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X-ray Diffraction Analysis of Crystals Containing Twofold Symmetric Nucleosome Core Particles

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Abstract

Nucleosome core particles containing a DNA palindrome and purified chicken erythrocyte histone octamer have been reconstituted and crystallized. The dyad symmetry of the palindrome extends the dyad symmetry of the histone octamer to result in a twofold symmetric particle. This ensures that the structure determined by X-ray diffraction will yield a true representation of the DNA strand rather than the twofold averaged structure which would result from using a non-palindromic DNA sequence. The crystals provide isotropic diffraction to 3.2 Å with observed reflections extending to d spacings of about 2.8 Å using a rotating-anode Cu$K\alpha$ X-ray source. Although the DNA palindrome is a factor contributing to the quality of the diffraction data, another significant factor is an improved preparative technique which enriches for correctly phased nucleosome core particles.

1. Introduction

The nucleosome is the fundamental repeating unit of chromatin structure. The complete nucleosome unit is composed of a repeat length of DNA organized by an octamer of core histone proteins and one molecule of histone H1 or an H1-like histone. The DNA repeat length consists of a variable-length linker segment and about 168 bp of DNA bound to the histone octamer as two full turns of a left-handed superhelix which further binds a molecule of histone H1 to form the chromatosome. The core particle of the nucleosome is defined based on nuclease digestion patterns of chromatin and consists solely of 146 bp of DNA organized in 1.8 superhelical turns around the histone octamer (van Holde, 1988). The histone octamer is further divided into a heterotetramer of two units each of histones H3 and H4 and two heterodimers of histones H2A and H2B.

This laboratory has previously described the structure of the nucleosome core particle to 8 Å resolution (Uberbacher & Bunick, 1985, 1989). The crystals used in that structure determination were composed of core particles isolated from chicken erythrocyte nuclei. The bulk core particles were composed of homogeneous histone octamers and heterogeneous DNA sequences. A closely similar structure at 7 Å resolution has been reported based on nucleosome core particles isolated from beef kidney (Richmond, Finch, Rushton, Rhodes & Klug, 1984). Significant improvement of the diffracting limit of nucleosome core particle crystals has required the use of defined-sequence DNA's which are capable of phasing precisely on the histone octamer. A number of candidate sequences were chosen by a theoretical analysis of DNA sequence-dependent phasing in published nucleosome sequences (Uberbacher, Wilkinson-Singley, Harp & Bunick, 1988). One of the sequences which was implemented successfully was derived from a sub-repeat selected from a dodecamer of 171 bp sub-repeats in a 2 kbp *Bam*HI repeat of α-satellite DNA from the human X chromosome (Yang, Hansen, Oishi, Ryder & Hamkalo, 1982). Crystals of core particles containing the α-satellite DNA sequence have been shown in preliminary experiments to diffract X-rays to a significantly higher resolution than the previous random-sequence crystals. Similar improvement using defined-sequence DNA in reconstituted nucleosome core particles has been reported (Richmond, Searles & Simpson, 1988). However, crystals containing such defined-sequence DNA's still possess a potential twofold packing disorder because the DNA sequence fails to reflect the dyad symmetry of the histone core.

2. Materials and methods

To extend the symmetry of the histone core to the DNA, a palindromic fragment was constructed from one half of a human α-satellite nucleosomal DNA sequence subcloned from plasmid pXBR2 (Yang *et al.*, 1982; Hauser, 1985). A theoretical analysis of the 2 kbp sequence indicated the most likely nucleosome phasing positions (Uberbacher *et al.*, 1988). A restriction map overlaid with the theoretical positions showed that a nucleosomal sequence could be conveniently subcloned

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as an *Rsa*I/*Sst*I fragment. The sequence was subcloned into the *Eco*RV site of pνAJE vector. The pνAJE vector was constructed from BLUESCRIPT II (Stratagene Cloning Systems) by deletion of the *Ava*I fragment from the multiple cloning site and insertion of a synthetic linker into the *Bam*HI site. The synthetic linker contains a single *Eco*RV site embedded within two identical, asymmetrical *Ava*I sites. This construction was used to verify nucleosome phasing by DNase I footprinting. A 145 bp fragment based on the precise nucleosome center, but with *Eco*RV ends, was synthesized using two 90-mer synthetic oligonucleotides with complementary ends matching the center 35 bp of the nucleosome sequence (Fig. 1). The synthetic 145 bp sequence was cloned into the *Eco*RV site of the pνAJE vector using *E. coli* XL1 Blue (Stratagene Cloning Systems) as the host cell. The transformant was then grown to produce a quantity of the fragment with asymmetrical *Ava*I ends which was ligated to construct a plasmid containing nine copies of the 145 bp nucleosome sequence in multiple direct repeats.

The palindrome was constructed by taking advantage of the *Alu*I site positioned 4 bp from the nucleosome center in the native sequence (Fig. 1). The native α-satellite fragment was cut with *Alu*I and the shorter fragment was purified by ion-exchange chromatography on Mono Q (Pharmacia LKB Biotechnology). The shorter fragment was blunt-end ligated to a 10 bp synthetic *Eco*RI linker (New England Biolabs) and cut with *Eco*RI. The half-nucleosome fragment was self-ligated to form the 146 bp palindrome which was then ligated into the *Eco*RV site of pνAJE vector. The construction was transformed into the *E. coli* strain *Epicurian coli* SURE (Stratagene Cloning Systems, La Jolla, CA). The resulting clone was used for production of the α-satellite palindrome fragment.

Serendipitous placement of the *Alu*I site in the native sequence allowed both the construction of a palindrome by addition of the synthetic linker fragment and the conversion of a 145 bp fragment to a 146 bp nucleosome sequence. The final construction was verified by sequencing and is shown with the native nucleosomal DNA sequence in Fig. 1. Production of the palindrome fragment has required large batch (50–400 l) fermentation. Purification of the palindrome fragment had to take into account the metastable nature of the 146 bp palindrome. Phenol and chloroform extractions and exposure to high temperature were avoided. Large-scale restriction enzyme digestions were carried out overnight at room temperature with enzyme concentrations calibrated to obtain maximum efficiency of cutting. Yield of palindrome using these procedures has been no higher than about 1 mg of the 146 bp fragment from 10 l of culture. This appears to be because of low yield of plasmid per cell and may be attributable to the decreased ability of the cells to replicate plasmid containing a large palindrome (manuscript in preparation). Repeated attempts to increase the number of copies of the palindrome in the plasmid construction have been unsuccessful.

Histone octamers used in reconstitutions were prepared from fresh packed chicken erythrocytes using standard preparation protocols (Hewish & Burgoyne, 1973; Lutter, 1978; von Holt *et al.*, 1989). Washed nuclei were digested with micrococcal nuclease. Soluble chromatin released from the digestion was bound to an hydroxylapatite column equilibrated with 10 m*M* sodium phosphate (pH 7.4). Very lysine rich histones were eluted with 0.65 *M* NaCl, 10 m*M* sodium phosphate (pH 7.4). Core histones were eluted with 3 *M* NaCl, 10 m*M* sodium phosphate (pH 7.4) and reassembled as octamers by dialysis against 2 *M* NaCl, 20 m*M* Tris–HCl (pH 8.0),

alpha satellite native

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EcoRV                                                                AluI
          10        20        30        40        50        60        70
ATCAATATCC ACCTGCAGAT TCTACCAAAA GTGTATTTGG  AAACTGCTCC ATCAAAAGGC ATGTTCAGCT CTG

     80        90        100       110       120       130       140   145
TGAGTGA AACTCCATCA TCACAAAGAA TATTCTGAGA ATGCTTCCGT  TTGCCTTTTA TATGAACTTC CTGAT
                                                                      EcoRV
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alpha satellite palindrome

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EcoRV                                                                EcoRI
          10        20        30        40        50        60        70
ATCAATATCC ACCTGCAGAT TCTACCAAAA GTGTATTTGG  AAACTGCTCC ATCAAAAGGC ATGTTCAGCG GAA

     80        90        100       110       120       130       140   146
TTCCGCT GAACATGCCT TTTGATGGAG CAGTTTCCAA ATACACTTTT  GGTAGAATCT GCAGGTGGAT ATTGAT
                                                                      EcoRV
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Fig. 1. Sequences of the human α-satellite DNA fragments used to reconstitute nucleosome core particles. The α-satellite native sequence is a 145 bp nucleosome phasing sequence from a human α-satellite *Bam*HI repeat. The sequence was modified to terminate in blunt, *Eco*RV ends. The α-satellite palindrome sequence is a subclone constructed by cutting the α-satellite native fragment near the nucleosome center, at the *Alu*I site, and ligating the shorter fragment to a synthetic *Eco*RI linker. The 146 bp palindrome was constructed by self-ligating the resulting half-nucleosome fragment to form an inverted repeat. The center of the *Eco*RI recognition site coincides with the nucelosome dyad axis when the fragment is bound in the correct, symmetric phase to the histone octamer core.

0.1 m*M* phenylmethyl sulfonyl fluoride (PMSF). The histone octamers were concentrated to 10 mg ml^{-1} and stored at 253 K. Histones were analyzed by sodium dodecyl sulfate polyacrylamide gel electrophoresis. Coomassie Blue-stained gels were scanned on a Molecular Dynamics Computing Densitometer Model 300A and the stoichiometry of the histone preparation was compared to a standard prepared by solubilizing total protein from whole chicken-erythrocyte nuclei. Peaks were fitted using *ImageQuant* (Molecular Dynamics) and *PeakFit* (Jandel Scientific) software. A dynamic equilibrium exists between the octamer and a dimer–hexamer pair (Stein & Page, 1980) which results in dimer-depleted histone stocks if gel-filtration chromatography or sucrose-gradient centrifugation is used for purification. Hydroxylapatite chromatography consistently produced pure histone octamer stocks that were virtually identical in subunit stoichiometry to octamer histones in the solubilized total histone standard.

Reconstitution of nucleosome core particles was initiated by adding together purified chicken erythrocyte histone octamers and purified 146 bp human α-satellite palindromic DNA in approximately equimolar amounts adjusted to make the final concentration of nucleosome core particles 1 mg ml^{-1}. The reactants were equilibrated overnight at room temperature by dialysis against a starting buffer of 2 *M* KCl, 20 m*M* Tris–HCl (pH 8.0), 1 m*M* disodium ethylenediaminetetraacetic acid (EDTA), 1 m*M* dithiothreitol (DTT), 0.02% sodium azide, 0.1 m*M* PMSF and 0.01% Nonidet P40. Nucleosome core particles were assembled using a modification of the slow salt gradient dialysis procedure used by Richmond *et al.* (1988). The slow salt dialysis gradient was produced by pumping an ending buffer of 0.3 *M* KCl, 20 m*M* Tris–HCl (pH 8.0), 1 m*M* EDTA, 1 m*M* DTT, 0.02% sodium azide and 0.01% Nonidet P40 into the starting dialysis buffer with stirring while maintaining a constant volume in the dialysis vessel. The rate at which ending buffer was added to the dialysis chamber was adjusted to lower the salt concentration over a period of 6 d at room temperature. Core particles were then dialyzed against a final buffer of 50 m*M* KCl, 20 m*M* Tris–HCl (pH 8.0), 0.02% sodium azide, 0.1 m*M* PMSF. Reconstitutions were analyzed by particle gel electrophoresis using a 5% polyacrylamide vertical slab gel run in 8.9 m*M* Tris–borate (pH 8.3), 1 m*M* EDTA. The reconstituted nucleosome core particles were concentrated to 10 mg ml^{-1} using a Centricon 30 microconcentrator (Amicon Division, W. R. Grace & Co.) prior to purification of the correctly phased nucleosome core particles.

Yields of correctly phased core particles were improved by increasing the dialysis time at moderately high salt (0.6–0.3 *M* NaCl). However, some core particles with DNA positions on the histone octamer differing from the correct, symmetrically phased core particles can be detected by particle gel electrophoresis in even the best reconstitutions. The translational position of DNA on the histone octamer affects nucleosome mobility (Meersseman, Pennings & Bradbury, 1992; Harp *et al.*, 1996) such that degenerately phased core particles can be removed by preparative gel electrophoresis.

The correctly phased core particles in the finished reconstitution were separated from free DNA, free protein and degenerately phased core particles by preparative polyacrylamide gel electrophoresis (Uberbacher & Bunick, 1986) using a Model 491 Prep Cell (Bio-Rad Laboratories) (Harp *et al.*, 1996). Correctly phased nucleosome core particles were concentrated and passed through a gel-filtration column equilibrated with 50 m*M* KCl, 20 m*M* potassium cacodylate (pH 6.0), 1 m*M* EDTA and 0.01% Nonidet P40. Peak fractions were pooled and concentrated as described previously to 10 mg ml^{-1} nucleosome core particles and stored at 277 K. Core particles prepared in this way were used for crystallization.

Crystals were grown by the method of Rhodes and coworkers (Rhodes, Brown & Klug, 1989), with modifications to use dialysis procedures. Solutions containing 4–6 mg ml^{-1} of purified nucleosome core particles were adjusted to 55 m*M* $MnCl_2$ in 10 m*M* potassium cacodylate (pH 6.0), 50 m*M* KCl and then dialyzed to lower (about 50 m*M*) $MnCl_2$ concentration in glass buttons at room temperature by adding buffer without $MnCl_2$. The method of double dialysis was also used to control the $MnCl_2$ gradient. A slow rate of crystallization is important to grow large, solid crystals. Crystal growth is most rapid along the *c* axis as the core particles stack along the axis of the DNA superhelix. The slower rate of packing along *a* and *b* axes can result in hollow crystals if solution parameters do not lie precisely along the solubility edge of the phase diagram. We have found that formation of hollow crystals can be largely prevented by manipulation of pH.

For initial X-ray diffraction experiments, crystals were mounted in standard X-ray capillary tubes (Charles Supper Co., Natick, MA) using artificial mother liquors containing 22.5% 2-methyl-2,4-pentanediol (MPD). Diffraction data were collected at 277 K using an X-ray Research GmbH MAR image-plate area detector mounted on a Rigaku RU200HB rotating-anode X-ray generator operated at 50 kV and 100 mA with a Cu anode and a 0.3 mm focal spot. Exposures were of the order of 20 min per 1/4 degree spindle rotation with collimating slits set at 0.4 mm. Rotation frames were processed using the X-ray *Data Streamer* software (Kabsch, 1988).

3. Results and discussion

For data-collection experiments, the MAR image-plate detector was positioned to record reflections at a maximum resolution of 3.2 Å. The data were processed using *MARXDS* and *MARSCALE* software (Kabsch, 1988) and *MOSFLM* 5.22 (Leslie, 1990). Approximately 27% of a

unique data set to 3.2 Å was collected. The data showed an overall R_{sym} of 5.3%. The percentage of observed reflections between 3.5 and 3.2 Å is 74% for $I > 1\sigma(I)$ and 54% for $I > 2\sigma(I)$. For all data to 3.2 Å, 88% of reflections are observed at $I > 1\sigma(I)$ and 79% at $I > 2\sigma(I)$. Radiation damage using Cu $K\alpha$ radiation is significant. Average intensity of reflections is reduced by 17% in ~12 h of exposure. Radiation damage appears relatively isotropic with resolution except for the 3.2 Å shell, which shows a 24% reduction in average intensity after ~12 h of exposure. Experiments with cryo-protective solutions and flash-cooling techniques are currently under way in an effort to retard radiation damage.

Although the crystals used for the 8 Å structure described previously (Uberbacher & Bunick, 1989) were produced using spermine, the method of Rhodes and coworkers (Rhodes *et al.*, 1989) was followed because it is more reproducible. That method produces crystals in the form of hexagonal rods (Fig. 2) with cross-section dimensions of about 0.25 × 0.3 mm and up to 4.5 mm long. Cell constants vary with hydration state as discussed by Struck, Klug & Richmond (1992). Unit-cell dimensions determined in 22.5% MPD are $a = 106.3$, $b = 183.7$, and $c = 110.3$ Å, and the space group is $P2_12_12_1$. Analysis of nucleosome core particle crystals containing the 145 bp α-satellite native sequence DNA indicates that unit-cell dimensions at 22.5% MPD are $a = 106.8$, $b = 175.0$, and $c = 109.8$ Å, also in $P2_12_12_1$. The difference in b-axis lengths reflects the 1 bp difference in the lengths of the two DNA's. Diffraction from the crystals is relatively isotropic to 3.2 Å resolution with some reflections extending to d spacings of 2.8 Å. This result contrasts with the anisotropic resolution reported in previous work (Richmond *et al.*, 1984, 1988; Struck *et al.*, 1992; Richmond, Rechsteiner & Luger, 1993) using synchrotron radiation. The difference may be due to the removal of degenerately phased nucleosome core particles by preparative gel electrophoresis as well as the symmetrical nature of the DNA palindrome containing nucleosome core particles.

Figs. 3(*a*) and 3(*b*) present X-ray diffraction patterns from a crystal of nucleosome core particles containing the DNA palindrome. An interesting feature of the X-ray

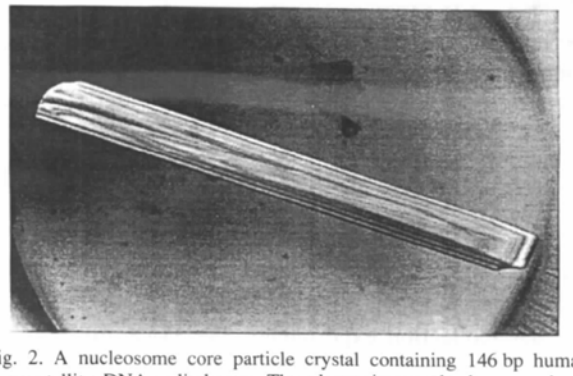
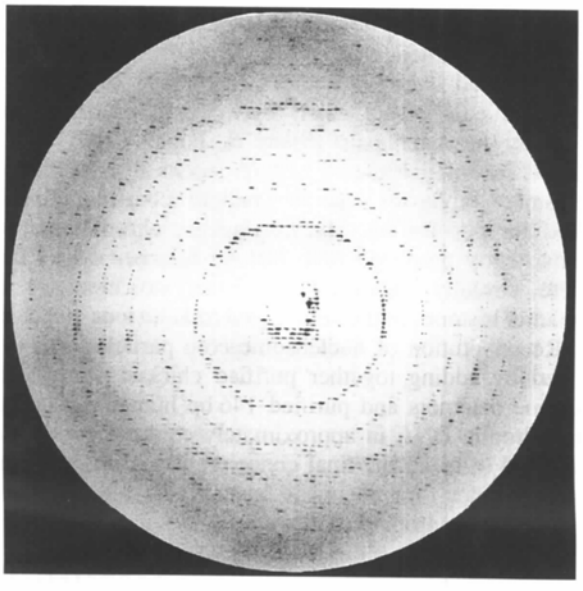
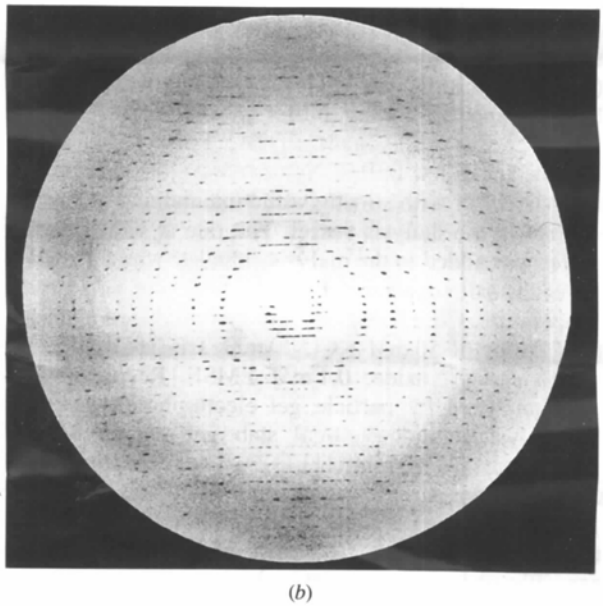


Fig. 2. A nucleosome core particle crystal containing 146 bp human α-satellite DNA palindrome. The photomicrograph shows a single hexagonal rod approximately 4.5 mm in length and 0.3 mm in diameter. Such crystals grow by the method of double dialysis in approximately 2 weeks.



(*a*)



(*b*)

Fig. 3. Small-angle rotation frames (0.25° spindle rotation) of diffraction from a crystal of nucleosome core particles containing the DNA palindrome. Exposures were made as described in the text. The crystal to detector distance was set to give a maximum resolution of 3.2 Å. (*a*) The frame was recorded with the a axis of the crystal approximately aligned with the incident X-ray beam. (*b*) This frame was recorded with the crystal b axis approximately aligned with the incident X-ray beam.

diffraction patterns obtained from crystals of nucleosome core particles is the diffuse scatter with strong meridional intensity at about 3.4 Å. This phenomenon was first reported by Finch *et al.* (1981) who noted its similarity to fiber diffraction from B DNA. In diffraction patterns from crystals of nucleosome core particles containing random-sequence DNA, diffuse scatter is most pronounced when the *b* axis is perpendicular to the incident beam. The sequence heterogeneity and conformational disorder of DNA is less in nucleosome core particles containing the DNA palindrome as seen in Fig. 3(*a*), where the crystal *a* axis was approximately parallel to the X-ray beam such that b^* is nearly vertical. This is a strong indicator that the use of palindromic DNA will provide structural data concerning the interactions between DNA and histones in the nucleosome core particle.

Analysis of the diffraction data indicates that nucleosome core particles containing the DNA palindrome crystallize in space group $P2_12_12_1$. Richmond *et al.* (1988) also assigned crystals of nucleosome core particles containing defined-sequence DNA to $P2_12_12_1$ with a single nucleosome in the crystallographic asymmetric unit. However, the assignment was later changed to an unspecified space group with two nucleosomes in the asymmetric unit (Richmond *et al.*, 1993). Assuming that the unit-cell dimensions are essentially unchanged, and thus the number of nucleosomes in the unit cell is unchanged, the simplest transformation of the crystal lattice which will result in two nucleosomes in the asymmetric unit is to the monoclinic system. The difference in crystal packing may be due to the homogeneity of the nucleosome core particles used to grow crystals with the more homogeneous particles packing in the higher symmetry orthorhombic lattice.

A significant problem has been producing enough DNA palindrome to make the quantity of nucleosome core particles necessary to refine the phase diagram for crystallization, explore alternative approaches to crystallization, and proceed with the structure determination. This problem has now been solved, and we are able to prepare adequate stocks of palindrome DNA (Palmer, Gewiess, Harp, York & Bunick, 1996). We expect that the use of a DNA palindrome to complete the dyad symmetry of the nucleosome core particle will result in a data set to a resolution of about 3 Å when collected at a synchrotron source. Solution of this structure should provide valuable information concerning the organization of the nucleosome which will be directly applicable to questions of the role of this basic structural unit in the regulation of gene expression and DNA packaging and replication. These studies will certainly complement and extend the observations of Arents & Moudrianakis (1993) who have simulated the binding of model DNA on the surface of their 3.1 Å histone octamer crystal structure (Arents, Burlingame, Wang, Love & Moudrianakis, 1991). The crystal structure of the nucleosome core particle at a similar resolution should yield some structural information on the histone tails which are disordered in the histone octamer structure but which are likely to be at least partially ordered in the nucleosome core particle structure incorporating the DNA palindrome.

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