STUDIES TOWARD *IN VITRO* RECONSTITUTION OF PLANT CHROMATIN

A Thesis presented to the faculty of the graduate school of
Western Carolina University in partial fulfillment
of the requirements for the degree of Master of Science in Biology

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LIST OF ABBREVIATIONS

bp	base pairs
DTT	DL-dithiothreitol
E-64	N-(trans-epoxysuccinyl)-L-leucine 4-guanidinobutylamide
EDTA	ethylenediaminetetraacetic acid
HEPES	4-(2-Hydroxyethyl)piperazine-1-ethanesulfonic acid
MNase	micrococcal nuclease
MOPS	3-(N-Morpholino)propanesulfonic acid
NE	nuclear extract prepared from etiolated seedlings
NEemb	nuclear extract prepared from germinated embryos
PMSF	phenylmethylsulfonyl fluoride
SDS-PAGE	sodium dodecyl sulfate polyacrylamide gel electrophoresis
Tris	tris(hydroxymethyl)aminomethane
WCE	whole cell extract prepared from etiolated seedlings
WCEemb	whole cell extract prepared from germinated embryos

ABSTRACT

To study the relation between chromatin structure and DNA function in detail it is necessary to have an *in vitro* procedure for assembling nucleosomes on a naked DNA template with properties similar to native chromatin. Such procedures exist for yeast and animal model systems but have not been developed for plants. The goal of this project was to lay the groundwork for developing a chromatin assembly extract from plants. Extracts from various plant materials were tested to determine their suitability for chromatin reconstitution. Tissues from plants are thought to have much higher levels of protease and nuclease activities than those of animals or yeast. Therefore, methods to determine the relative activity of proteases and nucleases had to be developed to determine if the template DNA, histones, and chromatin assembly proteins could survive the chromatin assembly reaction. Additionally, methods to streamline the isolation of maize nuclei and purification of histones were developed. This work lays the foundation for future research that could result in extracts to reconstitute plant chromatin *in vitro*.

INTRODUCTION

In eukaryotic cells, the genomic DNA is packaged with histone proteins into chromatin. The basic structural unit of chromatin is the nucleosome core particle, consisting of 146 bp of DNA wrapped 1.65 times around an octamer of histone proteins. The histone octamer consists of two each of the core histones H2A, H2B, H3, and H4, with H2A and H2B forming a tetramer and H3 and H4 forming heterodimers above and below (9). Nucleosomes occur down the length of DNA like "beads on a string" and are separated on average by 29-43 bp of linker DNA.

The assembly of regularly spaced nucleosome arrays is an ATP-dependent process, requiring histone chaperones and oligomeric ATP-dependent remodeling complexes (19). In the process, a single molecule of histone H1 may be associated with the nucleosome to secure the ends of the DNA where it enters and exits the core particle. Additional non-histone proteins and further folding contribute to the formation of the higher-order chromatin structures necessary for compaction of the genome within the nucleus.

The packaging of the genome into chromatin presents an obstacle to the functional processes of DNA. Transcription, replication, recombination, and DNA repair all require access to DNA by trans-acting factors. There are three basic mechanisms a cell may use to alter the chromatin structure in order to increase or decrease accessibility of the DNA for protein interactions. These include nucleosome disruption by ATP-dependent remodeling complexes, incorporation of core histone variants into nucleosomes, and reversible posttranslational modification of histones. Although poorly

understood, these processes allow for the selective activation and silencing of genes necessary to produce the specific transcriptional programs needed for controlling cellular differentiation, organogenesis, organismal development, and response of the organism to changing environmental conditions.

Gene expression is a dynamic process in which chromatin plays a pivotal role through its modifications and structure. This has lead many to view chromatin as an active participant in gene expression as opposed to a hindering element. Research has demonstrated that transcriptional promotion can differ on naked DNA templates versus those assembled into chromatin in otherwise identical systems (12). Also certain properties of transcription factors can be ascertained from chromatin based templates that cannot be seen from naked templates. Chromatin templates are more likely to require the specific transcriptional activators that are needed *in vivo* (12). These findings have led researchers to move towards systems that mimic *in vivo* conditions for investigating transcriptional regulation.

A study involving chromatin assembled through extracts elucidated a fundamental mechanism involved in gene transcription. Two modified forms of the Gal4 protein (a sequence specific DNA binding transcription-factor in yeast) had their transcriptional activity compared in systems with template DNA reconstituted into chromatin. Gal4(1-147) has had its transcriptional activation motif deleted. It demonstrated transcriptional promotion well on naked DNA templates, but not *in vivo* (18). A Gal4-VP16 chimera has had a transcriptional motif from the herpes virus added to it, that is known to recruit histone remodeling complexes (10). Gal4-VP16 had promoted transcription effectively both on naked templates and *in vivo* (16). The transcriptional activity of the two variants

was then tested on preassembled chromatin and the results were similar to results from *in vivo* experiments (4,12). This led to the conclusion that while Gal4(1-147) may bind to its target DNA, nucleosomes are likely blocking the promoter region, preventing transcription. Additionally, Gal4-VP16 was tested in the presence and absence of ATP. The level of transcription was 10× higher in the system containing ATP. This led to the deduction that transcriptional promoters must recruit chromatin remodeling factors, since the remodeling of chromatin is an ATP-dependent function.

Such experiments demonstrate the importance of having an *in vitro* procedure for assembling nucleosomes on a naked DNA template with properties similar to native chromatin. Procedures exist for yeast and animal model systems but have not been developed for plants. (18,5,6). Consequently, much of our understanding of chromatin function in plants comes from transformation studies. The results thus obtained, however, do not give sufficient understanding of the underlying mechanisms, and the gaps are often filled using the knowledge obtained from animal systems.

It was the goal of this project to facilitate future investigations in functional plant genomics by developing a procedure for producing defined plant chromatin templates *in vitro.* This work has the potential to contribute to a better understanding of plant growth and development and responses to the environment.

A greater understanding of plant chromatin structure and dynamics could lead to advancements in applied plant science. For instance, the plant histone 2A variant RAT5 has been shown in *Arabidopsis* to be essential for integration of foreign DNA into the plant's genome by *Agrobacterium*-meditated transformation (11). It has been hypothesized that incorporation of RAT5 into the nucleosome somehow makes chromatin

more accessible to the integration machinery. *In vitro* studies using well-defined chromatin templates could be used to determine the underlying mechanism. A full understanding of this process might one day give us the ability to transform even recalcitrant plant species with high efficiency.

METHODS

Germinated Maize Embryos

Seeds (*Zea mays*, cultivar NK199) were surface-sterilized in 10% commercial bleach with stirring for 10 minutes followed by extensive washing in sterile distilled water. The seeds were then germinated between moist paper towels at 24°C in complete darkness for 2 days. Embryos were manually dissected into ice-cold collection medium [20% w/v sucrose in 50 mM potassium phosphate buffer (pH 7.0)]. Each embryo was closely inspected and cleaned of any contaminating endosperm. Once the harvest was completed, the collection medium was decanted and the embryos were flash frozen in liquid nitrogen and stored at -80°C until use.

Etiolated Maize Seedlings

Seeds (*Zea mays*, cultivar NK199) were surface-sterilized in 10% commercial bleach with stirring for 10 minutes followed by extensive washing in sterile distilled water. The seeds were germinated between moist paper towels at 30°C in complete darkness for 3-5 days. The mesocotyl, coleoptile, radicle, and lateral roots were harvested, flash frozen in liquid nitrogen, and stored at -80°C until use.

Isolation of Nuclei

Intact nuclei were isolated from plant embryos or etiolated seedlings using a procedure adapted from Galbraith *et al* (8). Briefly, 30 g of plant material was pulverized under liquid nitrogen with a mortar and pestle. The pulverized material was then added

to 300 ml of cold Galbraith buffer [20 mM MOPS (pH 7.0), 30 mM sodium citrate, 45 mM $MgCl_2$, 1% v/v Triton X-100]. The suspension was allowed to incubate on ice with gentle stirring for 10 minutes. It was then filtered through a single layer of Miracloth^{TM} (Calbiochem) and centrifuged at 350×g for 15 minutes at 4°C. The pelleted nuclei were gently resuspended in 50 ml of a modified Galbraith buffer lacking Triton X-100 [20 mM MOPS (pH 7.0), 30 mM sodium citrate, 45 mM $MgCl_2$, 1 mM DTT, 0.2 mM PMSF, 2 μM E-64] and centrifuged at 800×g for 10 minutes at 4°C. The supernatant was again decanted and the wash was repeated until the supernatant became clear. The washed nuclear pellet was immediately processed for isolation of soluble chromatin fragments or preparation of crude nuclear extracts.

It was found to be essential to monitor the quality of each preparation of isolated nuclei in order to obtain reproducible results. Contamination with starch granules was assessed by taking a sample from each isolation and staining with an equal volume of I_2/KI (Lugol's solution). The condition of the nuclei and the degree of contamination of the nuclear suspension with cellular debris was assessed by light microscopy after staining with safranin O, which selectively stains the nuclei red.

Isolation of Soluble Chromatin Fragments and Histones

Chromatin was fragmented with micrococcal nuclease (MNase) while still inside the nucleus according to procedures outlined by Bonaldi *et al* (3). Nuclei isolated from 30 g of plant material were washed twice in 50 ml of cold MNase digestion buffer [10 mM Tris-HCl (pH 7.5), 250 mM sucrose, 1.5 mM $MgCl_2$, 1 mM $CaCl_2$, 1 mM DTT, 0.2 mM PMSF, 2 μM E-64], centrifuging at 800×g for 10 minutes at 4°C. After the final

wash, the pellet was resuspended in 5 ml of MNase digestion buffer containing 4000 gel units of MNase and incubated at 37°C for 30 minutes. The reaction was terminated by adjusting the solution to 5 mM EDTA, and the nuclei were pelleted by centrifugation at 800×g for 10 minutes at 4°C. The supernatant was discarded and the nuclei were lysed by resuspension in 6 ml of lysis buffer [1% v/v Nonidet P-40, 10 mM Tris-HCl (pH 7.6), 1 mM EDTA, 0.5 mM DTT, 0.2 mM PMSF, 2 μM E-64] and incubated at 4°C for 30 minutes with agitation. Insoluble material was removed by a 30 minute 10,000×g centrifugation at 4°C. The supernatant was added to 800 μl of a 1:1 slurry of hydroxylapatite and equilibration buffer [100 mM potassium phosphate (pH 6.7), 300 mM NaCl] and incubated at 4°C with agitation for 30 minutes. The hydroxylapatite with bound chromatin fragments was pelleted by a brief low speed spin and the supernatant was discarded. The hydroxylapatite was then washed with 5 ml equilibration buffer [100 mM potassium phosphate (pH 6.7), 300 mM NaCl, 0.5 mM DTT, 0.2 mM PMSF, 2 μM E-64], and the histones were eluted with 200 μl elution buffer [100 mM potassium phosphate (pH 6.7), 2 M NaCl, 0.5 mM DTT, 0.2 mM PMSF, 2 μM E-64]. The purity of the histones was evaluated by SDS-PAGE on a 15% acrylamide gel.

Preparation of Crude Whole Cell Extracts

Crude whole cell extracts for reconstituting chromatin were prepared according to procedures used to make a similar extract from commercial dry yeast (15). Briefly, a BeadBeater (BioSpec Products, Bartlesville, OK) with 2 mm diameter zirconia beads was used to homogenize 10 g of germinated maize embryos or etiolated seedlings in an equal volume of cold extraction buffer [20 mM HEPES-KOH (pH 7.5), 5 mM KCl, 2.5 mM

$MgCl_2$, 1 mM EDTA, 0.5 mM DTT, 10 mM β-glycerophosphate, 10% v/v glycerol, 0.2 mM PMSF, 2 μM E-64]. The cells were broken open by several cycles of 1 minute grinding and 3 minutes rest. The homogenate was filtered through a Buchner funnel, and the filtrate was then centrifuged at 800×g for 5 minutes at 4°C to remove any remaining tissue debris. The supernatant was recovered and centrifuged in a SW-28 Ti Beckman-Coulter rotor at 122,000×g for 2 hours at 4°C to isolate the soluble fraction. The soluble fraction was recovered, aliquoted and stored at -80°C until use. The total protein concentration was estimated by Peterson's modified Lowry assay using chicken ovalbumin as the standard (13).

Preparation of Crude Nuclear Extracts

The washed nuclear pellet was homogenized with a tight-fitting Dounce homogenizer in 6ml of Buffer A [20 mM HEPES (pH 7.9), 25% glycerol, 0.42 M NaCl, 1.5 mM $MgCl_2$, 0.2 mM EDTA, 0.5 mM DTT, 0.2 mM PMSF, 2 μM E-64]. The resulting suspension was incubated at 4°C with agitation for 30 minutes. The bulk of chromatin was then removed by centrifugation at 15,000×g for 30 minutes at 4°C. An ultracentrifuge rotor that could accommodate volumes less than 12 ml was not available, so the cleared homogenate had to be floated in 600 μl microcentrifuge tubes. The homogenate was transferred to microcentrifuge tubes; the tubes were capped, and the caps were rounded off with nail clippers. The microcentrifuge tubes were floated in Beckman 17 ml polyallomer tubes (16 × 102 mm) containing a 30% v/v glycerol, 0.5 M NaCl solution. The tubes were then centrifuged at 122,000×g for 2 hours at 4°C. The supernatant was recovered from each microcentrifuge tube and aliquots were stored at

-80°C until use. The total protein concentration was estimated by Peterson's modified Lowry assay using chicken ovalbumin as the standard (13).

Determining Kinetic Parameters for Proteolysis of Casein by Thermitase

Initial rates for the proteolysis of casein by thermitase were determined by a fluorometric assay using FTC-labeled casein (Pierce) as substrate. The reaction mixtures contained 25 mM Tris-HCl (pH 7.5), 150 mM NaCl, 2-150 μg casein, 0.5 μg FTC-labeled casein, and 14 mU thermitase in a final volume of 140 μl. Fluorescence was recorded at 30ºC at an excitation wavelength of 485 nm and an emission wavelength of 520 nm with a POLARstar OPTIMA fluorescence spectrophotometer. The data was fit with the Michaelis-Menten equation to determine the K_M and V_{max} values using the non-linear least squares regression algorithm Solver, an add-in for Microsoft Excel 2003

Testing Extracts for General Nuclease Activity

All extracts were tested for general deoxyribonuclease activity by a fluorometric assay using the DNaseAlert™ substrate from Ambion, a division of Applied Biosystems. Briefly, 20 μl of extract was combined with 10 μl of water and 37.5 μl of 2×DNase Buffer [10 mM Tris-HCl (pH 7.6), 2.5 mM $MgCl_2$, 0.5 mM $CaCl_2$]. Samples for determining fluorescence background were made using extracts heat-killed by a 20 minute incubation at 65ºC. The reaction was initiated by adding 7.5 μl 10 pmol/μl DNaseAlert™ substrate. Fluorescence was recorded over 2 hours at 30ºC at an excitation wavelength of 544 nm and an emission wavelength of 590 nm in a POLARstar OPTIMA fluorescence spectrophotometer.

The level of deoxyribonuclease activity in the extracts was also checked by agarose gel electrophoresis. Samples contained 2 μg of supercoiled pUC19 plasmid DNA with 10 or 20 μl of extract. The samples were brought to 40 μl with Buffer A and incubated for either 6 or 12 hours at 30ºC. Following phenol/chloroform extraction and ethanol precipitation, the samples were analyzed on a 1.5% Tris-glycine agarose gel to visualize the effects of nucleases within the extracts on the plasmid.

Testing Extracts for General Protease Activity

The level of protease activity was tested in all extracts by a fluorometric assay using FTC-labeled casein (Pierce) as substrate. For the whole cell extracts: 20 μl of extract was added to 1 μl of protease inhibitor cocktail [120 mM EDTA, 24 mM PMSF, 0.24 mM E-64, 64 mM DTT], and 100 μl of 10 ng/μl FTC-Casein in WCE working reagent [25 mM Tris-HCl (pH 7.5), 180 mM NaCl]. For the nuclear extracts: 20 μl of extract was added to 1 μl of protease inhibitor cocktail, and 100 μl of 10 ng/μl FTC-casein in NE working reagent [25 mM Tris-HCl (pH 7.5), 96 mM NaCl]. Samples for determining fluorescence baseline for each sample were made using extracts heat-killed by a 20 minute incubation at 65ºC. Fluorescence was recorded over a 2 hour period at 30ºC at an excitation wavelength of 485 nm and an emission wavelength of 520 nm in a POLARstar OPTIMA fluorescence spectrophotometer.

In Vitro Chromatin Assembly

Assembly of chromatin onto a naked DNA template was attempted according to the method of Rodriguez-Campos *et al* (2, 15). To prepare the chromatin assembly

reaction, 10 or 20 μl of the embryonic crude whole cell or nuclear extract was added to a reaction mix to give a final volume of 34 μl with the following final concentrations: 500 ng of purified core histones, 20 mM HEPES-KOH (pH 7.5), 5 mM KCl, 3 mM $MgCl_2$, 1 mM EDTA, 0.5 mM DTT, 10 mM β-glycerophosphate, 10% v/v glycerol, 40 mM disodium creatine phosphate, 3 mM ATP, 35 ng creatine phosphokinase (Sigma, ≥ 150 units/mg), 0.2 mM PMSF, and 2 μM E-64. Phage λ DNA (NEB 48,502 bps, 1 μl of 200 ng/μl) was then added and the reaction was incubated at 30°C for 8 hours.

Micrococcal Nuclease Digestion Assay

The chromatin assembly reaction was evaluated by partial MNase digestion products (2,18). In this procedure, 1 μl of 106.25 mM $CaCl_2$ was added to 35 μl of the chromatin assembly reaction. MNase (10 gel units) was added and the reaction was incubated at room temperature for 5 minutes. Digestion was stopped by adding an equal volume of MNase stop buffer [20 mM EDTA, 200 mM NaCl, 1% SDS], 4 μl of 20 mg/ml glycogen, and incubating on ice for 10 minutes. The sample was then deproteinized with 80 μg of proteinase K at 55°C for 30 minutes followed by an ethanol precipitation. The DNA was resuspended in $TE_{0.1}$ [10 mM Tris-HCl (pH 8.0), 0.1 mM EDTA] and fractionated on a 1.5% agarose gel in Tris-borate buffer at 5 V/cm. Bands were visualized by staining with ethidium bromide.

RESULTS

Preparation of Plant Tissue

The etiolated plant seedlings yielded about 20 g of usable plant material for every 100 seeds. Embryonic tissue was dissected from germinated seeds in much lower yields - about 20 g for every 300 seeds. Surface sterilization greatly lowered fungal contamination. However, if the seedlings were contaminated with a fungus it spread quickly and would often compromise the entire batch.

Isolation of Nuclei

Approximately 2.5 ml of pelleted nuclei were obtained for every 30 g of plant material. Nuclei obtained from either dissected embryos or etiolated seedlings were stained with Lugol's solution to determine starch contamination. After adding Lugol's solution to the nuclei, the nuclear suspension remained white, which indicated that the nuclear suspension was free of starch. Additionally, the nuclei were stained with safronin O for microscopic examination. The nuclei appeared to be intact and relatively free of debris.

Isolation of Histones

Chromatin was fragmented by MNase digestion while still inside the nucleus. The nuclei were then lysed. If the chromatin had not been fragmented sufficiently, the solution would have become viscous. However, it did not.

Hydroxylapatite strongly binds the positive charges on the histones even at high-salt concentrations. Non-histone proteins will not bind under this condition. This allows for a one-step purification.

As expected, the chromatin bound to hydroxylapatite, and at the salt concentration used, the hydroxylapatite did not appear to bind other proteins. Washing released some histones, indicating the binding sites on the hydroxylapatite were saturated. The SDS-PAGE gel shown in figure 1demonstrates that the all of the core histones and histone H1 could be purified in this manner.

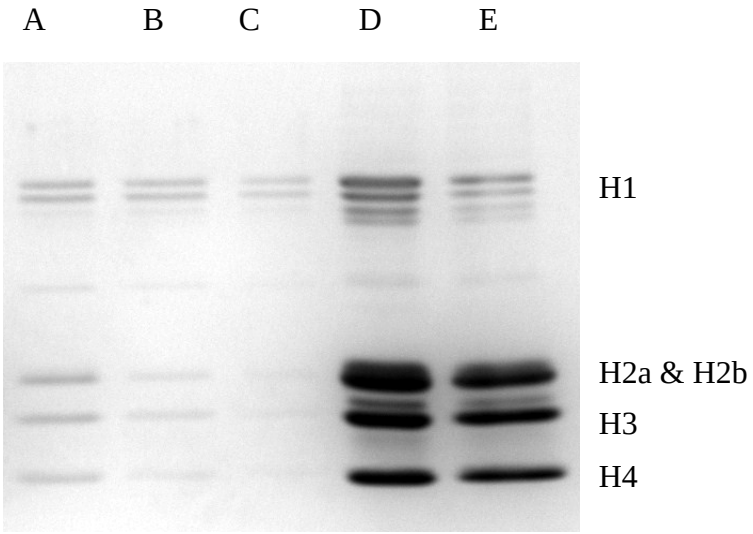

Figure 1. SDS-PAGE Analysis of Histone Purification.
A-C: 30 μl of supernatant from successive washes of hydroxylapatite. D: 15 μl of the first elution. E: 15 μl of second elution. Purified histones were fractionated on a 15% acrylamide gel and stained with Coomassie Blue R-250. Hydroxylapatite was washed with 100mM potassium phosphate (pH 6.7) and 300 mM NaCl. The histones were eluted with 100mM potassium phosphate (pH 6.7) and 2 M NaCl. Molecular weight markers were not used because the positive residues on histone proteins cause them to run aberrantly on gels.

Estimating Acceptable Nuclease and Protease Activities

The histones and DNA to be assembled into chromatin must survive the chromatin assembly reaction. To be useful, then, an extract must contain acceptable deoxyribonuclease and protease activities. The nuclease and protease activities of an extract can be estimated by comparison with standard enzyme preparations of known activity.

An acceptable level for the protease or nuclease activity can be calculated from the integrated Michaelis-Menten equation: $K_M \ln \frac{[S]_0}{[S]} + [S]_0 - [S] = V_{max} t$, where $[S]_0$ and [S] refer to the initial and final substrate concentrations respectively and t is the elapsed time. This calculation requires the K_M and V_{max} values of the standard enzymes for the substrates used in the protease and nuclease assays. The kinetic parameters for degradation of the DNaseAlert^{TM} substrate by DNase I were provided by Ambion, Inc. The kinetic parameters for the proteolysis of casein by thermitase were determined from the dependence of the initial reaction rate on the concentration of casein (figure 2). The results are given in Table 1.

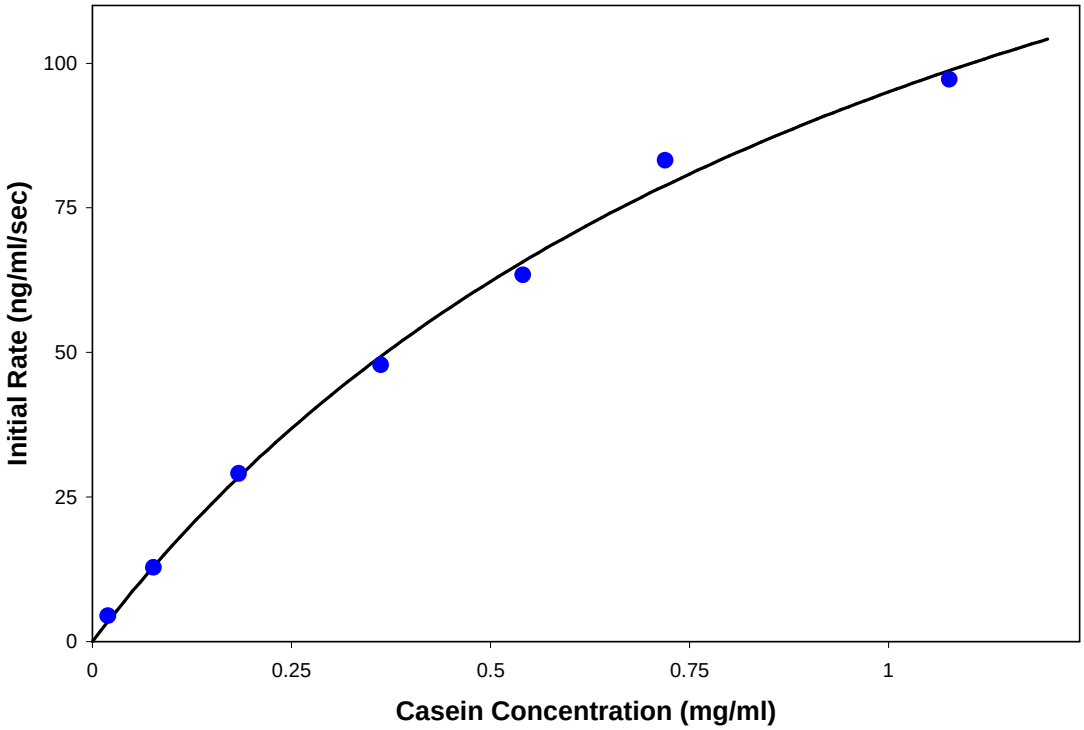

Figure 2. Initial rate of proteolysis as a function of casein concentration. The data was fit with the Michaelis-Menten equation to determine the K_M and V_{max} for the proteolysis of casein by thermitase at 30°C.

Table 1. K_M and V_{max} values for Dnase I and Thermitase

Enzyme	**Substrate**	**K_M (mg/ml)**	**V_{max} (mg/ml/sec)**
DNase I (EC 3.1.21.1)	DNaseAlert™	4.47×10^{-3} [a]	5.80×10^{-6} [a] (for 2 mU/µl)[b]
Thermitase (EC 3.4.21.66)	casein	1.11	2.0×10^{-4} (for 0.1 mU/µl)[c]

[a] Ambion, Inc. (personal communication)
[b] One unit of DNase I is defined as the amount of enzyme required to completely degrade 1 µg DNA in 10 min at 37°C, and is equivalent to 0.04 Kunitz units.
[c] One unit of thermitase is defined as the amount of enzyme required to decrease the absorbance of a 1% (v/v) nonfat powdered milk solution by 1 absorbance unit at 595 nm in 60 minutes at 37°C in a total reaction volume of 200 µl.

It was decided that to be useful, the chromatin assembly reaction should at the very least retain 50% of the DNA and protein after 8 hours of incubation. By these criteria, extracts exhibiting the equivalent of 2.5 mU or less DNase I activity in the nuclease assay and 0.5 mU or less thermitase activity in the protease assay could potentially be useful for chromatin assembly reactions.

Nuclease Activity

Two methods were used to assess the level of deoxyribonuclease activity in the extracts, with varying results. In the fluorometric assay the level of nuclease activity was observed to be high in the nuclear and the whole cell extracts produced from etiolated seedlings (NE and WCE, respectively), while the nuclear and whole cell extracts made from germinated embryos exhibited relatively low levels (NEemb and WCEemb, respectively) as shown in figure 3. The fluorescent signal reached a plateau in assays of

the NE and WCE. This indicates the nuclease activity was great enough in both extracts to deplete the substrate within a relatively short incubation time.

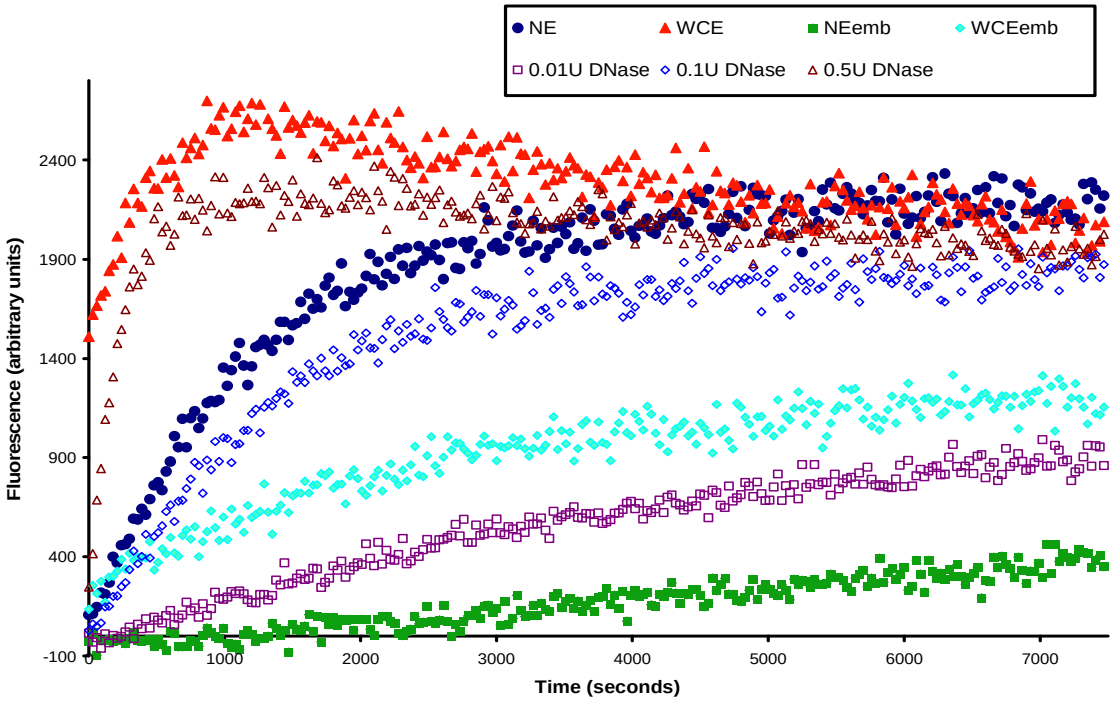

Figure 3. Fluorometric Nuclease Assay for Determining General Nuclease Activity. Briefly, 20 µl of extract was combined with 10 µl of water and 37.5 µl of 2×DNase Buffer [10 mM Tris-HCl (pH 7.6), 2.5 mM $MgCl_2$, 0.5 mM $CaCl_2$] and fluorescence was measured every 30 seconds for a 2 hour period at an excitation wavelength of 544 nm and an emission wavelength of 590 nm at 30° C.

When nuclease activity was analyzed by agarose gel electrophoresis, the WCE appeared to have little nuclease activity, with the exception of light smears at the higher concentration of extract (figure 4). All other results of the gel electrophoresis assay were consistent with results from the fluorometric assay as shown in figures 4 and 5.

NE	-	-	-	-	+	+	+	+
WCE	+	+	+	+	-	-	-	-
10µl	-	+	-	+	-	+	-	+
20µl	+	-	+	-	+	-	+	-
6 hr	-	-	+	+	-	-	+	+
12 hr	+	+	-	-	+	+	-	-

pUC19

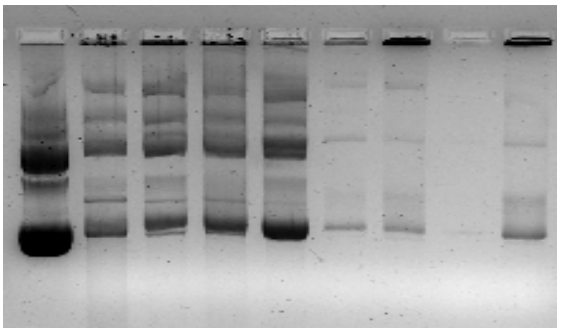

Figure 4. Gel Nuclease Assay for Whole Cell and Nuclear Extracts from Etiolated Seedlings. 2 µg of pUC19 was incubated with either 10 or 20 µl of the extract for either 6 or 12 hours at 30° C.

NEemb	-	-	-	-	+	+	+	+
WCEemb	+	+	+	+	-	-	-	-
10ul	-	+	-	+	-	+	-	+
20ul	+	-	+	-	+	-	+	-
6 hr	-	-	+	+	-	-	+	+
12 hr	+	+	-	-	+	+	-	-

pUC19

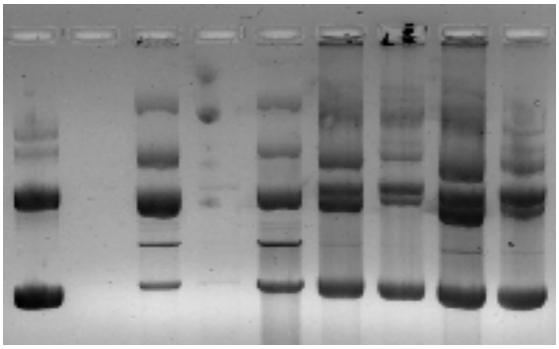

Figure 5. Gel Nuclease Assay for Whole Cell and Nuclear Extracts from Germinated Embryos. 2 μg of pUC19 was incubated with either 10 or 20 μl of the extract for either 6 or 12 hours at 30° C.

Protease Activity

A fluorometric assay (figure 6) was also used to assess the level of protease activity in the extracts. The assay was unable to detect any protease activity in the WCE, NE, or NEemb. The only extract with observable protease activity was the WCEemb, and its activity was much less than 0.1 mU of thermitase.

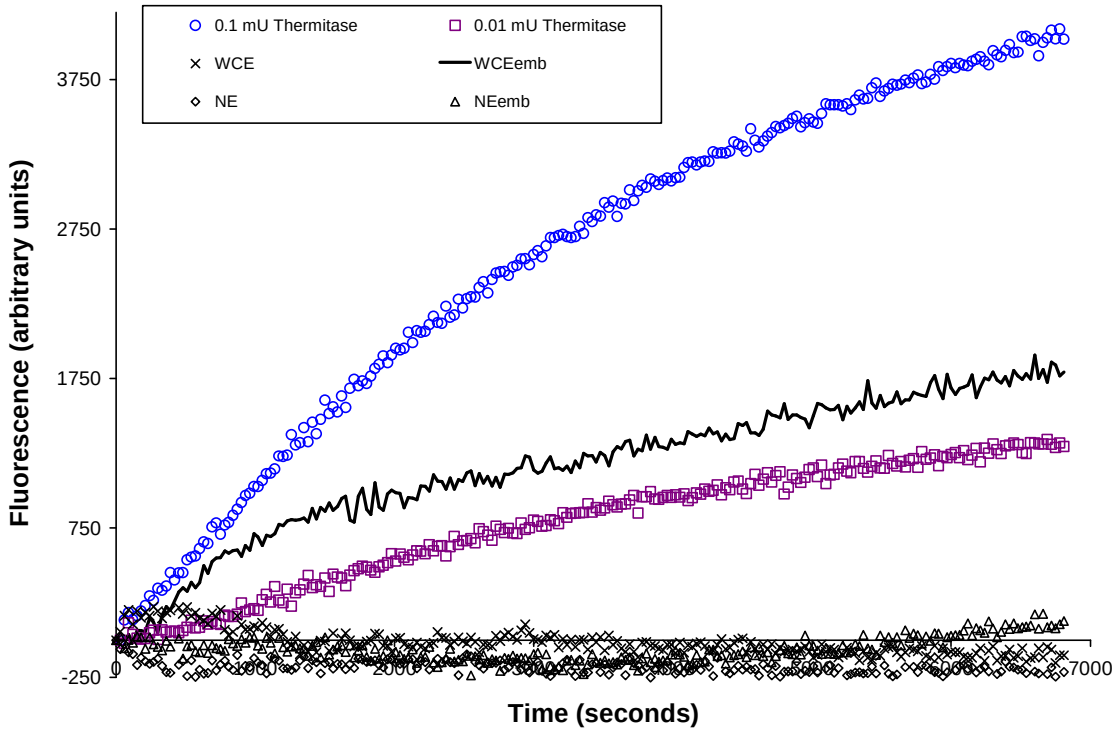

Figure 6. Fluorometric Protease Assay for Testing General Nuclease Activity. Fluorescence was measured every 30 seconds for a 2 hour period at an excitation wavelength of 485 nm and an emission wavelength of 520 nm at 30° C. For whole cell extracts: 20 µl of extract was added to 1 µl of protease inhibitor cocktail [120 mM EDTA, 24 mM PMSF, 0.24 mM E-64, 64 mM DTT], and 100 µl of 10 ng/µl FTC-Casein in WCE working reagent [25 mM Tris-HCl (pH 7.5), 180 mM NaCl]. For nuclear extracts: 20 µl of extract was added to 1 µl of protease inhibitor cocktail, and 100 µl of 10 ng/µl FTC-casein in NE working reagent [25 mM Tris-HCl (pH 7.5), 96 mM NaCl].

Micrococcal Nuclease Digestion Assay

The MNase digestion assay (figure 7) showed that chromatin assembly did not occur. If the bacteriophage λ DNA had been assembled into chromatin, the gel would have shown regularly spaced bands approximately 200 base pairs apart. Instead, the gel shows a band that is likely the uncut phage λ DNA with a smear of DNA below.

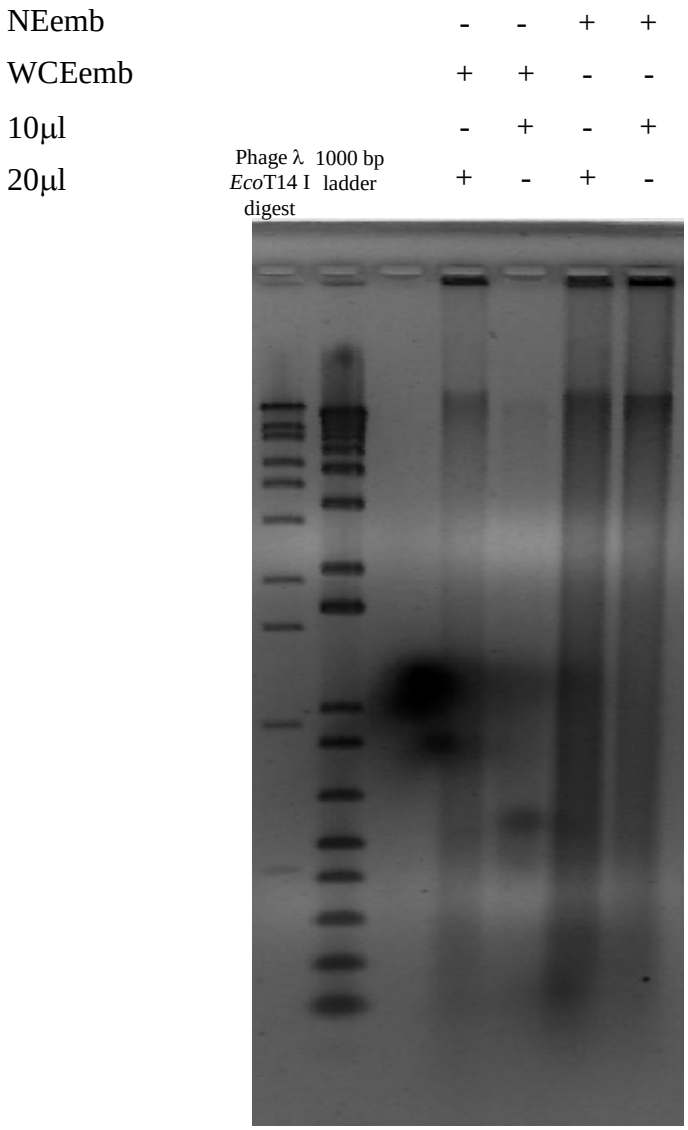

Figure 7. Micrococcal Nuclease Digestion Assay for Determination of Chromatin Assembly. Either 10 or 20 µl of extract was incubated at 30° C for 8 hours in a 35 µl chromatin assembly reaction containing 200 ng phage λ DNA, 500 ng of purified core histones, 20 mM HEPES-KOH (pH 7.5), 5 mM KCl, 3 mM $MgCl_2$, 1 mM EDTA, 0.5 mM DTT, 10 mM β-glycerophosphate, 10% v/v glycerol, 40 mM disodium creatine phosphate, 3 mM ATP, 35 ng creatine phosphokinase, 0.2 mM PMSF, and 2 µM E-64. The presence of assembled chromatin was tested by partial micrococcal nuclease digestion. A ladder of bands ~200 bp apart would have indicated the assembly of chromatin with regularly spaced nucleosomes.

DISCUSSION

Preparation of Plant Tissue

Large amounts of usable material can be generated from etiolated seedlings. With this method sufficient plant material plant material could be harvested in five minutes. Isolating embryos is considerably more laborious. The embryos must be dissected out of a germinated seed and individually inspected and cleaned of any endosperm. Consequently, it can take upwards of 3 hours to harvest 20 g of germinated embryonic tissue. The benefit of using embryonic tissue is that the majority of cells are actively dividing and should have high levels of chromatin remodeling proteins. Exclusively harvesting the meristems of the radicle and coleoptile from etiolated seedlings would likely produce tissue with equal amounts of chromatin assembly proteins, with considerably less effort involved.

Isolation of Nuclei

A variety of methods were tested for releasing intact nuclei. These included bead beating, manually slicing etiolated seedlings with a razor blade, pulverizing tissue under liquid N_2 with a mortar and pestle, and the use of various buffers. Pulverizing the tissue gave the best results. It produced high amounts of nuclei that were free of starch and largely free of cellular debris. Nuclei from 30 g of plant material could be isolated in less than 2 hours, making it possible to perform downstream applications the same day as the nuclei were extracted.

Isolating Histones

When purifying histones, the chromatin contained within the isolated nuclei must be solubilized. This is done by breaking or digesting the chromatin into smaller fragments. Two different methods were tested to solubilize chromatin. Sonicating lysed nuclei failed to adequately relieve the solution of its viscosity. With this method the chromatin was not sufficiently solubilized, so the majority of the chromatin was pelleted with the unlysed nuclei and large chromatin fragments when centrifuged in preparation for chromatography. Consequently, the supernatant which was applied to a hydroxylapatite column did not contain many histones, and upon elution few or no histones were observed by SDS-PAGE (data not shown).

It was later found that treating intact nuclei with MNase consistently solubilized the chromatin. After lysing the nuclei to release the solubilized chromatin, the viscosity of the solution was never observed to have noticeably increased with this method. However, determining an adequate method of lysing the nuclei proved an arduous task. After conducting an extensive literature review several different nuclear lysis buffers were tested. Hypotonic buffers failed to efficiently lyse the nuclei, so a number of different detergents were tested. Sufficient nuclear lysis did not occur in buffers containing 1% w/v deoxycholate, 0.8-5% w/v SDS, or with the two in combination at concentrations of 0.1% and 1%, respectively. Interestingly, Nonidet P-40 at 1% v/v consistently lysed the nuclei. Nonidet P-40 is a generally mild detergent. It is often used for its ability (at concentrations below 0.5% v/v) to selectively lyse the cytoplasmic membrane while leaving the nuclear membrane of cells intact. As it is a nondenaturing nonionic detergent, Nonidet P-40 did not interfere with the binding of nucleosomes by

hydroxylapatite. Nonidet P-40 absorbs strongly at 260 nm which prevented the accurate measurement of DNA present in the solution. Though not critical, the amount of hydroxylapatite to use had to be estimated based on previous experiments using other detergents (data not shown).

During the first attempts to purify histones, solubilized chromatin was applied to a small hydroxylapatite column (1 ml bed volume). After extensive washing, the histones were eluted by increasing the salt concentration. Yet even with the use of a step gradient, the eluted histones were much too dilute for this study.

A batch method was deemed more suitable. The batch method is only possible because histones bind strongly to hydroxylapatite under conditions in which other proteins will not bind. Consequently, in the batch method a minimal amount of hydroxylapatite can be used and the dilution of the histones can be avoided. In this method the solubilized chromatin is incubated with the hydroxylapatite. The hydroxylapatite could then be spun down and transferred to a smaller tube, from which the histones could be eluted. Instead of passing a solution through a column slowly eluting the histones, the hydroxylapatite could be incubated with the elution buffer and have the histones elute simultaneously.

Nuclease Activity

Cellular extracts for the reconstitution of chromatin have been developed using animal embryos, but tissues from plants are thought to have much higher levels of protease and nuclease activities (17,19,14). This is one of the reasons that an *in vitro* transcription assay had not been developed with plant extracts until 16 years after the first

one was developed with animal extracts (17). Nucleases within the extract cannot simply be inactivated by chelating agents. The same ions used by nucleases may be cofactors for chromatin assembly proteins. For example Mg^{2+} is known to be necessary for the production of properly spaced nucleosomes (1). With these problems known it was clear that an assay for determining nuclease activity within extracts had to be developed. This assay would at least help establish the suitability of the extracts in a chromatin assembly reaction.

The first assay attempted used thiazole orange, which fluoresces upon intercalation into double stranded DNA. The expected outcome in an extract containing nucleases would be high fluorescence initially that curtailed as nucleases cleaved the DNA and released the fluorophore into solution. This method worked well with standards. However, because there was additional DNA in the extracts, the fluorescent signal decreased much more slowly and could not be quantified by comparison to standards. This was particularly true for the nuclear extracts.

To circumvent this problem a cleavable fluorescent-labeled DNase substrate that would fluoresce upon its cleavage was added to the extracts. The results from this assay were consistent and revealed that the all of the extracts, except possibly the nuclear extract prepared from germinated embryos, contained too much nuclease activity to be useful.

The plasmid to be assembled into chromatin must be able to remain intact after up to 8 hours in solution with the extracts. To visualize the effects of nuclease activity in the extracts, an assay was performed that involved incubating a plasmid with the extracts and determining whether sufficient levels were left intact. The results were consistent with

those of the fluorescence assay with the exception of the WCE extract. The reason for this discrepancy is unknown. However, the DNaseAlert™ substrate used in the fluorescence assay is designed to detect the presence of nucleases capable of cutting either single- or double-stranded DNA. It may be, then, that the WCE extract contained significant single-stranded nuclease activity, which would not be detected with the agarose gel assay.

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Protease Activity

The histones and chromatin assembly proteins must be able to survive extended incubations with the extract. A fluorescent assay was performed using FTC-casein to determine the amount of protease activity relevant to the protease thermitase. Serine and cysteine proteases were inhibited by PMSF and E-64, respectively. Additional protease activity appeared to be absent in all the extracts with the exception of the WCEemb extract. In the WCEemb extract the protease activity was still well below the acceptable level.

Chromatin Assembly and Micrococcal Nuclease Digestion Assay

Due to the results of the nuclease assay, we tested only the embryonic extracts for the ability to promote chromatin folding on a naked DNA template. The reaction was evaluated by a partial MNase digestion. With DNA incorporated into chromatin, MNase can cleave only the linker DNA between the nucleosomes. Analyzing the product of this digestion on a gel should produce a ladder of bands ~200 base pairs apart if regularly spaced nucleosomes were produced (18, 2). Instead a single band (the template DNA)

was observed followed by a smear. Since the digestion created a smear instead of a ladder of bands there must not have been an obstacle to the MNase's cleavage of the DNA, suggesting that no chromatin was assembled.

Conclusion

Embryonic extracts show the most promise for continued research. Most cells within the embryonic tissue are undergoing rapid division and thus high levels of chromatin assembly proteins should be present. Also, protease activity in the extracts is completely inhibited by the irreversible protease inhibitors PMSF and E-64. To be useful, however, the nuclease activity of the extracts must be lowered.

While the extracts may have adequate amounts of chromatin assembly proteins, it is also possible that secondary metabolites present in the extracts could have inhibited the process. This is believed to have been a problem in early attempts at developing extracts for plant *in vitro* transcription assays (17). Such compounds, undoubtedly present in the crude extracts, may inhibit chromatin assembly proteins as well.

Perhaps ammonium sulfate precipitation could be used to remove the chromatin assembly proteins from nucleases and interfering compounds. This approach was used in the development of an *in vitro* transcription assay for plants (17). To guide the process, ammonium sulfate cuts of a simple chromatin assembly extract from yeast could be followed in parallel. This strategy should be successful if the chromatin assembly proteins are as highly conserved between plants and yeast as the histones are.

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