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ARTICLE *in* JOURNAL OF BIOSCIENCES · MARCH 1987

Impact Factor: 2.06 · DOI: 10.1007/BF02716952

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J. Biosci., Vol. 12, No. 1, March 1987, pp. 41–50. © Printed in India.

Analysis of nucleosome arrangement on satellite DNA of rat liver chromatin

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MS received 28 July 1986; revised 18 November 1986

Abstract. The arrangement of nucleosomes on the nucleotide sequence of satellite DNA of Oceanian rat (*Rattus rattus*) has been studied. Nucleosome cores were prepared from rat liver nuclei with micrococcal nuclease, exonuclease III and nuclease Sl. From the total population of core DNA fragments, the satellite-containing fragments were selected by molecular cloning and the complete nucleotide sequence of these clones was determined. The data show that nucleosomes occupy a number of preferred positions on satellite DNA. These positions are strictly defined. Thus location of nucleosomes along the satellite sequence is non-random. Such finding may have important biological significance.

Keywords. Nucleosome positioning; satellite chromatin; rat liver; *Rattus rattus.*

Introduction

Although nucleosomes were detected and characterized more than a decade ago (Hewish and Burgoyne, 1973; Olins and Olins, 1974), their arrangement on DNA with possible functional implications is still disputed (Igo-Kemenes *et al.,* 1982; Simpson, 1986; Wu *et al.,* 1986). A noteworthy feature of the nucleosome is its ability to serve diverse functions. It compacts DNA and causes the formation of a highly organized superstructure. At the same time, it also leaves DNA freely accessible to different regulatory molecules. Probably the sequence-dependent positioning of nucleosomes along a given DNA plays a role in the execution of such diverse functions.

Several workers have conducted nuclease digestion experiments to study the nucleosome arrangement (Cartwright *et al.,* 1982; Bock *et al.,* 1984; Eissenberg *et al.,* 1985). Here the DNA in question is usually identified by hybridization with appropriate DNA probes. These experiments have several limitations. Firstly, the precise position of a nucleosome core cannot be deduced from a determination of individual nuclease cuts in the linker region. Secondly, the micrococcal nuclease generally used to map the inter-nucleosomal linker regions has a pronounced sequence specificity (Hörz and Altenburger, 1981). It cuts at preferential recognition sequences ($\frac{\text{CATA}}{\text{GTAT}}$ and $\frac{\text{CTA}}{\text{GAT}}$). As a consequence, under partial digestion conditions the nuclease cleaves selectively only those linkers that contain particular susceptible DNA sequences. Such experiments, therefore, do not reveal the location of all nucleosome cores along a given DNA. Rather they make specific selection and thereby create the inappropriate impression of precise nucleosome positioning (McGhee and Felsenfeld, 1983). Smith and Lieberman (1984) have also shown that micrococcal nuclease is. of limited value for mapping nucleosome positions with respect to specific DNA sequences.

The present study addresses the question of nucleosome arrangement on liver

satellite DNA of Oceanian rat (*R. rattus*). This satellite is highly abundant and constitutes about 3% of the haploid genome of the rat (Sealy *et al.,* 1981). Analyses with restriction nucleases had established that the repeat unit of satellite DNA of *R. rattus* is approximately 185 bp, while that of *R. norvegicus* is 370 bp long. However, both satellites are composed of 93 and 92 bp internal subrepeats (Witney and Furano, 1983).

Materials and methods

Preparation of rat liver nuclei and nuclease digestion

Nuclei were prepared from the liver of Oceanian type male rat (*R. rattus,* 2n = 38) according to Greil *et al.* (1976). For digestion with micrococcal nuclease, nuclei were suspended at 0°C in buffer A (65 mM KCl, 15 mM NaCl, 0·5 mM spermine, 0·15 mM spermidine, 0·2 mM EDTA, 0·2 mM EGTA, 1 mM PMSF, 10 mM Tris-HCl, pH 7·4) and pelletted. The pellet was resuspended in buffer A to give a final concentration of 0·4–0·5 mg DNA/ml. $CaCl_2$ (1·6 mM) was added and the reaction mixture allowed to stand for 2 min at 37°C. The reaction was started by the addition of 32, 64 and 128 units of micrococcal nuclease/ml. Aliquots were taken at different times and their reaction terminated by the addition of EGTA to a final concentration of 5 mM, followed by centrifugation of the nuclei. The nuclear pellet was washed several times with bufer A.

Lysis of nuclei and chromatin extraction

The nuclei were suspended in 0·2 mM EDTA (pH 7·9). When the nuclei were completely lysed and dispersed in the extraction buffer, the suspension was centrifuged. The insoluble chromatin pelleted as a gel. The amount of soluble chromatin present in the supernatant fraction was determined from their DNA content by measuring the absorbance at 260 nm.

Exonuclease III treatment of solubilized chromatin

A 10 times concentrated buffer was added under vortexing to the soluble chromatin ($5A_{260}$ units/ml). This was followed by addition of 11 and 22 units of exonuclease III/ml. The mixture was incubated for 1 h at 37°C with occasional shaking. The reaction was terminated by the addition of 5 mM EDTA, and precipitation with ethanol. The ethanol precipitate was deproteinized. Proteinase K and sodium dodecyl sulphate were removed by 3 rounds of ethanol precipitations. DNA was finally dissolved in 10 mM Tris-HCl (pH 7·4).

Sl nuclease treatment of exonuclease III-trimmed DNA

Exonuclease III-treated DNA ($1A_{260}$/ml) was incubated at 20°C for 30 min in a buffer containing 200 mM NaCl, 25 mM potassium acetate (pH 4·5), 0·1 mM $ZnSO_4$

and 2·7 units of Sl nuclease/μg DNA (Vogt, 1973). The reaction was terminated by the addition of 100 mM Tris-HCl (pH 8·8) followed by precipitation with ethanol. DNA was dissolved in 10 mM Tris-HCl (pH 7·4) and applied to preparative 5% (w/v) Polyacrylamide tube (0·6 × 18 cm) gels. Gels were stained for 3 h with 40 μg toluidine blue/ml in distilled water. The band corresponding to core DNA (145 bp) was excised and eluted (Maxam and Gilbert, 1980).

Preparation of satellite probe

Total DNA prepared from the liver nuclei of *R. rattus* was partially digested with *Eco*RI (1 U/μg DNA) and analysed on 1·5% agarose gel. Monomer satellite bands corresponding to 185 bp DNA was isolated. This was ligated with linear *Eco*RI-digested and dephosphorylated pBR 322. This preparation was used to transfect *Escherichia coli* JM 490A cells by CaCl2 procedure using a top agar.

After the bacterial colonies were well grown, replicas for 25 colonies were made using ampicillin containing LB plates with or without nitrocellulose filters. From the plate containing filters, the filter was carefully removed, treated with lysozyme and the colonies were fixed on filter with different steps of washing with buffer and finally baked at 80°C under vacuum. From the plate (without filter) containing 25 colonies, DNA was extracted from each colony. This was digested with *Eco*RI and analysed on 0·6% agarose gel to find out the clone containing insert of correct size. Such Plasmids were amplified and DNA was purified using CsCl gradient.

Screening of satellite-containing colonies was done by colony hybridization. The filter containing 25 colonies of *E. coli* (with inserts) were hybridized against [^{32}P]-labelled satellite probe (1·8 × 10^8 cpm/μg). This clone containing pBR with satellite insert was further used for screening the plaques having satellite DNA.

Cloning of core DNA and sequence analyses

Core DNA was cloned according to the procedure described in a laboratory manual (Maniatis *et al.,* 1982). For cloning, the core DNA was first ligated with commercially available Bam Hl linker d(C-G-G-G-A-T-C-C-C-G) which was prephosphorylated. The linker-core DNA complex was cleaved with Bam Hl. Finally, the mononucleosome containing 5+145 + 5 = 155 bp DNA was separated on a preparative 5% Polyacrylamide slab gel. Bam Hl fragments of linker core DNA were ligated into the Bam Hl site of the multipurpose cloning Vector M13 mp7 (Messing *et al.,* 1981). M13 plaques were obtained on *E. coli* K12 JM 103. They were transferred from agar plates to nitrocellulose paper. This was baked and hybridized with [^{32}P]-labelled satellite probe (1·8 × 10^8 cpm/μg DNA). The positive clones were selected and DNA was isolated.

Sequencing was according to the dideoxyribonucleotide chain termination reaction of Sanger *et al.* (1977). A 17-nucleotide primer (P. L. Biochemical Inc.) was used. After autoradiography, the order of nucleotides was read and compared with known satellite sequences to find out the position of nucleosomes. Later the data were analysed by computer.

Results and discussion

Preparation of nucleosome core DNA

Rat liver nuclei were digested with micrococcal nuclease to convert chromatin into mononucleosomes (figure 1). It was important at this stage to minimize selection for or against any particular DNA fraction and obtain a core DNA population as representative as possible of the total DNA. This was achieved because the final processing of mononucleosomes to core particles was obtained by exonuclease III rather than by micrococcal nuclease. In contrast to micrococcal nuclease, exonuclease III has less sequence specificity (Linxweiler and Hörz, 1982). Digestion was carried out to such an extent that mononucleosomes represented the major chromatin fraction, but some dinucleosomes and higher oligonucleosomes still persisted (figure 2). No attempt was made to drive the digestion further, since it was observed that oligonucleosomes are only slowly processed to mononucleosomes during prolonged incubation times, while degradation to subnucleosomal particles proceeds at a considerable rate (Greil *et al.,* 1976). Trimming of the mononucleosomes to obtain nucleosomes cores was performed by digesting the soluble chromatin with exonuclease III and treatment of the deproteinized DNA with nuclease Sl. We used conditions of rather extensive digestion in order to minimize the amount of material longer than core length, since the presence of such material obscures the results. After treatment with nuclease Sl to obtain blunt ends, core DNA was fractionated on preparative Polyacrylamide gels. Following staining, the core DNA was

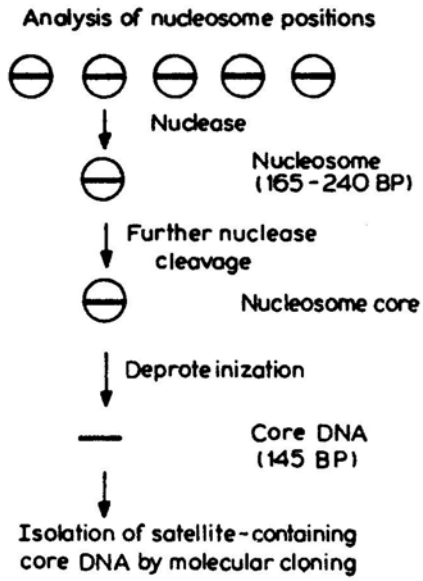

Figure 1. Protocol for the analysis of nucleosome positioning. A population of mono-, di- and oligonucleosomes is obtained after digestion of chromatin with micrococcal nuclease. This is further treated with exonuclease III and nuclease Sl to obtain core DNA. Techniques of molecular cloning are used to screen satellite-containing core DNA from this total population.

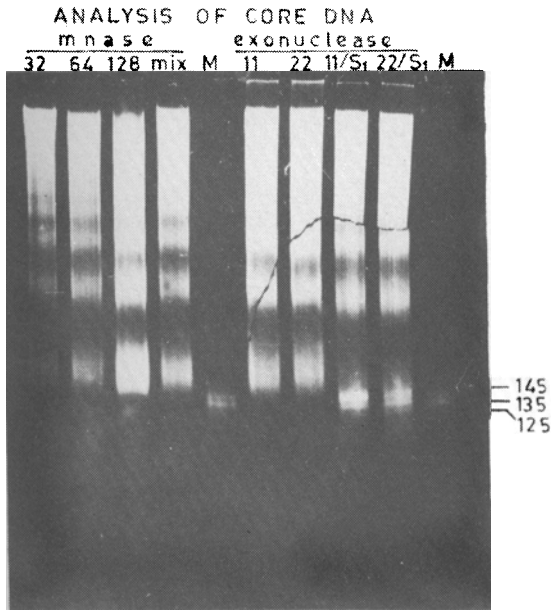

Figure 2. Isolation of nucleosome core DNA on a preparative Polyacrylamide gel. Methodology is described in the text. The final core DNA of 145 bp length is excised from the gel and eluted for further processing.

excised and eluted. As shown in figure 2, the final core DNA preparation was 143 ± 2 bp long.

Mapping of nucleosome positions by cloning and sequence analysis of nucleosome core DNA

Restriction nucleases have been used to prepare satellite DNA-containing probe. Most satellite DNAs are either completely resistant to certain restriction nucleases or give regular patterns consisting of small fragments, quite different from the continuous fragment distribution found with nonrepetitive eukaryotic DNA. After the digestion of nuclei with restriction nucleases (Pfeiffer *et al.*, 1975), satellite DNA-containing chromatin can be separated accordingly either as high or as a low molecular weight fraction from the rest of the chromatin (Igo-Kemenes *et al.*, 1977). Figure 3 represents the preparation of satellite probe using digestion of DNA with *Eco*RI. Determination of the sequence of core DNA presents a picture of the location of nucleosomes on DNA. One major problem concerning this approach is that any DNA sequence in the core preparation, even highly abundant repetitive DNA sequences, comprises only a small proportion of the total population of DNA sequences. Furthermore, it is impossible to perform sequence analysis with a mixture of fragments. This problem was, however, overcome by the application of methods of gene technology. Cloning linkers were attached by blunt-end ligation to the core DNA fragments (figure 4) and cloned in *E. coli* cells using M13 mp7 DNA as a vector. By the use of colony hybridization techniques, a number of clones containing satellite DNA were identified.

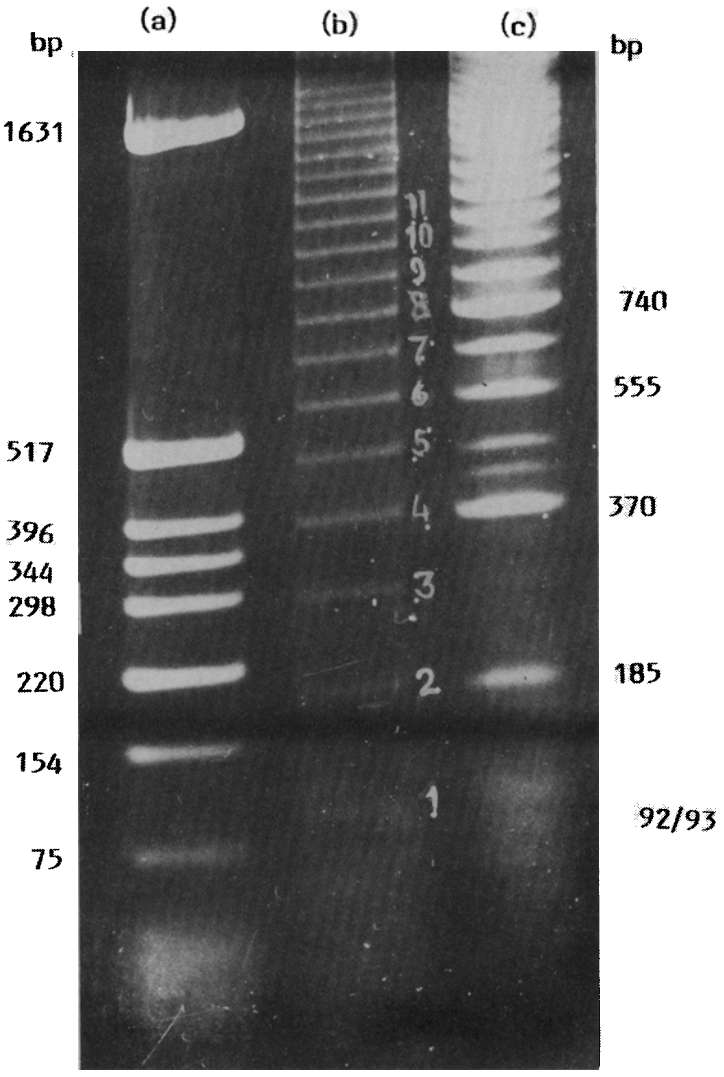

Figure 3. Partial digestion of rat liver DNA with *Eco*RI. The digest was subjected to 1·5% agarose gel electrophoresis. This displays a 92/93 bp ladeer (b). For comparison (a) pBR digested with HinfI, and (c) an *Eco*RI digest of enriched satellite DNA shown for chain length markers are included. Numbers on the left: DNA sizes in base pairs; on the right: multiples of 185 bp (the nucleosomal repeat in satellite-containing chromatin). Fragment lengths are given in base pairs.

The complete nucleotide sequence of a satellite DNA clone is depicted in figure 5. The results of sequence analyses of satellite DNA clones are presented in figure 6. It is evident that groups of nucleosornes are positioned in particular sets of preferred and strictly defined positions. Clearly, the location of nucleosomes along the satellite sequence is non-random. However, the present data give little information about the long range organization of nucleosomes within the chromatin fibre. Of course, the clones under study display another kind of heterogeneity. They show some variation

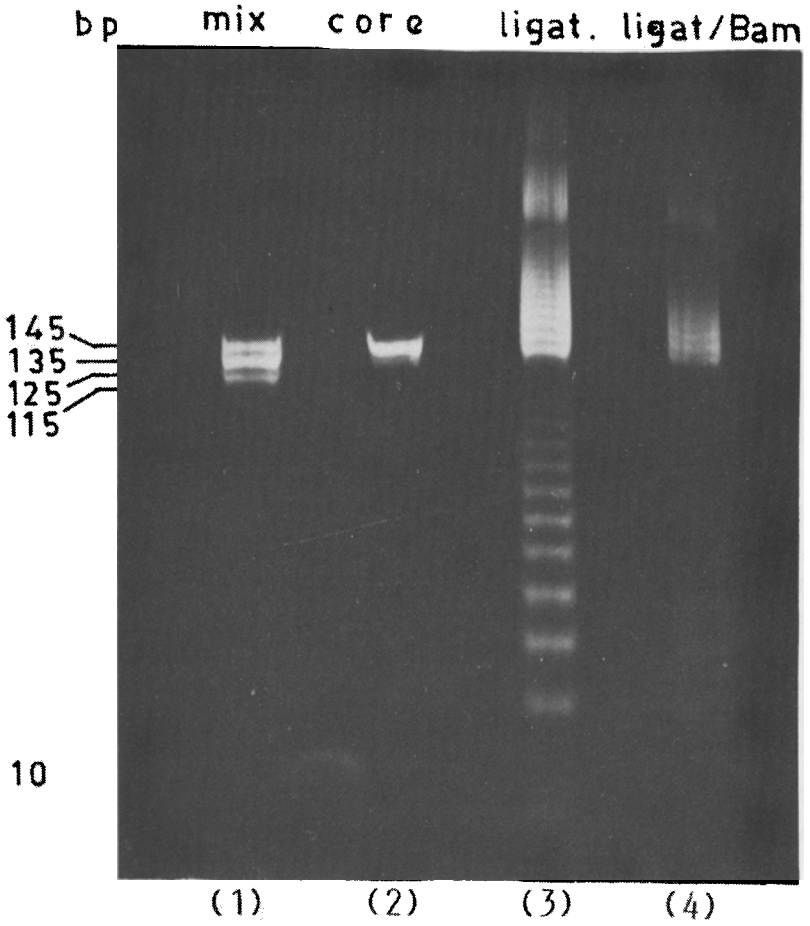

Figure 4. Ligation of core DNA to Bam Hl linker and its cleavage with Bam Hl. Lane 1: Mixture of mono- and subnucleosomal particles as a marker. Lane 2: Isolated core DNA. Lane 3: Core DNA ligated with Bam Hl linker. Lane 4: Core DNA-linker complex digested with Bam Hl.

in length. However, this may be attributed to over-digestion with exonuclease III. In addition, most of the satellite core DNA show a shift in the position on one end which is compensated by almost an equal shift at the other end. Thus this indicates the positioning of nucleosomes in particular frames. To confirm that our findings on nucleosome positions in satellite chromatin is not an artifact of core isolation and cloning conditions, nucleosome arrangement has also been mapped using digestion of nuclei with different restriction nucleases (unpublished). Both the data are in agreement with the multiple framing model of nucleosome arrangement.

Mechanism of nucleosome positioning and its biological significance

Distribution of nucleosomes exhibits certain degree of preference for DNA sequences (Kornberg, 1981). Short consensus sequences or boxes, like the T-A-T-A

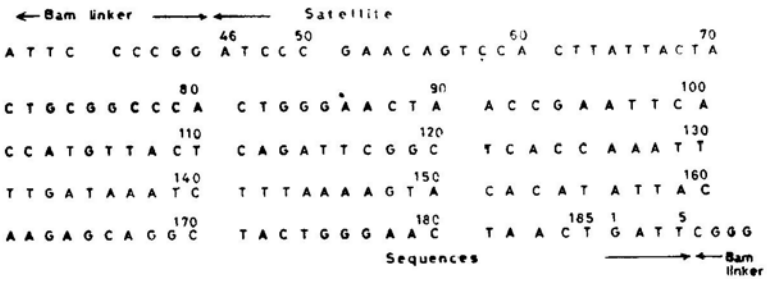

Figure 5. Sequencing of satellite DNA by sanger method. The complete nucleotide sequences of a core DNA clone (R 3701) is given. The sequences of satellite DNA are flanked by nucleotide sequences of Bam Hl linker which helps in identification of the start and end of the core DNA.

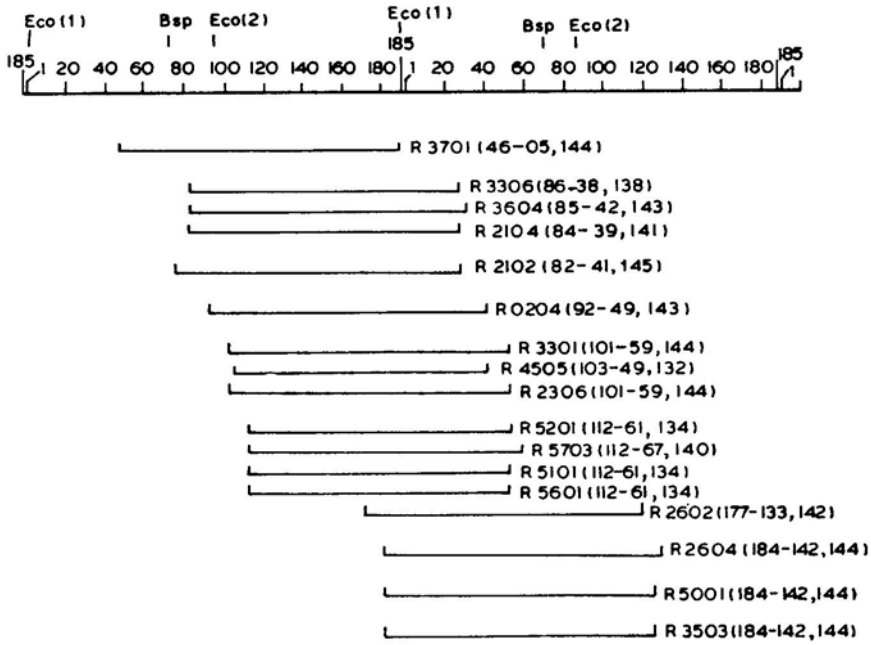

Figure 6. Positioning of nucleosome cores on liver satellite DNA of *R. rattus.* The first row represents satellite unit of 185 bp. The numbering of the satellite sequence is according to Pech *et al.* (1979). Restriction sites that occur once (Bspl) or twice (*Eco*RI) within the satellite unit are appropriately labelled. The bars indicate the regions covered by each clone. In the extreme right column the designation of the clones is given. The number of first and last base, and the total number of nucleotides present in each core DNA clone is mentioned in parentheses.

box, may direct the placement of nucleosomes. Unfortunately, the experimental data available at present are insufficient to make a search for such consensus sequences. Nevertheless, computer analysis has revealed a nonrandom distribution of certain dinucleotides in eukaryotic DNA that may be involved in nucleosome positioning (Trifonov and Sussman, 1980). Also the specific protein-DNA interactions involving

either histones or nonhistone proteins and nuclease sensitive regions of chromatin may determine the nucleosome positioning in yeast plasmids (Strauss and Varshavsky, 1984; Thoma and Simpson, 1985; Neubauer *et al.,* 1986; Thoma, 1986).

Our results on satellite containing chromatin support the view that the arrangement of nucleosomes along a DNA chain is dependent, at least to some extent, on the DNA sequence. Other factors, however, may also contribute to the preferential location of nucleosomes. This might include the influence of sequence-specific proteins, specificity of the histones themselves and possible changes in specificity upon histone modifications, constraints on the length of linker DNA (Mengeritsky and Trifonov, 1983), action of nucleoplasmin (Earnshaw *et al.,* 1980), other assembly factors and so forth. It is difficult at present to evaluate the contribution of any of these possible factors separately.

Concerning the functional significance of positioned nucleosomes, various proposals have been made. Regular nucleosome arrangements on satellite DNA may, by modulating chromatin superstructure, affect chromosome recognition and pairing. On protein coding sequences, nucleosome arrangement could be related to the interconversion between active and inactive states of a gene. The location of nucleosomes on RNA genes has been found to be important for rapid transcription (Wittig and Wittig, 1982). It is a reasonable possibility that positioned nucleosomes facilitate organization of chromatin into higher order structure.

Conclusions

The *in vivo* location of nucleosomes on satellite DNA of rat liver chromatin has been mapped using the method of molecular cloning. The data indicate that nucleosome cores may assume a defined position on satellite sequences. Thus the present studies suggest that multiple but strictly defined frames may be a general mode of arrangement of nucleosomes on chromatin containing highly repetitive DNA.

Acknowledgements

This investigation was carried out in the laboratory of Dr. Tibor Igo-Kemenes at the Institute of Physiological Chemistry, University of Munich, West Germany. The author expresses his appreciation for a research fellowship from the University Grants Commission-sponsored Indo-German Cultural Exchange (DAAD) Programme.

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