

A pea homologue of human DNA helicase I is localized within the dense fibrillar component of the nucleolus and stimulated by phosphorylation with CK2 and cdc2 protein kinases

Narendra Tuteja^{1,*}, Alison F. Beven², Peter J. Shaw² and Renu Tuteja¹

¹International Centre for Genetic Engineering & Biotechnology, Aruna Asaf Ali Marg, New Delhi 110 070, India, and

²Department of Cell Biology, John Innes Centre, Colney, Norwich NR4 7UH, UK

Received 15 August 2000; accepted 6 October 2000.

*For correspondence (fax +91 11 6162316; e-mail narendra@icgeb.res.in).

Summary

DNA helicases catalyse the transient opening of duplex DNA during nucleic acid transactions. Here we report the isolation of a second nuclear DNA helicase (65 kDa) from *Pisum sativum* (pea) designated pea DNA helicase 65 (PDH65). The enzyme was immunoaffinity purified using an antihuman DNA helicase I (HDH I) antibody column. The purified PDH65 showed ATP- and Mg²⁺-dependent DNA and RNA unwinding activities, as well as ssDNA-dependent ATPase activity. The direction of DNA unwinding was 3' to 5' along the bound strand. Antibodies against HDH I recognized the purified PDH65, and immunodepletion with these antibodies removed the DNA and RNA unwinding and ATPase activities from purified preparations of PDH65. The DNA and RNA unwinding activities were upregulated after phosphorylation of PDH65 with CK2 and cdc2 protein kinases. By incorporation of BrUTP into pea root tissue, followed by double immunofluorescence labelling and confocal microscopy, PDH65 was shown to be localized within the dense fibrillar component of pea root nucleoli in the regions around the rDNA transcription sites. These observations suggest that PDH65 may be involved both in rDNA transcription and in the early stages of pre-rRNA processing.

Keywords: plant DNA helicase, *Pisum sativum*, plant RNA helicase, rDNA transcription, rRNA processing, nucleolus.

Introduction

DNA unwinding is an essential step in many molecular processes, including DNA replication, repair and recombination, and transcription and translation (Kornberg and Baker, 1991). DNA helicases provide duplex unwinding functions to all these basic processes (Boroweic, 1996; Lohman and Bjornson, 1996; Matson *et al.*, 1994; Tuteja, 1997; Tuteja, 2000; Tuteja and Tuteja, 1996). Helicase action is an energy-driven process, the energy for which is provided by hydrolysis of NTPs or dNTPs. Although many DNA helicases have been characterized from bacteriophage, bacterial, viral, animal and fungal systems (Matson *et al.*, 1994; Thommes and Hubscher, 1992; Tuteja and Tuteja, 1996), little is yet known about plant DNA helicases. In plants, so far only two DNA helicases have been purified to homogeneity

from pea chloroplasts (Tuteja and Phan, 1998a; Tuteja *et al.*, 1996) and one from pea nuclei (Pham *et al.*, 2000).

A clear-cut biological role has not yet been defined for many eukaryotic DNA helicases. Recently, the first isolated plant (pea) nuclear DNA helicase of 45.5 kDa (PDH45) was shown to be homologous to translation initiation factor and to stimulate topoisomerase I activity (Pham *et al.*, 2000). This report describes the isolation of a second, 65 kDa nuclear pea DNA helicase, designated PDH65, which is shown to be a potential plant homologue of human DNA helicase I (Tuteja *et al.*, 1990). It contains both DNA and RNA duplex unwinding activities, and is localized in the nucleolus within the dense fibrillar component in regions around the rDNA transcription sites. The enzyme is

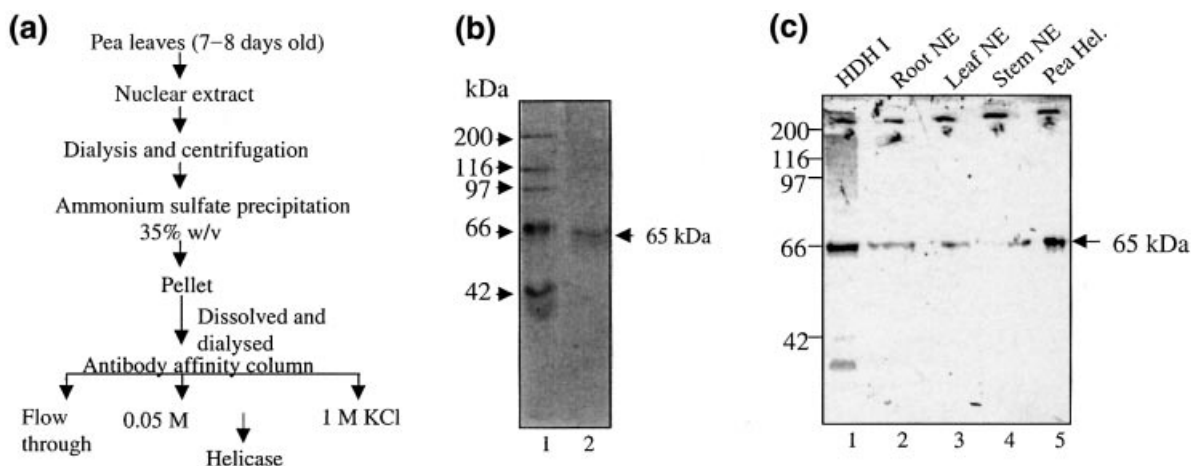


Figure 1. Purification and Western blot of PDH65.

(a) Immuno-affinity purification scheme for pea nuclear DNA helicase II (PDH65).

(b) SDS-PAGE of purified PDH65 (40 ng, lane 2) and protein size markers (lane 1) separated on a 10% gel and visualized by silver staining.

(c) Immunoblot of pea nuclear proteins extracted from root (20 μg, lane 2), leaf (20 μg, lane 3), stem (20 μg, lane 4) and purified PDH65 (0.1 μg, lane 5) and HDH I (0.1 μg, lane 1). The membrane was probed with antiserum raised against HDH I.

also shown to be upregulated by phosphorylation with CK2 and cdc2 protein kinases.

Results

Immunoaffinity purification of pea homologue of HDH I

All the purification steps were carried out at 0–4°C. The purification scheme is outlined in Figure 1(a). Briefly, nuclei were isolated from 700 g leaves of 7–8-day-old pea plants. The proteins of the pea nuclear extract were precipitated with ammonium sulphate (0.35 g mL⁻¹) and the pellet was collected by centrifugation. The pellet was resuspended and dialysed in buffer A. The dialysed fraction was loaded onto an antibody (anti-HDH I) affinity column equilibrated with buffer A. The flow-through was collected and again passed through the same column. After a thorough wash of the column with buffer A, the proteins bound to the beads were eluted with a linear gradient of 0.05–1 M KCl in buffer A. Fractions eluting at around 0.4 M KCl contained a single protein (1.10 μg) of 65 kDa, as checked on SDS-PAGE (Figure 1b, lane 2). This pea protein contained DNA helicase activity similar to HDH I, and has been designated PDH65.

Recognition of pea PDH65 by an anti-HDH I antiserum

The identity of pea PDH65 as a homologue of HDH I was further confirmed immunologically by Western blotting. This showed that the anti-HDH I antiserum recognize the purified pea PDH65 (Figure 1c, lane 5) as well as HDH I (Figure 1c, lane 1). The antiserum also recognizes a single

band of 65 kDa in total nuclear extracts from root, leaf and stem (Figure 1c, lanes 2, 3 and 4, respectively).

Characterization of DNA helicase activity of pea PDH65

The DNA unwinding activity of PDH65 was characterized by assaying the displacement of radiolabelled DNA from a partial duplex DNA substrate. The structure of the DNA duplex substrate used to detect DNA helicase activity is shown on the left of Figure 2(a). These assays showed that HDH I and PDH65 both had similar DNA helicase activity (Figure 2a, lanes 2 and 3, respectively). In the absence of either ATP or Mg²⁺, no DNA unwinding activity was observed (Figure 2a, lanes 4 and 5). The activity was inhibited by the DNA intercalating ligands nogalamycin and daunorubicin (Figure 2a, lanes 6 and 7). The properties of PDH65 are summarized and compared with those of PDH45 (Pham *et al.*, 2000) in Table 1. The order of activity of nucleotides and divalent cations for PDH65 was ATP > dATP and Mg²⁺ > Mn²⁺ > Ca²⁺, respectively (Table 1). The enzyme activity was inhibited by trypsin, EDTA and M13ssDNA (data not shown). PDH65 can also unwind longer duplexes (>17 bp), but with lower efficiency (data not shown).

RNA unwinding and ATPase activities of PDH65

The structure of the RNA duplex substrate used is shown on the left of Figure 2(b). These results showed that both the HDH I and PDH65 contained RNA helicase activity (Figure 2b, lanes 2 and 3, respectively). The RNA helicase activity of pea PDH65 was also ATP- and Mg²⁺-dependent

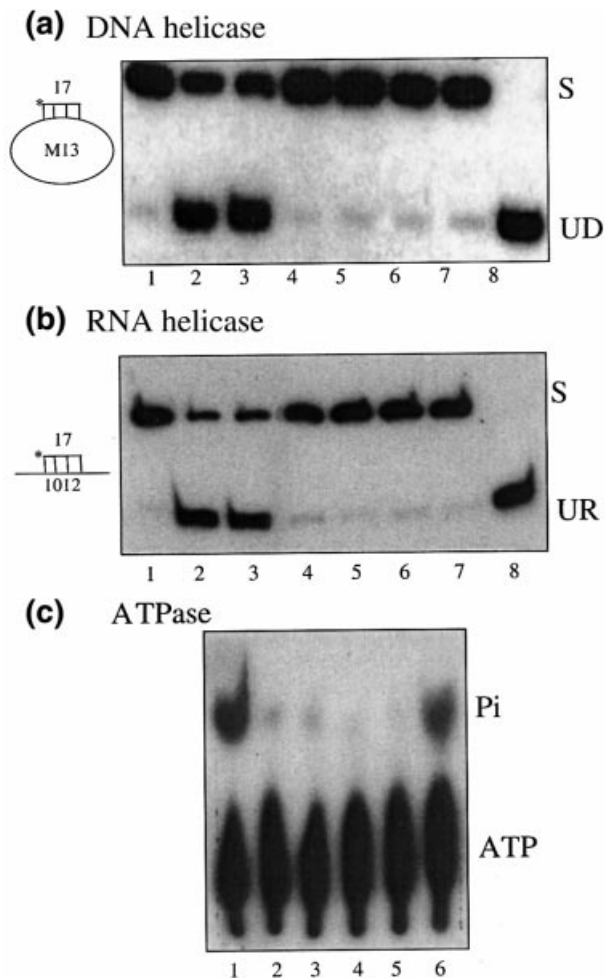


Figure 2. DNA helicase, RNA helicase and DNA-dependent ATPase activities of PDH65.

(a) DNA helicase activity detected by displacement of ^{32}P -labelled 17-mer DNA oligonucleotide annealed to M13ssDNA. The structure of the substrate is shown on the left; the positions of the substrate (S) and unwound DNA (UD) on the right of the gel. Asterisk denotes the ^{32}P -labelled end. Lane 1, no enzyme; lane 2, HDH I (5 ng); lane 3, purified PDH65 (6 ng); lane 4, reaction without ATP; lane 5, reaction without Mg^{2+} ; lane 6, reaction in presence of $10\ \mu\text{M}$ nogalamycin; lane 7, reaction in presence of $10\ \mu\text{M}$ daunorubicin; lane 8, heat-denatured substrate.

(b) RNA helicase activity. Structure of RNA duplex substrate shown on left; positions of substrate (S) and unwound RNA (UR) on right side of gel. Lanes 1–8 as in (a).

(c) ATPase activity. Released radiolabelled Pi from $[\gamma\text{-}^{32}\text{P}]$ ATP was separated by TLC and exposed to film. The standard reaction contained 100 ng ssDNA, 6 ng purified PDH65 and 3 mM MgCl_2 . Lane 1, reaction with PDH65; lane 2, reaction without ssDNA; lane 3, reaction without Mg^{2+} ; lane 4, reaction in presence of $10\ \mu\text{M}$ nogalamycin; lane 5, reaction in presence of $10\ \mu\text{M}$ daunorubicin; lane 6, HDH I control (5 ng).

(Figure 2b, lanes 4 and 5). The DNA intercalators nogalamycin and daunorubicin inhibited RNA helicase activity of PDH65 (Figure 2b, lanes 6 and 7). PDH65 showed ssDNA-dependent and Mg^{2+} -dependent ATPase activity (Figure 2c, lane 1). If ssDNA and Mg^{2+} were omitted from the reaction, the enzyme showed no ATPase activity

(Figure 2c, lanes 2 and 3). Nogalamycin and daunorubicin inhibited the ATPase activity (Figure 2c, lanes 4 and 5). The ssDNA- and Mg^{2+} -dependent ATPase activity of HDH I is shown in lane 6.

Kinetics and titration of DNA helicase activity

The rate of DNA unwinding under standard assay conditions with 6 ng purified PDH65 was linear up to 30 min (Figure 3a). Over longer periods it deviated from the linearity. Titration of DNA helicase activity with increasing amounts of PDH65 in a 30 min reaction showed a sigmoidal curve and a maximum value of unwinding close to 90% with 10 ng of the enzyme (Figure 3b).

Direction of DNA unwinding by PDH65

In order to determine the direction of unwinding, two different substrates were prepared with long ssDNA bearing short stretches of duplex DNA at the ends. Diagrams of the directional substrates are shown above each autoradiogram in Figure 4(a,b). The strand to which the enzyme binds and moves defines the direction of unwinding. The results show that PDH65 moves unidirectionally in the 3' to 5' direction (Figure 4a, lane 2) and not in the 5' to 3' direction (Figure 4b, lane 2).

Immunodepletion of DNA helicase and ATPase activities of PDH65 by an anti-HDH I antiserum

Purified PDH65 was reacted separately with IgG purified from the sera of a pre-immune rabbit and a rabbit immunized with HDH I. The antigen–antibody complex was removed by protein A–Sepharose. The supernatant was analysed for DNA helicase and ATPase activities. This showed that both the DNA helicase (Figure 5, lane 2) and ATPase (Figure 5, lane 3) activities were depleted with anti-HDH I antibodies, whereas there was no reduction of DNA helicase (Figure 5, lane 3) and ATPase (Figure 5, lane 2) activities in pre-immune IgG-treated samples. Similarly, the RNA helicase activity of PDH65 was also immunodepleted (data not shown).

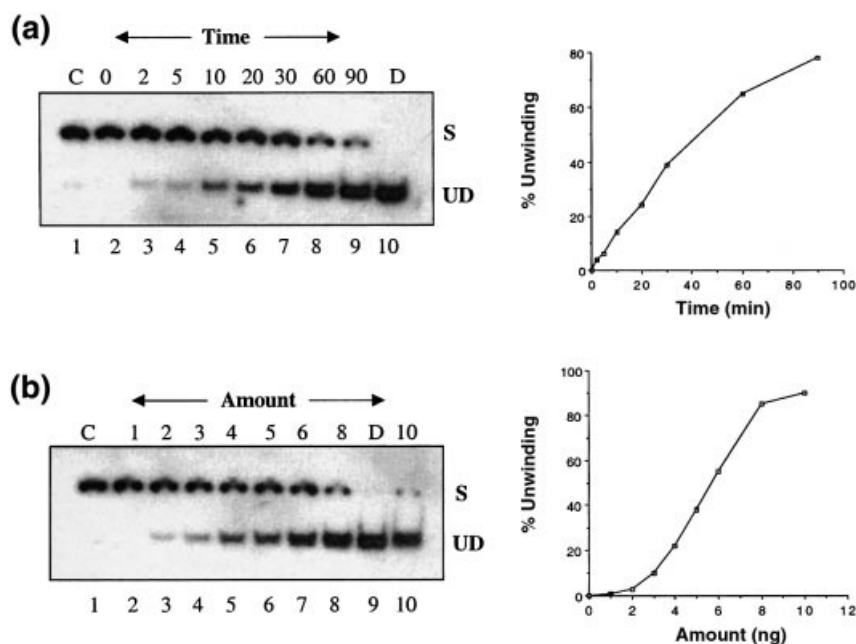
In vitro phosphorylation of PDH65 and HDH I by CK2 and cdc2 protein kinases

We have previously shown (Tuteja *et al.*, 1995; Tuteja and Tuteja, 1998) that human DNA helicase IV (HDH IV, identical to nucleolin) is a substrate for CK2 and cdc2 protein kinases. Here we have used HDH IV as a positive control and BSA as a negative control for both kinases. The *in vitro* phosphorylation is based on incubating proteins with $[\gamma\text{-}^{32}\text{P}]$ ATP in presence of CK2 or cdc2 kinase. After incubation, the phosphorylation of the proteins was

Table 1. Differences between pea nuclear DNA helicase I (PDH45) and II (PDH65)

Property	PDH45*	PDH65
Molecular mass (kDa, on SDS-PAGE)	45.5	65
Behaviour on ssDNA-column chromatography	Eluted at 0.2 M salt	Eluted at 0.4 M salt
Optimum concentration:		
ATP (mM)	0.6	3.0
MgCl ₂ (mM)	0.6	3.0
KCl (mM)	150	10.0
Divalent cation requirements	Mg ²⁺ > Mn ²⁺ >> Ca ²⁺	Mg ²⁺ > Mn ²⁺ > Ca ²⁺
Nucleotide requirements	ATP > dATP > dCTP > CTP, GTP > UTP > dTTP	ATP > dATP
Inhibition by:	Yes	No
(NH ₄) ₂ SO ₄ (45 mM)		
M13RFI DNA (30 mM as P)	No	Yes
Pea leaves total RNA (30 mM as P)	No	Yes
Unwinding of longer duplexes (>17 bp)	No	Yes
Enzyme concentration curve	Not sigmoidal	Sigmoidal
Homologous to human DNA helicase I	No	Yes
Homologous to tobacco eIF-4A	Yes	No
Stimulation of topoisomerase I	Yes	No
<i>In vitro</i> translation inhibition by the antibodies	Yes	No
Substrate for CK2 protein kinase	No	Yes
Substrate for cdc2 protein kinase	No	Yes
Localization	Nucleus and cytosol	Nucleolus

* Pea DNA helicase of 45.5 kDa, Pham *et al.* (2000). P = phosphate.

**Figure 3.** Kinetics (a) and concentration dependence (b) of PDH65.

Positions of annealed DNA substrate (S) and unwound DNA (UD) are indicated. Autoradiogram shown on left; quantitative data plotted as graph on the right.

(a) Standard reaction carried out with 6 ng of purified PDH65 for the times indicated. (b) Increasing amounts (indicated at the top of each lane) of PDH65 used in the standard 30 min reaction. Lanes C and D are control reactions without enzyme and using heat-denatured substrate, respectively. The structure of DNA helicase substrate used for the helicase assay is shown in Figure 2(a).

examined by gel electrophoresis. As shown in Figure 6(a,b), both the 65 kDa HDH I (lane 1) and 65 kDa PDH65 (lane 2) were phosphorylated with CK2 and cdc2 protein kinases. A 100 kDa radiolabelled band of HDH IV was also seen in both gels (Figure 6a,b, lanes 3) as a positive control

of CK2 and cdc2 kinases. These kinases did not phosphorylate either BSA (Figure 6a,b, lanes 4) or pea chloroplast DNA helicase (Tuteja *et al.*, 1996; data not shown).

The effect of phosphorylation of PDH65 by these kinases was also examined. Both DNA and RNA helicase activities

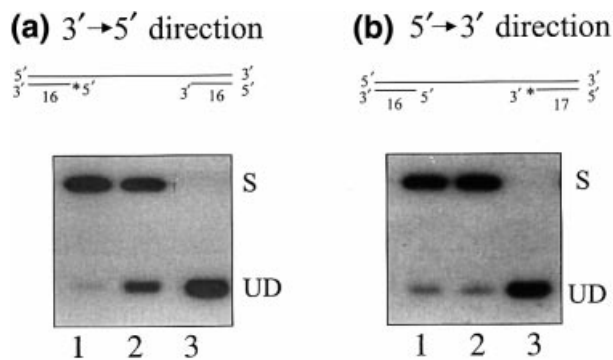


Figure 4. Direction of unwinding by PDH65. Structure of the linear substrate for 3' to 5' direction (a) and 5' to 3' direction (b) is shown on top of the autoradiograms of the gels. In each gel: lane 1, reaction without enzyme; lane 2, reaction with 6 ng purified PDH65; lane 3, is the heat-denatured substrate. S, position of substrate; UD, position of unwound DNA.

of PDH65 were stimulated after phosphorylation by either CK2 (Figure 6c,d; lane 3) or cdc 2 (Figure 6c,d, lane 4) as compared to untreated PDH65 (Figure 6c,d, lane 1). Similarly, the ATPase activity of PDH65 was also stimulated (data not shown).

Immunofluorescence labelling of BrUTP incorporation and localization of pea PDH65

The localization of PDH65 was analysed by immunofluorescence labelling followed by confocal microscopy, using vibratome sections of the terminal 3–5 mm of pea roots (Thompson *et al.*, 1997). Immunofluorescence labelling with anti-HDH I antibodies showed very clear and specific labelling of the nucleoli in all cells. The pattern of labelling was very similar to that previously obtained for antigens which are located in the dense fibrillar component of the nucleolus (Shaw *et al.*, 1995). To confirm this, double labelling of transcription sites using BrUTP incorporation was used (Thompson *et al.*, 1997). Images from these experiments are shown in Figure 7(a,b). This confirms that the helicase labelling substantially overlaps the BrUTP labelling, although it is different in detail. The labelling with anti-HDH I is rather more diffuse than that of BrUTP, suggesting that it surrounds and includes the BrUTP-labelled transcription sites, rather than being restricted to the transcription sites. The nucleolar region labelled by anti-HDH I corresponds broadly to the dense fibrillar component of the nucleolus.

Discussion

Helicases play an important role by providing duplex unwinding functions that may be central to the control of cell proliferation, and thus to plant growth and develop-

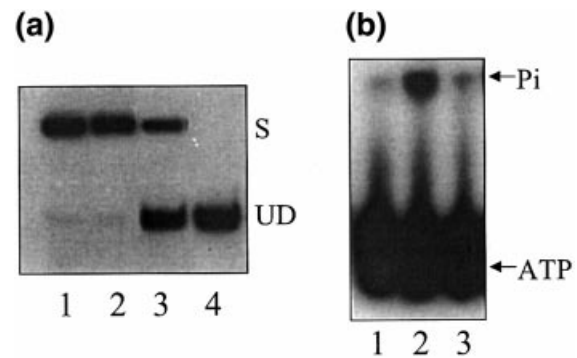


Figure 5. Immunodepletion of DNA helicase and DNA-dependent ATPase activities of PDH65.

(a) Immunodepletion of DNA helicase activity. Lane 1, control without enzyme; lane 2, PDH65 pretreated with anti-HDH I IgG; lane 3, PDH65 pretreated with pre-immune IgG; lane 4, heat-denature substrate. S, position of substrate; UD, position of unwound DNA.

(b) Immunodepletion of ATPase activity. Lane 1, control without enzyme; lane 2, PDH65 pretreated with pre-immune IgG; lane 3, PDH65 pretreated with anti-HDH I IgG. Structure of DNA helicase substrate used for the helicase assay is shown in Figure 2(s).

ment. In this study we have purified and characterized the pea homologue of a human helicase, HDH I, and have shown that it is located at and around the rDNA transcription sites within the nucleolus. It is therefore likely that this helicase is involved in rDNA transcription or the early stages of pre-rRNA processing. A detailed study of this and similar helicases may help unravel the mechanisms of rDNA transcription, rRNA processing and replication, processes which are as yet poorly understood.

Using an HDH I antibody affinity column we purified a 65 kDa protein (PDH65) from pea nuclei to apparent homogeneity. The purified protein was recognized by anti-HDH I antibodies which confirmed this protein as a pea homologue of HDH I. As is the case with HDH I (Tuteja *et al.*, 1990), PDH65 contained DNA unwinding activity in the 3' to 5' direction which was completely Mg^{2+} - and ATP-dependent. A 3' to 5' unwinding activity was also reported for pea chloroplast DNA helicases (Tuteja and Phan, 1998a; Tuteja *et al.*, 1996); SV-40 large T antigen helicase (Stahl and Knippers, 1987); HDH I, II, III, V and VI (Tuteja and Tuteja, 1996); and NDH I and II from calf thymus (Zhang and Grosse, 1991). PDH65 required ATP or dATP as cofactor although dATP was less active, which again is similar to HDH I (Tuteja *et al.*, 1990) and pea chloroplast DNA helicase I (Tuteja *et al.*, 1996). As the non-hydrolysable analogue of ATP ($ATP\gamma S$) could not replace ATP, ATP hydrolysis is clearly required for the DNA unwinding activity of PDH65. The ATPase activity of PDH65 was also completely Mg^{2+} - and ATP-dependent.

The DNA unwinding reaction rate of PDH65 at limiting enzyme concentration showed a sigmoidal curve similar to HDH I (Tuteja *et al.*, 1990). This behaviour suggests cooperation in the enzyme reaction, which is probably due to

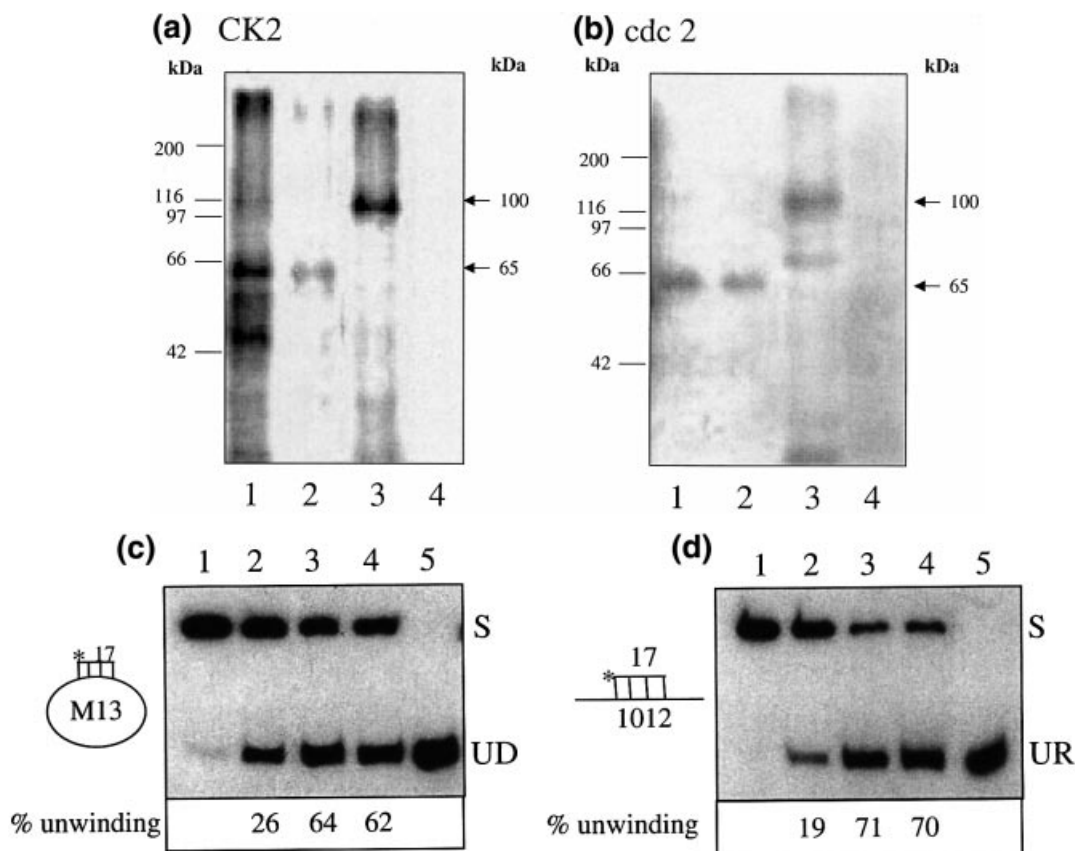


Figure 6. Phosphorylation and stimulation of DNA and RNA helicase activities of PDH65 by CK2 and CDC2 protein kinases. *In vitro* phosphorylation of PDH65 and HDH I with CK2 (a) and cdc2 (b) protein kinases. Lane 1, HDH I; lane 2, PDH65; lane 3, 100 kDa HDH IV as a positive control; lane 4, BSA as a negative control. (c) DNA helicase and (d) RNA helicase activities of PDH65 after phosphorylation with CK2 and cdc2 protein kinases. Lane 1, control without PDH65; lane 2, untreated PDH65 (6 ng); lane 3, PDH65 (6 ng) pretreated with CK2; lane 4, PDH65 (6 ng) pretreated with cdc2; lane 5, heat-denatured substrate. Percentage unwinding in lanes 2–4 of both panels (c,d) are shown at the bottom of the gel. Structure of substrate used is on the left of each gel. S, substrate; UD, unwound DNA; UR, unwound RNA.

interactions between enzyme molecules or subunits. Many helicases have been shown to be homo- or heterodimers, or multimers (Borowiec, 1996; Kornberg and Baker, 1991; Matson *et al.*, 1994).

Many DNA intercalating ligands have been shown to modulate DNA metabolism by inhibiting the enzymes that interact with it (Tuteja and Phan, 1998b). We found that the major groove intercalators, nogalamycin and daunorubicin, were inhibitory to both the DNA helicase and ATPase activities of PDH65, suggesting that the binding of these inhibitors to DNA generated a complex that impeded the translocation of the helicase. These ligands were previously also reported to be inhibitory for the DNA helicase and ATPase activities of pea chloroplast DNA helicase and I and II (Tuteja and Phan, 1998a; Tuteja and Phan, 1998b).

Anti-HDH I antibodies removed the DNA and RNA helicase and ATPase activities of the PDH65, which

shows that HDH I and PDH65 are very similar proteins and might have the same function in both human and plant cells. It is interesting that PDH65 and HDH I both also show RNA helicase activity, which is not a common property of DNA helicases. Among the few examples of DNA helicases with RNA helicase activity are the SV-40 T antigen (Borowiec, 1996) and HDH IV, which has been shown to be identical to nucleolin (Tuteja *et al.*, 1995). This property of PDH65 suggests that it may be involved in both rDNA transcription and rRNA processing.

Casein kinase 2 (CK2) and cdc2 are serine/threonine protein kinases which are known to be involved in gene expression, the regulation of cell growth and proliferation, and the regulation of the cell cycle in eukaryotes (Allen, 1992; Norbury and Nurse, 1989; Pinna, 1990). Very few proteins are known to be substrates for both CK2 and cdc2 protein kinases. Here we show that PDH65 and HDH I are substrates for both CK2 and cdc2 protein kinases, and that

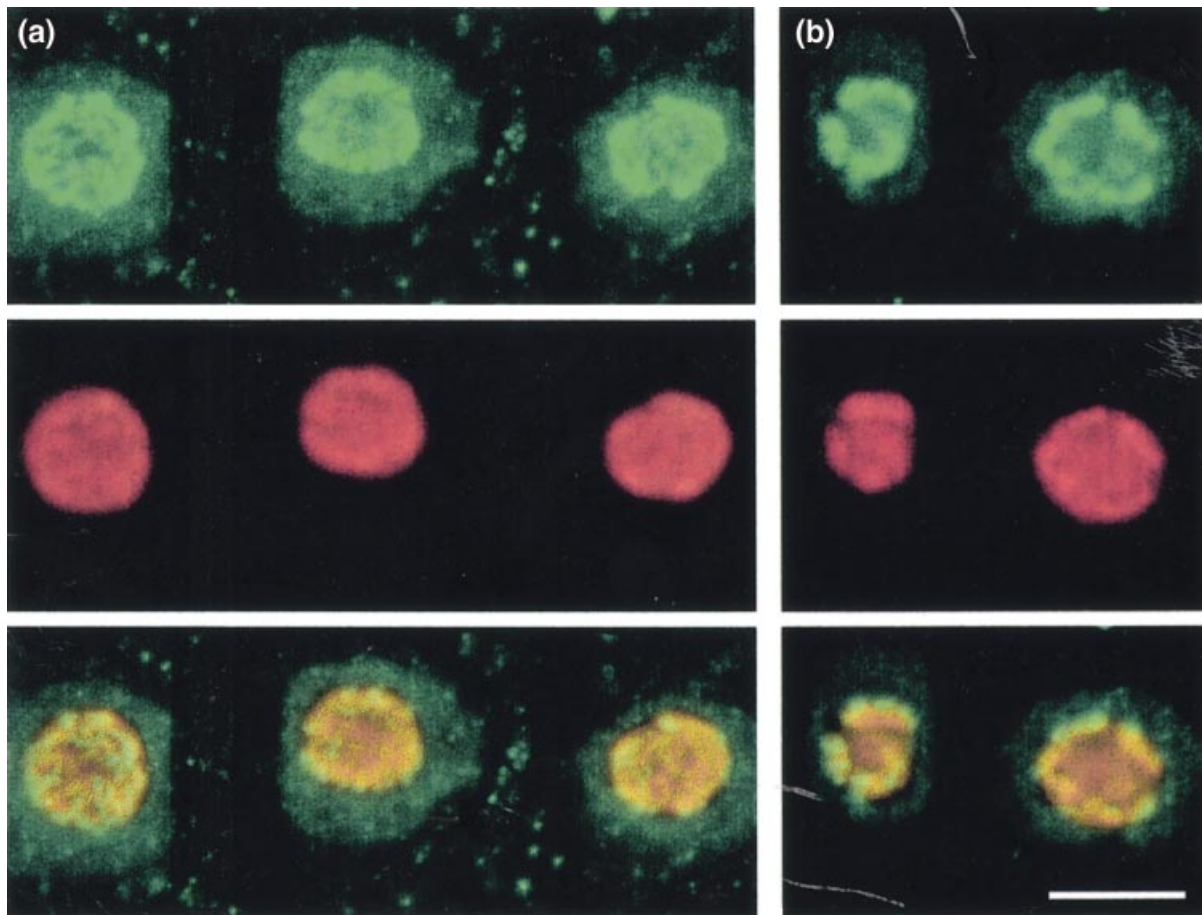


Figure 7. Immunofluorescence and confocal microscopy.

(a) Projection of the entire stack of confocal images through a group of three nucleoli.

(b) A single confocal section from another series of confocal images. In each case the results from specimens labelled with both BrUTP, for transcription sites (green) and anti-HDH I (red) are shown. BrUTP labelling is restricted to many punctate sites in the nucleolus (and fainter sites in the nucleoplasm). Anti-HDH I labelling is restricted to the nucleolus and shows a broadly similar distribution, but detailed examination shows that it is more diffuse and surrounds the BrUTP sites. Bar = 10 μm .

the helicase activity is markedly increased after phosphorylation of PDH65 by both these kinases. This clearly suggests that these phosphorylations play an important role in regulating the enzyme activity of PDH65. It has previously been shown that nucleolin is phosphorylated by CK2 (Belenguer *et al.*, 1989) and cdc2 (Belenguer *et al.*, 1990), and that phosphorylation of nucleolin by CK2 was related to the control of rDNA transcription (Belenguer *et al.*, 1990). The unwinding activity of HDH IV has also been shown to be stimulated by phosphorylation with CK2 and cdc2 protein kinases (Tuteja *et al.*, 1995).

PDH65 is the second DNA helicase that has been purified from pea nuclei, and differs from the first reported pea DNA helicase, PDH45 (Pham *et al.*, 2000). Specific antisera against either of these two helicases do not cross-react with the other helicase, indicating that they recognize different proteins. Further differences between PDH65 and PDH45 are detailed in Table 1, confirming that these two

are different proteins. Helicases are involved in various cell functions, and most organisms have been reported to contain several different helicases. For example, at least 13 different helicases have been reported in *Escherichia coli* (Lohman and Bjornson, 1996; Matson *et al.*, 1994); nine in human cells (Tuteja and Tuteja, 1996); and two in pea chloroplasts (Tuteja, 2000).

The most striking finding of this study was the localization of PDH65 in the dense fibrillar component of the nucleolus, surrounding the transcription sites. BrUTP incorporation showed a bright, punctate distribution within the dense fibrillar component of the nucleolus. The anti-HDH I antibody labelling was observed around the transcription sites, but was not restricted to them. It occupied a subregion of the dense fibrillar component of the nucleolus, and probably included the initial transcription sites and the region of early transcript processing. These observations suggest that PDH65 may be involved

both in the regulation of rDNA transcription, and in the early events of pre-rRNA processing.

Experimental procedures

Materials

Seeds of *Pisum sativum* L. were imbibed in aerated water for 12 h, then germinated at 18°C for 2 or 7–8 days. M13ss and dsDNA and total RNA from pea leaves were prepared using standard methods. NTPs and ATP γ S were obtained from Boehringer-Mannheim, and [γ -³²P] ATP and [α -³²P]dCTP were purchased from Amersham. The RNA and DNA oligonucleotides were synthesized chemically and purified electrophoretically. Daunorubicin and nogalamycin were purchased from Topogene (Columbus, OH, USA) and Sigma, St. Louis, MO, USA, respectively. The composition of buffer A is as follows: 50 mM Tris–Cl pH 8.0, 50 mM KCl, 1 mM DTT, 1 mM EDTA, 1 mM PMSF, 1 mM sodium metabisulphite, 1 μ M pepstatin, 1 μ M leupeptin, 1 mg ml⁻¹ benzamidine and 10% glycerol.

Preparation of nuclear extract

Pea nuclear extracts were prepared from 700 g pea leaves as described below. Pea seedlings 7–8 days old were harvested in cold buffer containing 0.55 M sucrose, 50 mM Tris–Cl pH 8.0 and 10 mM MgCl₂ (STM buffer). The tissue was washed once and ground in STM buffer. Thereafter the homogenate was passed through two layers of cheesecloth and two layers of Miracloth (Cal Biochem, San Diego, CA, USA). The filtrate was then centrifuged at 1000 *g* for 10 min at 4°C in a Sorvall RC 5B centrifuge. The pellet was slowly resuspended in STM containing 2.5% Triton \times 100, and incubated at 4°C with slow shaking followed by centrifugation at 2000 *g* for 30 min at 4°C. The resulting nuclear pellet was then resuspended in a buffer containing 600 mM KCl, 50 mM Tris–Cl pH 7.9, 1.5 mM MgCl₂, 0.2 mM EDTA, 0.5 mM DTT, 25% glycerol, 0.5 mM PMSF, 50 μ M leupeptin and 1 mM pepstatin, and homogenized in a Potterelvehjham homogenizer. Then the homogenate was spun at 12 000 *g* for 30 min at 4°C, and the clear supernatant (nuclear extract) was dialysed against buffer containing 50 mM KCl, 50 mM Tris–Cl pH 8, 20% glycerol and protease inhibitors, and stored at –80°C.

Preparation of DNA and RNA helicase substrates

The DNA helicase substrate used in the DNA unwinding assay was prepared as described (Tuteja *et al.*, 1990). It consisted of a ³²P-labelled 17-mer DNA oligonucleotide (5'-GTTTTCCAGTCAC-GAC-3') annealed to M13mp19 phage ssDNA. For direction specific substrates a DNA oligonucleotide 32-mer (5'-TTCGAG-CTCGGTACCCGGGATCCTCTAGAGT-3') was annealed with M13mp19 phage ssDNA, and prepared as described (Tuteja *et al.*, 1990; Tuteja *et al.*, 1996). The RNA duplex substrate for the RNA unwinding assay was prepared as described (Tuteja *et al.*, 1995). It consisted of a ³²P-labelled 17-mer RNA oligonucleotide (5'-CUGGCCAAUAUGGCAU-3') annealed to 1 kb RNA.

DNA unwinding assay

The DNA helicase assay measures the displacement of a labelled DNA oligonucleotide from a partial duplex DNA helicase substrate, catalysed by the helicase. The reaction was performed in a 10 μ l reaction mixture consisting of 20 mM Tris–HCl pH 8.0, 3.0 mM ATP, 3.0 mM MgCl₂, 10 mM KCl or NaCl, 8 mM DTT, 4% (w/v) sucrose, 80 μ g ml⁻¹ BSA, \approx 1 ng ³²P-labelled 17 mer–M13 annealed substrate (\approx 1000–2000 cpm) and the helicase fraction. The reaction mixture was incubated for 30 min at 37°C and the reaction was stopped, analysed and quantified as previously described (Tuteja and Phan, 1998a; Tuteja *et al.*, 1990).

RNA unwinding assay

The reaction was performed in the presence of ³²P-labelled RNA duplex substrate and 1 unit of RNase block, as described (Tuteja *et al.*, 1995).

DNA-dependent ATP hydrolysis assay

The ATP hydrolysis catalysed by PDH65 was assayed by measuring the formation of radiolabelled Pi from [γ -³²P]ATP. The reaction conditions were identical to those described above for the helicase reaction, except that the ³²P-labelled helicase substrate was replaced by a mixture of 1665 Bq [γ -³²P] ATP and cold ATP (1 mM). The reaction was performed for 2 h at 37°C and analysed as described (Tuteja *et al.*, 1996).

Preparation of anti-HDH I antibodies and Western blotting

Human DNA helicase I (HDH I) was purified as previously described (Tuteja *et al.*, 1990). The polyclonal antibodies against 65 kDa HDH I were raised in rabbit and purified as IgG fractions using protein A–Sepharose as described (Harlow and Lane, 1988). Western blotting was performed by a standard method (Harlow and Lane, 1988) using alkaline phosphatase-conjugated goat anti-rabbit IgG H+I (Bio Rad, Hercules, CA, USA) as a secondary antibody.

Preparation of antibody-affinity column

The purified anti-HDH I IgG was coupled to cyanogen bromide-activated Sepharose-4B (Pharmacia, Uppsala, Sweden) according to the manufacturer's instructions.

Immunodepletion of pea DNA helicase and ATPase activities of PDH65

Aliquots (20 μ l) of immunoaffinity-purified pea helicase fraction were incubated separately at 4°C for 16 h with a 10-fold excess of purified anti-HDH I IgG and rabbits' pre-immune IgG (control). Immunodepletion was performed by adding 10 μ l protein A–Sepharose (Pharmacia) beads equilibrated in 100 mM Tris–HCl (pH 7.5) buffer. After 4 h at 4°C, the beads were removed by centrifugation and supernatants were assayed for DNA helicase and ATPase activities.

Phosphorylation by CK2 and cdc2 protein kinases

Phosphorylation of PDH65 and HDH I was performed with animal CK2 and cdc2 protein kinases (kindly provided by Dr Lorenzo Pinna, Padova, Italy and Dr Sandor Pongor, Trieste, Italy, respectively) as described by Tuteja *et al.* (1995). Human DNA helicase IV (HDH IV) was used as a positive control and BSA as a negative control. The PDH65 was pretreated with CK2 and cdc2 protein kinases as described (Tuteja *et al.*, 1995).

BrUTP incorporation, in situ labelling and confocal microscopy

The terminal 3–5 mm of pea radicle was excised and used to cut 30–40 µm vibratome sections, and treated as described (Thompson *et al.*, 1997). BrUTP incorporation into the nuclei in tissue sections was performed as described (Thompson *et al.*, 1997). After the transcription reaction the sections were washed, fixed in 4% formaldehyde and permeabilized by cellulase. BrUTP incorporation was detected using anti-BrdU antibody and PDH65 was detected by labelling using anti-HDH I antibodies, in each case followed by either cy3- or fluorescein-labelled secondary antibodies as described previously (Shaw *et al.*, 1995; Thompson *et al.*, 1997). Confocal optical section stacks were collected using a BioRad MRC-1000 UV confocal microscope. Double-labelled images were collected sequentially so that maximum discrimination between the two fluorescent probes was obtained. In control experiments one or other secondary antibodies was omitted; no significant cross-talk was present between the two fluorescent channels. In other control experiments, BrUTP or one or other primary antibody was omitted, and this also abolished the relevant fluorescent labelling. Images were analysed as described (Shaw *et al.*, 1995; Thompson *et al.*, 1997) using NIH-IMAGE, an image-processing package written by Wayne Rasband, and ADOBE PHOTOSHOP.

Acknowledgements

We thank Drs Lorenzo Pinna (Padova, Italy) and Sandor Pongor (Trieste, Italy) for the kind gift of CK2 and cdc2 protein kinases, respectively, and Professor A. Falaschi (Trieste, Italy) for critically reading the manuscript. We also thank Dr Dinesh Gupta for his help in preparation of illustrations and Ms R. Radha for secretarial assistance. This work was partly supported by the Biotechnology and Biological Sciences Research Council of the UK (P.J.S. and A.F.B.).

References

- Allen, J.F. (1992) How does protein phosphorylation regulate photosynthesis? *Trends Biochem. Sci.* **17**, 12–17.
- Belenguier, P., Baldin, V., Mathieu, C., Prats, H., Bensaid, M., Bouche, G. and Amalric, F. (1989) Protein kinase NIII and the regulation of rDNA transcription in mammalian cells. *Nucl. Acids Res.* **17**, 6625–6635.
- Belenguier, P., Caizergues-Ferrer, M., Labbe, J.-C., Doree, M. and Amalric, F. (1990) Mitosis-specific phosphorylation of nucleolin by p34 cdc2 protein kinase. *Mol. Cell. Biol.* **10**, 3607–3618.
- Boroweic, J.A. (1996) DNA helicases. In *DNA Replication in*

- Eukaryotic Cells* (Melvin, L. and De Pamphilis, eds). Cold Spring Harbor: Cold Spring Harbor Laboratory Press, pp. 545–574.
- Harlow, E. and Lane, D. (1988). *Antibodies: A Laboratory Manual*. Cold Spring Harbor: Cold Spring Harbor Laboratory Press.
- Kornberg, A. and Baker, T.A. (1991) DNA helicases. In *DNA Replication*, 2nd edn. New York: W.H. Freeman, pp. 355–378.
- Lohman, T.M. and Bjornson, K.P. (1996) Mechanism of helicase-catalyzed DNA unwinding. *Ann. Rev. Biochem.* **65**, 169–124.
- Matson, S.W., Bean, D. and George, J.W. (1994) DNA helicases: enzymes with essential roles in all aspects of DNA metabolism. *Bioessays*, **16**, 13–21.
- Norbury, C.J. and Nurse, P. (1989) Control of the higher eukaryote cell cycle by p34 cdc2 homologues. *Biochem. Biophys. Acta*, **989**, 85–95.
- Pham, X.H., Reddy, M.K., Ehtesham, N.Z., Matta, B. and Tuteja, N. (2000) A DNA helicase from *Pisum sativum* is homologous to translation initiation factor and stimulates topoisomerase I activity. *Plant J.* **24**, 219–230.
- Pinna, L.A. (1990) Casein kinase 2: an 'eminence grise' in cellular regulation. *Biochem. Biophys. Acta*, **1054**, 267–284.
- Shaw, P.J., Highett, M.I., Beven, A.F. and Jordan, E.G. (1995) The nucleolar architecture of polymerase I transcription and processing. *EMBO J.* **14**, 2896–2906.
- Stahl, H. and Knippwers, R. (1987) The simian virus 40 large tumor antigen. *Biochem. Biophys. Acta*, **910**, 1–10.
- Thommes, P. and Hubscher, U. (1992) Eukaryotic DNA helicases: essential enzymes for DNA transaction. *Chromosoma*, **101**, 467–473.
- Thompson, W.F., Beven, A.F., Wells, B. and Shaw, P.J. (1997) Sites of rDNA transcription are widely dispersed through the nucleolus in *Pisum sativum* and can comprise single genes. *Plant J.* **12**, 571–581.
- Tuteja, N. (1997) Unraveling DNA helicases from plant cells. *Plant Mol. Biol.* **33**, 947–952.
- Tuteja, N. (2000) Plant cell and viral helicases: essential enzymes for nucleic acid transactions. *Crit. Rev. Plant Sci.* **19**, 449–478.
- Tuteja, N. and Phan, T.N. (1998a) A chloroplast DNA helicase II from pea that prefers fork-like structures. *Plant Physiol.* **118**, 1029–1039.
- Tuteja, N. and Phan, T.N. (1998b) Inhibition of pea chloroplast DNA helicase unwinding and ATPase activities by DNA-interacting ligands. *Biochem. Biophys. Res. Commun.* **244**, 861–867.
- Tuteja, N. and Tuteja, R. (1996) DNA helicases: the long unwinding road. *Nature Genet.* **13**, 11–12.
- Tuteja, R. and Tuteja, N. (1998) Nucleolin: a multifunctional major nucleolar phosphoprotein. *Crit. Rev. Biochem. Mol. Biol.* **33**, 407–436.
- Tuteja, N., Tuteja, R., Rahman, K., Kang, L.-Y. and Falaschi, A. (1990) A DNA helicase from human cells. *Nucl. Acids Res.* **18**, 678–6792.
- Tuteja, N., Huang, N.W., Skopac, D. *et al.* (1995) Human DNA helicase IV is nucleolin, an RNA helicase modulated by phosphorylation. *Gene*, **160**, 143–148.
- Tuteja, N., Phan, T.N. and Tewari, K.K. (1996) Purification and characterization of a DNA helicase from pea chloroplasts that translocates in the 3' to 5' direction. *Eur. J. Biochem.* **238**, 54–63.
- Zhang, S. and Grosse, F. (1991) Purification and characterization of two DNA helicases from calf thymus nuclei. *J. Biol. Chem.* **266**, 20483–20490.