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Characterization of topoisomerase I and II activities in nuclear extracts during callogenesis in immature embryos of *Zea mays*

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Abstract

We have characterized the topoisomerase I and II activities in nuclear extracts from immature embryos of *Zea mays* and the effect of the treatment with 2,4-dichlorophenoxyacetic acid (2,4-D) and abscisic acid (ABA). These extracts were shown to be essentially devoid of protease and nuclease activities and they were tested for their ability to relax supercoiled DNA, unknotting P4 DNA and catenate circular duplex DNA under catalytic conditions. Unknotting and catenation reactions are strictly magnesium- and ATP-dependent, but not the relaxation of circular supercoiled DNA allowing the detection of both topoisomerase I and II activities. Two cytotoxic drugs, camptothecin, a plant alkaloid that inhibits eukaryotic topoisomerase I, and epipodophyllotoxin VM-26 (teniposide) that inhibits topoisomerase II, have been assayed in our extracts showing similar inhibitory effects on topoisomerase enzymes. Alkaline phosphatase treatment of nuclear extracts abolishes both topoisomerase activities. Nuclear extracts from embryos treated with 2,4-D showed 200% increase on topoisomerase II activity as compared with untreated ones, but only residual activity was detected in ABA-treated embryos. Nuclear extracts from hormone-treated and untreated embryos showed similar topoisomerase I activity with deviations of less than 25%. These differences are discussed in terms of possible post-translational modifications of the enzymes associated with the increase in proliferation activity of calli.

Introduction

Regeneration of intact plants from *in vitro* cultures is a prerequisite in many plant transformation methodologies. Cereals are in general a difficult material for *in vitro* manipulations. Calli from these species have been obtained using explants from immature organs such as embryos, leaves and inflorescences which contain undifferentiated cells (reviewed in [32]). Thus, treatment of *Zea mays* immature embryos with the synthetic auxin 2,4-dichlorophenoxyacetic acid (2,4-D) at the appropriate concentration is necessary for the induction of callus and the regeneration of the plants takes place when the hormone is removed from the culture medium [14, 29, 31]. *In vitro* treatment of *Zea mays* immature embryos with abscisic acid (ABA) abolishes its normal germination and embryos are led to dormancy. In these no cell proliferation is observed for long periods of time and when ABA is removed from culture medium normal germination takes place.

The molecular mechanisms mediated by the auxin that lead to undifferentiated plant cell proliferation giving rise to callus structure, or the effect of abscisic acid on embryo dormancy rather than normal embryo germination are unknown, but it seems reasonable to suppose that in these divergent developmental processes, important changes in nuclear function occur.

DNA topoisomerases are enzymes that control different biological processes in prokaryotic and eukaryotic cells related to nucleic acid function such as DNA replication, gene transcription, nuclear matrix organization, chromosome structure and segregation (for review see [33]). Two main types of DNA topoisomerases (topoisomerase I and topoisomerase II) have been isolated from prokaryotic and eukaryotic cells and extensive studies on their *in vitro* catalytic reactions have been reported [33]. Thus eukaryotic topoisomerase I catalyzes the relaxation of circular supercoiled DNA in an ATP-independent manner by making a transient single-strand break. Mammalian topoisomerase II catalyzes the passage of one duplex DNA segment through another one via a transient double-strand break. The enzymatic activity of topoisomerase II requires divalent cations (Mg^{2+}) and an energetic cofactor such as ATP or ADP. It has been reported that eukaryotic DNA topoisomerases may exist *in vivo* as phosphoproteins [2, 9, 27]. Some protein kinases are able to *in vitro* phosphorylate both DNA topoisomerases with high efficiency increasing significantly their catalytic activity [1, 8, 28]. This suggests that post-translational modifications may play an important role in the control of eukaryotic DNA topoisomerases.

Little is known about DNA topoisomerases in plants. The catalytic activity of topoisomerase I has been reported in different plant systems such as wheat embryo mitochondria [11], carrot and spinach cells [30], pea chloroplast [19, 24] and the enzyme has been isolated from wheat germ [10]. Topoisomerase II activity has been reported in cauliflower inflorescences [13] and gyrase activity has been found in extracts from pea chloroplasts [19]. Here we report the characterization of topoisomerase activity in maize embryonic nuclear extracts. We have used the different reaction requirements for both types of DNA topoisomerases to study in the same nuclear extracts topoisomerase I and topoisomerase II activities. The variation of these activities upon treatment with two hormones that produce effects on cell proliferation (2,4-D and ABA) has also been studied.

Material and methods

Nucleic acids and topoisomerase inhibitor drugs

Negatively supercoiled duplex circular DNA from plasmid pBR322 was prepared by alkaline lysis of bacterial cultures followed by double banding in cesium chloride-ethidium bromide gradient [21]. Knotted DNA from bacteriophage P4 was a gift of Drs. J. Roca and J.R. Lecea (Dept. of Physiology, Faculty of Medicine, University of Barcelona). Histone H1 from calf thymus was obtained as described [4]. The auxin analogous 2,4-dichlorophenoxyacetic acid (2,4-D), abscisic acid and camptothecin were purchased from Sigma. Epipodophyllotoxin VM-26 was from Bristol-Myers.

Embryo culture and nuclear extract preparations

Immature embryos (W64A, pure inbred line), 13 days after pollination were cultivated in Petri dishes (for 48 h) with 20 ml of modified Murashige and Skoog (MS) medium, [31] solidified with Gelrite and 2 mg/l of 2,4-dichlorophenoxyacetic acid (2,4-D), or 10 μM of abscisic acid (ABA) in treated embryos. Embryo size was 2.5–3 mm in length at the time of culture. They were maintained in a controlled room at 26–28 °C and with a photoperiod of 16 h light, 8 h dark (light intensity of 800 lux).

All the steps for protein extraction were carried out at 0–4 °C unless otherwise stated. Immature embryos removed from the MS solid medium were suspended in 50 ml of homogenization buffer (50 mM Tris-HCl pH 7.9, 10 mM KCl,

5 mM $MgCl_2$, 5 mM 2-mercaptoethanol, 1 mM PMSF (phenylmethylsulfonyl fluoride)) and homogenized with a Dounce homogenizer by ten passes with a loose pestle of 76–152 μm clearance. The resultant homogenate was passed through three gauze layers. After filtration, nuclei were collected by centrifugation at 1500 × *g* for 10 min. Two additional washes were made with homogenization buffer containing 0.25 M sucrose. Light microscopy controls of nuclei were made in different steps.

The nuclear pellet was resuspended in 5 ml of extraction buffer (10 mM Tris-HCl pH 7.9, 0.4 M NaCl, 5 mM 2-mercaptoethanol, 1 mM EDTA, 1 mM PMSF, 20% glycerol), homogenized using a Dounce homogenizer with a loose pestle of 25–76 μm clearance until total nuclear disruption was obtained. The nuclear suspension was slowly stirred for 30 min and the bulk of chromatin was removed by centrifugation at 15000 × *g* for 30 min. Supernatant was carefully removed and dialyzed against 1 liter of 10 mM Tris-HCl, pH 7.9, 50 mM NaCl, 50 mM KCl, 5 mM 2-mercaptoethanol, 1 mM EDTA, 20% glycerol, for 4–6 h with three changes of dialysis buffer. The dialyzed extract was centrifuged at 30000 × *g* for 30 min and the supernatant was removed and used for further analysis.

Nuclear extract analysis

Protein concentration of nuclear extracts was analyzed by the method of Bradford (Bio-Rad) [3], using bovine serum albumin as a standard for the reaction. Protein concentration of different samples was adjusted to 100 μg/ml by dilution with dialysis buffer. Protein gel electrophoresis was carried out on 7.5% polyacrylamide gels as described by Laemmli [17]. Gels were silver-stained according to the method of Morrisey [21]. High molecular weight protein markers were purchased from Sigma. Exo- and endonuclease activity in nuclear extracts was checked by incubating for 3 hours 1 μg of pBR322 DNA digested with *Eco* RI and ^{32}P-labelled using DNA polymerase I, in 100 μl of reaction solution (50 μl nuclear extracts, 50 μl relaxation standard buffer) and analyzed in 1% agarose gel.

Topoisomerase assays

Circular supercoiled pBR322 DNA was incubated in standard relaxation buffer containing 10 mM Tris-HCl pH 7.9, 50 mM KCl, 50 mM NaCl, 5 mM $MgCl_2$, 5 mM 2-mercaptoethanol, 1 mM EDTA, 10% glycerol. Relaxation kinetic reactions were carried out by incubating at 30 °C 2 μg of circular supercoiled pBR322 DNA in 50 μl of reaction solution that contained 25 μl of nuclear extract and 25 μl of standard relaxation buffer made 10 mM $MgCl_2$ and without glycerol. When topoisomerase II relaxation activity was assayed, the reaction mixture also contained 1.5 mM ATP. Aliquots of 10 μl were taken at different intervals of time and placed into Eppendorf tubes containing 2 μl of 2.5% SDS (sodium dodecyl sulfate). One μl of 10 mg/ml proteinase K solution was added and the mixtures were incubated at 37 °C for 30 min. Samples were made 5% sucrose, 0.01% xylene cyanol and 0.01% bromophenol blue and analyzed in 1% agarose slab gels.

Standard topoisomerase II reaction buffer contained 10 mM Tris-HCl, pH 7.9, 50 mM NaCl, 50 mM KCl, 5 mM $MgCl_2$, 5 mM 2-mercaptoethanol, 1.5 mM ATP, 0.5 mM EDTA, 10% glycerol. Catenation reaction was carried out by incubating 0.5 μg of circular supercoiled pBR322 DNA at 30 °C for 60 min in 50 μl of reaction solution containing 25 μl of embryo nuclear extract solution, 25 μl of standard topoisomerase II reaction buffer made 10 mM $MgCl_2$, 3 mM ATP without glycerol and varying quantities of calf thymus histone H1. Reactions were stopped as described before and the DNA samples were analyzed in 1% agarose slab gels. Unknotting reactions were obtained by incubation of 2 μg of knotted P4 DNA at 30 °C in 50 μl of standard topoisomerase II reaction buffer as described for the catenation reaction, except that histone H1 was omitted. Aliquots of 10 μl were taken at different intervals of time and the reaction was stopped as in previous experiments.

Agarose gel electrophoresis

Standard electrophoresis was carried out at 2 V/cm in 1% or 0.6% (for unknotting reactions) agarose slab gels immersed in 90 mM Tris-borate, 2 mM EDTA pH 8.3 buffer. After the run, gels were stained in aqueous solution of 1 μg/ml ethidium bromide for 30 min. DNA bands were visualized with UV light and photographed with a Polaroid camera. Negative films were scanned in a Chromoscan 3 microdensitometer (Joyce-Loebl) and the band integrals were obtained. The amount of supercoiled DNA in the relaxation reactions and the consumption of knotted P4 DNA substrate were quantitated [25].

Alkaline phosphatase treatment

50 μl of embryonic nuclear extracts were mixed with 50 μl of 50 mM Tris-HCl pH 8.0 containing 25 units alkaline phosphatase (Boehringer Mannheim at 25 u/μl in 3 M NaCl, 1 mM $ZnCl_2$, 1 mM $MgCl_2$, 1 mM triethanolamine pH 7.6, storage buffer) and incubated at 30 °C for 20 min. Aliquots of 25 μl were added to Eppendorf tubes that contained the appropriate buffer with 0.5 μg of circular supercoiled DNA or knotted P4 DNA to finally obtain the standard reaction conditions. These topoisomerase reactions were carried out at 30 °C for 60 min. The reactions were stopped and analyzed as described above.

Results

Characterization of nuclear extracts from maize embryos

Immature embryos from *Zea mays* (W64A pure inbred line) obtained 12–15 days after pollination were placed for 48 h in Murashige and Skoog (MS) solid medium [30] in the presence or in the absence of 2 μg/ml of 2,4-dichlorophenoxyacetic acid (2,4-D) or 10 μM abscisic acid (ABA). Nuclear extracts from embryos untreated and treated with 2,4-D or ABA, were obtained in parallel preparations as described in the Material and methods section. The extracts were dialyzed against the same buffer (10 mM Tris-HCl, pH 7.9, 50 mM NaCl, 50 mM KCl, 5 mM 2-mercaptoethanol, 1 mM EDTA, 20% glycerol) and the protein concentration of each preparation was measured by the Bradford method [3]. The samples were diluted with the last dialysis buffer and adjusted to a final concentration of 100 μg/ml, aliquoted and stored frozen at − 70 °C. An additional control on the state of the proteins extracted was made by SDS gel electrophoresis. Figure 1 shows a silver-stained polyacrylamide gel electrophoresis of 0.40 M NaCl-soluble nuclear proteins of both treated and un-

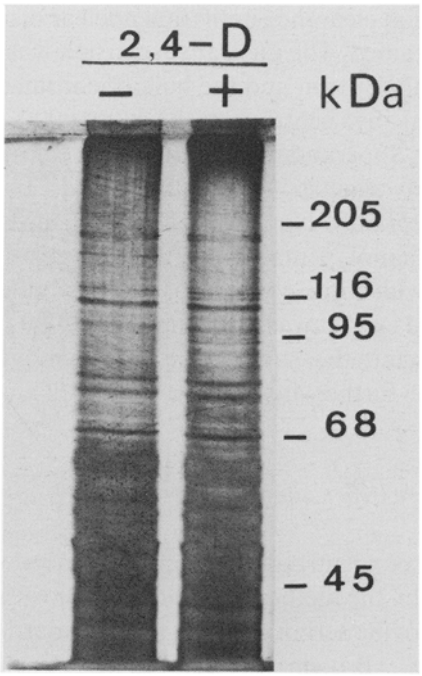

Fig. 1. SDS-polyacrylamide gel electrophoresis of proteins present in maize embryo nuclear extracts.
Nuclear extracts from *Zea mays* immature embryos treated and untreated with 2,4-dichlorophenoxyacetic acid, were obtained in parallel extractions. Protein concentration was measured by the method of Bradford, and adjusted to 100 μg/ml. 25 μl (2.5 μg) of extract solution of both treated (+ 2,4-D) and untreated (− 2,4-D) embryos were loaded on 7.5% SDS-polyacrylamide gel electrophoresis. Protein markers were loaded in the same gel which correspond to myosin (205 kDa), β-galactosidase (116 kDa), phosphorylase (95 kDa), bovine serum albumin (68 kDa) and ovalbumin (45 kDa).

treated immature embryos. It can be seen that the protein quantities are similar in both samples and high molecular weight polypeptides are visible indicating that no major degradative activities are present. The studies described below were made with three different sets of embryos, obtaining results that showed a deviation of less than 10%. ^{32}P-labelled DNA was used to check exo- and endonuclease activities in the extracts which in our assay conditions were not detected after three hours of incubation (result not shown).

DNA topoisomerase I activity in embryo nuclear extracts

To evaluate topoisomerase I activity in nuclear extracts the kinetics of DNA relaxation were measured in the absence of ATP. In these conditions only topoisomerase I can relax supercoiled DNA [33]. Figure 2 shows the gel electrophoresis of the products of the incubation of plasmid pBR322 supercoiled DNA at different times with nuclear extracts from embryos treated and untreated with 2,4-D. The percentage of relaxed DNA was quantitated by scanning densitometry and plotted as a function of time in Figure 2. Most of the supercoiled DNA was relaxed during the linear period and the average in relaxation activity was 25% higher in nuclear extracts from treated (+ 2,4-D) embryos than in untreated ones. When topoisomerase I activity was assayed in nuclear extracts from embryos treated with ABA similar activity to the untreated embryos was found (see Fig. 4A). Assays with different extract preparations give quantitative and reproducible results with a difference of less than 5%. The presence of intermediate DNA circular forms and the absence of linear forms after incubation shows that in our reaction conditions no endonuclease activity is detected.

Characterization of DNA topoisomerase II activity in embryo nuclear extracts

Eukaryotic DNA topoisomerase II catalyzes the relaxation of supercoiled DNA, knotting/un-

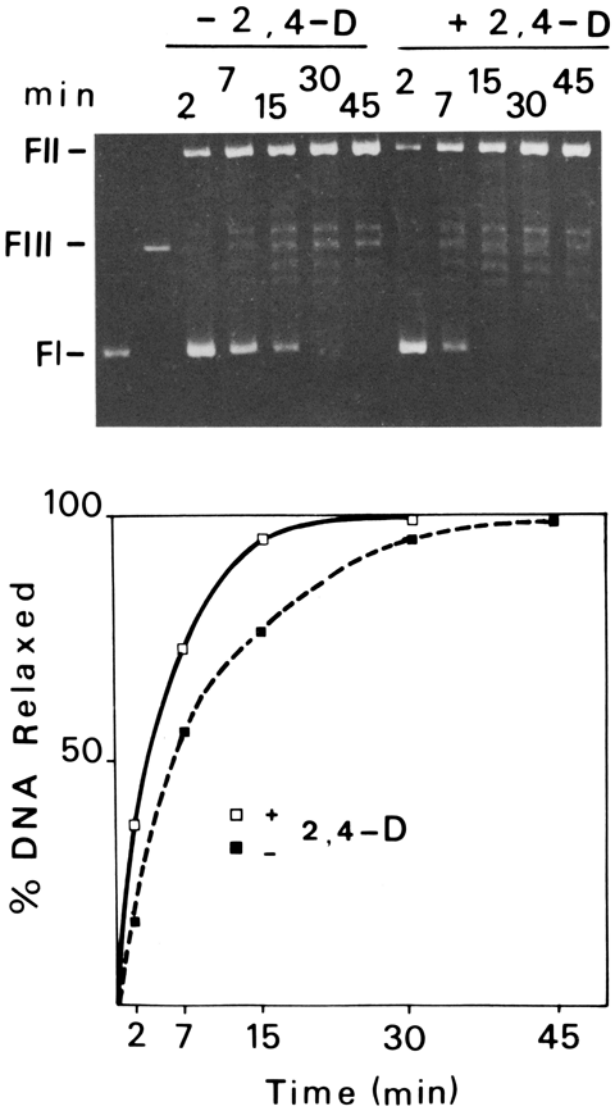

Fig. 2. DNA relaxation reaction in embryo nuclear extracts. Time course of relaxation of 2 μg of circular supercoiled pBR322 DNA incubated with 25 μl of nuclear extract solution from embryos treated (+ 2,4-D) and untreated (- 2,4-D) with 2,4-dichlorophenoxyacetic acid, in a total of 50 μl of reaction solution. Aliquots of 10 μl (400 ng of DNA) of relaxation reaction mixture were taken at indicated times and electrophoresed in 1% agarose gel as described in Material and methods. FI are supercoiled, FIII linear and FII nicked and relaxed forms of circular DNA. The relaxation of pBR322 DNA was quantitated by scanning densitometry; the values are given in per cent of final activity and are plotted in the lower figure.

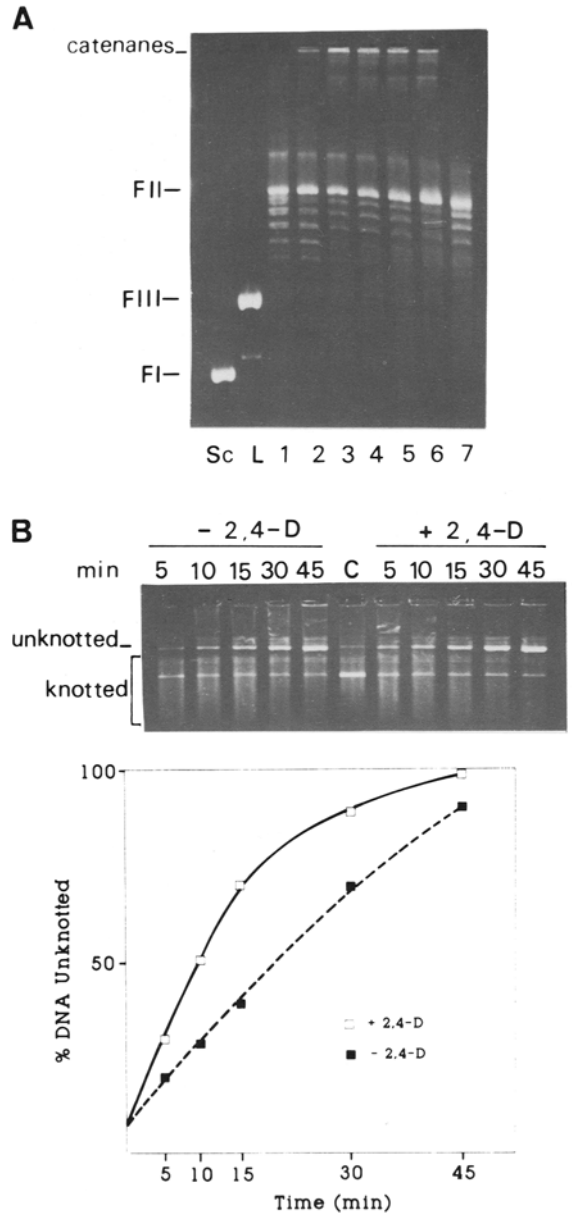

Fig. 3. Catenation and unknotting DNA reaction in embryo nuclear extracts.

A. Catenation reaction was made in 25 μl of standard topoisomerase II reaction buffer containing 0.5 μg of pBR322 circular supercoiled DNA and different quantities of calf thymus histone H1. Electrophoresis in 1% agarose gel of the catenation products is presented in panel A. Lanes 1–7 contained 0, 45, 90, 120, 150, 200 and 200 ng of histone H1 respectively. In lane 7 ATP was omitted in the reaction. B. Electrophoresis of unknotting product reaction. 2 μg of knotted P4 DNA were incubated with 25 μl of nuclear extracts (2.5 μg of extracted protein) from embryos treated (+ 2,4-D) and untreated (– 2,4-D) with 2 mg/l 2,4-D in 50 μl of topoisomerase II reaction solution. 10 μl (400 ng of DNA) aliquots of reaction solution are taken at indicated times, and analyzed in 0.6% agarose gel. Lane c is a control of initial untreated knotted P4 DNA sample. Unknotted P4 DNA was quantitated by scanning densitometry, compared with the knotted P4 DNA present in the reaction and the per cent values plotted as function of time (lower graph).

knotting and catenation/decatenation reactions of circular duplex DNA in an ATP-dependent manner [33]. To investigate the presence of topoisomerase II activity in embryo nuclear extracts, catenation of duplex circular DNA and unknotting the P4 knotted DNA reactions were assayed. It has been described that DNA topoisomerase II catalyzes the catenation of circular duplex DNA in the presence of a condensing agent such as spermidine or histone H1 [17]. Figure 3A shows the agarose gel electrophoretic pattern of the products obtained by incubation of circular supercoiled pBR322 DNA with a nuclear extract in the presence of different quantities of histone H1. The maximum catenation activity was achieved under our reaction conditions at a concentration of 90–120 μg/ml of histone H1. At H1 concentrations higher than 500 μg/ml catenation and even relaxation reactions became completely inhibited (not shown). When histone H1 or ATP is omitted in the incubation medium, no catenation of DNA is observed and only relaxation of supercoil DNA is obtained (lanes 1 and 7). This is due to the presence of DNA topoisomerase I in the extracts that is active in these conditions.

In order to evaluate the DNA topoisomerase II activity in embryo nuclear extracts, unknotting of P4 knotted DNA was assayed. Figure 3B shows the electrophoresis products obtained by incubation of knotted P4 DNA at different times with nuclear extracts from treated and untreated 2,4-D immature maize embryos. The extension of the reaction (consumption of knotted DNA) may be plotted as a function of the time (Fig. 3). The evaluation of the unknotting activity of different nuclear extracts was made by densitometric scanning and the average obtained shows an increase of 200% in 2,4-D-treated embryos as compared to the untreated ones. When topoiso-

merase II activity was assayed in nuclear extracts from ABA-treated embryos, only residual activity was found even at 5-fold quantities of nuclear protein (Fig. 4B).

Effects of camptothecin and epipodophyllotoxin VM-26 in maize embryo nuclear extracts

Camptothecin and epipodophyllotoxin VM-26 are two cytotoxic drugs that cause in mammalian cells an extensive breakage on chromosomal DNA [6, 7, 15]. It has been demonstrated that camptothecin acts over topoisomerase I inhibiting its catalytic activity, whilst VM-26 acts specifically on topoisomerase II inhibiting also its enzymatic activity [6, 15]. Neither of the two drugs interacts or intercalates directly with DNA.

Figure 5A shows the effect of camptothecin on the catalytic activity of nuclear extracts upon relaxation of supercoiled DNA in conditions (absence of ATP) where only topoisomerase I is active. The relaxation activity is partially inhibited when quantities of drug are increased as evidenced by the presence of supercoiled pBR322 DNA after the reaction. In our reaction conditions we have a maximum inhibition (40%) at 500 μM camptothecin: higher drug concentrations do not increase the extension of inhibition. This result shows that nuclear topoisomerase I from maize has a similar behavior to its homologous mammalian enzyme. Thus when the relaxation reaction is carried out in the presence of low quantities of topoisomerase I inhibition of relaxing supercoiled DNA rather than nicking is obtained [15]. On the other hand camptothecin does not inhibit the unknotting reaction of nuclear extracts in our reaction conditions (not shown).

To investigate whether epipodophyllotoxin VM-26 is an inhibitor of the topoisomerase II activity in maize embryo nuclear extracts, we have performed two types of assays. One consisted of the incubation of pBR322 supercoiled DNA with embryo nuclear extracts in the presence of ATP. In these conditions both topoisomerases I and II are active in relaxation of supercoiled DNA. Figure 5B shows a relaxation assay with

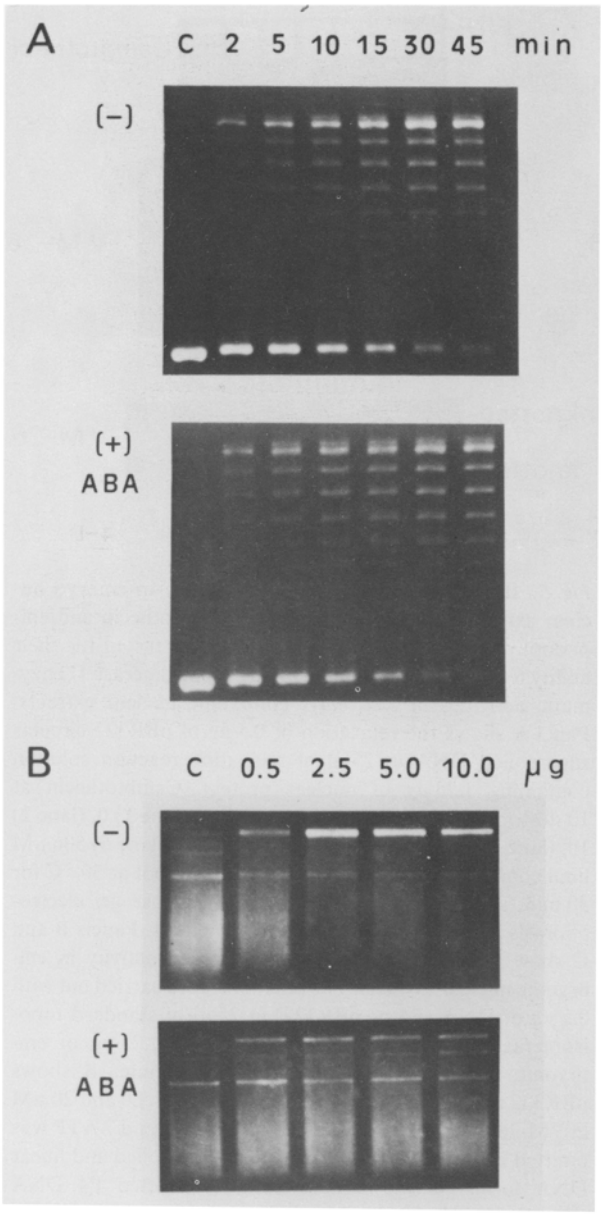

Fig. 4. Topoisomerase activity in nuclear extracts from ABA-treated embryos. Panel A shows the 1% agarose gel electrophoresis of relaxation reaction of supercoiled pBR322 DNA by nuclear extracts from embryos untreated (−) and treated (+) with ABA. Reaction conditions are identical to those in Fig. 2 except that 2.5 μg of DNA were relaxed. Panel B shows the 0.6% agarose gel electrophoresis of unknotting reaction of knotted P4 DNA by nuclear extracts untreated (−) and treated (+) with ABA. Reactions were carried out at 30 °C for 60 min and each fraction contains 0.5 μg of knotted P4 DNA and increasing quantities of nuclear extract proteins. Lane c is a control of pBR322 (A) or P4 (B) DNA incubated in reaction buffer 45 and 60 min respectively and processed as the rest of the samples.

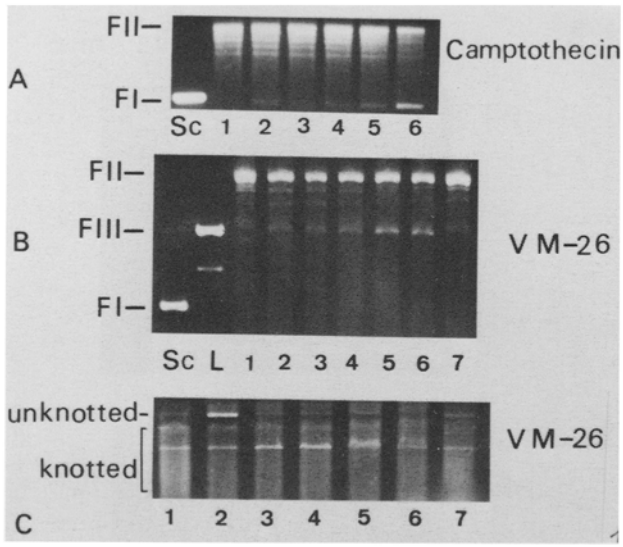

Fig. 5. Inhibition of topoisomerase activity in embryo nuclear extracts. Two cytotoxic drugs, camptothecin and epipodophyllotoxin VM-26 (teniposide), were tested for their ability to inhibit topoisomerase I and topoisomerase II enzymatic activities in *Zea mays* embryonic nuclear extracts. Panel A shows the relaxation of 0.5 μg of pBR322 circular supercoiled DNA in 25 μl of relaxation reaction solution containing 1.25 μg of nuclear protein. Camptothecin at 10 mM in DMSO solution was added to (lane 1) 0, (lane 2) 10, (lane 3) 25, (lane 4) 50, (lane 5) 250 and (lane 6) 500 μM final concentration. Reactions were carried out at 30 °C for 30 min, stopped and analyzed in 1% agarose gel electrophoresis as indicated for relaxation reactions. Panels B and C show the inhibition of topoisomerase II activity in embryonic nuclear extracts. The reactions were carried out with 0.5 μg of DNA (P4 or pBR322) in 25 μl of standard topoisomerase II reaction solution containing 1.25 μg of embryonic nuclear protein, at 30 °C for 60 min. B shows pBR322 relaxation reaction with 0, 0, 0.3, 1, 5, 20 and 20 μM in VM-26 (lanes 1–7) respectively. In lanes 1 and 7 ATP was omitted in the reaction. Sc and L are supercoiled and linear DNA forms. C shows unknotting of knotted P4 DNA reaction at 0, 0, 10, 50, 250, 1500, and 1500 μM in VM-26 (lanes 1–7) respectively. In lane 1 ATP was omitted and in lane 7 no protein extract was present in the reaction.

increasing quantities of VM-26. It can be seen that there is a correlation between the appearance of the linear DNA form and the quantity of VM-26 in the reaction. When ATP is omitted and VM-26 is present (lane 7) in the reaction no linear form of the DNA is visible, indicating that the appearence of this form is due to the topoisomerase II catalytic activity associated to the presence of VM-26 and not to the presence of the drug alone. On the other hand, in these reaction conditions (– ATP and VM-26) relaxation of circular supercoiled DNA is visible, indicating that topoisomerase I activity is not affected by VM-26. This result suggests that VM-26 stimulates the enzymatic DNA cleavage as in mammalian topoisomerase II [7] and probably by a similar mechanism. In this sense VM-26 was tested for its capacity to inhibit the P4 unknotting reaction. As is shown in Fig. 5C, the incubation of knotting P4 DNA with nuclear extracts containing increasing quantities of VM-26 inhibits the reaction, confirming that this drug acts in a similar manner over plant topoisomerase II as in its homologous mammalian enzyme [6].

The effect of alkaline phosphatase on the topoisomerase activity present in embryonuclear extracts

Topoisomerase I and topoisomerase II activities from mammalian cells can be regulated '*in vitro*' by phosphorylation [1, 8, 28]. It has been published that casein kinase II and protein kinase C can phosphorylate topoisomerase II increasing its *in vitro* activity [1, 28]. The catalytic activity of topoisomerase I can also be increased by phosphorylation. On the other hand, both topoisomerase activities can be reversed by treatment with alkaline phosphatase [1, 8]. Figure 6 shows the relaxation reaction of pBR322 supercoiled DNA and unknotting reaction of knotted P4 DNA by embryonic nuclear extracts treated with alkaline phosphatase. When these reactions are compared with controls it can be seen that phosphatase considerably reduces both topoisomerase activities. This result suggests that maize embryonic topoisomerases are phosphoproteins and could be regulated by post-translational modifications. Attempts to modify the topoisomerase activity in the extracts by endogenous kinase activity were made in different conditions and in the presence of kinase cofactors such as Ca^{2+} or phospholipids but no significant and reproducible results were obtained.

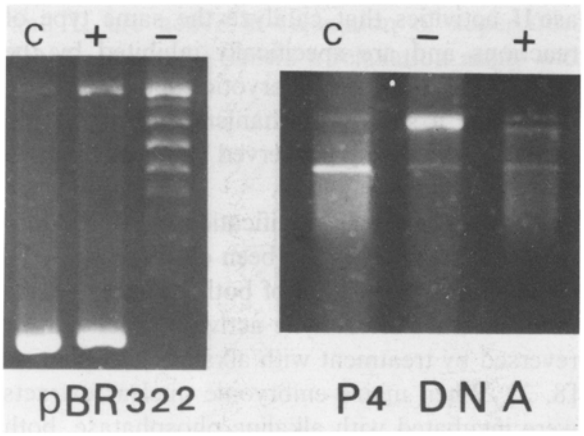

Fig. 6. Topoisomerase activity in embryonic nuclear extracts after alkaline phosphatase treatment.
50 μl of embryonic nuclear extracts (5 μg protein) were incubated in 100 μl final reaction volume with 25 units of concentrated bovine intestinal alkaline phosphatase (25 u/μl) at 30 °C for 20 min. An identical control reaction was made without alkaline phosphatase treatment of the extracts. After incubation aliquots of 25 μl of reaction solution were adjusted to standard topoisomerase reaction buffers and 0.5 μg of supercoiled pBR322 or knotted P4 DNA were mixed and the reactions carried out at 30 °C for 30 min. Panel A shows the relaxation of supercoiled pBR322 by embryonic nuclear extracts treated (+) or untreated (−) with alkaline phosphatase. Panel B: the same as A for unknotting P4 reaction. Lane C is control DNA.

Discussion

The topoisomerase activities in *Zea mays* embryo nuclear extracts were investigated by means of specific reactions. Thus, topoisomerase I is able to relax circular supercoiled DNA [33] in the absence of energetic cofactors and divalent cations (Mg^{2+}) stimulate topoisomerase I [20] but they are not strictly necessary for its activity. In embryonic nuclear extracts relaxation reactions occur even in the presence of 10 mM EDTA (not shown). Eukaryotic topoisomerase II can relax circular supercoiled DNA by a transient double-strand break [33]. For this reaction divalent cations and an energetic cofactor (ATP) are needed. Another way to distinguish the two activities is by using specific enzyme inhibitors. The relaxation of circular supercoiled pBR322 DNA by nuclear extracts was measured in the presence of the cytotoxic drug epipodophyllotoxin VM26 (teniposide) that interferes with the breakage-reunion reaction of mammalian DNA topoisomerase II [6]. When this drug was tested in embryonic nuclear extracts, no difference in relaxation was detected in reactions where ATP was omitted. When ATP and the drug were added, the linear form of pBR322 was visible in the relaxation products correlating with the quantities of drug present in the reaction. These results indicate the existence of two different mechanisms of relaxation of circular supercoiled DNA, suggesting the presence of two catalytic enzymatic activities, topoisomerase I and topoisomerase II. Two additional specific reactions were made to characterize topoisomerase II activity in maize embryonic nuclear extracts. Catenation of circular supercoiled DNA was obtained in a narrow range of histone H1 concentrations and only when ATP was present, indicating the need for specific conditions in this type of reaction. The fact that the catenation reaction was not complete, may be attributed to the reversibility of the catenation/decatenation reaction or to the competition of topoisomerase I for supercoiled DNA. The second specific reaction to test topoisomerase II activity in embryonic nuclear extracts consisted in unknotting of knotted P4 DNA. This reaction is also ATP-dependent in mammalian topoisomerase II [16]. Maize embryonic nuclear extracts were able to produce the unknotting reaction and this catalytic activity could only be observed when the reaction was carried out in the presence of ATP. Gyrase activity has been described in pea chloroplast [19]. In our maize embryo nuclear extracts we were not able to detect such activity, suggesting that gyrase activity in higher plants is only present in chloroplast, supporting the endosymbiotic theory for the evolution of such organelles [19].

Many antitumor drugs are known for their ability to inhibit the catalytic action of mammalian topoisomerases *in vivo* and *in vitro*. Some of them are natural or semi-synthetic products of plant origin. Two cytotoxic drugs were tested *in vitro* for their ability to inhibit topoisomerase activity in maize embryo nuclear extracts. Camptothecin is a plant alkaloid extracted from *Camptotheca*

acuminata, that inhibits the nucleic acid synthesis in mammalian cells and induces extensive strand breaks in chromosomal DNA. *In vitro* DNA topoisomerase I produces single-strand DNA breaks in the presence of camptothecin. A low concentration of the mammalian enzyme and relatively high drug concentrations inhibit the catalytic activity of mammalian topoisomerase I [15]. It has recently been reported [5] that wheat germ DNA topoisomerase I is not inhibited *in vitro* by camptothecin but nicking activity is detected even at low enzyme concentrations. In our reaction conditions (low enzyme concentration, 50 mM KCl, 50 mM NaCl and 5 mM $MgCl_2$) similar to the assays performed with purified mammalian enzyme, partial inhibition of topoisomerase I activity by camptothecin is obtained in a similar degree to mammalian topoisomerase I [15] except that a higher drug concentration (500 μM) was required for maximal inhibition. No significant DNA topoisomerase I nicking activity induced by the drug was detected (not shown). As judged by the results obtained, camptothecin is also able to specifically inhibit maize topoisomerase I because only this catalytic activity was altered in our assays.

The second substance assayed was the nonintercalative drug epipodophyllotoxin VM-26 that specifically inhibits mammalian topoisomerase II [6]. As mentioned above, VM-26 induces the appearance of the linear form of pBR322 in the relaxation of supercoiled DNA reaction in the presence of ATP suggesting its capacity to interfere with the breakage-reunion reaction of topoisomerase II in embryonic nuclear extracts, but it does not interfere with the relaxing topoisomerase I activity. A similar result has been obtained with the intercalative antitumor drug acridine m-AMSA with purified topoisomerase II from cauliflower [13]. When VM-26 was tested in the P4 DNA unknotting assay by embryonic nuclear extracts, inhibition was also obtained. These results show that epipodophyllotoxin VM-26 is able to inhibit the catalytic activity of maize topoisomerase II. It is then possible to conclude that in embryonic nuclear extracts from *Zea mays* we have detected topoisomerase I and topoisomerase II activities that catalyze the same type of reactions and are specifically inhibited by the same drugs as other eukaryotic topoisomerases, suggesting a similar mechanism and arguing in favour of the highly conserved catalytic function of these nuclear enzymes.

Post-translational modifications of both topoisomerases I and II have been described [9, 27]. *In vitro* phosphorylation of both enzymes results in an increase in catalytic activity, which can be reversed by treatment with alkaline phosphatase [8, 2]. When maize embryonic nuclear extracts were incubated with alkaline phosphatase, both topoisomerase I and topoisomerase II activities were considerably reduced, suggesting that both enzymes are phosphoproteins in *Zea mays* embryos.

Previous studies have demonstrated increases in topoisomerase II activities, during tumor tissue and cell proliferation [23]. We have evaluated the topoisomerase activity in the proliferative calli induced in immature maize embryos by 2,4-dichlorophenoxyacetic acid and the results show a two-fold increase in topoisomerase II activity and a 25% increase in topoisomerase I for the same nuclear extracts when compared with the activity found in immature embryo nuclear extracts (in the absence of 2,4-D). In contrast, when embryos are treated in a culture medium containing abscisic acid, which is known to inhibit cell proliferation, we have not detected topoisomerase II activity in nuclear extracts whereas topoisomerase I activity is similar to that in untreated embryos. These results suggest that a change in topoisomerase II nuclear activity may be associated with cell proliferation occurring during the callogenesis process in *Zea mays*.

Little is known about the mechanisms that regulate the topoisomerase II activity in eukaryotic cells. One possibility is post-translational modifications such as phosphorylation or ADP-ribosylation, that can modulate its catalytic activity. It is known that external cell stimulation by hormones, growth factors, chemicals etc. may activate specific kinases that modulate post-translational modifications of factors that control cell proliferation events. The tumor promoter

phorbol ester stimulates phosphorylation of topoisomerase II in *Geodia* cells by protein kinase C [27]. This protein kinase is transiently activated as a consequence of diacylglycerol produced from the turnover of inositol phospholipids as response to a membrane stimulatory effect [25]. It has been reported that 2,4-dichlorophenoxyacetic acid is able in plant cells to liberate inositol phospholipids [11]. Our results indicate an increase in topoisomerase II activity in maize embryos when they are incubated with 2,4-D suggesting that similar regulatory pathways may be present in proliferative plant cells.

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