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Eight different highly specific nucleosome phases on α-satellite DNA in the African green monkey

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ABSTRACT

The question of nucleosome phasing on African Green Monkey (AGM) α-satellite DNA has been addressed by employing a new approach. Nucleosome cores were prepared from AGM nuclei with micrococcal nuclease, exonuclease III and nuclease S1. The core DNA population derived from α-satellite DNA containing chromatin was purified from total core DNA by denaturation of the DNA, reassociation to a low Cot value, and hydroxyapatite chromatography to separate the renatured satellite fraction. After end-labeling the termini of the α-satellite containing core DNA fragments were mapped by high resolution gel electrophoresis relative to known restriction sites along the 172 bp repeat unit of the satellite DNA. The results show that nucleosomes occupy eight strictly defined positions on the α-satellite DNA which could be determined with an accuracy of ± 1 base pair. Approximately 35 % of all nucleosomes are organized in one of these frames while the other seven registers contribute about 10 % each.

INTRODUCTION

Most investigations addressing the question of nucleosome phasing in chromatin can be broadly grouped into two categories differing in the experimental design (for a review see refs. 1-4). The first category of experiments involves partial digestion of nuclei with micrococcal nuclease and mapping of the micrococcal cuts relative to known restriction sites. The DNA in question is usually visualized by Southern transfer and hybridization. The underlying rationale is that micrococcal nuclease cuts would reflect the location of all linkers in a repeating nucleosome structure. This strategy has been seriously questioned when it was realized that micrococcal nuclease has a much greater sequence specificity than previously thought (5,6). As a consequence, under partial digestion conditions the nuc-

lease cleaves selectively only those linkers that contain particularly susceptible DNA sequences usually of the type $\frac{CATA}{GTAT}$ and $\frac{CTA}{GAT}$ (5). The experiments therefore do not uncover the location of all nucleosome linkers along a given DNA but only a rather specific selection, thereby frequently creating the false impression of specific nucleosome positions.

In the second experimental design, micrococcal nuclease digestion is carried almost to a limit in an effort to convert the entire chromatin to core particles containing 145 bp DNA. This DNA is then digested with restriction nucleases and the resulting fragments again visualized after Southern transfer by hybridization techniques, thereby effectively mapping core positions relative to known restriction sites. This second approach largely circumvents the complications arising from the sequence specificity of micrococcal nuclease. Its major drawback, however, is the difficulty of visualizing by hybridization techniques the very short DNA fragments that are generated after digestion with restriction nucleases.

For a special class of DNA like satellite DNA that is highly abundant in the genome there is a way out of this dilemma. Such DNA can often times be physically isolated by a variety of techniques (reviews 7,8). We argued that it should be possible to isolate the satellite DNA fraction from total core DNA by renaturation kinetics, exploiting its much higher rate of reassociation. Purified satellite DNA containing core fragments could then be endlabeled and subjected to the high resolution electrophoretic techniques that have been so successfully used in DNA sequencing.

We have applied this new approach to the study of α-satellite DNA containing chromatin from AGM[1)] cells. There have been claims that a strict phase relationship between α-satellite DNA sequence and nucleosome positions exists (9-11). As described in the accompanying paper (12) we have questioned these claims since the results stemmed from experiments basically conforming to the first category described above and therefore subject to the same criticisms. In fact, protein-free α-satellite DNA is highly preferentially cleaved at certain sites by micrococcal nuclease (12), and we therefore concluded that the direct mapping of

micrococcal nuclease cuts in α-satellite DNA containing chromatin is not a feasible approach for the investigation of nucleosome phasing on this DNA. By using our new approach we could show that the organization of α-satellite DNA containing chromatin is more complex since nucleosomes were found to reside in eight highly specific positions on AGM α-satellite DNA.

MATERIALS AND METHODS

Enzymes and DNA. HindIII, ClaI, S1 nuclease, polynucleotide kinase, and alkaline phosphatase from calf intestine were from Boehringer Mannheim, HphI, MboII, and exonuclease III from New England Biolabs, Beverly, MA., micrococcal nuclease from Worthington, Freehold, N.J., and proteinase K from E. Merck, Darmstadt. EcoRI was prepared by T. Igo-Kemenes according to Greene et al. (13) and kindly donated. It was used under EcoRI* conditions as described by Polisky et al. (14). Bsp was purified from Bacillus sphaericus R according to Kiss et al. (15). pBR322 DNA, a gift of G. Combriato, was used as a molecular weight reference. It was linearized with ClaI, 3'-endlabeled with DNA polymerase I (large fragment) as described (16), cleaved with EcoRI, and subjected to partial degradation at purine residues. pBR322 DNA digested with HpaII and 5'-endlabeled was kindly donated by W. Linxweiler. Fragment lengths were calculated from the published sequence of pBR322 DNA (17).

AGM cell culture and isolation of nuclei. AGM cells (BSC-1, CV-1, and Vero) were grown to confluency in roller bottles using minimal essential medium (Dulbecco's modification). Nuclei were prepared by three different methods: the first procedure is as described before (18) except that cells were washed in phosphate buffered saline instead of Tris/$CaCl_2$ buffer and that 3.0 mM rather than 3.3 mM $CaCl_2$ was used afterwards. 1.0 mM phenylmethane sulfonyl fluoride (PMSF) was present in all buffers except in the final 2.2 M sucrose containing buffer in which the PMSF concentration was lowered to 0.1 mM. The second procedure was identical to the first except that a spermine/spermidine containing buffer (60 mM KCl, 15 mM NaCl, 0.15 mM spermine, 0.5 mM spermidine, 0.2 mM EDTA, 0.2 mM EGTA, 15 mM Tris-HCl, pH 7.4) based on the one described by Hewish and Burgoyne (19) was used

instead of the Tris/$CaCl_2$ buffer. The third procedure is that described by Brown et al. (10) using sodium phosphate/$CaCl_2$ buffers with the inclusion of 80 mM NaCl.

Preparation of nucleosomal core DNA. The preparation of core DNA will be described in detail elsewhere (manuscript in preparation). Briefly, it involves digestion of AGM nuclei with micrococcal nuclease and preparation of soluble chromatin as described (20). The soluble chromatin was trimmed with exonuclease III, DNA isolated and further digested with nuclease S1 similarly as described by Igo-Kemenes et al. (21). The resulting DNA was digested with alkaline phosphatase and core DNA isolated by preparative polyacrylamide gel electrophoresis.

Terminal labeling and gel electrophoresis. 5'-terminal labeling with polynucleotide kinase and analysis on sequencing gels was performed according to Maxam and Gilbert (22). Partial degradation of DNA fragments at purine residues was according to Gray et al. (23). Analysis by agarose gel electrophoresis was as described (20), and the same buffer system was used for non-denaturing polyacrylamide gels. Histone analysis by SDS gel electrophoresis was described before (24).

Enrichment of α-satellite DNA from total core DNA. 5'-end-labeled core DNA digested with different restriction nucleases was denatured by boiling for 10 min in 0.08 M sodium phosphate buffer, pH 6.75, and allowed to reassociate at 55-60^{o} to a Cot value of 2 - 5x10^{-3} (26) as described by Kohne and Britten (25). The DNA was then fractionated by hydroxyapatite column chromatography at 55-60^{o}. Single stranded DNA was washed off the column at 0.12 M phosphate buffer and renatured DNA was recovered by elution with 0.4 M phosphate buffer. DNA was dialyzed against water and ethanol precipitated using sheared salmon sperm DNA as carrier.

RESULTS

Enrichment of α-satellite DNA containing nucleosomal core DNA. The first step in our protocol for the mapping of nucleosome positions on AGM α-satellite DNA involves the preparation of nucleosome cores from AGM nuclei. It was important at this stage to minimize selection for or against any particular DNA

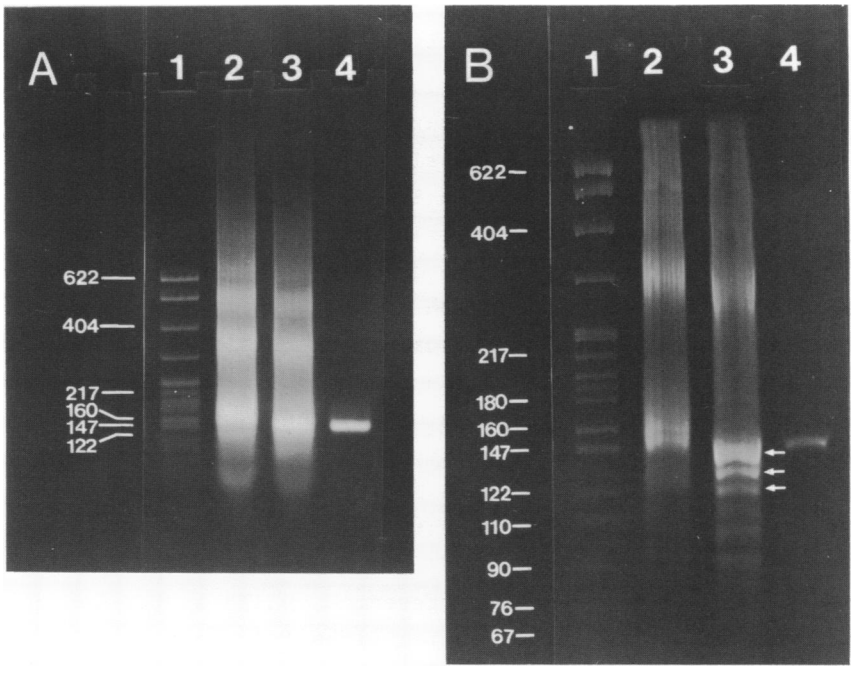

Figure 1. Preparation of nucleosomal core DNA from AGM chromatin. BSC-1 nuclei were digested with 150 U/ml micrococcal nuclease for 5 min at 37°, soluble chromatin was extracted and further digested with 50 U/ml exonuclease III for 5 min at 37°. DNA was isolated by proteinase K treatment, incubated with 1000 U/ml S1 nuclease at 37° for 30 min, ethanol precipitated, dissolved in 50 mM Tris-HCl, pH 8.0, 0.2 mM EDTA and treated with 5 U/ml alkaline phosphatase for 30 min at 37°. DNA was analyzed in a 2 % agarose gel (A) or a 5 % polyacrylamide gel (B) before as well as after exonuclease/S1 treatment (lanes 2 and 3, respectively). The arrows denote core DNA of 145 bp and subnucleosomal fragments 135 bp and 125 bp long. Lane 4 shows core DNA isolated by preparative polyacrylamide gel electrophoresis from the digest depicted in lane 3. HpaII digested pBR322 DNA (lane 1) served as a molecular weight reference. Fragment sizes are listed in base pairs.

fraction and obtain a core DNA population as representative as possible of the total DNA. We started out with a micrococcal nuclease digestion step, prepared soluble chromatin, isolated DNA and obtained the familiar nucleosomal ladder upon gel electrophoresis (Fig. 1A). Despite the fact that digestion with micrococcal nuclease was quite extensive not all of the chromatin could be converted to mononucleosomes, but some dinucleo-

somes and higher oligonucleosomes still persisted.

Trimming of the nucleosomes to obtain nucleosome cores was accomplished by digesting the soluble chromatin with exonuclease III and treatment of the purified DNA with nuclease S1. The DNA shown after electrophoresis in a 2 % agarose gel in Fig. 1A was also analyzed in a 5 % polyacrylamide gel where resolution in the 145 bp range is better (Fig. 1B). It is apparent that 145 bp core DNA is the dominant product of exonuclease III/S1 digestion, yet there is in addition some subnucleosomal DNA. Also by changing the digestion conditions it was not possible to convert all chromatin to mononucleosomes and at the same time have no or only little DNA in the subnucleosomal range. This raises the possibility that a sequence dependent bias is introduced into our core DNA population either by too limited or by too extensive digestion with micrococcal nuclease. In the former case cores flanked by micrococcal nuclease insensitive linkers would be underrepresented, while in the latter case, cores containing preferential sites for micrococcal nuclease would be selectively lost. In view of this situation we have used in the following a wide range of chromatin preparations extending from early to very late micrococcal digests.

145 bp core DNA was isolated by preparative gel electrophoresis, 5'-endlabeled with polynucleotide kinase, digested with restriction nucleases and then used for the satellite enrichment procedure by exploiting the rapid rate of reassociation of highly repetitive DNA. It proved to be preferable to carry out restriction nuclease digestion prior to the satellite enrichment procedure because after the denaturation step the satellite core fragments reassociated in many different registers therefore precluding subsequent mapping studies with restriction nucleases.

Endlabeled core DNA fragments digested with restriction nucleases were denatured and allowed to reassociate to a Cot value of $2 - 5 \times 10^{-3}$ (Methods). Separation of the renatured fraction from the bulk of single stranded DNA was achieved by hydroxyapatite chromatography. Usually about 10-15 % of the core DNA were found in the double stranded fraction, roughly in keeping with the proportion of highly repetitive DNA in AGM nuclei.

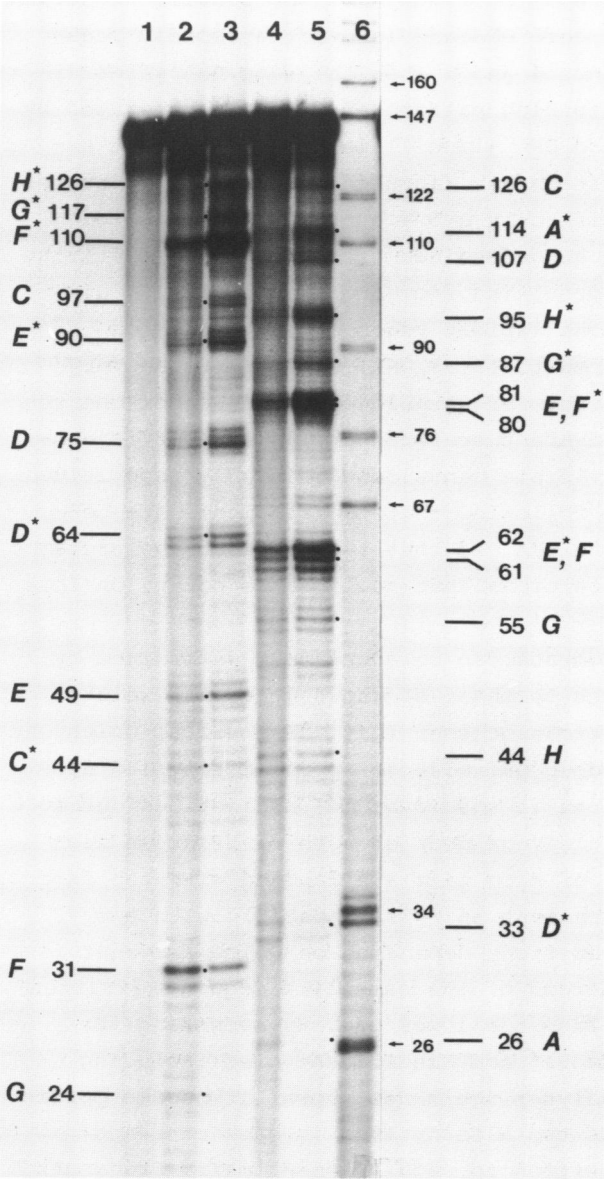

Figure 2. Nucleosome cores occupy defined positions on the α-satellite DNA repeat unit. 5'-endlabeled core DNA was digested with HindIII or EcoRI* and analyzed in an 8 % polyacrylamide sequencing gel (lanes 2 and 4, respectively). The satellite enriched DNA fractions in the HindIII and EcoRI* digests are shown in lanes 3 and 5, respectively. Unrestricted core DNA is shown in lane 1. The most prominent fragment in each cluster of bands is marked by a dot and its size listed to the left for HindIII and right for EcoRI*. The letters designate the nucleosome frames that these fragments correspond to (see text and Fig. 3). A 5'-endlabeled HpaII digest of pBR322 DNA was used as a size marker (lane 6) with fragment lengths given in nucleotides.

Mapping nucleosome core positions on the AGM α-satellite repeat unit. Endlabeled core DNA digested with either HindIII or EcoRI* was analyzed in 8 % high resolution sequencing gels

(Fig. 2). Lanes 2 and 3 show the results of the HindIII digestion experiment (lane 2 total core DNA and lane 3 the rapidly renatured fraction) while lanes 4 and 5 show the same DNA after digestion with EcoRI*. A striking pattern of bands is generated by both nucleases. Undigested DNA (lane 1) is composed almost exclusively of 143-147 bp fragments (apparent from short exposures of the autoradiograms) with only a 5 % contamination due to approximately 135 bp DNA as determined from direct radioactivity measurements. All bands resulting from EcoRI* and HindIII digestion are enriched in the rapidly reassociating fraction (lanes 3 and 5) as compared to total DNA, except for the very short fragments which do not form stable complexes under the renaturation conditions and are therefore partly lost. It is concluded that all clearly visible bands contain highly repetitive DNA. We will show in the following that the fragments are derived from the α-satellite DNA and that they reflect the presence of a number of defined positions that nucleosomes occupy on this DNA.

The sizes of all fragments can be accurately determined by comparing their mobilities to those of known molecular weight markers (e.g. lane 6). It can be seen that these sizes, which are listed in Fig. 2, add up pairwise to approximately 140 nucleotides. (For 145 bp cores it should be 141 nucleotides due to the 4 bp stagger in the cleavage mode of both restriction nucleases.) The two members of each pair are labeled with the same letter in Fig. 2. Each fragment pair is compatible with two alternative nucleosome positions depending on which fragment is derived from the upper strand of the satellite sequence (written as in Fig. 4) and which from the lower one. By comparing the patterns obtained with EcoRI* and HindIII and also including MboII and HphI in the analyses (gels not shown) it became possible to decide between the two alternatives in each case and assign one nucleosome position to each fragment pair. Fragments from the lower strand are consistently marked with an asterisk in Fig. 2.

The sizes of all fragments found in digests with each of the four restriction nucleases are listed in Table 1. The most prominent fragments in any band cluster (corresponding to those

Table 1. Determination of the nucleosome positions on AGM α-satellite DNA. The first two columns list every fragment between 15 and 135 nucleotides long that is found in HindIII, EcoRI*, MboII, and HphI digestion experiments together with the strand it is derived from (see text). Fragments corresponding to the strongest band in each band cluster (see Fig. 2) are underlined. Nucleosome start and end points were calculated for each fragment (columns 3 and 4), underlined positions correspond to underlined fragments. As described in the text, eight defined nucleosome positions (A-H) are proposed to account for all data. They are listed in the last two columns.

	fragment sizes		calculated positions		proposed positions	
	upper strand	lower strand	start	end		
HindIII EcoRI* MboII HphI	26, 27	113,114	5, 6	148,149	5 - 149	A
HindIII EcoRI* MboII HphI	 109,110,111 113,114,115	 34, 35, 36 31	 32, 33, 34 32, 33, 34	 3, 4, 5 4	32 - 4	B
HindIII EcoRI* MboII HphI	97, 98, 99 125,126 65, 66 69, 70	43, 44, 45 78, 79 74, 75	74, 75, 76 78, 79 77, 78 77, 78	47, 48, 49 47, 48 47, 48	76 - 48	C
HindIII EcoRI* MboII HphI	75, 76, 77 106,107,108 46, 47 49, 50, 51	63, 64, 65 32, 33, 34 98, 99,100 95, 96	96, 97, 98 96, 97, 98 96, 97 96, 97, 98	67, 68, 69 67, 68, 69 67, 68, 69 68, 69	96 - 68	D
HindIII EcoRI* MboII HphI	49, 50 79, 80, 81 20 23, 24	90, 91, 92 60, 61, 62 125,126 121,122	123,124 123,124,125 123 123,124	94, 95, 96 95, 96, 97 94, 95 94, 95	123 - 95	E
HindIII EcoRI* MboII HphI	30, 31 60, 61, 62	110,111 79, 80, 81	142,143 142,143,144	114,115 114,115,116	142 - 114	F
HindIII EcoRI* MboII HphI	24 54, 55, 56	116,117 86, 87	149 148,149,150	120,121 121,122	149 - 121	G
HindIII EcoRI* MboII HphI	43, 44, 45	126,127 95, 96	159,160,161	130,131 130,131	159 - 131	H

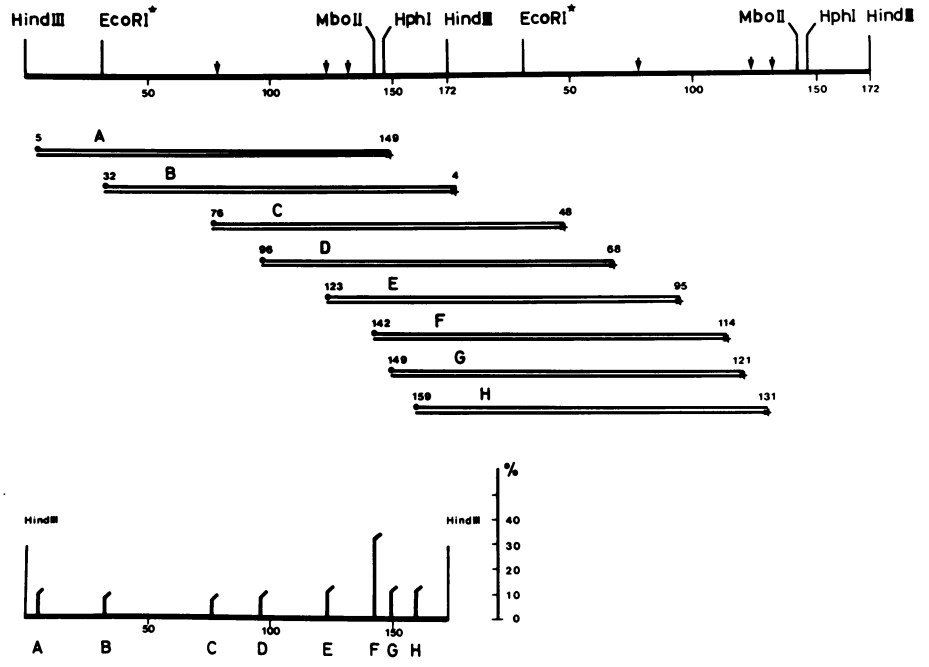

Figure 3. Location and relative abundance of the eight nucleosome frames on α-satellite DNA. Two tandem α-satellite repeat units are shown at the top with the numbering and the location of the restriction sites based on the published sequence of the satellite repeat unit (27). Arrows denote preferential micrococcal nuclease cleavage sites (12). The positions of the eight nucleosome frames (A-H) are depicted below with the numbers referring to the first and last nucleotide pair of a 145 bp nucleosome core. The 5'-end of the lower strand of each frame (based on the sequence as presented in Fig. 4) is marked with an asterisk. Correspondingly, fragments in the sequencing gel of Fig. 2 derived from the lower strand are marked with a starred letter. At the bottom, the relative percentage of each of the frames is shown, as determined by excising bands from sequencing gels and measuring the radioactivity.

bands marked with a dot in the gel of Fig. 2) are underlined in Table 1. The nucleosome positions corresponding to each of the bands detected in the sequencing gels were calculated and are listed in the center two columns of Table 1. Again positions corresponding to the most prominent band in each band cluster are underlined. It is apparent that the use of the four restriction nucleases yields a completely self-consistent picture. The core DNA used in the mapping studies is not precisely 145 bp

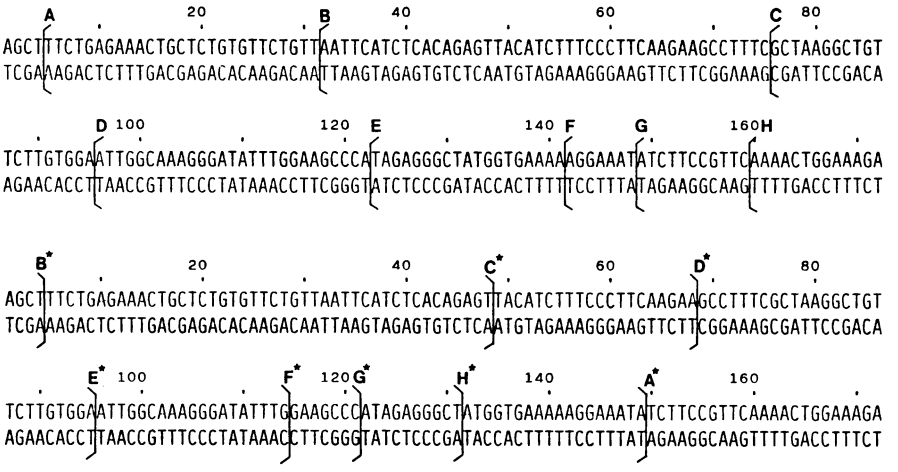

Figure 4. Nucleosome core boundaries for the eight frames on the sequence of the α-satellite repeat unit. The start ([) and end points (]) of the nucleosome cores relative to the nucleotide sequence of the α-satellite repeat unit taken from Rosenberg et al. (27) are shown.

long but ranges from 143 - 147 bp due to imprecise trimming of the nucleosomes during exonuclease III/S1 treatment. This size heterogeneity of the core DNA can in principle fully account for the finding of band clusters rather than just two bands for each nucleosome position. Core sizes computed from all combinations of nucleosome start and end positions listed in Table 1 vary between 141 and 148 bp. If we base our position assignments on a value of 145 bp for the true core size and take all experimental values into consideration we can arrive at eight precisely defined nucleosome positions (A-H) that provide a best fit to the values (see last two columns of Table 1 and Figs. 3 and 4). We would like to emphasize, though, that a microheterogeneity in the nucleosome positions themselves might also contribute to the generation of multiple bands and cannot be ruled out by the data. If such a microheterogeneity does exist, however, it cannot be more than 1 or 2 bp in either direction of the positions listed in Table 1.

Direct proof that the fragments present after HindIII digestion of core DNA are derived from the α-satellite component. It could be argued that the bands shown in Fig. 2 might actually

be derived from a different repetitive component in AGM DNA. Even though this possibility is very unlikely, since that repetitive component would need to have the same arrangement of restriction sites as the α-satellite DNA, we still wished to provide direct evidence for the origin of the bands in Fig. 2. To that end a number of bands were isolated from the sequencing gel in Fig. 2 and subjected to partial degradation at purine residues (Fig. 5). The resulting fragment ladders are exactly those predicted by the sequence of the α-satellite repeat unit and the assignments of the nucleosome positions as described in the preceding paragraph. For fragments F and F* the corresponding nucleotide sequences are shown in Fig. 5. The 110 nucleotide long F* fragment as isolated from the sequencing gel contains in addition some material shorter by one nucleotide but otherwise identical as shown by the degradation pattern at purine residues. In the case of E*, two fragments differing in size by one nucleotide could be isolated separately as shown in Fig. 5. Fragments D, D*, and E can be similarly identified without ambiguity. This type of analysis does not only confirm that the fragments in question are derived from the α-satellite DNA but also verifies the strand assignments for those fragments and permits more accurate size determinations especially for the larger fragments since the purine specific fragment ladders can be sized against known marker fragments along the entire length of the gel.

Relative abundance of the eight nucleosome frames on the α-satellite DNA. For a number of reasons the actual contribution of any particular nucleosome frame in α-satellite containing chromatin is difficult to assess. As stated at the beginning, the strong sequence specificity of micrococcal nuclease is a serious hindrance. We have therefore analyzed very early and very late digests by micrococcal nuclease. All eight frames and only those eight frames were always detected. There were, however, quantitative variations in their relative contributions. With core preparations that were judged optimal, i.e. few oligonucleosomes left and little intranucleosomal cutting, we arrived at the values for the eight frames shown at the bottom of Fig.3. As a first approximation, frame F amounted to 35 % and all other frames were around 10 %. In very early as well as very late

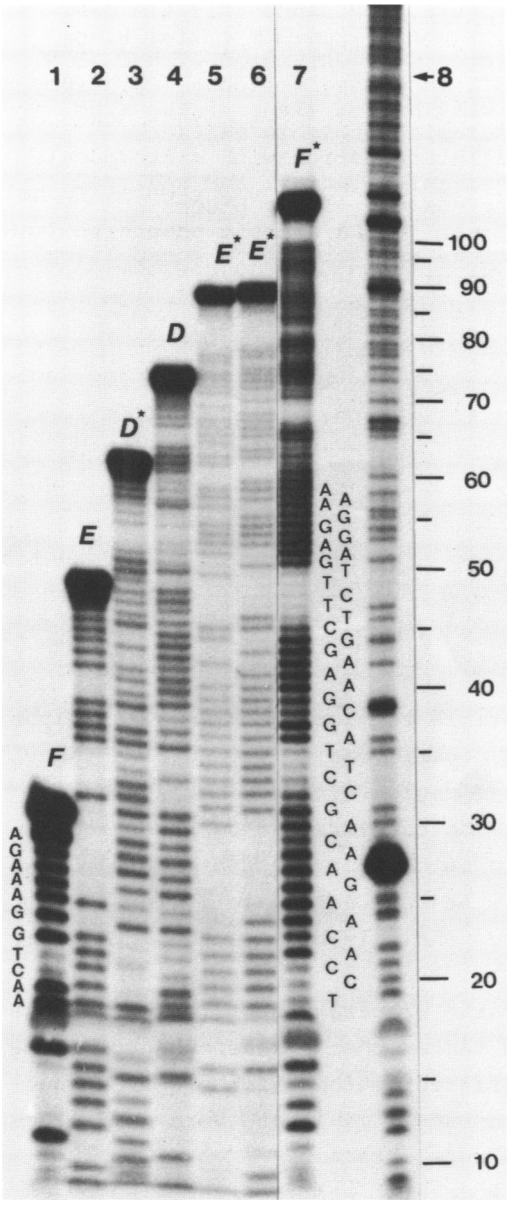

Figure 5. Purine specific degradation patterns of fragments present after HindIII digestion of core DNA. Individual bands were cut out of the HindIII digestion pattern of core DNA shown in Fig.2, lane 3, subjected to partial degradation at purine residues and analyzed in an 8 % sequencing gel (lanes 1-7, designation of the fragments (F,E etc.) as on the left in Fig. 2). The nucleotide sequences shown next to lanes 1 and 7 are taken from the published sequence of the α-satellite DNA (27) and are based on the position assignments of Fig. 3. The sequences are seen to fully agree with the actual patterns. The size marker (lane 8) was prepared from ClaI digested pBR322 DNA as described in Methods.

micrococcal nuclease digests, frame F tended to adopt higher values (40 - 60 %) while the contributions of all other frames decreased in parallel. We interpret this to be due to the fact that F is the only frame in which the two sites of the α-satel-

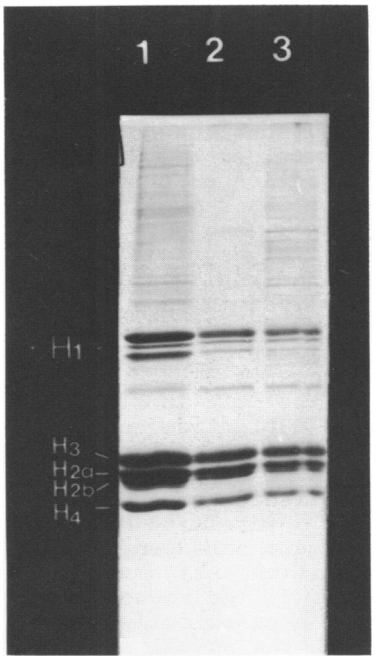

Figure 6. Histone analysis of chromatin from AGM cells. Histones were extracted with 0.25 N HCl according to Johns (29) from total nuclei (lane 3) and from soluble chromatin extracted after digestion with micrococcal nuclease (lane 2) and analyzed by SDS gel electrophoresis. Lane 1 contains reference histones extracted from mouse liver nuclei.

lite DNA that are most susceptible to micrococcal nuclease (pos. 123 and 132, see accompanying paper) come to lie outside the core region, i.e. in the linker region. Frame F nucleosomes would therefore be expected to be the first to be liberated by micrococcal nuclease and would be overrepresented in early digests. Conversely, they should be the last to fall prey to overdigestion and should correspondingly be enriched also in very late digests.

Since the method used for the preparation of the nuclei has been invoked in the past as a factor that influences nucleosome phasing (10) we used three different methods for preparing nuclei (see Methods) and compared BSC-1, CV-1, and Vero cell lines. In all cases the same results were obtained.

Histone degradation as a source of artifact can also be excluded since histones were found to be intact in the chromatin fractions used for our analyses (Fig. 6).

In order to exclude that the nucleosome positioning found here might be a peculiarity of tissue culture cells, we performed the same experiments starting with nuclei obtained from

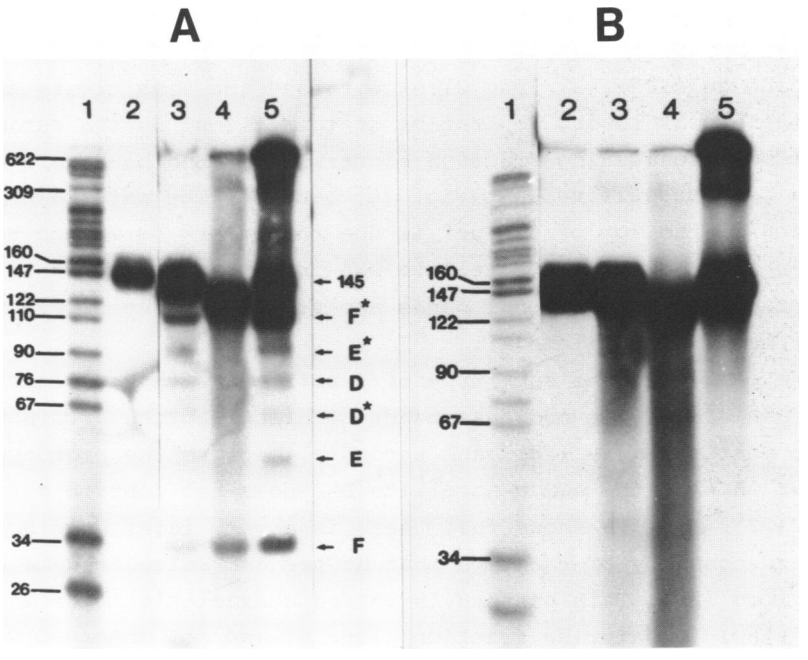

Figure 7. Comparison of the slowly and the rapidly renaturing fraction of Hind III and Bsp digested core DNA. 5'-endlabeled core DNA digested with HindIII (A) or Bsp (B) was analyzed in 8 % nondenaturing polyacrylamide gels. 1, HpaII digested pBR322 DNA; 2, undigested core DNA; 3, core DNA after restriction nuclease digestion; 4 and 5, satellite depleted and enriched fraction, respectively, of the sample in lane 3. The bands in the HindIII digestion experiment are designated with letters as in Fig. 2.

the brain of an African Green Monkey. Again the same results were obtained. Also the proportion of the individual frames was very much the same.

As a final control we wished to determine if our core preparations would be prone to the same artifact that McGhee and Felsenfeld have cautioned against (28). In their hands, the preparation of nucleosome cores by the exclusive action of micrococcal nuclease introduces a pronounced bias that manifests itself in the generation of a 10 bp ladder upon subsequent digestion of endlabeled core DNA with certain restriction nucleases.

First, we analyzed our HindIII digested core DNA by the type of nondenaturing polyacrylamide gels that McGhee and Felsenfeld

have used (28). The same kind of pattern was obtained (Fig. 7A, lane 3) as after electrophoresis of this material in the sequencing gel (Fig. 2, lane 2) except that the sensitivity and resolution was lower. Separation of this digest into a rapidly and slowly renaturing fraction confirms that the bands are derived from repetitive DNA (Fig. 7A, lane 5). The material migrating at the top of the gel is due to network formation because of renaturation of the satellite DNA in different registers. Importantly, no additional bands are visible in the slowly renaturing fraction (lane 4).

We performed the same type of experiment with Bsp digested core DNA since this nuclease tends to bring out the artifactual bands most strongly (28), and secondly, because the prototype repeat unit of the AGM α-satellite DNA does not contain a Bsp site (27). Fig. 7B shows that there is a very faint banding pattern possibly of the type that McGhee and Felsenfeld have described (28). This pattern is indeed characteristic of the more slowly renaturing fraction (lane 4) and not observed with the satellite component (lane 5). We ascribe the very low intensity of this pattern in our analyses to our procedure for the preparation of the core particles. Instead of relying only on micrococcal nuclease to generate directly the core particles, we use more gentle conditions and trim the nucleosomes in a subsequent step with exonuclease III and S1 to obtain the core particles. We conclude from these experiments that the banding pattern that we observe with the satellite DNA has nothing to do with the 10 bp ladder of McGhee and Felsenfeld.

DISCUSSION

By using a new approach for the determination of nucleosome phasing we have been able to demonstrate the existence of eight defined nucleosome positions on AGM α-satellite DNA. The resolution afforded by our method has permitted to delineate these positions with an accuracy of about ± 1 bp. The low level of variability observed in the location of the nucleosomes for each frame is largely due to imperfect trimming of the nucleosomes in the generation of the nucleosome cores. It is possible, however, that the positions themselves are not absolutely invariant.

Even then, a microheterogeneity, if present, can encompass no more than 1 - 2 bp for each position. One frame contributes approximately 35 % of the nucleosomes on the α-satellite chromatin while the remaining seven frames amount to about 10 %, each. It should be noted that the approach taken yields no direct information on the relative order of the individual frames along the satellite DNA. It is equally possible from our results that any one frame is tandemly repeated many times or that transitions from one frame to another occur frequently.

We feel certain that we have not overlooked any additional frames present in significant amounts nor do we consider it likely that we have grossly misjudged the relative contribution of any particular frame. The micrococcal nuclease digestion step appears to be the most critical factor as far as selective loss or enrichment of any nucleosome frame is concerned. We have obtained very similar results, however, for the relative contributions of the eight frames over a wide range of digestion conditions. Only in very early or very late digests did these values begin to change. Separate control experiments have also demonstrated that our core DNA preparations show no evidence of an artifactual bias due to micrococcal nuclease overdigestion as described by McGhee and Felsenfeld (28). We attribute this fact to the use of exonuclease III in addition to micrococcal nuclease for the preparation of cores rather than micrococcal nuclease alone; this makes it possible to use micrococcal nuclease under fairly gentle conditions in our procedure. For exonuclease III we have previously shown that it discriminates to a much lesser extent at the nucleotide sequence level (16) and is therefore highly suited for the mapping of the boundaries of nucleoprotein complexes as the nucleosome cores investigated here. Changing the cell lines and using different methods for the preparation of the nuclei also made no difference. We even prepared nuclei from a solid tissue and have in all cases obtained very similar results.

The question of phasing on α-satellite containing chromatin from AGM cells has a long history that is more fully described in the accompanying paper. The claim by Musich et al. first made in 1977 (9) and recently renewed (11) that there exists unique

phasing, i.e. phasing in just one frame, has been questioned by us (18,12). The results presented in this and the accompanying paper make it clear why the approach used by Musich et al., partial digestion of nuclei with micrococcal nuclease and mapping of the cuts, has revealed the existence of only one of the frames thus leading to the false conclusion of unique phasing. Only frame F is positioned in such a way that the two sites of the satellite repeat unit which are most susceptible to micrococcal nuclease are exposed in the linker region. Under partial digestion conditions and at low temperature, i.e. conditions at which micrococcal nuclease attacks only at such strongly preferred sites, frame F will be the only one attacked to an appreciable extent. Also in our hands, the relative contribution of this frame is overestimated if digestion with micrococcal nuclease is very light.

Wu and Varshavsky have recently addressed the question of nucleosome phasing on AGM α-satellite DNA mostly by examining a satellite chromatin fraction that is solubilized by EcoRI (pers. commun.). They digested this chromatin fraction with various restriction nucleases and determined the accessibilities of the corresponding restriction sites to the nucleases. Their results are difficult to compare to ours, however, since that chromatin subfraction constitutes only 8 % of the total α-satellite chromatin.

Results obtained in this laboratory with mouse satellite DNA containing chromatin (X.Y. Zhang and W. Hörz, manuscript in preparation) and rat satellite DNA containing chromatin (21, 30, and T. Igo-Kemenes, manuscript in preparation) indicate that multiple but strictly defined frames may be a more general phenomenon for chromatin containing highly repetitive DNA.

A question of obvious interest that has been the subject of much discussion lately addresses the mechanism that leads to the specific positioning of nucleosomes along the DNA. It is unclear at present if nucleosome positioning on nonrepetitive DNAs is subject to the same precision as on satellite DNAs. By conventional techniques, multiple specific registers that are closely spaced would probably not be distinguished from completely random arrangements, and cases where strict phasing has been claim-

ed must be viewed with some reservation due to experimental pitfalls mostly caused by the properties of micrococcal nuclease (1-4). On the other hand, techniques that circumvent these problems are presently not available for unique DNAs.

It has been argued that the binding of sequence specific proteins to a DNA might passively align the adjacent nucleosomes at fixed positions (2). The positions of the nucleosomes further away would be propagated from one nucleosome to the next one via the linker. However the high degree of precision in the nucleosome positioning on α-satellite DNA renders a mechanism rather unlikely that only specifies where nucleosomes should not be rather than where they should be.

Alternatively the histones might themselves bind DNA with a low level of sequence specificity or, more generally, there might be factors that affect histone binding in certain ways and bring out the frames that we have detected. Even though the eight frames share no homologies that are obvious in direct sequence comparisons there might be structural similarities not apparent from a scrutiny of the nucleotide sequence alone. It is also not known if there is any connection between the heterochromatic nature of satellite DNA containing chromatin and specific nucleosome arrangements.

The reconstitution of nucleosomes from DNA and pure histones as well as DNA transfer involving α-satellite DNA and other cell types, experiments that are currently under way in this laboratory, might provide some insight into these intriguing questions.

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[1] AGM: African Green Monkey (Cercopithecus aethiops)

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