Analysis of Nucleosomal Deoxyribonucleic Acid in a Higher Plant

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Comparisons of nuclease digestions of chromatin in nuclei from rye embryos and rat liver show that their nucleosomal DNA is similar, i.e. DNA subunits consist of 200 base-pair repeats with 140 base-pair cores of identical substructure. The identical nucleosome structure is present in nuclei from cells of rye embryos that have been non-viable (i.e. dead) for more than 7 years. These findings indicate a high degree of stability of the DNA–histone complex and are consistent with conservation of the nucleosomal structure of chromatin during evolution.

The proposed substructure of chromatin envisages repeating nucleoprotein subunits or ‘nucleosomes’, each nucleosome consisting of DNA associated with a histone core of two molecules each of histones F2A1, F3, F2A2 and F2B (Kornberg, 1974; Van Holde *et al.*, 1974; Baldwin *et al.*, 1975).

The presence of nucleosomal structures in such divergent groups as vertebrates (Burgoyne *et al.*, 1974; Shaw *et al.*, 1976), a dicotyledonous plant, *Pisum sativum* (pea) (McGhee & Engel, 1975), eukaryotic micro-organisms (Lohr & Van Holde, 1975; Noll, 1976; Morris, 1976a) and viruses (Griffith, 1975; Corden *et al.*, 1976) indicates the stability and conservation of the histone–DNA assembly during evolution.

The nucleosome structure of chromatin is based on evidence from: (a) electron micrographs of isolated chromatin, which reveal uniformly spaced beaded structures (Woodcock, 1973; Olins & Olins, 1974; Oudet *et al.*, 1975; Finch *et al.*, 1975; Woodcock *et al.*, 1976a,b); (b) biochemical analysis of histone–histone interactions (Kornberg & Thomas, 1974; D’Anna & Isenberg, 1974); and (c) characterization by polyacrylamide-gel electrophoresis of the size classes of the DNA digestion products from isolated nuclei incubated with endogenous nuclease (Williamson, 1970; Hewish & Burgoyne, 1973; Burgoyne *et al.*, 1974) or with added micrococcal or staphylococcal nuclease (Noll, 1974a; Axel *et al.*, 1974; McGhee & Engel, 1975; McGhee & Kimmel, 1975).

The DNA of rat liver nucleosomes has been carefully characterized by calibration with DNA fragments produced by restriction-endonuclease digestion and found to be about 200 base-pairs in length [205 ± 15 (Noll, 1974a); 198 ± 6 (Noll & Kornberg, 1976); 200 ± 4.5 (Morris, 1976b)] with a

relatively nuclease-resistant DNA core of 140 base-pairs in length (Noll & Kornberg, 1976). By using the same methods and markers, a shorter nucleosomal repeat has been found in yeast (165 ± 6 ; Thomas & Furber, 1976), in *A. nidulans* (154 ± 9 ; Morris, 1976a) and in *N. crassa* (170 ± 5 ; Noll, 1976), but in each the nucleosomal DNA core is similar to that in rat (Morris, 1976a; Noll, 1976). For both the pea plant and for mouse myeloma cells, McGhee & Engel (1975) have reported a DNA repeat of 170 ± 30 base-pairs. The value for the mouse myeloma monomer (and for the pea) was subsequently revised to 140 base-pairs (McGhee & Kimmel, 1975). Therefore, although shorter nucleosome repeats exist in fungi, the low value obtained for a higher plant has yet to be confirmed.

For an accurate determination of the length of DNA within the nucleosome, reliable DNA marker fragments are required for comparison. In addition, the method of this determination is important (Noll *et al.*, 1975). Noll & Kornberg (1976) have demonstrated that exonuclease activity of micrococcal nuclease produces smaller DNA fragments from chromatin at 37° than at 2°C . This effect on nucleosomal-DNA-length determinations can be eliminated by measuring differences between successive DNA multiples rather than by determining the size of the smallest fragments from known markers.

A study of the integrity of DNA during the loss of viability in embryos of the monocotyledon, *Secale cereale* (rye) has established the subunit nature of rye chromatin (K. S. E. Cheah & D. J. Osborne, unpublished work). The present paper reports: (1) that by comparison with precisely calibrated DNA standards from rat and *A. nidulans* nucleosomes, the length of nucleosomal DNA in a higher plant is similar to that of rat, i.e. 200 ± 4.5 base-pairs

(Morris, 1976b); (2) that the nucleosome structure remains fully conserved in nuclei from embryo cells of grain that has been dead for at least 7 years.

Experimental

Materials

Embryos from *Secale cereale* (rye), either fully viable, i.e. 100% germination (Nunns Corn and Coal Co., Woodbridge, Suffolk, U.K.) or from dead grain, i.e. 0% germination for previous 7 years (a gift from Welsh Plant Breeding Station, Aberystwyth, Dyfed, Wales, U.K.), were isolated by the method of Johnston & Stern (1957).

Micrococcal nuclease (EC 3.1.4.7) from *Staphylococcus aureus* was obtained from Sigma (London) Chemical Co., Kingston-upon-Thames, Surrey, U.K. (1 unit will produce 1 μ mol of acid-soluble polynucleotides from DNA/min at pH 8.6, 37°C). DNAase I (deoxyribonuclease I; EC 3.1.4.5; Worthington) from bovine pancreas (ribonuclease-free) was purchased from Cambrian Chemicals, Croydon, Surrey, U.K.; 1 unit causes an increase in A_{260} of 0.001/min per ml at 25°C, pH 5.0, with highly polymerized DNA as substrate.

RNAase I (ribonuclease A; EC 3.1.4.22) from bovine pancreas was also from Cambrian Chemicals (3000 units/mg; 1 unit causes an increase in A_{260} of 1.0/min at 37°C, pH 5.0). Agarose for electrophoresis was from Sigma, and metrizamide [2-(3-acetamido-5-*N*-methylacetamido-2,4,6-tri-iodobenzamido)-2-deoxy-D-glucose], used in the preparation of nuclei, was obtained from Nyegaard and Co. A.S., Oslo, Norway. All other chemicals were AnalaR grade from BDH, Poole, Dorset, U.K.

Nucleosome DNA fragments from rat liver and *A. nidulans* were a gift from Dr. N. R. Morris, and rat liver nucleosome core DNA and DNAase I-digestion products were a gift from Dr. L. Lutter, both at the MRC Laboratory of Molecular Biology, Cambridge.

Methods

Preparation of rye-embryo nuclei. Nuclei from isolated viable and dead rye embryos were prepared by a modification of the method of A. J. Trewavas (personal communication). All procedures were carried out at 0–2°C. Hand-selected rye embryos were homogenized in a mortar and pestle in 1.0 ml of sucrose/Buffer A' (after Hewish & Burgoyne, 1973), consisting of 0.3 M-sucrose in 10 mM-KCl/15 mM-NaCl/15 mM- β -mercaptoethanol (Sigma)/15 mM-Tris/HCl (pH 7.4 at 20°C)/0.15 mM-spermine/0.5 mM-spermidine (both from Sigma). The homogenate was made up to 10.0 ml and filtered through three layers of Miracloth (Calbiochem Ltd., Hereford, U.K.). The residue was washed with sucrose/Buffer A, Triton X-100 (BDH) added to a final concentration of 1% (v/v), and the nuclei were pelleted at 625g for

6 min at 2°C in an MSE bench centrifuge. The pellet was drained, resuspended in 1.0 ml of 40% (w/v) metrizamide (in 0.3 M-sucrose/Buffer A) and layered on a two-step gradient consisting of 40 and 50% (w/v) metrizamide/0.3 M-sucrose/Buffer A solution (0.75 ml of each). The samples were centrifuged for 15 min at 44 720g ($r_{av.}$ = 6.4 cm) and 2°C in the SW65 rotor of a Beckman model L2-65B ultracentrifuge. After centrifugation, the nuclear layer formed at the 40–50% (w/v) metrizamide interphase was removed, and re-pelleted at 625g. Viable-embryo nuclei prepared in this way were free from cytoplasmic and cell-wall contamination, as judged by electron microscopy and fluorescence optical microscopy, and from RNA as shown by polyacrylamide-gel electrophoresis of the nuclear DNA samples. Nuclear preparations from dead embryos contained some cytoplasmic components, but as a routine were treated with RNAase A (preheated at 90°C for 10 min to remove DNAase activity) before electrophoresis.

Preparation of viable-embryo nucleosome DNA.

(a) Micrococcal nuclease digestion products. Nuclei prepared from 600 viable rye embryos were incubated at 37°C for 30 s–7 min with 10 units of micrococcal nuclease in 1.0 ml of 0.3 M-sucrose/Buffer A containing 1.0 mM-CaCl₂. The reaction was stopped by the addition of sodium dodecyl sulphate to 2.5% (w/v), then an equal volume of 0.3 M-NaCl/0.2 M-EDTA (pH 8.0 at 20°C), and NaClO₄ to 0.5 M. The samples were deproteinized three times by shaking with an equal volume of chloroform/3-methylbutan-1-ol (24:1, v/v) and the aqueous layer was dialysed against electrophoresis buffer [40 mM-Tris/acetate (pH 7.8 at 20°C)/20 mM-sodium acetate/20 mM-EDTA] at 2°C. The DNA samples were concentrated to approx. 0.1 ml by surrounding the dialysis bag with Carbowax 20000 (Searle Diagnostic, High Wycombe, Bucks., U.K.). Glycerol was added to 5% (v/v) and Bromophenol Blue to 2.5% (v/v).

(b) DNAase I digestion products. Nuclei from 600 viable rye embryos were incubated at 37°C for 30 s with 300 units of DNAase I in 1.0 ml of sucrose/Buffer A containing 5 mM-MgSO₄ and 5 mM-CaCl₂. The samples were deproteinized as described above, the DNA was dialysed overnight at 2°C against 1000 vol. of water, concentrated, sucrose added to 20% (w/v) and the mixture denatured in boiling 50% (v/v) formamide.

Gel electrophoresis. Large DNA fragments were separated on vertical 2.0% (w/v) agarose slab gels (0.3 cm $\times$ 14.5 cm $\times$ 15.5 cm) prepared by dissolving agarose in boiling electrophoresis buffer. Lower-molecular-weight DNA fragments were separated in 6% (w/v) polyacrylamide slab gels. Denatured DNA fragments produced by DNAase I digestion of chromatin were separated under denaturing conditions on 10% (w/v) polyacrylamide slab gels con-

EXPLANATION OF PLATE I

Comparison by agarose-gel electrophoresis of rat and rye nucleosomal DNA from brief micrococcal-nuclease digests
 Rye nuclei were incubated with micrococcal nuclease (10 units/ml) for 30s at 37°C, and DNA fragments were prepared as described in the text. Slots contained 10–15 µg of DNA from (a) rye, (b) rat and (c) rye.

EXPLANATION OF PLATE 2

Time course of micrococcal-nuclease digestion of rye chromatin and comparison with rat liver and A. nidulans
Rye nuclei were incubated with micrococcal nuclease (10 units/ml) for 1–7 min at 37°C. The DNA fragments prepared as before were separated on 6% (w/v) polyacrylamide gels. DNA samples (10 µg, except where indicated) in each slot were from: (a) rye (7 min digest); (b) rat liver markers; (c) rat liver purified 140-base-pair DNA core marker; (d) *A. nidulans* DNA core marker; (e) rye (7 min digest) 5 µg load only; (f), (g), (h) and (i) rye, 7 min, 4 min, 2 min and 1 min digests respectively; markers (b) and (d) were a gift from N. R. Morris, and marker (c) was a gift from L. Lutter.

EXPLANATION OF PLATE 3

Comparison of DNAase-I digests of rat and rye chromatin

Rye nuclei were incubated with DNAase I as described in the text, and the fragments were denatured and separated under denaturing conditions in 10% (w/v) polyacrylamide gels containing 7M-urea. DNA samples in the gel slots were from: (a) rye (15 μ g); (b) rat; (c) rye (10 μ g).

EXPLANATION OF PLATE 4

Comparison of nucleosomal DNA lengths in nuclei from fully viable and dead rye embryos by agarose-gel electrophoresis

Nuclei were incubated with 2 units of micrococcal nuclease/ml first for 30s at 37°C, and then transferred to 0°C for 10min. The reaction was stopped with EDTA (pH 7.0, at 20°C) to 20mM. Digests were incubated with 100µg of ribonuclease A for 10min/ml at 37°C, and the DNA was extracted and separated on 2.0% (w/v) agarose gels as described. Gel slots contained DNA from nuclei of (a) viable (20µg), (b) dead (10µg) and (c) viable (10µg) rye embryos.

taining 7M-urea. Electrophoresis conditions for both agarose and polyacrylamide gels were 25 mA and 35 V for 16 h at room temperature (20–24°C).

Determination of DNA lengths. Determination of DNA fragment lengths in rye nucleosomes produced by micrococcal-nuclease and DNAase -I digestions was by comparison with nucleosomal DNA from rat liver and *A. nidulans*. These DNA markers had been previously calibrated with fragments produced by digestion with restriction-endonuclease. Rat liver and *A. nidulans* micrococcal nuclease digests were performed as for viable rye embryos, at 37°C (Morris, 1976a).

Results and Discussion

Electrophoresis of DNA fragments produced by brief micrococcal-nuclease digestions of nuclei of viable rye embryos and rat liver show a nucleosomal DNA repeat of similar size (i.e. 200 ± 4.5 base-pairs), up to the 8×200 base-pair unit (Plate 1). That differs from that in *A. nidulans*. With time, the DNA within the nucleosome is digested to a relatively resistant fragment, the nucleosome 'core' (Plate 2), which is similar in size to that of both rat and *A. nidulans*, i.e. 140 base-pairs. This nucleosome core of rye is further cleaved into three smaller size classes, of approx. 125, 100 and 90 base-pairs. Thus rye nucleosomes contain a basic DNA unit of 200 base-pairs, of which 140 are relatively more resistant to nuclease. This estimate for rye differs from that of another higher plant, the pea (170 ± 30 , revised to 140; McGhee & Engel, 1975; McGhee & Kimmel, 1975). The nucleosome DNA repeat for pea may not, however, be different from rye, for the following reasons. As discussed in the introduction, the method of determining DNA length is critical. That used for determining the pea repeat was by estimation of the length of the smallest DNA fragment produced after extensive (20 min) digestion with micrococcal nuclease at 37°C (McGhee & Engel, 1975). At 37°C the exonuclease activity of micrococcal nuclease would give a monomer fragment of smaller size (Noll & Kornberg, 1976). Since the DNA repeat was not determined by difference between the different repeating units, but by comparison with the smallest fragment, a low estimate would be derived. In addition, the long digest times would generate nucleosome cores, not monomers, which would again result in a low estimate.

DNAase-I digests of rat nuclei (Noll, 1974b) and *A. nidulans* (Morris, 1976a) yield DNA fragments which can be separated as an increasing arithmetical series of ten nucleotides. The same pattern of DNA fragments (20–120 nucleotides) is produced in rye (Plate 3), indicating a similar regular internal structure of the rye nucleosome.

Although in both rat and rye the nucleosomal DNA repeat is different from that of *A. nidulans* (Morris, 1976a) and *N. crassa* (Noll, 1976), the relatively nuclease-resistant DNA core and its internal substructure are the same. This difference is presumed to lie in the length of the DNA linking nucleosome cores (Morris, 1976a; Noll, 1976). The physiological function of different lengths of DNA linker is as yet unknown. However, differences in nucleosomal DNA lengths within a single organism have been found, e.g. in erythrocyte and liver of chick (Morris, 1976b; N. R. Morris, personal communication) and may be related to regulatory mechanisms of gene function. As yet there is no comparable information available for plants.

Evolutionary conservation of the histone structure is least in histone F1 (Elgin & Weintraub, 1975). Since histone F1 is absent from the core (Kornberg & Thomas, 1974), but is associated with the linker (Noll & Kornberg, 1976), it has been suggested (Noll, 1976; Morris, 1976a) that the different DNA linker length found in fungi may be related to the difference in size and structure of histone F1. In higher plants, histone F1 does not appear to differ qualitatively from that of higher animals (Gofshtein, 1968; De Lange & Smith, 1971); where differences have been found, they have been attributed to proteolysis during the extraction procedure (Towill & Nooden, 1973). Macro- but not micro-nuclei in *Tetrahymena* do, however, appear to lack detectable histone F1 (Gorovsky & Keevert, 1975). Despite the absence of histone F1, it is claimed that the nucleosome repeat of both types of nuclei is similar to that of calf thymus. However, small differences in the nucleosomal DNA repeat are difficult to evaluate when the numbers of DNA fragments generated are few. Further work is therefore necessary to determine whether these findings are at variance with the suggestions of Noll (1976) and Morris (1976a). Embryos of dry seeds that have lost viability ('dead') afforded an opportunity to assess the stability of the histone-DNA complex within naturally senescent cells that have not been exposed to conditions of chemical or low-temperature preservation. Comparisons of the DNA fragments produced from micrococcal-nuclease digests of nuclei from viable and dead rye embryos show a similar digestion pattern and nucleosomal DNA repeat (Plate 4). Thus it can be inferred that the nucleosome structure is stable long after cell death.

Our results from rye are evidence from plants for a characteristic longer nucleosomal DNA repeat in higher eukaryotes, and for an evolutionary conservation of the nucleosome core and its internal structure, common to all eukaryotes. There is no reason therefore to believe that the nucleosomal DNA repeat differs between higher plants and animals. Further, in nuclei from embryo cells that

are known to have been dead for at least 7 years, the nucleosome structure is retained intact and the DNA repeat is unaltered. This stability of the nucleosome structure within chromatin may have an important bearing on the ubiquity of such repeating units in the conservation of the genetic material in living organisms.

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