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Relation of nucleosomes to nucleotide sequences in the rat

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The relation of nucleosomes to nucleotide sequences is random for most single copy sequences in rat liver. This could be due to variation in the DNA content of nucleosomes, and a procedure for detecting such variation is described.

Introduction

The structure of chromatin in eukaryotes is based on units termed nucleosomes, which consist of a set of eight histone molecules surrounded by about 200 base pairs of DNA. The work described here is concerned with the question whether nucleosomes occur in a specific relation to nucleotide sequences in DNA. We studied this question because it seemed crucial to the functional significance of histones in chromatin. If the function of histones were simply to package DNA then the relation of nucleosomes to nucleotide sequences would probably be random, whereas if histones were to play a rôle in gene expression then the relation of nucleosomes to nucleotide sequences might well be specific. Such a specific relation would arise if nucleosomes were laid down from fixed starting points in sequence during the assembly of chromatin, and further if the DNA content of a nucleosome were the same from one to the next so that a specific relation, once established, could be maintained for a considerable distance along the DNA.

The relation of nucleosomes to nucleotide sequences in the chromatin of Simian Virus 40 has been investigated by the use of restriction enzymes and it is found to be random (Cremisi, Pignatti & Yaniv 1976). These studies further showed that nucleosomes are essentially fixed in place on the viral genome in physiological conditions, so the random location of nucleosomes on the genome is unlikely to be due to sliding or exchange. We report here on the relation of nucleosomes to nucleotide sequences in cellular chromatin, where we thought the result might be different from that found for viral chromatin, due to a difference in structure. The nucleosomes in cellular chromatin lie in close apposition along the DNA, and thus appear to be more regularly arranged than those in viral chromatin, which are spaced a variable distance apart (Bellard, Oudet, Germond & Chambon 1976).

Experimental methods

The preparation of rat liver nuclei, digestion with micrococcal nuclease and DNase I, fractionation of nucleosome monomer and dimer, and extraction of DNA were as described (Noll & Kornberg 1977). *Escherichia coli* exonuclease III (exo III) was a gift from Michael Goldberg. Exo III digestion of 140–160 base-pair DNA fragments was performed according to Richardson, Lehman & Kornberg (1964) and allowed to continue until a plateau value of absorbance at 260 nm corresponding to a 48 % increase over the initial value was reached.

The resulting single stranded DNA was fractionated in 98 % formamide – 8 % polyacrylamide gels (Maniatis, Jeffrey & Van deSande 1975), with a DNase I digest of chromatin for size calibration, and 40–70 residue material was cut from the gel and recovered by electroelution. DNA reassociation was carried out in 0.24 M sodium phosphate, pH 6.8, at 51 °C and monitored by hydroxyapatite chromatography at 50 °C (Britten, Graham & Neufeld 1974).

Exo III digestion of nucleosome monomer and dimer was carried out at 37 °C in 100 mM sodium phosphate, pH 7.4, containing 0.5 mM $MgCl_2$ and 1 mM 2-mercaptoethanol. The product was passed through a column of Sephadex G-100 in 50 mM sodium acetate, pH 5.5, and the eluate was made 0.1 mM with respect to $ZnCl_2$ and digested with single strand specific nuclease S1 (Sigma) at 37 °C. Further details of all methods are described elsewhere (Prunell & Kornberg 1977).

Relation of nucleosomes to nucleotide sequences in rat liver chromatin

The distribution of nucleosomes in cellular chromatin is not so readily analysed by the use of restriction enzymes as the distribution in viral chromatin, due to the vast difference in genome size (10^7 nucleosomes in the genome of a mammalian cell compared with 25 in Simian Virus 40). We have therefore taken an alternative approach based on extensive digestion of cellular chromatin with micrococcal nuclease, which results in 140 base-pair fragments of DNA that are quite homogeneous in size and represent the middle, or 'core', region of the nucleosomes (see Kornberg (1977) for references). If the relation of nucleosomes to nucleotide sequences is specific then the same set of 140 base-pair fragments will be produced from every copy of the genome, whereas if the relation is random then the fragments derived from one copy of the genome will partly overlap in sequence the fragments derived from another. Procedures for distinguishing in a qualitative way between unique and overlapping sets of fragments, involving melting, reannealing, and treatment with single strand specific nuclease, are described elsewhere (Prunell & Kornberg 1977). A procedure that enables a quantitative distinction to be made is as follows. The 140 base-pair fragments are digested with exonuclease III (exo III) from *E. coli*, which degrades DNA from the 3′ end and is specific for double stranded DNA (Richardson *et al.* 1964). The digestion proceeds until 70 bases have been removed from both 3′ ends of a 140 base-pair fragment, at which point two single strands of 70 residues remain, and these are resistant to further digestion due to the requirement of the enzyme for double strand DNA. Now, in the case of a unique set of 140 base-pair fragments, every single strand produced by exo III digestion will have lost its complement and will remain single stranded. In the case of an overlapping set of 140 base-pair fragments, the single strands produced by exo III will also overlap and will reassociate with one another.

There are two difficulties with this approach, both of which can be circumvented. The first arises in the digestion with micrococcal nuclease for the production of 140 base-pair fragments, where nicks are introduced. Nicks pose a problem by enabling exo III to act within as well as at the ends of 140 base-pair fragments. Since nicks become numerous only late in the digestion with micrococcal nuclease, they may be avoided by terminating the digestion at a stage where the fragments have not yet been converted to homogeneous 140 base-pair material but range in size from 140 to 160 base pairs.

The second difficulty arises from a lack of synchrony in the exo III degradation of a 140

base-pair fragment from the two 3′ ends. Single strands both longer and shorter then 70 residues will be found in a limit digest. Strands longer than 70 residues may overlap in sequence and reassociate, even if they are derived from a unique set of 140 base-pair fragments. Strands shorter than about 40 residues may be unable to form stable duplexes, even if the 140 base-pair fragments are random. Strands both longer than 70 and shorter than 40 residues are therefore removed by preparative polyacrylamide gel electrophoresis.

In summary, the relation of nucleosomes to nucleotide sequences can be evaluated by a procedure involving micrococcal nuclease digestion of chromatin to convert the DNA to 140–160 base-pair fragments, exo III digestion and gel electrophoresis to produce 40–70 residue single strands, and reassociation analysis. The proportion of nucleosomes that occur in a random relation to nucleotide sequences is given by the percentage of single strands that reassociate. This interpretation of reassociation behaviour applies to nucleotide sequences that are represented in a single copy in the genome. It does not apply to repeated sequences, since the relation of nucleosomes to these sequences could vary from one location of a sequence in the genome to another and even if the relation were specific at every location, the single strands produced by exo III digestion would reassociate.

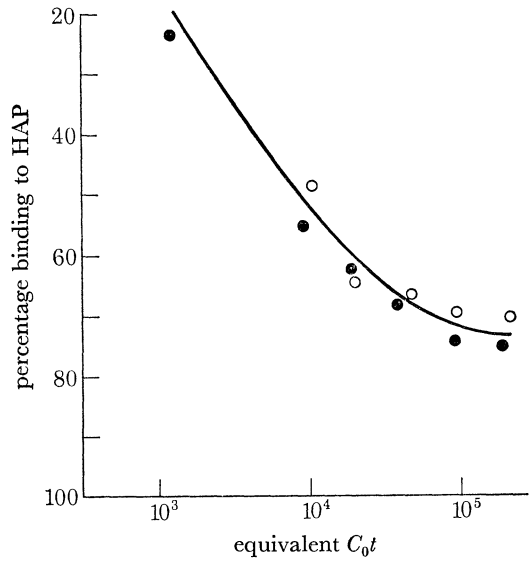

FIGURE 1. Reassociation kinetics of single strands derived from chromatin in rat liver nuclei (●) and from reconstituted chromatin (○) by the procedure described in the text. The percentage of binding to hydroxyapatite (HAP) represents the proportion of strands that participate in duplex formation. The equivalent C_0t represents the time allowed for duplex formation multiplied by the overall concentration of DNA and referred to standard DNA reassociation conditions (Britten *et al.* 1974).

We have carried out the procedure described above, starting from chromatin in rat liver nuclei, and also, as a control, starting from chromatin that had been released from rat liver nuclei and carried through two cycles of dissociation in 2 M NaCl and reconstitution by removal of the salt to ensure a completely random location of nucleosomes on the DNA. Single strands derived from chromatin in nuclei reassociate extensively, and their time course of reassociation is virtually indistinguishable from that of random, control material (figure 1). The failure of either type of material to reassociate completely is due to a preference of exo III for degrading certain sequences as compared with their complements (Prunell & Kornberg 1977). We

conclude that the relation of nucleosomes to single copy DNA sequences in rat liver is random. Single copy sequences represent two thirds of the total in rat liver (Holmes & Bonner 1974). Of course, a small proportion of these sequences, for example the 5% or so believed to code for proteins, could be packaged in nucleosomes in a specific way but escape detection in our experiment.

It may be asked whether rat liver is sufficiently heterogeneous in cell type that a specific relation of nucleosomes to nucleotide sequences, different in each cell type, could give the appearance of a random relation. Electron micrographs of thin sections of rat liver (Blouin, Bolender & Weibel 1977) show that nuclei of hepatocytes account for 87% of the total volume of nuclei in the tissue. If the volume of a nucleus is roughly the same for all cell types represented (hepatocytes, endothelial cells, Kupffer cells, fat-storing cells) then hepatocytes account for approximately 90% of the total number of cells and the tissue may be regarded as essentially homogeneous in cell type.

Do nucleosomes vary in DNA content within a cell?

The finding of a random relation of nucleosomes to nucleotide sequences casts doubt on the assumptions required for a specific relation (see Introduction). We are led to ask, for example, whether the DNA content of nucleosomes is constant within a cell. Inasmuch as a nucleosome contains both 'core' and 'linker' DNA (see Kornberg (1977) for references) and the core length is constant (140 base pairs), the question becomes whether the linker length is constant within a cell. This question also arises in connection with the breadth of the bands formed when chromatin is cleaved in the linker region with micrococcal nuclease and the resulting DNA fragments are analysed in a polyacrylamide gel. The breadth of the bands may be due to variation in the site of cleavage in the linker region, or to variation in length of the linker DNA, or to a combination of these effects (Noll & Kornberg 1977; Lohr, Corden, Tatchell, Kovacic & Van Holde 1977).

A possible approach for detecting variation in length of the linker DNA is to digest chromatin with micrococcal nuclease for a time sufficient to cleave between some but not all nucleosomes, isolate the nucleosome dimer by sucrose gradient sedimentation, and degrade the linker DNA from the ends to obtain a trimmed dimer comprising two cores connected by a linker. If the linker length is constant then DNA extracted from the trimmed dimer will form a sharp band in a polyacrylamide gel. The difficulty with this procedure is to degrade the linker DNA at the ends of the dimer without cleaving the linker in the middle. We have investigated the use of a combination of exo III and S1 nuclease for this purpose. Exo III should degrade from the 3′ termini and expose single strand regions which are digested by S1. When nucleosome monomers are trimmed with these enzymes, the DNA is converted from a broad distribution of average size 175 base pairs to nearly homogeneous material of about 140 base pairs (figure 2). In preliminary experiments where nucleosome dimers were trimmed in this way, the breadth of the band obtained in a polyacrylamide gel was virtually unaffected. While this suggests that the length of linker DNA does vary within a cell, the conclusion cannot be firmly stated until control experiments have been done to establish that exo III and S1 act on nucleosome dimers in the same manner as they do on monomers.

In the course of this work we found that exo III and S1 are capable of degrading the core as well as linker DNA. The resulting DNA fragments form a pattern of bands in a

polyacrylamide gel similar to that produced by digestion with DNase I (figure 3). Although the band patterns are similar, their significance is quite different. The DNase I pattern arises from nicking of the DNA at intervals of 10 bases along both strands and reveals sites of exposure of the DNA (Noll 1974). In contrast, exo III invades the core particle from the ends (followed by S1), and the band pattern reveals impediments to exo III digestion, that is, sites where the DNA is protected, presumably bound to histones, at intervals of 10 base pairs along the DNA.

FIGURE 2. Trimming of nucleosome monomer with exo III and S1. Two lanes of a 98% formamide–8% polyacrylamide gel are shown, which contain DNA extracted from nucleosome monomer before (top) and after (bottom) treatment with exo III and S1. Direction of electrophoresis is from left to right.

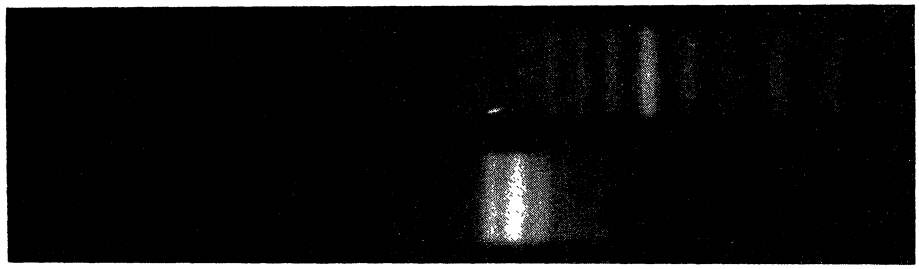

FIGURE 3. Digestion of core DNA by exo III and S1. Gel as in figure 2, showing DNA fragments from nucleosome monomer treated with exo III and S1 (bottom) or DNase I (top).

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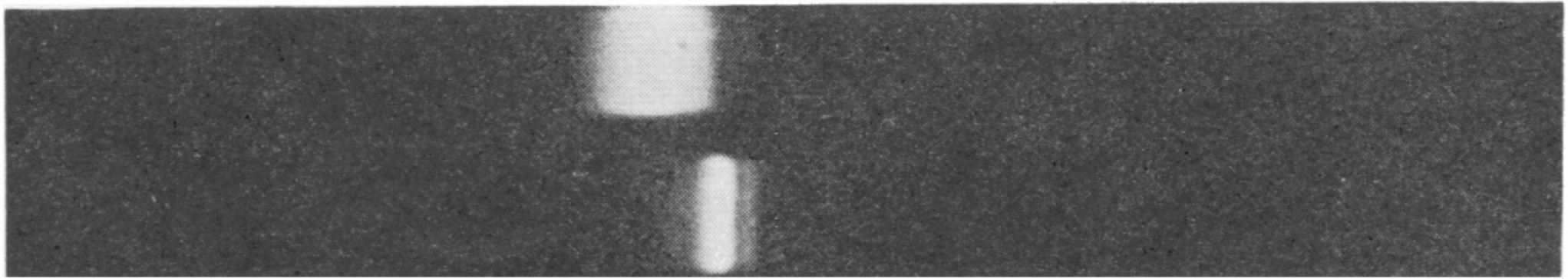

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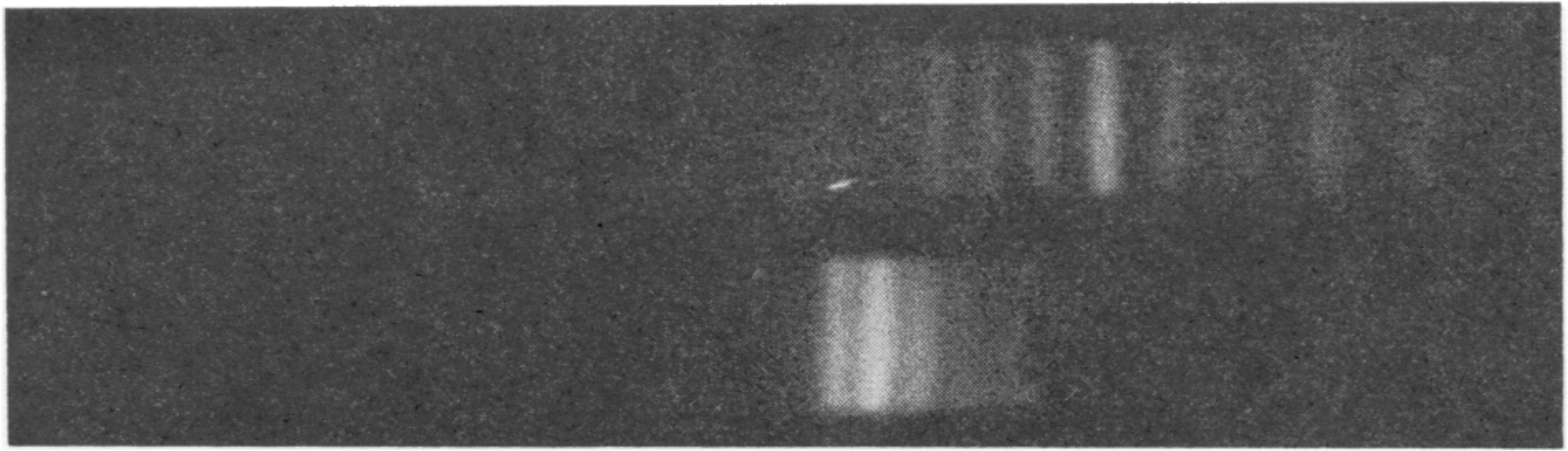

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