTP:dNTP(10:1)	2.00
(e)	Same as (c)	4.0 µg p43	25.0
(f)	Same as (c)	4.0 µg p43 +ddNTP:dNTP(10:1)	9.20
(g)	Same as (c)	0.25 µg *E.coli* ssB	3.50
(h)	Same as (c)	1.0 µg *E.coli* ssB	2.90
(i)	Same as (c)	4.0 µg *E.coli* ssB	0.40
(j)	Same as (c)	0.25 µg histone H1	3.40
(k)	Same as (c)	1.0 µg histone H1	2.90
(l)	Same as (c)	4.0 µg histone H1	0.40
(m)	Same as (c)	4.0 µg BSA	4.50
(n)	Same as (c)	10.0 µg BSA	5.40

Components of a standard DNA synthesis reaction included 1 µg M13ssD, 100 µM each of rNTPs, 50 µM each of dATP, dCTP, dGTP and 1 µCi of [³H]TTP in a 100 µl reaction volume as described in (14). A dose of 0.2 U Fraction 2A DNA polymerase was employed for primase mediated DNA synthesis. p43, 43 kDa protein.

Figure 4 shows that the polystyrene-bound 43 kDa protein was able to trap ctDNA polymerase. When 1.5 U Fraction 2A was used, 0.25 U DNA polymerase activity could be retained by 43 kDa protein under the experimental conditions. Interestingly, addition of 1 µg of M13mpl9ssDNA along with the Fraction 2A enzymes increased the retention of ctDNA polymerase 3-fold. On the other hand, addition of 40 µg of polyclonal anti-43 kDa antibodies inhibited the interaction to the extent of 50%. Similar amount of pre-immune antibodies did not have any effect. In a separate assay, the above mentioned eluant was found incapable of DNA synthesis with unprimed ssDNA template. This suggests that no significant interaction between the coated 43 kDa protein and the DNA primase could be detected by this technique. Furthermore, no polymerase binding could be detected in control wells coated with 1% BSA alone.

The possibility of interaction of DNA polymerases from other sources with purified 43 kDa protein was also examined. The results are summarized in Table 3. Commercially available DNA polymerases of bacteriophages T4 and T7 did not interact with the 43 kDa protein. Similarly a crude preparation of aphidicolin sensitive (26) pea nuclear DNA polymerase (ctDNA polymerase is aphidicolin resistant) also did not show any binding. In these cases, even a 1.0 M NaCl eluant did not show any DNA polymerase activity, thereby ruling out the possibility of any stronger interaction. On the other hand, binding could be observed with *E.coli* DNA *Pol*I. Here also, addition of 1 µg of ssDNA along with the *E.coli Pol*I enzyme resulted in an increased recovery of the enzyme. It is worthwhile to note that 5 µg of 43 kDa protein also stimulated the *E.coli* DNA *Pol*I (1 U) activity by at least 1.5-fold (not shown). Weak binding to 43 kDa protein was also observed with Klenow fragment of *E.coli Pol*I. (Table 3).

In co-immunoprecipitation studies, a complex of 43 kDa and the ctDNA polymerase along with other Fraction 2A proteins could be specifically precipitated by anti-43 kDa antibodies but not with pre-immune antibodies. The presence and molecular weight of ctDNA polymerase in the immunoprecipitate was identified by a DNA polymerase activity gel (22) and the polymerase activity remaining in the supernatant was measured as mentioned in Materials and Methods. Densitometric quantitation of the autoradiogram shown in Figure 5A demonstrated that about 0.4 U 70 kDa ctDNA polymerase was present in the immuno-precipitate (lane 2) when 1.5 U Fraction 2A enzyme was mixed with 5 µg 43 kDa protein. Figure 5B shows that a comparable amount was depleted in the supernatant fraction (lane 2). When BSA instead of the 43 kDa protein was present during co-immuno-precipitation, almost no DNA polymerase could be detected in the precipitate as expected (Fig. 5A, lane 1). If anti-43 kDa antibody was omitted in the reaction with the purified protein, very little DNA polymerase activity was detected in the precipitate (Fig. 5A, lane 3) whereas the DNA polymerase activity of the supernatant increased (Fig. 5B, lane 3). The latter increase in activity could be attributed to the accessory activity of 43 kDa protein present in the supernatant.

Table 3. Interaction of various DNA polymerases with the 43 kDa protein

| DNA polymerases	Amount of ³H TMP incorporated (c.p.m.)			R* (%)	Interaction
	Input (×10⁻⁵)	Eluant/50 µl	Unbound (×10⁻⁵)		
Fraction 2A	1.81	24012	1.02	13.3	++
T4 phage	1.75	710	1.71	0.4	–
T7 phage	1.84	620	1.73	0.3	–
Klenow	1.56	8000	1.42	5.1	+
E.coli	1.73	22000	1.34	12.7	++
Crude preparation of pea nuclear DNA polymerases	1.42	832	1.21	0.6	–

R*, retention of DNA polymerase activity in the wells of an ELISA plate coated with the 43 kDa protein. Approximately 7.5 µg 43 kDa protein was added to coat each of the wells.
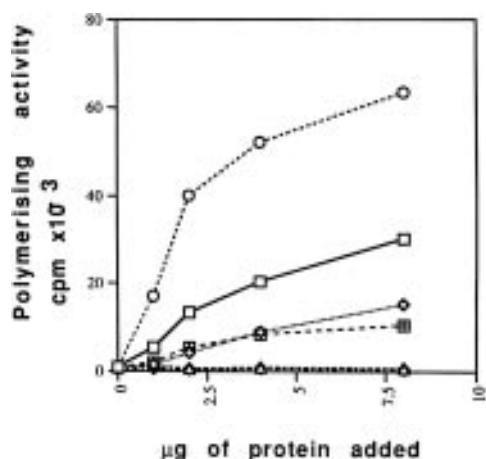
Figure 4. Interaction between the ctDNA polymerase and the 43 kDa protein. 1.5 U Fraction 2A DNA polymerase was incubated with the indicated amount of 43 kDa protein added in presence of either no extra reagents (Normal), 40 µg anti-43 kDa antibody (Ab), or 1 µg of M13ssDNA (ssD). Bound DNA polymerase was eluted with 400 mM NaCl and the eluant was assayed for activity using templates which were either activated calf thymus DNA (ACTD), unprimed M13ssDNA (ssD), or singly primed M13ssDNA (SP ssD). When BSA alone was used for coating, incubation condition with DNA polymerase was normal and the 400 mM NaCl eluant was assayed employing ACTD template. Symbols are as follows: (□) ACTD, Normal; (◇) ACTD, Ab; (○) ACTD, ssD; (△) UPssD, Normal; (▣) SP ssD, Normal; (◆) ACTD, Normal (only for BSA). Templates followed by conditions of incubation are indicated in the symbols. The primer used in the case of SP ssD was the universal 17 mer. Calculated activity of 50 µl eluant is shown.

Since the Fraction 2A contained several other contaminating polypeptides, the direct interaction between the DNA polymerase and the 43 kDa protein was studied using the far western technique. Proteins of various fractions containing the pea ctDNA polymerase (Materials and Methods) were resolved on a polyacrylamide gel and then transferred onto a nitrocellulose membrane. Transferred proteins were probed with ^{125}I labelled 43 kDa protein. In this procedure, the membrane bound proteins interacting with the radioiodinated protein were readily identifiable by acquisition of the radiolabel, thus allowing their detection by autoradiography. The autoradiographic bands in lanes 1–7 of Figure 6 represent proteins capable of interacting with the 43 kDa protein. The banding position of the ctDNA polymerase in lane 1 was determined separately by a DNA polymerase activity gel (22) and located at a molecular weight of 70 kDa as shown in lane 8 of Figure 6.

Though Fractions 2A and 2B contained protein(s) other than the 70 kDa DNA polymerase which bound to the 43 kDa protein, the direct interaction between the polymerase and labelled protein was nevertheless obvious (lanes 1 and 6 of Fig. 6). Two
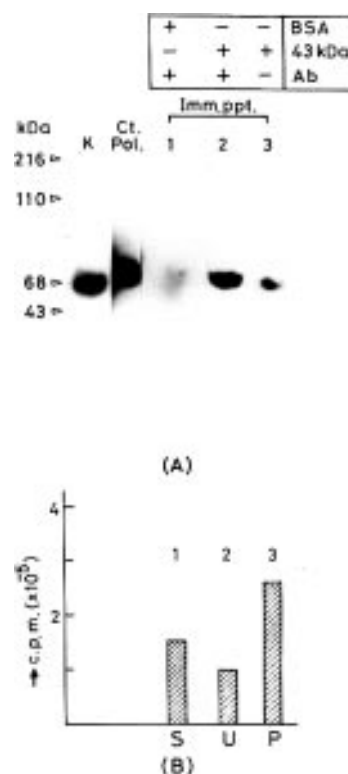

Figure 5. Coimmunoprecipitation of the ctDNA polymerase and the 43 kDa protein. (**A**) An autoradiogram of DNA polymerase activity gel of the immunoprecipitate along with control DNA polymerase. Presence of the 70 kDa ctDNA polymerase is detected with 5 µg BSA (lane 1), 5 µg 43 kDa protein (lane 2), no anti-43 kDa antibody (lane 3). 0.05 U Klenow and 0.5 U Fraction 2A ctDNA polymerase were analysed in lanes marked respectively, as K and Ct-pol. (**B**) DNA polymerase activity of corresponding supernatant (SUP) fractions. The amount of [³H]TMP incorporation is displayed in the form of hatched bars for lanes 1–3.

micrograms of each of the marker protein (lane M) and 5 µg of BSA (lane 3) failed to bind. Only the DNA Polymerase component of the Fractions 3 and 4 could bind to the labelled protein (lanes 2, 4 and 5). The Fraction 4 DNA polymerase when stored with BSA (lane 5) showed enhanced binding compared to the enzyme stored in absence of BSA (lane 4). Hence the stability of nearly purified DNA polymerase could be improved quite significantly by addition of BSA. No significant self interaction of 43 kDa protein was observed (lane 7), a fact consistent with the observation that the protein is a monomer in its native state. Collectively these results strongly suggested the possibility of a direct interaction between the 43 kDa protein and 70 kDa pea ctDNA polymerase.

Table 4. Template preference for activation of Fraction 2A DNA polymerase

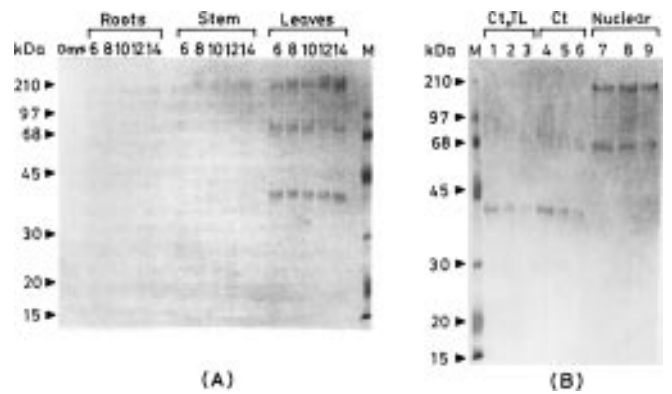| Template (1 µg)	[³⁵S]dAMP incorporation (c.p.m.) +		Activation of DNA synthesis
	No protein	5 µg p43	(-fold)
(i) ACTD	1.47×10^5	2.32×10^5	1.6
(ii) Unprimed M13 mp19ssDNA	3.0×10^4	6.1×10^4	2.0
(iii) Poly dT₂₅₀			
(a) Unprimed	2.3×10^3	3.1×10^4	13.4
(b) Singly primed by oligo dA	8.1×10^3	3.0×10^4	3.7
(c) Doubly primed by oligo dA	14.2×10^3	3.4×10^4	2.4

ACTD, activated calf thymus DNA; p43, 43 kDa protein.
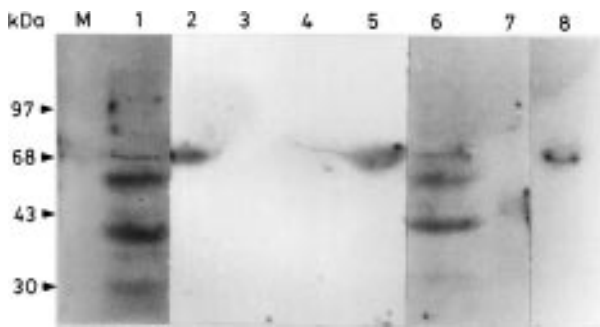
Figure 6. Identification of [^{125}I]-43 kDa binding proteins. Autoradiogram of the far western blot containing various ctDNA polymerase fractions. Two micrograms of each of the marker proteins was loaded in lane M. 0.2 U Fraction 2A ctDNA-polymerase enzymes (lane 1), 0.8 U polymerase of Fraction 3 enzyme plus 2 μg BSA (lane 2), 5 μg BSA (lane 3), 0.4 U polymerase of Fraction 4 without BSA (lane 4), 0.8 U polymerase of Fraction 4 with 4 μg BSA (lane 5), 0.2 U Fraction 2B enzyme (lane 6) and 6 μg of 43 kDa protein (lane 7) were resolved by SDS–10% PAGE. Lane 8 shows an autoradiogram of the DNA polymerase activity gel of Fraction 2A enzyme. Part of the blot containing the lanes M, 1, 6 and 7 was exposed for a longer time to identify weaker interactions whereas the part containing the lanes 2–5 was subjected to a short term exposure to reduce the background.

Figure 7. Distribution of the 43 kDa protein. (**A**) Western blot analysis of crude fractions of proteins (75 μg/lane) from indicated tissues of the pea plant. Rabbit anti-43 kDa antisera were used at 1:20 000 dilution. Age of the plant tissues has been indicated on top of the figure. Molecular weight of the prestained markers is shown in lane M. (**B**) Western blot analysis of the crude proteins from subcellular fractions of cells derived from 7 day old leaf tissue. Thermolysin treated chloroplast (Ct,TL), untreated chloroplast (Ct) and nuclear proteins were analysed in lanes 1–3, 4–6 and 7–9 respectively; 50 μg of protein was used in lanes 1, 4 and 7; 25 μg in lanes 2, 5 and 8; 15 μg in lanes 3, 6 and 9.

The size of the nascent DNA chains increases in the presence of 43 kDa protein

The mechanism of accessory activity of 43 kDa protein was analysed by measuring the amount and length of the synthesised DNA products. Unprimed poly dT$_{250}$ template was used in these experiments as the maximum stimulation of DNA synthesis was observed with this template (Table 4). Nascent radiolabelled DNA products of the synthesis reaction in the presence or absence of 43 kDa protein were analysed electrophoretically in a denaturing polyacrylamide gel. Average molecular weight was calculated from the size distribution of synthesised DNA chains. The calculated average molecular size of the DNA chains in presence of 5 μg of 43 kDa protein was increased from 40 to 108 nt. Similarly, using the same template singly primed with oligo dA$_{20}$, the average size of the chain length increased from 53 to 128 nt. In addtion to the increase in size, the 43 kDa protein dependent increase in the amount of DNA synthesis was also observed (data not shown).

Tissue-specific and subcellular localization of the 43 kDa protein

Although the protein was purified from pea chloroplasts, it was of interest to study its distribution within various parts of the plant. Proteins from various tissues of the plant pea were examined at different growth stages by western blotting using anti-43 kDa polyclonal antisera (dilution 1:20 000). Figure 7A shows that the 43 kDa form of the protein could be detected only in the leaf extracts. In order to further localize this protein to subcellular compartments, proteins from intact chloroplasts and nuclei of pea leaves were similarly examined. As shown in Figure 7B, the 43 kDa protein could be identfed only within the chloroplasts. It remains to be seen whether the crossreacting 68 kDa and 200 kDa nuclear proteins observed in Figure 7B represent the precursor forms of the 43 kDa protein or similarly acting additional proteins. These proteins were not observed when the blot was probed with preimmune serum (not shown). Since the protease thermolysin is known to digest the cytosolic proteins adhering to the surface of the

chloroplasts (16), thermolysin treated chloroplasts were also included in the study. Western blot data did not vary significantly with or without the thermolysin treatment (compare lanes 1 and 4 of Fig. 7B), confirming that the 43 kDa protein is localized within the chloroplast.

DISCUSSION

This work represents the first report of identification, purification and characterization of a DNA polymerase accessory protein from a plant source. The purified 43 kDa protein from pea chloroplasts is a strong DNA binding protein but could bind to RNA very poorly, indicating its specificity for DNA-related processes rather than RNA-related events such as post-transcriptional modifications, etc. Though the DNA–protein complex was stable to the harsh conditions of electrophoresis, it was disrupted by even moderate salt concentration. This suggests the importance of ionic interaction in the protein–DNA recognition.

Besides binding to DNA, the protein also activates the cognate DNA polymerase. Since other DNA binding proteins namely *E.coli* SSB, histone H1, failed to activate pea ctDNA polymerase, the observed activation of pea ctDNA polymerase seems to be specific for the 43 kDa protein. The extent of activation depended on the amounts of the protein and ctDNA polymerase and also on the nature of the template employed. Interestingly, under conditions where primase activity was not required, i.e. either with excess primers or in the absence of rNTPs, the activation was reduced. It is possible, therefore, that optimal activation requires an interaction of the 43 kDa protein with both DNA polymerase and DNA primase.

The 43 kDa protein, therefore, has two distinct activites, one for DNA binding and another for stimulation of DNA polymerase activity. While the polyclonal antibodies to the 43 kDa protein could successfully repress DNA polymerase activation, they showed little inhibitory effect on DNA binding. These results may imply that the domains of these two activities are spatially non-overlapping.

Three independent approaches were taken to address the possibility of interaction between the DNA polymerase and the purified protein. While the approach of a solid phase assay could reveal the interaction between the partners, no binding of the 43 kDa protein was observed with DNA polymerases from either T4 or T7 bacteriophages. Furthermore, the binding of pea ctDNA polymerase was inhibited by polyclonal anti-43 kDa antibody. Thus the solid phase assay detected a somewhat specific interaction between pea ctDNA polymerase and the 43 kDa protein. Binding was also observed between *E.coli* DNA *Pol*I and the 43 kDa protein. The latter observation is indicative of the evolutionary linkage (27) between the two polymerases.

In co-immunoprecipitation studies, a 70 kDa DNA polymerase was observed to interact with the 43 kDa protein. It is important to mention that a prolonged autoradiographic exposure of the polymerase activity gel containing only the Fraction 2A enzymes revealed a weak band of DNA polymerase at 90 kDa (15) in addition to the one at 70 kDa (A. Gaikwad, unpublished). At present, it is unclear how these two DNA polymerases are related but it could be safely assumed that the domain for interaction with 43 kDa protein resides in the 70 kDa form.

The involvement of other protein(s) within the Fraction 2A mediating the interaction cannot be ruled out by any of the two approaches above. Hence the direct interaction was established on a far western blot. In spite of this direct interaction, one could argue about the non-obligatory participation of intermediary protein(s) for the stimulatory activity.

The mechanism of activation by 43 kDa protein remains to be elucidated. The average size of DNA chains synthesized in presence of the 43 kDa protein increased by about 2.5-fold while the total DNA synthesis increased by a factor of 13. These data indicate that either higher amounts of DNA polymerases were made available at all stages of DNA synthesis or the affinity of the DNA polymerase for the template increased in presence of the 43 kDa protein resulting in increased processivity. These possibilities can be explored with the purified DNA polymerase and primase from the pea chloroplast.

An obvious explanation for the accessory activity would be that the 43 kDa protein is itself a component of the DNA polymerase holoenzyme. It does not appear to be true for various reasons. Firstly, the protein was not detectable in the Fraction 2A DNA polymerase which was fully competent at carrying out DNA synthesis including pea ct-ori specific DNA replication (14). The DNA synthesis was also unaffected by anti-43 kDa antibodies. Secondly, the 43 kDa protein, unlike most subunits of polymerase holoenzyme from other sources (28), was never observed to copurify with the polymerase activity. Rather, its high pI of ~9 permitted its complete separation from DNA polymerase activity by a DEAE–cellulose chromatographic step. Thirdly, the subunit components of DNA polymerase are usually present in low abundance (29) whereas the supplementary accessory activities like SSBs (30) are produced in high quantities within the relevant cells. Prior estimates have determined that about 5×10^7 cells (31) with about 70 chloroplasts per cell (32) are present in 1 g tissue from 7–8 day old pea leaves. Taking into account about 88% loss of 43 kDa protein during purification (this manuscript, Table 1), the calculated copy number of 43 kDa protein in each chloroplast will be at least 10 000 which is a large excess over that of pea

ctDNA polymerase. Thus for the reasons outlined above, the 43 kDa protein probably represents a nonholoenzyme accessory factor for the pea ctDNA polymerase.

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