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Subunit structure of α-satellite DNA containing chromatin from African green monkey cells

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ABSTRACT

α-satellite DNA containing chromatin from African green monkey cells (CV-1 cells) has been used to study the question whether or not nucleosomes are arranged in phase with the 172 bp repeat unit of the satellite DNA. Digestion experiments with DNAase II led us to exclude a simple phase relationship between the nucleosomal and the satellite DNA repeats. Digestion of CV-1 nuclei with micrococcal nuclease and endogenous nuclease(s) produced a series of sharp bands in the satellite DNA register over a background of heterogeneous length fragments. This observation is explained by a preferential cleavage of certain nucleotide sequences by these nucleases and is not in contradiction to our conclusion that a simple phase relationship does not exist.

INTRODUCTION

Although in recent years an understanding of the general architecture of the nucleosome has emerged (review ref. 1) little is known about the structure and properties of the internucleosomal linkers. One aspect of this problem has attracted the attention of several groups of workers: does the nucleotide sequence of the DNA influence the arrangement of the nucleosomes leading to a unique positioning on the DNA, or are the nucleosomes distributed randomly?

Reassociation experiments with exonuclease III treated DNA from nucleosomal core particles of rat liver allowed the conclusion that the relation of nucleosomes to nucleotide sequences is random for most single copy sequences (2). Experiments with SV40 chromatin have also been interpreted in favor of a random distribution of nucleosomes (3,4) while, using another approach, it was observed that in the region of the EcoRI and BamH1 sites

of SV40 DNA, the nucleosomes occupy a small number of distinct positions (5). In still another approach, a specific region in the DNA of compact SV40 minichromosomes was found to be uniquely exposed to nucleases (6-8) which means that at least some nucleosomes are in distinct positions relative to the SV40 DNA. A quite different system should also be mentioned in this context: in reconstitution experiments with procaryotic DNA fragments of known sequence two out of many possible arrangements of the core histone particle were found to be highly favored (11).

In mouse satellite chromatin no evidence of a possible phase relationship between DNA sequence and nucleosome positioning was detected in studies employing restriction nucleases (9) or micrococcal nuclease (10). One satellite DNA which is particularly suitable for studying such a possible relationship is the α-satellite of the African green monkey (12). According to an analysis with restriction nucleases it comprises about 13 % (13) and according to reassociation experiments about 20 % (14,15) of the genome and has been thoroughly characterized by restriction nucleases (13,16). Sequence analysis has revealed a repeat length of 172 bp (15). The results of HindIII digestion experiments with micrococcal nuclease prepared mononucleosomes and the results of other experiments were considered by Musich et al. to be strongly indicative of a phase relationship between the nucleosomal and the satellite DNA repeats (17). Other experiments with micrococcal nuclease and subsequent hybridization studies led D. Singer recently to the opposite conclusion (18). The experiments described in the present paper deal with this question, but it should be kept in mind that they allow only conclusions as to a possible phase relationship between the nucleosomal and the satellite DNA repeats and cannot be used as basis for general statements about a random or non-random distribution of nucleosomes on the DNA.

MATERIALS AND METHODS

Cell culture, isolation of nuclei and DNA. CV-1 and BSC-1

cells (African green monkey) were obtained originally from American Type Culture Collection and from G. Sauer (Heidelberg), respectively. Cells were grown to confluency in minimal essential medium supplied with nonessential amino acids, 110 mg/l pyruvate, 1.5 g/l glucose, 0.1 mg/l Fe $(NO_3)_3 \cdot 9\ H_2O$ and 10 % fetal calf serum.

In a typical preparation of nuclei the monolayers of two roller bottles (550 cm^2) were washed with Tris/Ca^{2+} buffer (15 mM Tris-HCl, 3.3 mM $CaCl_2$, pH 7.0) and scraped off the culture vessel in the same buffer. After centrifugation for 5 min at 1000 g the cells were suspended in 15 ml Tris/Ca^{2+} buffer containing 0.1 % Triton X-100 and 0.34 M sucrose and treated in a tightly fitting Dounce homogenizer until most of the nuclei appeared free from cytoplasmatic particles as seen in the phase contrast microscope. The nuclei were centrifuged (10 min, 1200 g) and the pellet suspended in 1 ml of the same buffer. 6 ml Tris/Ca^{2+} buffer in 2.2 M sucrose were added and after thorough mixing the nuclei were centrifuged through a cushion of Tris/Ca^{2+} buffer in 2.2 M sucrose (5 ml) for 60 min at 100 000 g. The pelleted nuclei were washed in Tris/Ca^{2+} buffer and stored at -80° C or used immediately. Amounts of nuclei are given in A_{260} units measured after dissolving them in 0.2 % SDS.

The DNA from BSC-1 cells was isolated as in ref. (13) and α-satellite DNA as in ref. (12). For isolation of the HindIII dimer BSC-1 DNA was digested with an excess of HindIII and after separation by electrophoresis on a 4 % acrylamide gel the dimer eluted as described in (19). The cloned HindIII monomer was isolated from the hybrid plasmid pHG20 (20) by digestion with EcoRI and BamH1; a fragment containing one 172 bp HindIII monomer and 377 bp derived from the plasmid pBR313 (21) was used in hybridization experiments.

Enzymes, digestions, gel electrophoresis, hybridization. Micrococcal nuclease and DNAase I and II were purchased from Worthington, proteinase K from Merck, Darmstadt. HindIII and EcoRI were kindly provided by R. Streeck and BamH1 by U. Hänggi of our laboratory.

Digestions with micrococcal nuclease were carried out in a

polyamine containing buffer similar to that described in ref. (22) (0.15 mM spermine, 0.5 mM spermidine, 15 mM Tris-HCl, 60 mM KCl, and 15 mM NaCl, 0.2 mM EDTA and 0.2 mM EGTA, adjusted to pH 7.4). Digestions were terminated by the addition of an equal volume of 15 mM EDTA, 1 % SDS, and 10 mM Tris-HCl, pH 7.8, and mixtures then incubated in the presence of 0.2 mg/ml proteinase K for about 14 h. After precipitation with ethanol the DNA was dissolved in a buffer containing 10 mM Tris-HCl, 1 mM EDTA, pH 7.7. Aliquots were applied to agarose gels as specified in the legends. Gel electrophoresis and reference substances were as described in (19). Electrophoresis buffers were Tris-phosphate as in ref. (23) (without SDS) except for Tris-borate (24) in Fig. 2.

Nick translation of α-satellite DNA was as described (25). In the case of cloned HindIII monomer the DNAase I treatment was omitted. Transfer to nitrocellulose filters and hybridization conditions were as in (26,27). The last three wash steps before exposition were for 30 min in 1xSSC containing 0.1 % SDS.

RESULTS AND DISCUSSION

Digestion with micrococcal nuclease of nuclei and satellite DNA from African green monkey cells. Digestion of nuclei from CV-1 cells with micrococcal nuclease and subsequent deproteinization is known to result in the usual series of nucleosomal DNA fragments which are multiples of a unit length fragment. Determinations of the unit length have yielded values between 189 bp (28) and 185 bp (17,18). Our measurements agree best with a value of 188 bp, which will be used in the following. We were surprised to find superimposed on the 188 bp repeat of the nucleosomal pattern a pattern of sharp bands in the 172 bp repeat of the α-satellite DNA, both after digestion at 37^{o} (Fig. 1a) and 0^{o} (Fig. 1c). The bands are as sharp as is usually found only in restriction nuclease digests and moreover coincide in their mobilities with bands of a HindIII digest of α-satellite DNA (Fig. 1b and d). The length of the repeat unit is 172 bp according to sequence data (15) which were recently confirmed with cloned satellite DNA fragments (20) in our laboratory (H. Graf, unpublished).

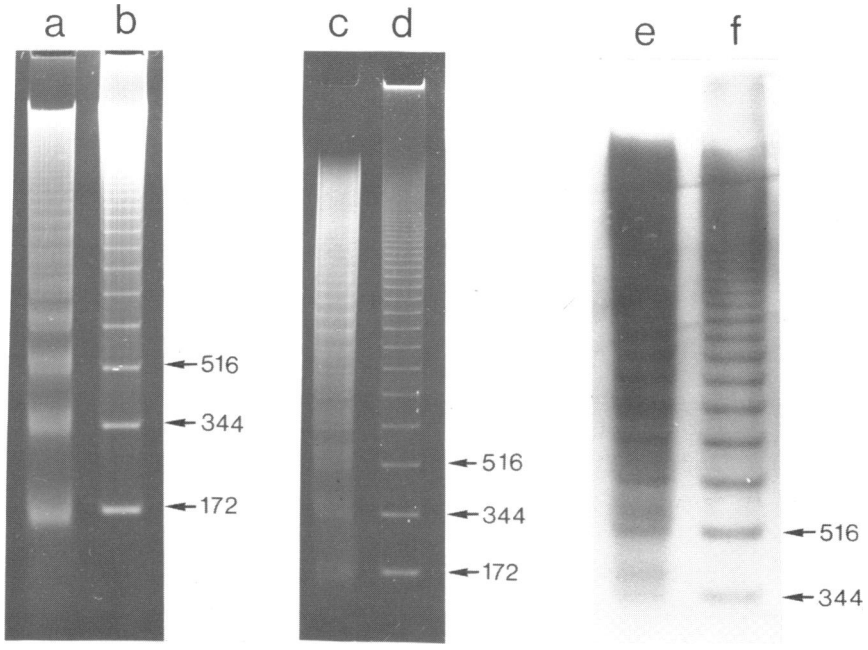

Figure 1. Micrococcal nuclease digestion of CV-1 nuclei produces DNA fragments of discrete length which are derived from the α-satellite component. a. 0.75 A_{260} units nuclei (stored at -80° C) were incubated with 2.5 units micrococcal nuclease in 60 µl polyamine buffer, supplemented with 1.5 mM $CaCl_2$ and 50 mM NaCl for 1 min at 37° C. An aliquot containing 0.14 A_{260} units DNA was electrophoresed on a 2 % agarose gel. c. 4.2 A_{260} units nuclei were incubated with 110 units micrococcal nuclease in 360 µl buffer as in a. for 20 min at 0° C. An aliquot containing 0.13 A_{260} units DNA was applied to a 1.5 % agarose gel. b. and d. Reference samples for calibration: 0.04 A_{260} units α-satellite DNA partially digested with HindIII. e. and f. DNA of the gels c. and d. respectively, was transferred to nitrocellulose filters and hybridized against ^{32}P-labelled cloned HindIII monomer.

The superimposed nucleosome and satellite DNA periodicities (Fig. 1) were readily observed on the gels and also on the original photographs. The same holds for the band patterns of the subsequent figures. Necessarily some of the contrast is lost on reproduction.

In order to localize the distribution of the α-satellite DNA in the nuclear digestion pattern, DNA from the gels shown in Fig. 1c and d was transferred to nitrocellulose filters and hybridized against a plasmid derived ^{32}P-labelled DNA fragment

containing one α-satellite repeat unit (Fig. 1e and f). Hybridization to DNA fragments in the register of the satellite repeat can be seen clearly up to higher multiples. At higher multiples hybridization leads to a continuous background due to length heterogeneity of DNA fragments in these bands. If in an analogous experiment (not shown) the fragments were separated on a 2 % instead of a 1.5 % agarose gel, hybridization appeared to occur also in the register of the nucleosomal repeat.

On first sight one might interpret these results by saying that in the CV-1 nuclei a certain fraction of the nucleosomes is arranged in phase with the satellite repeat unit, leading to the series of bands in the register of the satellite DNA, while the other part of the nucleosomes is arranged at distances of about 188 bp as in the main band DNA. If on the other hand protein-free α-satellite DNA itself possesses sites of preferential cleavage by micrococcal nuclease, the sharp bands in the satellite DNA register (Fig. 1a,c,e) could be explained without invoking a phase relationship.

In control experiments we therefore incubated protein-free α-satellite DNA with micrococcal nuclease under partial digestion conditions. Separation by gel electrophoresis yielded a series of sharp bands in the register of the α-satellite repeat unit, as well as a background of heterogeneous length fragments (Fig. 2b,c). Two additional bands of intermediate size are seen in between each pair of multiples, one being 46 bp longer than the lower full multiple, the other 46 bp shorter than the next higher multiple. Apparently α-satellite DNA is preferentially cleaved by micrococcal nuclease in a 172 bp register at two sets of sites which are 46 bp apart.

Experiments with the 344 bp HindIII dimer of the α-satellite DNA confirm the notion of preferential cleavage sites for micrococcal nuclease: between the 344 bp dimer and the 172 bp monomer bands four major and two minor bands are clearly visible, at 294, 265, 245, and 221 bp and at 314 and 194 bp, respectively (Fig. 2e,f). These data are not sufficient to unequivocally locate the micrococcal cleavage sites relative to the HindIII cleavage site of the repeat unit. However, one can say that the results of the satellite DNA and the HindIII dimer cleavages are

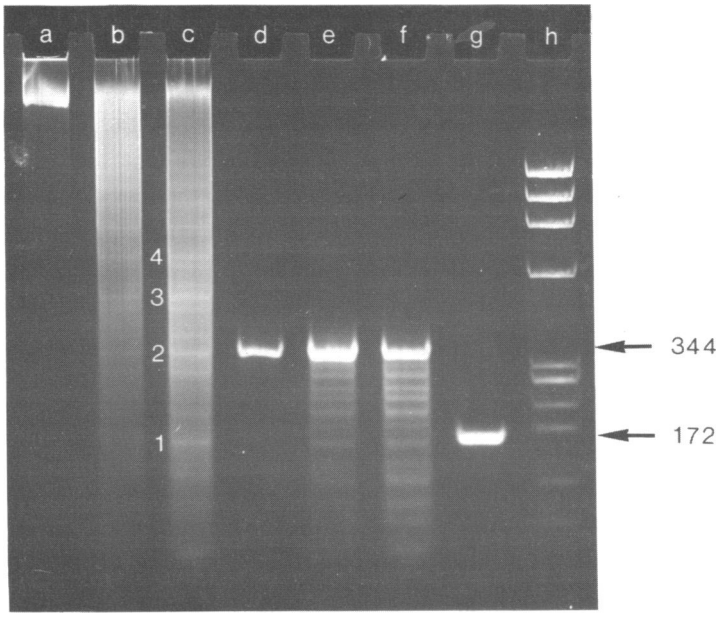

Figure 2. Sharp bands are also produced on digestion with micrococcal nuclease of α-satellite DNA (b,c) and HindIII dimer (e,f). 0.09 A_{260} units α-satellite DNA were incubated with 0.25 units micrococcal nuclease in 270 µl polyamine buffer (supplemented with 2 mM $CaCl_2$ and 50 mM NaCl) at 37° C for 2.5 min (b) and 6 min (c). (The numbers 1,2,3, and 4 designate the positions of the monomer, dimer, trimer, and tetramer of the α-satellite repeat, respectively.) A control sample (0.02 A_{260} units) was incubated under identical conditions without nuclease (a). 0.075 A_{260} units HindIII dimer were incubated with 0.2 units micrococcal nuclease for 2.5 min (e) and 6 min (f). A control sample (0.015 A_{260} units) was incubated for 6 min without nuclease (d). Reference samples: the 172 bp HindIII monomer (g) and 0.06 A_{260} units of ØX 174 RFI DNA digested to completion with BspRI (h).

consistent with a map in which the preferential micrococcal nuclease cleavage sites are located approximately at positions 50 and 96 or at positions 76 and 122, respectively, of the HindIII repeat unit. A preference of micrococcal nuclease for A/T rich sequences is well known (29). There are in the satellite DNA sequence A/T clusters at all possible positions of preferential cleavage, but there are other A/T clusters where apparently no preferential cleavage takes place under our conditions. Thus the known nucleotide sequence of the DNA (15) does

not help in a definite assignment of the micrococcal cleavage sites.

The finding of superimposed nucleosomal and satellite DNA periodicities in micrococcal digests of CV-1 nuclei (Fig. 1) can be explained on the basis of the preferential but not sequence specific mode of cleavage by this nuclease: sharp bands in the 172 bp register are formed when two of the preferential cleavage sites are located in internucleosomal linker regions; on the other hand, when cleavage occurs in linker regions without a preferential cleavage site diffuse bands in the nucleosomal register arise.

The finding of sharp bands in the nuclear digest offers also an alternative explanation of the results of Musich et al. (17) which were mentioned in the Introduction: if their micrococcal nuclease prepared mononucleosomes contained a fraction which had been formed by cleavage at the preferential sites, subsequent HindIII cleavage of the DNA would be expected to yield sharp bands; in other words, the sharp bands would not be the result of a phase relationship between the satellite and nucleosomal register. It may be mentioned that we confirmed the main experimental results of Musich et al. (17): when micrococcal nuclease prepared mononucleosomal DNA was redigested with HindIII we found a small amount of a 135-140 bp DNA together with some smaller DNA fragments. (However, corresponding redigestion experiments with EcoRI$^+$, which yielded two bands and with Hph which gave no sharp bands are at variance with the detailed model of ref. 17.) We also confirmed the observation (17) that some satellite DNA containing chromatin is solubilized by EcoRI but in the absence of a quantitative determination this finding can be accounted for as well on the basis of a random distribution of nucleosomes on the satellite DNA.

In summary, all our results with micrococcal nuclease digestions can be explained without postulating a phase relationship between the nucleosomal and the satellite DNA repeats but they do not exclude such a relationship. A nuclease without preference for certain sequences would be a better tool for studying this problem. We therefore turned first to digestion with endogenous nuclease(s) and then to DNAase II.

Digestion of nuclei from CV-1 cells with endogenous nuclease(s). Nuclei from CV-1 cells showed a remarkably low activity of endogenous nuclease(s). Autodigestion of such nuclei for 30 or 60 min did not lead to the appearance of oligosomal material; in comparison, mouse and rat liver nuclei were appreciably degraded under these conditions. For nuclei from CV-1 cells 27 h of incubation in the presence of Ca^{2+} and Mg^{2+} were required to yield the digestion pattern shown in Fig. 3a. Cleavage with endogenous nuclease(s) led also to a series of faint but distinct bands in the register of the satellite repeat unit plainly visible over a background of heterogeneous length fragments. These bands are only observed in the range of higher multiples. They were more clearly seen when the DNA was transferred to a nitrocellulose filter and hybridized against a cloned ^{32}P-labelled α-satellite monomer (Fig. 3c). Better blotting results were obtained when ^{32}P-labelled α-satellite DNA of high molecular weight was used because in this case filters could be washed under more stringent conditions (Fig. 3e).

The rather high background makes the interpretation of the autodigestion patterns difficult, but as with micrococcal nuclease, one could attribute the appearance of distinct bands to an endogenous nuclease with a preference for certain nucleotide sequences, possibly to the one found by Maio et al. (30) in African monkey testes and CV-1 cells. Thus, these results too are consistent with our general conclusion (see below).

Digestion of nuclei from CV-1 cells with DNAase II. Incubation of CV-1 nuclei with DNAase II led to the pattern of Fig.4a. The lengths of the DNA fragments fit into the register of the nucleosomal repeat in CV-1 cells with an average of 188 bp, thus clearly differing from the satellite repeat of 172 bp. No DNA bands could be detected in the register of the satellite repeat unit. A coelectrophoresis experiment with a DNAase II digest of nuclei and a partial HindIII digest of α-satellite DNA (not shown) showed that the sharp bands would have been detected in the nuclear digest, if they had been present even in a modest amount. In order to detect the distribution of the α-satellite DNA in the DNAase II digestion pattern, the DNA of the pattern of Fig. 4a was transferred to a nitrocellulose filter and hybri-

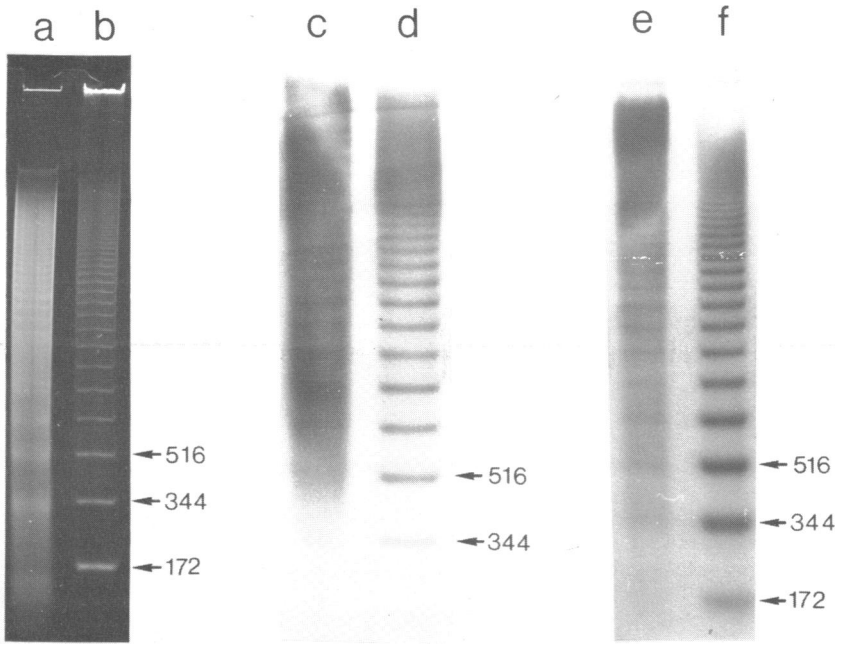

Figure 3. Digestion of CV-1 nuclei by endogenous nuclease(s) produces fragments of discrete length in the register of the α-satellite repeat, superimposed upon a background. Nuclei were prepared as described in Methods using a buffer of 10 mM Tris-HCl, pH 7.5, 2 mM $CaCl_2$, and 3 mM $MgCl_2$ instead of the Tris/Ca^{2+} buffer. a. 4.2 A_{260} units freshly prepared CV-1 nuclei were incubated in 400 µl of the same buffer for 27 h. The reaction was terminated by the addition of an equal amount of buffer (20 mM EDTA, 1 % SDS and 10 mM Tris-HCl, pH 7.8), treated with proteinase K and precipitated with ethanol as described in Methods. An aliquot containing 0.16 A_{260} units DNA was electrophoresed on a 1.5 % agarose gel. b. 0.03 A_{260} units of a partial HindIII digest of α-satellite DNA. c. and d. DNA from the patterns a. and b. respectively, was transferred to a nitrocellulose filter and hybridized against ^{32}P-labelled cloned HindIII monomer of α-satellite DNA. e. Nuclei were prepared, incubated (for 19 h), and electrophoresed as in a. and after transfer to nitrocellulose filters, hybridized against ^{32}P-labelled high molecular weight α-satellite DNA. The final wash steps were with 0.1xSSC containing 0.1 % SDS. f. Partial HindIII digest of α-satellite DNA, electrophoresed and hybridized as in e.

dized against the ^{32}P-labelled cloned BSC-1 monomer. Hybridization is only seen in the 188 bp register, none in the 172 bp register of the satellite DNA (Fig. 4c).

The results of the DNAase II digestion experiments practically exclude a simple phase relationship between the nucleo-

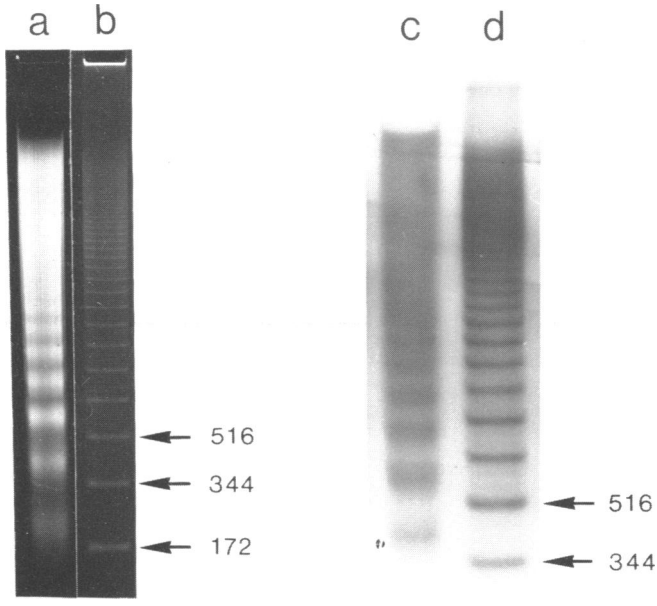

Figure 4. DNAase II digestion of CV-1 nuclei produces only diffuse bands in the nucleosome register. a. 2 A_{260} units freshly prepared CV-1 nuclei were washed twice in 0.3 ml 10 mM Tris-HCl, pH 7.0, at 4° C and incubated in 0.2 ml of the same buffer in the presence of 20 units DNAase II for 6 min at 37° C. An aliquot containing 0.14 A_{260} units DNA was applied to a 1.5 % agarose gel. b. 0.02 A_{260} units α-satellite DNA partially digested with HindIII. c. and d. DNA of the gels a. and b. respectively, was transferred to a nitrocellulose filter and hybridized against a ^{32}P-labelled cloned HindIII monomer of α-satellite DNA.

somal and the satellite DNA repeats. Thus, this nuclease yields the clearest results. One may argue, however, that in the low salt concentration (10 mM Tris-HCl) used for the incubation with DNAase II, the nucleosomes might have been rearranged. Therefore in a control experiment nuclei were first washed and incubated under the conditions of DNAase II digestion (10 min, 37° C) but without nuclease and afterwards digested with micrococcal nuclease as described in the legend to Fig. 1c. In the digestion pattern (not shown) series of bands in both the satellite and nucleosomal register appear, indistinguishable from those of Fig. 1a and 1c. The conversion of a homogeneous 172 bp nucleosomal repeat to a homogeneous 188 bp repeat during the 10 min

in a low salt buffer seems to be a rather unlikely supposition. Control experiments with DNAase II in the presence of divalent cations (not shown) yielded an approximately 95 bp repeat as expected (31).

CONCLUDING REMARKS

The experiments described in this paper deal with a basic question in chromatin research: does the DNA sequence influence the positioning of nucleosomes? For the system under investigation here, the α-satellite DNA component of the African green monkey cell, we come to the conclusion that there is no simple phase relationship between the nucleosomal and the satellite DNA repeats, in other words, no influence of the DNA sequence on the nucleosomal repeat is detected. This conclusion is based partly on the studies described in this paper where micrococcal nuclease is used and also on the few experiments on autodigestion of nuclei. The results with DNAase II digestion leave no other possibility but to assume that the nucleosomal repeat is essentially independent of the repeat of the satellite DNA sequence. This does not exclude the possibility that the arrangement of the nucleosomes is the product of several superimposed phase relationships or that certain distinct DNA sequences, e.g. at the transition from satellite DNA to other DNA sequences, determine nucleosomal positions over a short range. Such special types of phase relationships have been observed in other systems (5-8) and may be operative also in the satellite DNA system of African green monkey cells.

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