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The isolation of high molecular weight DNA from wheat, barley and rye for analysis by pulse-field gel electrophoresis

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Abstract

A method is presented for the preparation of large DNA molecules from protoplasts embedded in agarose blocks of three different cereals – hexaploid bread wheat (*Triticum aestivum*), barley (*Hordeum vulgare*) and rye (*Secale cereale*). Pulse-field gel electrophoresis (PFGE) analysis of these DNA preparations using a contour-clamped homogeneous field (CHEF) apparatus indicated that the size of the DNA molecules was greater than 6 Mb. DNA samples prepared by this method were shown to be useful for restriction analysis using both frequent and rare cutting enzymes.

Introduction

As increasingly dense RFLP-based genetic maps of plant genes become available, the possibility of physical mapping and eventual gene isolation by genome walking is becoming a reality. However, in cereals, the physical distances involved are great, for example in wheat an average chromosome is 750 Mb long and a recombination unit is, on average, about 3.8 Mb. Thus the ability to prepare unsheared DNA of very high molecular weight (HMW), for analysis by pulse-field gel electrophoresis (PFGE), or for use in the production of DNA libraries in yeast artificial chromosomes (YAC) [1], is a necessity.

Conventional methods of DNA preparation involve physical grinding of tissue followed by extraction in solutions containing proteinase K, detergent and EDTA. These procedures generate molecules of average size less than 500 kb, which are not suitable for PFGE. Schwartz and Cantor [2] developed a method in which yeast cells are embedded in agarose prior to lysis. In this approach the agarose matrix keeps large DNA molecules intact, while permitting the free diffusion of the detergent and protease. We have used this method to obtain nuclear DNA 0.2 to 2 Mb by *in situ* lysis of intact wheat nuclei embedded in agarose. However, Southern blot analysis of the preparations indicated that no discrete signals could be detected with any probe. Other methods of isolating HMW DNA from dicotyledonous plants based on that of Schwartz and Cantor have been reported for *Arabidopsis thaliana* [3] and tomato (*Lycopersicon esculentum*) [4]. We report here a method to obtain high molecular weight DNA by *in situ* lysis of protoplasts embedded in agarose from the three cereal crop species, hexaploid wheat, barley and rye.

Materials and methods

Plant materials and growth conditions

Seeds of hexaploid wheat cv. Chinese Spring, barley cv. Betzes, and rye cv. King II were surface-sterilized for 20 minutes in a solution of 10% Domestos (Lever), washed six times with sterile distilled water, and germinated on three pieces of water-saturated Whatman 3 MM paper in a 1 litre jar in the dark at 25 °C for seven days in sterile conditions.

Isolation of high molecular weight (HMW) DNA

HMW DNA was isolated from protoplasts obtained from leaf material of seven-day old seedlings. The entire shoots were surface-sterilized for 20 minutes in 10% Domestos, followed by two washes in sterile distilled water. After removing the coleoptile, the leaf tissue was sliced longitudinally into very fine pieces. These were then transferred to 10 ml of washing solution (0.7 M mannitol, 0.01 M $CaCl_2$ pH 5.8) in a 9 cm Petri dish. This solution was then replaced by 10 ml of enzyme solution consisting of 2% cellulase (Onuzuka RS), 0.5% rhozyme and 0.05% macerozyme (Onuzuka R10) in washing solution. Cell wall digestion proceeded for three hours at 25 °C with gentle shaking (25 rpm) on an orbital platform and was checked by microscopic observation. The protoplasts released were sequentially filtered through 40 μm and 20 μm nylon sieves and pelleted at 26 × *g* for 10 minutes. After one wash in washing solution, a small aliquot of the sample was counted. The protoplasts were then repelleted and suspended at a concentration of 1.5×10^6 ml^{-1} for wheat, and 4.5×10^6 ml^{-1} for rye and barley. This three-fold difference was necessary to provide approximately similar DNA concentration in the agarose blocks from the diploid and the hexaploid species.

The protoplast suspension was then mixed with an equal volume of 2% low melting point agarose in washing solution (kept at 45 °C). The final concentration of protoplasts in the block was approximately 0.7×10^6 protoplasts per ml (equivalent to 7×10^4 protoplasts per 100 μl block) for wheat and 2×10^6 protoplasts per ml (equivalent to 2×10^5 protoplasts per block) for rye and barley. The molten mixture was aliquoted into 2 mm × 5 mm × 10 mm plastic moulds and was allowed to solidify at 4 °C for 1 hour. The blocks were incubated for 48 hours at 50 °C in lysis buffer, ESP (0.5 M EDTA pH 8.0, 1% sodium N-lauroylsarcosine, 2 mg ml^{-1} proteinase K) to lyse the protoplasts and stored at 4 °C until use. A typical block of a volume of 100 μl prepared by this method would yield conveniently about 5 μg of good quality unsheared DNA for restriction digestion.

Restriction endonuclease digestion

Prior to digestion, DNA samples were washed twice in $T_{10}E_{10}$ (10 mM Tris, 10 mM EDTA pH 8.0) with 1 mM of phenylmethylsulphonyl fluoride (PMSF) at 50 °C for 30 minutes each time and twice more at room temperature. The blocks were then incubated with two changes of 1 ml of restriction digestion buffer in the presence of 8 mM spermidine for 1 hour each at room temperature. Digestions with *Not* I and *Bss* HII (NEB), *Bam* HI, *Nru* I, *Mlu* I, *Sma* I and *Sal* I (BRL) were carried out in 250 μl of fresh restriction buffer and 20 units of enzyme overnight under conditions recommended by the manufacturers. After digestion, the restriction endonuclease in each sample was inhibited by incubation in $T_{10}E_{10}$ containing 1 mg ml^{-1} of proteinase K for one hour at 50 °C. The blocks were then ready for electrophoresis.

Pulse-field gel electrophoresis

CHEF gels [5], 1% agarose in 1 × TBE (100 mM Tris-Cl, 100 mM boric acid, 2 mM EDTA pH 8.3) were run in TBE at 10 °C and 80 V (for 7-day runs) or 150 V (for 2-day runs) with pulse times between 40 seconds and 15 minutes in a LKB Pulsaphore 2015 system with the HEX

electrode. Several pulse phases, used to expand ranges of resolution, are described in the figure legends.

Alkaline transfer

Gels were stained in 1 mg/ml ethidium bromide in 1 × TBE. They were then irradiated at 254 nm for 1 minute, depurinated with 0.25 M HCl for 20 minutes and denatured with 0.5 M NaOH, 1.5 M NaCl for 20 minutes. Alkaline transfer [6] to GeneScreen Plus membrane was carried out for 24 hours.

Probe labelling and hybridization

The probes, the 0.75 kb *Sst* I fragment of the wheat α-amylase-1 (α-Amy1) genomic clone p501 [7] (a gift from D.C. Baulcombe) and chloroplast and maize mitochondria and chloroplast DNA (a gift from D.R. Marsh), were 'oligo-labelled' [8] with α-^{32}P-dCTP to a high specific activity of 1–10 × 10^8 cpm/μg.

Prehybridization was carried out for 2 hours at 65 °C in 0.6 M NaCl, 20 mM PIPES (pH 6.8), 4 mM EDTA, 0.2% gelatin, 0.2% Ficoll 400, 0.2% PVP-360, 1% SDS, and 0.5% sodium pyrophosphate containing 0.5 mg/ml autoclaved salmon testis DNA. Hybridizations were carried out overnight with labelled probe in the same buffer after removing the prehybridization solutions.

After hybridization, filters were washed at 65 °C twice in 2 × SSC with 1% SDS and twice in 0.2 × SSC with 1% SDS for 15 minutes each and then exposed to Kodak XAR-5 films with intensifier screens for 7 days at −70 °C.

Yeast strains and lambda concatemers used as molecular size markers

Chromosomal DNA markers were prepared from *Saccharomyces cerevisiae* strain YP184 and *Schizosaccharomyces pombe* strain 972 h [9]. Lambda concatemers were prepared from intact lambda phage precipitated by polyethylene glycol (PEG) from plate lysates [10].

Results and discussion

PFGE analysis using a contour-clamped homogeneous electric field (CHEF) [5] apparatus, of undigested DNA prepared by this method from wheat, barley and rye protoplasts showed that the DNA molecules were at least greater than 6 Mb in length (i.e. greater than the largest 5.7 Mb chromosome I of *S. pombe* [11] with gel conditions designed to resolve the three chromosomes of *S. pombe* (Fig. 1A)). Most of the uncut DNA (indicated by the arrow in Fig. 1A) did not enter the gel and the amount of degraded DNA due to premature lysis of protoplasts was less than 10%. The 0.75 kb *Sst* I fragment from the α-Amy1 genomic subclone, when probed onto a Southern blot of this uncut DNA, detected a band in the HMW DNA region. This indicated that although most of the uncut DNA was too big to enter the gel, free DNA molecules had been released from the embedded protoplasts and were available for transfer to the membrane and for hybridization with the probe. The wheat samples in Fig. 1 had been stored in the lysis buffer for either one month or two months after lysis. Thus there is no indication of degradation of the DNA during the period of storage.

The accessibility of this DNA to restriction endonucleases was checked by a time course experiment, during which wheat sample blocks were taken out of the lysis mixture (ESP) after 0, 0.5, 18 and 44 hours of incubation at 50 °C. The blocks were washed in $T_{10}E_{10}$ exhaustively in the presence of 1 mM PMSF. Each block was then sub-divided into 50 μl half-blocks. One half-block was digested by *Not* I, and the other half-block was digested by *Bam* HI and the digests of the time samples by both enzymes were analysed by PFGE. The ethidium bromide stained gel (Fig. 2A) showed that accessible DNA molecules were available for endonuclease digestion after 0.5 hour lysis in ESP, while they were not

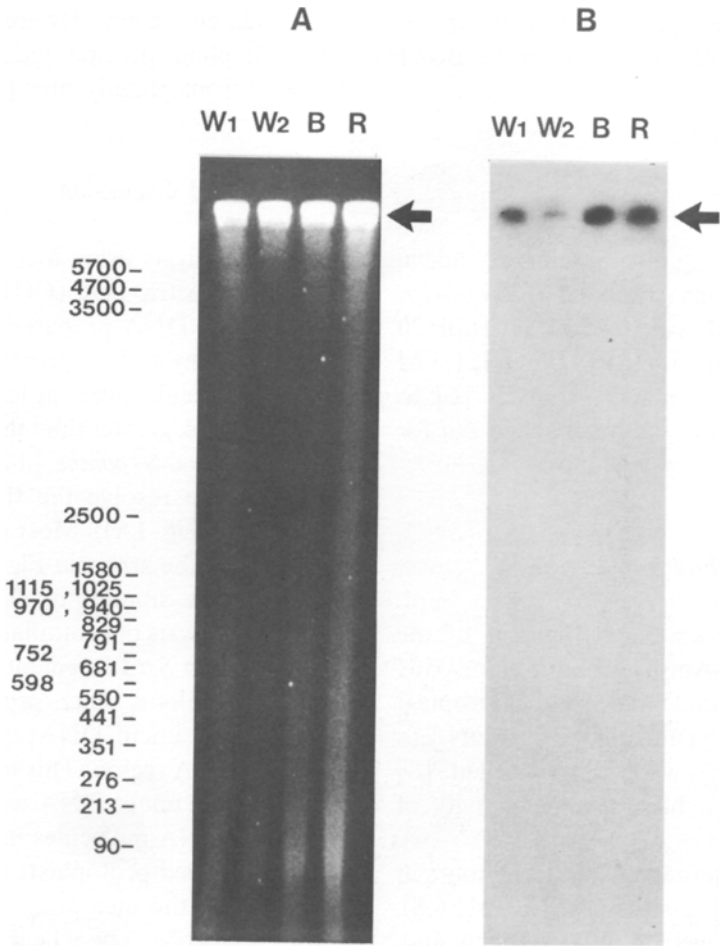

Fig. 1. Pulse-field gel analysis of HMW DNA isolated from wheat, barley and rye protoplasts. The two wheat tracks (W1 and W2) were undigested DNA after one and two months of storage in lysis buffer respectively. The barley (B) and rye (R) tracks were undigested DNA just after lysis for 48 hours. The gel was run at 80 V with a pulse regime of 15 minutes for 5 days, 80 seconds for 1 day and 40 seconds for 1 day. Chromosomes of *S. cerevisiae* strain YP148 and *S. pombe* strain 975h$^-$ were used as size markers and their sizes are indicated in kb on the left-hand side of the gel. The position of the HMW DNA remaining at the loading slots is marked with an arrow. A is the ethidium bromide-stained gel and B is the Southern hybridization results with the α-Amy1 probe.

available before lysis (0 time). The majority of the *Not* I fragments were larger than 100 kb, while those of *Bam* HI fragments were less than 23 kb. Also, unlike the undigested DNA, most of the *Not* I and all of the *Bam* HI fragments could enter into the gel. Nevertheless, because of the large size of some fragments, a significant portion of the *Not* I digest, and a very small portion of the *Bam* HI digest were not resolved and remained in the limited mobility region under the pulse conditions used for this gel. Both *Not* I and *Bam* HI digests on the blot of this gel showed well defined hybridization signals with the α-Amy1 probe except those of the samples taken out at 0 time of lysis (Fig. 2B). This confirmed that the quality of DNA released by this preparative method was excellent both for digestion by rare-cutting restriction endonucleases and enzymes that cut more frequently.

A series of rare-cutting enzymes used in animal systems [12, 13, 14] was tested on wheat DNA to select those gathering large fragments (between

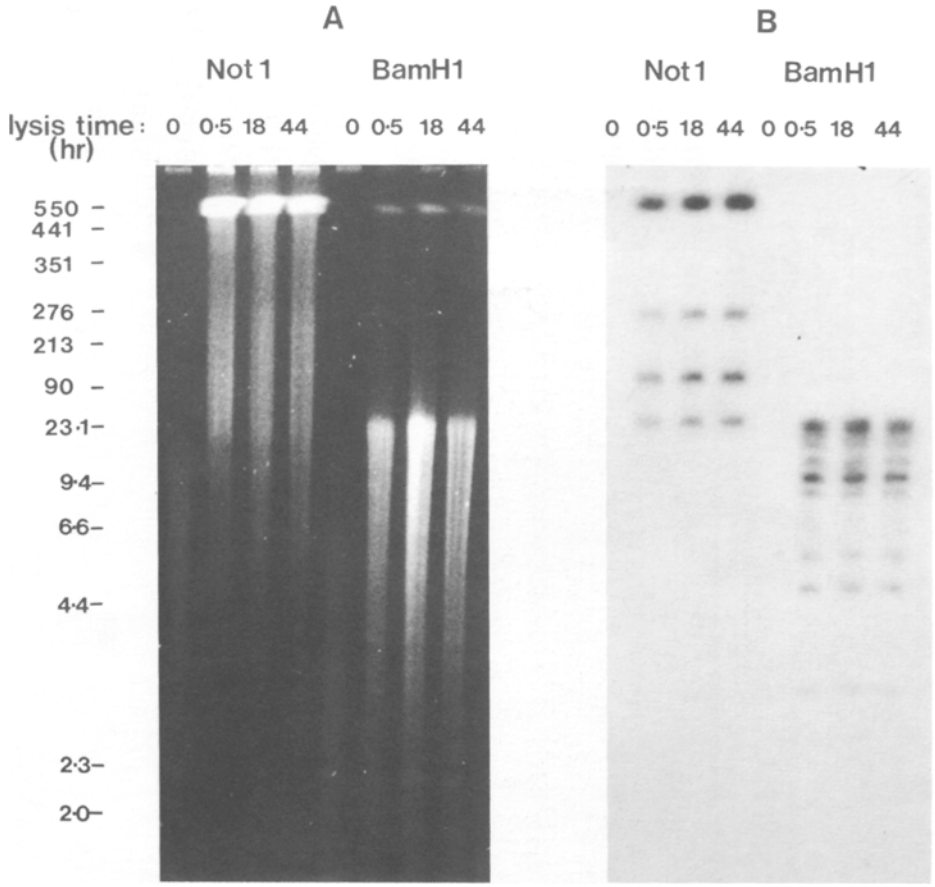

Fig. 2. Release and restriction digestion of DNA from agarose-embedded wheat protoplasts following incubation in lysis buffer at 50 °C for various times. Two series of agarose embedded wheat protoplasts were incubated in lysis buffer for the period in hours indicated at the top of each lane. One series was digested with *Not* I and the other series was digested with *Bam* HI and subsequently analysed by PFGE at a voltage of 150 V with a pulse time of 40 seconds for 24 hours. The ethidium bromide-stained gel is shown in A. Chromosomal DNA of *S. cerevisiae* strain YP148 and *λ-Hin* dIII digests were used as size markers and their sizes in kb are indicated on the left side of the gel. Southern blot analysis of these digests probed with the α-Amy1 probe is shown in B.

100–1000 kb). They contain either 8 bp recognition sites (eg. *Not* I) or C-methylation sensitive 6 bp recognition sites (eg. *Nru* I, *Mlu* I, *Bss* HII, *Sma* I and *Sal* I). The overall G-C contents of higher animals and plants may be similar, but the pattern of DNA methylation can be significantly different [15]. In plant systems, methylation occurs at CG as well as CNG sites and hence larger fragments can be generated using the C-methylation sensitive enzymes. Digestion of wheat DNA by these enzymes generated fragments ranging in size from 50 kb to 2 Mb as revealed by ethidium bromide staining of the gel (Fig. 3A). Southern blot analysis of these digests indicated that bands were detected by the α-Amy1 probe (Fig. 3B).

Subsequently, it was found that the same method could also be applied successfully to obtain barley and rye HMW DNA. A typical gel with *Nru* I digests of wheat, barley and rye DNA is shown in Fig. 4A, and hybridization results with the α-Amy1 probe are shown in Fig. 4B. This type of DNA preparation from the leaf protoplasts yields total DNA including organellar DNA from both chloroplasts and mitochondria. These organellar DNA molecules are un-

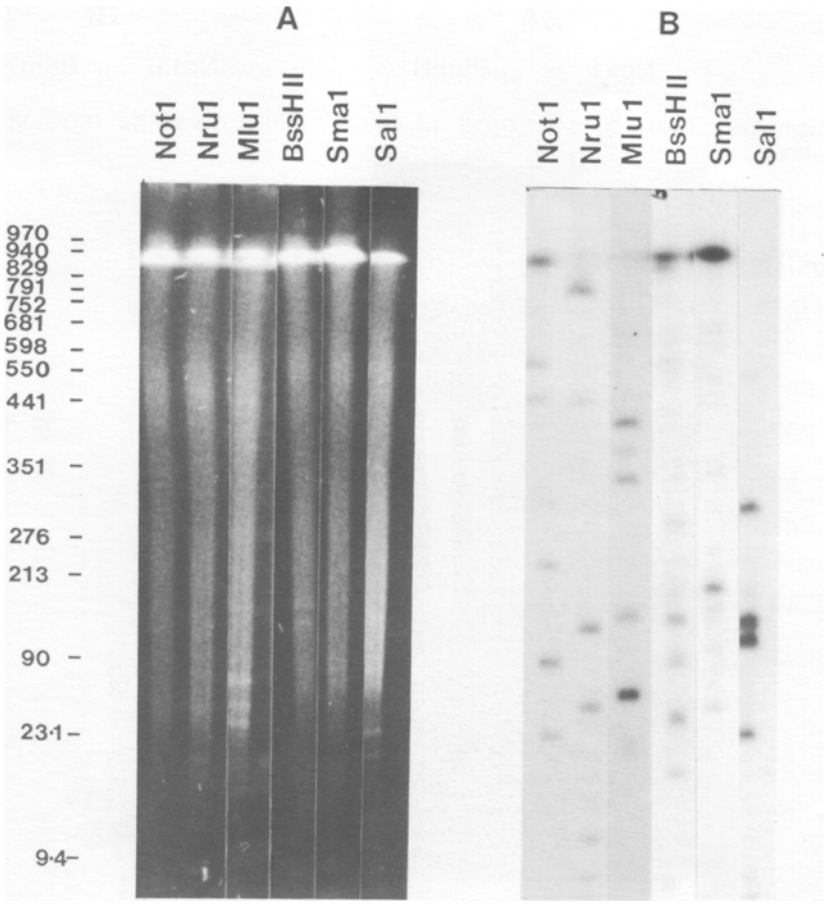

Fig. 3. Restriction digestion of DNA from wheat protoplasts embedded in agarose and hybridization analysis using the α-Amy1 probe. HMW wheat DNA was cut with the restriction endonucleases as indicated at the top of each track and separated by PFGE running at 150 V with a pulse regime of 80 seconds for 24 hours and 40 seconds for 20.5 hours. A is the gel stained with ethidium bromide. Chromosomal DNA of *S. cerevisiae* strain YP148, and *λ-Hin*d III digests are used as size markers and their sizes are indicated on the left side of the gel. Southern blot analysis of these digests probed with the α-Amy1 probe is shown in B.

methylated, so in general would usually be digested into fragments smaller than 50 kb if sites for a particular restriction enzyme were present. With the *Nru* I digests they appear as the more intense bands at the lower molecular weight ends of the genomic smears on the ethidium bromide stained gel (Fig. 4A). The banding pattern obtained after probing with a mixture of radioactively labelled mitochondria and chloroplast DNA (Fig. 4C) coincides with this distinctive pattern of ethidium bromide bands. The presence of organellar DNA in general would not interfere with the interpretation of the hybridization results with genomic DNA.

Conclusions

The method described here can generate unsheared HMW DNA from wheat, barley and rye. Extra methylation at CNG sites in addition to CG sites in plant results in larger restriction fragments with C-methylation sensitive restriction endonucleases. These large restriction fragments can be

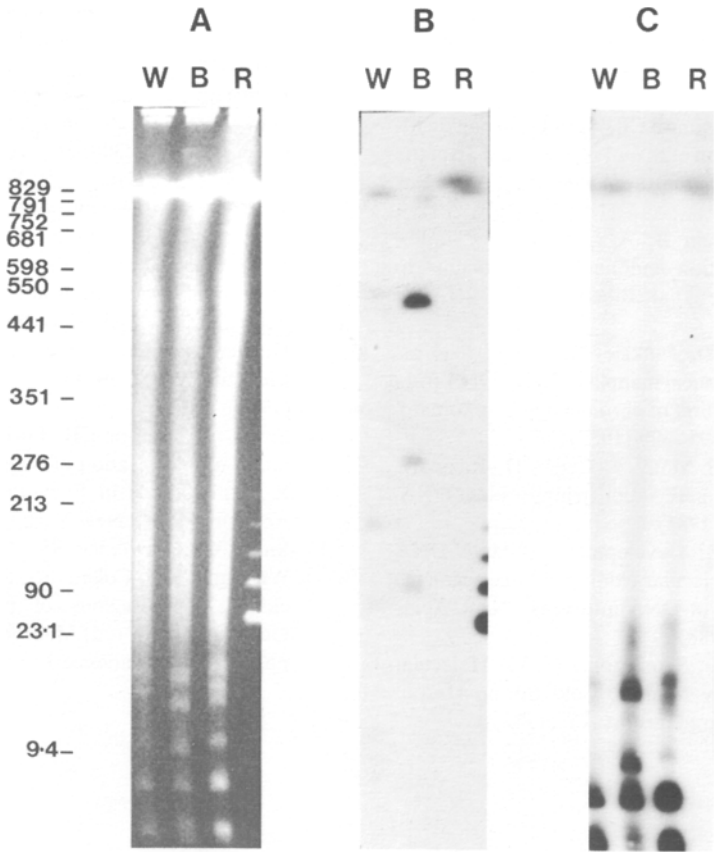

Fig. 4. PFGE of *Nru* I digests of HMW DNA isolated from wheat, barley and rye protoplasts and hybridization analysis using α-Amy1 probe and organellar DNA. *Nru* I digests of wheat (W), barley (B) and rye (R) DNA were separated at 150 V with a pulse regime of 40 seconds for 24 hours and 40 seconds for 20.5 hours. The ethidium bromide stained gel is shown in A. Chromosomal DNA of *S. cerevisiae* strain YP148, and λ-*Hin*d III digests are used as size markers and their sizes in kb are indicated on the left side of the gel. Southern blot analysis of these digests probed with α-Amy1 is shown in B and those to a mixture of maize chloroplast and mitochondrial DNA are shown in C. The autoradiogram in B was exposed for 7 days with intensifying screens at −70 °C, while that in C was exposed for 2 days under the same conditions.

separated by PFGE and are useful in linking DNA probes closely spaced on the genetic maps as well as for YAC cloning. As a result, macro-physical maps of loci extending over distances of several hundred kb to Mb can be generated, which also open up the possibilities of transformation of genetic linkage maps into physical maps.

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