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Activity of single-stranded DNA endonucleases in mung bean is associated with cell division

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Abstract

A single-strand-specific endonuclease from mung bean sprouts is widely used in molecular biology. However, the biological role of this enzyme is unknown. We studied the spatial and temporal activity of single-stranded DNA endonucleases in mung bean seedling by following enzyme activity that linearizes supercoiled plasmid DNA, a characteristic of this type of enzyme. The formation of a linear molecule from supercoiled DNA was found to occur in two distinguishable steps. The first, which involves introducing a nick into the supercoiled DNA and relaxing it, is very rapid and complete within a few seconds. The second step of cleaving the opposite strand to generate a unit-length linear duplex DNA is a relatively slow process. Analysis of the DNA cleavage sites showed the nuclease preferentially cuts supercoiled DNA at an AT-rich region. Varying levels of nuclease activity could be detected in different tissues of the mung bean seedling. The highest activity was in the root tip and was correlated with histone H1 kinase activity. This implies a link between nuclease activity and cell division. Induction of cell division in mung bean hypocotyls with auxin promoted formation of root primordia and considerably increased the activity of single-stranded DNA endonucleases. The nuclease activity and histone H1 kinase activity were reduced in mung bean cuttings treated with hydroxyurea, but not in cuttings treated with oryzalin. The potential function of single-stranded DNA endonucleases is discussed.

Introduction

Single-stranded DNA endonucleases have been characterized and purified from a variety of eukaryotic and prokaryotic cells. [13, 22, 29]. Some of these enzymes have proved to be useful tools in molecular biology research [8], but their biological significance is unknown. Mung bean nuclease and other single-strand-specific endonucleases can cleave both strands of circular covalently closed supercoiled DNA to generate unit-length linear duplex DNA [2, 18, 19, 29]. This activity suggests that unpaired or weakly hydrogen-bonded regions occur, or may be induced, in the superhelical DNA [4]; such regions are presumed to be rich in dA + dT [10, 16]. Indeed, cleavage sites for this type of nuclease are often found within AT-rich regions [17, 20, 24].

AT-rich regions are dispersed throughout the genome of prokaryotes and eukaryotes and play an important role in DNA structure and function. For example, AT-rich regions are composed of scaffold attachment regions (SARs) and are a feature of SARs among eukaryotic organisms [3,

12]. Initiation of DNA replication in prokaryotic and eukaryotic systems requires the presence of AT-rich sequences [1, 11, 15], but the mechanism by which these sequences exert their effects is unknown.

We initiated experiments to examine the function of single-stranded DNA endonucleases in mung bean plants. We hypothesized that the activity of these enzymes may be associated with AT-rich regions in the superhelical DNA and may be involved in DNA synthesis and repair. We show that the activity of these enzymes is associated with cell division. Their possible function in DNA synthesis and repair is discussed.

Materials and methods

Plant material and rooting of mung bean cuttings

Mung bean (*Vigna radiata* (L.) Wilcz.) seedlings were grown under controlled conditions (12 h photoperiod; temperature 26 °C). Cuttings were prepared from 10-day old seedlings, and consisted of the apical bud, one pair of primary leaves, epicotyl and a 3 cm long segment of the hypocotyl. Cuttings were incubated for 16 h with 50 mM α-naphthaleneacetic acid (NAA, Sigma) to promote the formation of root primordia, after which cuttings were washed with distilled water and incubated for various times either in water or in a solution containing 25 μM oryzalin (a gift from Dr W. T. Molin, Plant Sciences, University of Arizona) or 5 mM hydroxyurea (Sigma). Cuttings were sampled at various times; the 3 cm hypocotyl was removed and homogenized in buffer containing 25 mM Hepes pH 7.5, 1 mM PMSF, 5 μg/ml leupeptin and 5 μg/ml pepstatin. Homogenates were centrifuged (12000 × *g*, 15 min, 4 °C), the supernatant was collected, and aliquots stored at −80 °C until used. Protein concentration was determined with the Coomassie Plus Protein Assay Reagent (Pierce).

Nuclease assay

Single-stranded DNA endonuclease activity was tested by incubating 5–10 μg of total proteins with 0.5–1 μg of supercoiled plasmid DNA in buffer containing 50 mM sodium acetate pH 5.2, 30 mM NaCl and 1 mM $ZnCl_2$. Samples were incubated at room temperature for various times and reactions were stopped by adding EDTA to a concentration of 50 mM. The conversion of supercoiled plasmid DNA into a linear form was analyzed by electrophoresis in 1% agarose gel containing ethidium bromide. Mung bean nuclease, Klenow fragment of DNA polymerase I and restriction enzymes were purchased from Gibco-BRL.

Analysis of single-stranded DNA endonuclease cleavage sites

Supercoiled pUC18 (10 μg) was mixed with mung bean root tip extract (50 μg) for 1 min at room temperature, after which the sample was separated by electrophoresis in 1% agarose gel. The linear form was electroeluted from the gel as described [23]. The DNA was extracted with phenol:chloroform and precipitated with ethanol. The DNA pellet was resuspended with 20 μl of water and treated with Klenow prior to digestion with *Sau* 3AI. The digestion reaction was extracted with phenol/chloroform, precipitated and ligated into *Bam* HI/*Hinc*II sites of pBluescript KS^+ (Strategene). Clones containing pUC18 *Sau* 3AI fragments were sequenced using the T3 promoter primer and a Sequenase kit (USB).

Histone H1 kinase assay

Histone H1 kinase assay was used to monitor the mitotic activity in mung bean tissues [7]. Ten μg of total proteins in a volume of 20 μl was incubated in kinase buffer (25 mM Tris-HCl, 10 mM $MgCl_2$) containing 2.5 μg histone H1 (Sigma, Type-III), 0.2 mM ATP and 250 μCi/ml [γ^{32}P]ATP. Samples were incubated for 20 min at room temperature after which SDS buffer was added, the samples were heated to 95 °C for 5 min and resolved by 12.5% SDS/PAGE. Phosphoproteins were visualized by autoradiography of the dried gel.

Results

In order to monitor single-stranded DNA endonuclease activity in mung bean plants, we first compared the activity of purified mung bean nuclease with the activity of single-stranded DNA endonucleases in protein extracts from various tissues. Figure 1A shows that mung bean nuclease has a progressive mode of action on the supercoiled plasmid DNA. First, a nick is made in the superhelical DNA molecule that causes it to relax. This is a very rapid process and is completed within less than 1 min. The second step, cleaving the opposite strand to generate a unit-length linear duplex DNA, is a relatively slow process. In the assay illustrated in Fig. 1A, the supercoiled plasmid DNA was completely converted into the relaxed form by purified mung bean nuclease within less than 1 min. Linear form was detected after 1 min, and it continued to accumulate for the next 30 min. Varying levels of nuclease activity could be detected in different tissues of the mung

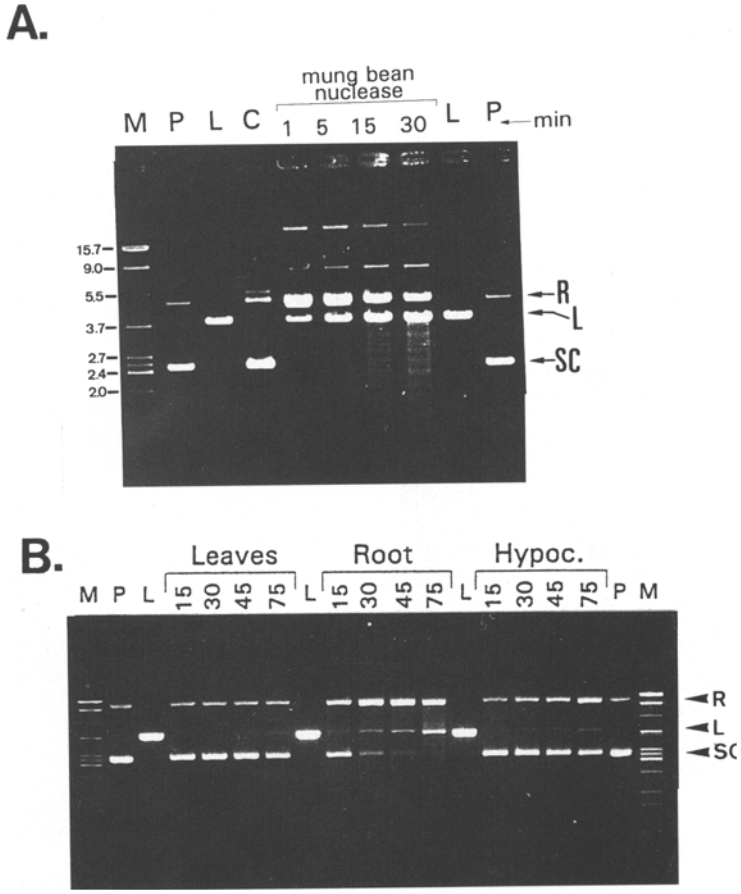

Fig. 1. Conversion of supercoiled plasmid DNA into a linear form. A. Mung bean nuclease activity on supercoiled (about 80% supercoiled) plasmid DNA (3 units/μg DNA) was determined at room temperature for various times and analyzed in 1% agarose gel containing ethidium bromide. B. Extracts from various tissues of mung bean seedlings were incubated for the indicated times (in seconds) with supercoiled plasmid DNA (10 μg total proteins/μg DNA) and analysed on 1% agarose gel. Extracts were made from the primary leaves (Leaves), root (Root, the lower 2 cm) and hypocotyls (Hypoc.) of 10-day old mung bean seedlings. M indicates size marker of lambda DNA digested with *Bam* HI, *Eco* RI and *Hin*d III, P and L at the top of the figure indicate input supercoiled plasmid DNA and *Eco* RI linearized plasmid, respectively and C is a control of input supercoiled DNA treated with the reaction buffer for 30 min. The R, L and SC on the right indicate the position of the relaxes, linear and the supercoiled plasmid DNA respectively.

bean seedlings, but the highest activity was in the roots (Fig. 1B).

Nuclease activity from root extract showed the same mode of action with plasmid DNA as the purified mung bean nuclease, implying that the same enzyme was being assayed. However, the possibility existed that the activity exhibited by the root extract was due to enzymes that randomly nicked the supercoiled DNA. Although the reaction condition favored the activity of single-stranded DNA endonucleases (low pH and Zn^{2+} ions as cofactors), we wanted to better characterize the enzyme activity in mung bean extracts by analyzing the cleavage sites. The activity of single-stranded DNA endonucleases toward supercoiled DNA has been described previously; the nuclease cleaves supercoiled DNA at specific sites and often at A + T-rich regions [2, 13, 18, 19, 29]. An analysis of the linear form plasmid DNA generated by the root extract showed (Fig. 2A, lane 3) that there are at least three different species resulting from cleavage at three different sites. When paired, these fragments add up to 2.7 kb (a + f; b + e; c + d), which is about the size of the linear form of pUC18. For sequence analysis of the cleavage sites, the linear form was eluted from the gel, treated with Klenow, and digested with *Sau* 3AI. The resulting fragments were then subcloned into the *Bam* HI/*Hinc* II sites of pBluescript KS + and sequenced. Although we expected to recover DNA fragments corresponding to three different cleavage sites, the clones that were isolated corresponded to different positions at the same region between 1512 to 1612 [31]. This region is very A + T-rich (75%). The sequence of the cleavage site at position 1556 is

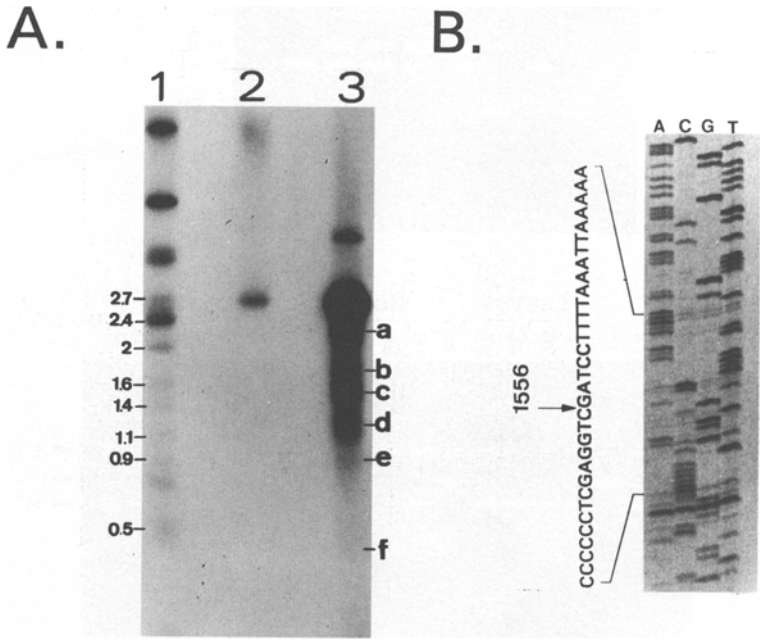

Fig. 2. Analysis of cleavage sites formed by root tip extract. A. Root tip extract (50 μg) was incubated with 10 μg of supercoiled pUC18 DNA for 1 min and further digested with *Hind* III followed by end-labeling using [α^{32}P]dCTP and Klenow. The resultant ^{32}P-labeled fragments were separated by 1% agarose gel, the gel was dried and exposed to X-ray film. Lane 1 is the ^{32}P-labeled lambda DNA marker (as in Fig. 1), lane 2 is ^{32}P-labeled *Hind* III-linearized pUC18, and lane 3 is the root tip-generated linear plasmid after digestion with *Hind* III. Lower-case letters on the right indicate the fragments identified. B. Sequence analysis of cleavage sites. The pUC18 linear form generated by extract from root tips was eluted from the gel, treated with Klenow, digested with *Sau* 3 AI and subcloned into *Bam* HI/*Hinc* II sites of pBluescript KS$^+$. Positive clones were sequenced using T3 promoter primer adjacent to the *Hinc* II site. The AT-rich cleavage region (above the arrow) and the linker of the cloning vector (below the arrow) are projected from the sequencing data. The number 1556, indicates the cleavage site position within pUC18.

illustrated in Fig. 2B. This region corresponds with fragments c and d (Fig. 2A) and appeared to be the preferential cleavage site. Thus, the results in Figs. 1 and 2 confirmed that the nuclease activity in mung bean extracts is indeed related to single-stranded DNA endonuclease.

Since AT-rich sequences have been shown to play an important role in initiation of DNA replication, we wanted to test whether the activity of this type of nuclease is linked to cell division. Analysis of root tissue (Fig. 3A) showed very high enzyme activity at the root tip (segment III), a region of high mitotic activity. Tissues above this region, which are undergoing cell enlargement and maturation (segment I and II), showed much reduced levels of nuclease activity. The spatial distribution of this enzyme activity was highly correlated with the activity of histone H1 kinase, an indicator of mitotic activity [7]. To corroborate the correlation between single-stranded DNA endonuclease activity and cell division, we next tested the nuclease activity in mung bean hypocotyls after treatment with auxin (NAA), which promotes the formation of root primordia. Very low levels of nuclease activity were detected in hypocotyl extracts before induction with auxin, or two days after preparation of the cuttings (Fig. 4A, times 0 and 2). However, the activity of endonuclease increased considerably in hypocotyl extract from 5-day-old cuttings (Fig. 4A), a stage where adventitious roots begin to emerge.

To further examine the correlation of nuclease activity with the cell cycle, mung bean cuttings were also treated with cell cycle inhibitors: oryzalin (a mitotic inhibitor) and hydroxyurea (an S-phase inhibitor). Cuttings that were first induced for the formation of root primordia with auxin (NAA) for 16 h were incubated with water, 5 mM hydroxyurea or with 25 mM oryzalin. The nuclease activity in cuttings treated with oryzalin was comparable to that of the control (Fig. 5A), although no visible roots could be detected after

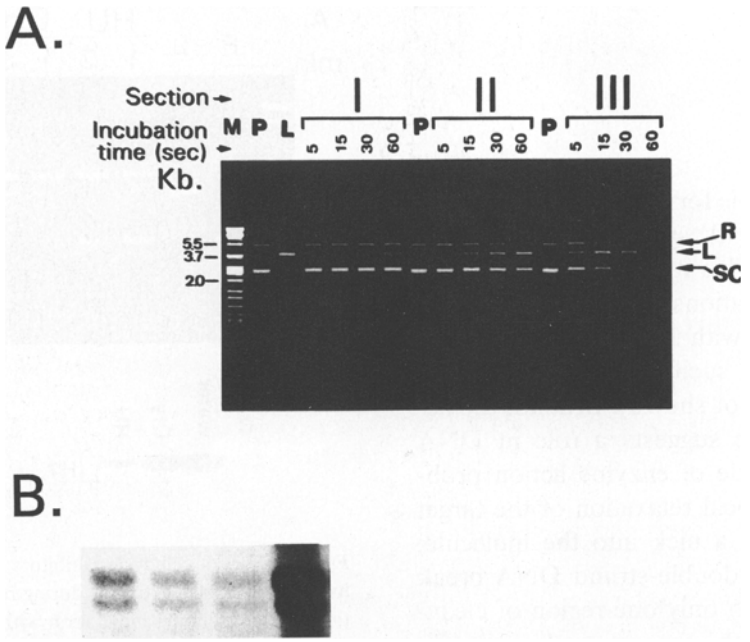

Fig. 3. Single-stranded DNA endonuclease activity associated with tissue displaying high levels of cell division. A. The lower segment of the root (2 cm long) was divided into three parts: I, the upper 1 cm; II, the middle 0.7 cm; III, the lower 0.3 cm of the root tip. Teach segment was extracted and incubated for the indicated times with supercoiled plasmid DNA (10 μg total proteins/μg DNA) and analyzed on 1% agarose gel. P and L at the top indicate input supercoiled DNA and *Eco* RI-linearized plasmid respectively. R, L and SC on the right indicate the position of relaxed, linear and supercoiled respectively. B. Histone H1 kinase activity in extracts from the corresponding root segments. C indicates control for no extract.

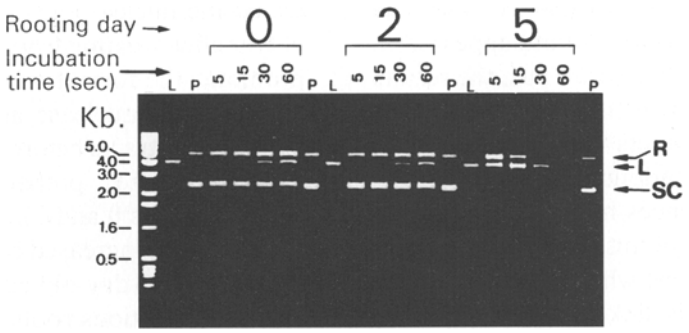

Fig. 4. Single-stranded DNA endonuclease activity during the course of adventitous root formation on hypocotyls of mung bean cuttings. Cuttings were prepared as described in Materials and methods. Cuttings were treated with auxin (NAA, 5×10^{-5} M) for 16 h to promote the formation of root primordia and then transferred to water. Samples were taken at various times (indicated at the figure), hypocotyls were removed, extracted and analyzed for nuclease activity. P and L at the top of the figure, indicate input supercoiled DNA and *Eco* RI-linearized plasmid, respectively. R, L and SC on the right indicate the position of relaxed, linear and supercoiled DNA, respectively.

5 days (Fig. 5C lane 2). In contrast, very low nuclease activity was observed in cuttings treated with hydroxyurea (Fig. 5A, HU). Interestingly, histone H1 kinase activity (Fig. 5B) in oryzalin-treated cuttings was similar to that of the control. Low activity was observed in hydroxyurea-treated cuttings.

Discussion

Our results imply a role for single-stranded DNA endonucleases in DNA synthesis mediated by AT-rich sequences. This hypothesis is based on the following observations. First, this nuclease activity is associated with tissues displaying high levels of cell division, such as root tips and apical meristems (data not shown). Second, the activity of the nuclease suggests a role in DNA metabolism. The mode of enzyme action probably gives rise to a local relaxation of the target DNA by introducing a nick into the molecule; less frequently will a double-strand DNA break occur. Third, although only one region of cleavage was identified in the present work, it is AT-rich, in agreement with previous reports [17, 20, 24]. Such regions have been implicated to play a role in DNA structure and function.

Single-stranded DNA endonucleases in mung bean may be involved in the first steps of the initiation of DNA replication. The mechanism by which an origin of replication is first recognized and then denatured is unknown for any prokaryotic or eukaryotic system. We suggest that AT-

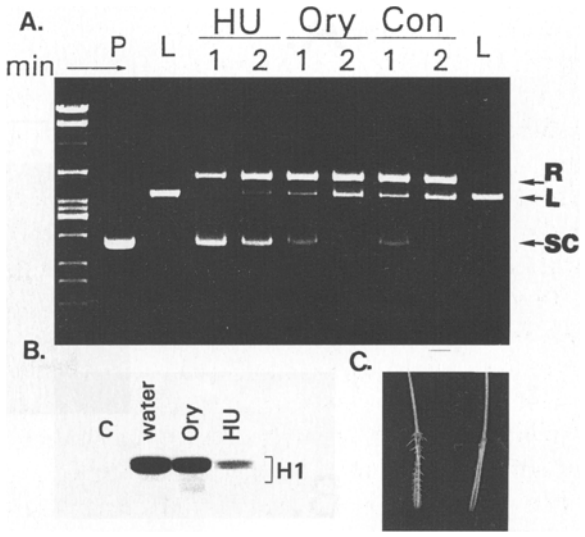

Fig. 5. Effect of cell cycle inhibitors on the endonuclease and histone H1 kinase activities during rooting of mung bean cuttings. After 16 hours incubation with auxin (as in Fig. 4) cuttings were either incubated in water (Con), in 5 mM hydroxyurea (HU) or in 25 μM oryzalin (Ory). Cuttings were harvested at 84 h (at this stage hydroxyurea-treated cuttings seemed to suffer) and analyzed for: (A) endouclease activity, (B) histone H1 kinase activity, (C) root formation (after 132 h) on cuttings treated with water (lane 1) or with oryzalin (lane 2).

rich sequences, which are characteristic of origins of replication both in prokaryotes and eukaryotes, form weakly hydrogen-bonded regions which tend to open easily and can be targeted by single stranded-DNA endonucleases. As a result, the superhelical DNA is nicked, and this may mark the region for initiation of replication. Indeed, endonucleases were found to be essential for the initiation of replication in bacteriophage [5]. Also, the autonomously replicating sequence (ARS) elements in yeast show sensitivity to single-strand-specific endonucleases [11], although these enzymes have never been implicated in the initiation of replication.

We found the activity of this type of nuclease is associated with tissues displaying high levels of cell division and is correlated with the activity of histone H1 kinase. This enzyme is routinely used to monitor the activity of the M-phase promoting factor [MPF, 7], which is composed of a CDC2 protein as a catalytic subunit and a cyclin as a regulatory subunit [28]. Interestingly, nuclease activity as well as histone H1 kinase activity decreased as a consequence of hydroxyurea treatment, which inhibits DNA synthesis, but not with oryzalin, a cell cycle inhibitor that disrupts the polimerization of microtubules [26]. This suggests that the activity of the nuclease may be induced between S and M phases, and that $p34^{cdc2}$/cyclin kinase remains active in mung bean plants, at least until metaphase. MPF activity fluctuates with a pattern similar to that of the mitotic cyclin, reaching a maximum at metaphase and disappearing as cells enter anaphase [27]. Induction of cyclin destruction was found to be microtubule-dependent [21]. Our results imply that the control of MPF activity in mung bean follows the same pattern as in mammalian cells, and its inactivation may require the microtubule-dependent destruction of mitotic cyclin.

The link between nuclease activity and the cell division cycle could suggest an involvement of single-stranded DNA endonucleases in DNA mismatch repair. Mismatched base pairs arise during the course of normal DNA metabolism. In *Escherichia coli*, three gene products are involved in the initial steps of long patch mismatch repair [6, 25]. One of these, Mut H, is presumed to function as a single-stranded DNA endonuclease. Also, certain gene products that have been implicated in nucleotide excision repair [9] were recently found to function as single-strand-specific endonucleases [14, 30]. Collectively, our results suggest a function for single-stranded DNA endonucleases during the course of the cell division cycle. Isolation of the genes encoding these enzymes will help to unravel their exact function.

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