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Nucleosome Arrangement in Green Monkey α-Satellite Chromatin

Superimposition of Non-Random and Apparently Random Patterns

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We have studied the structure of tandemly repetitive α-satellite chromatin (α-chromatin) in African green monkey cells (CV-1 line), using restriction endonucleases and staphylococcal nuclease as probes.

While more than 80% of the 172-base-pair (bp) α-DNA repeats have a *Hin*dIII site, less than 15% of the α-DNA repeats have an *Eco*RI site, and most of the latter α-repeats are highly clustered within the CV-1 genome. *Eco*RI and *Hin*dIII solubilize approximately 8% and 2% of the α-chromatin, respectively, under the conditions used. *Eco*RI is thus approximately 30 times more effective than *Hin*dIII in solubilizing α-chromatin, with relation to the respective cutting frequencies of *Hin*dIII and *Eco*RI on α-DNA.

*Eco*RI and *Hin*dIII solubilize largely non-overlapping subsets of α-chromatin. The DNA size distributions of both *Eco*RI- and *Hin*dIII-solubilized α-chromatin particles peak at α-monomers. These DNA size distributions are established early in digestion and remain strikingly constant throughout the digestion with either *Eco*RI or *Hin*dIII. Approximately one in every four of both *Eco*RI- and *Hin*dIII-solubilized α-chromatin particles is an α-monomer.

Two-dimensional (deoxyribonucleoprotein→DNA) electrophoretic analysis of the *Eco*RI-solubilized, sucrose gradient-fractionated α-oligonucleosomes shows that they do not contain "hidden" *Eco*RI cuts. Moreover, although the *Eco*RI-solubilized α-oligonucleosomes contain one *Eco*RI site in every 172-bp α-DNA repeat, they are completely resistant to redigestion with *Eco*RI. This striking difference between the *Eco*RI-accessible *Eco*RI sites *flanking* an *Eco*RI-solubilized α-oligonucleosome and completely *Eco*RI-resistant *internal Eco*RI sites in the same α-oligonucleosome indicates either that the flanking *Eco*RI sites occur within a modified chromatin structure or that an altered nucleosome arrangement in the vicinity of a *flanking Eco*RI site is responsible for its location in the nuclease-sensitive internucleosomal (linker) region.

Analogous redigestions of the *Eco*RI-solubilized α-oligonucleosomes with either *Hin*dIII, *Mbo*II or *Hae*III (both before and after selective removal of histone H1 by an exchange onto tRNA) produce a self-consistent pattern of restriction site accessibilities. Taken together, these data strongly suggest a preferred nucleosome arrangement within the *Eco*RI-solubilized subset of α-oligonucleosomes, with the centers of most of the nucleosomal cores being ~20 bp and ~50 bp away from the nearest *Eco*RI and *Hin*dIII sites, respectively, within the 172-bp α-DNA

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repeat. However, as noted above, the clearly preferred pattern of nucleosome arrangement within the *Eco*RI-solubilized α-oligonucleosomes is invariably violated *at the ends* of every such α-oligonucleosomal particle, suggesting at least a partially statistical origin of this apparently non-random nucleosome arrangement. We discuss the relative contributions of deterministic and statistical factors to the observed patterns of nucleosome arrangement on the α-DNA and also possible influence of specific non-histone components, such as a hypothetical DNA-binding protein that may recognize and bind the 172-bp α-DNA repeat.

1. Introduction

Eukaryotic chromosomes contain stretches of nucleotide sequences from about 10 to more than 10^3 bp† in length that are repeated thousands to millions of times per haploid genome. Highly repeated DNA is largely arranged in long tandem arrays ("satellite" DNA), which in some cases can be separated from the bulk DNA by isopycnic centrifugation (reviewed by John & Miklos, 1979; Brutlag, 1980; Igo-Kemenes *et al.*, 1982; Singer, 1982; Bouchard, 1982). Highly repeated, tandemly arranged DNA sequences are found mostly, but not exclusively, within centromeric heterochromatin (Jones, 1970; Pardue & Gall, 1970; Brutlag, 1980). They are apparently not transcribed in somatic cells (Singer, 1982; but see also Sealy *et al.*, 1981); however, their transcription has been observed in developing oocytes (Varley *et al.*, 1980; Diaz *et al.*, 1981).

No function of highly repeated, tandemly arranged DNA sequences has been identified (for reviews see Walker, 1968; John & Miklos, 1979; Brutlag, 1980; Orgel & Crick, 1980; Doolittle & Sapienza, 1980; Cavalier-Smith, 1982). Examples of virtually total elimination of the satellite DNA from precursors of somatic but not of germ-line nuclei during development in a variety of species (Hernick & Wesley, 1978; John & Miklos, 1979; Beerman & Meyer, 1980; Cavalier-Smith, 1982) suggest that any essential function of the bulk of satellite DNA may be confined to germ cell lineages.

The high relative abundance of the satellite heterochromatin with its well-defined DNA component, makes such systems useful for structural studies on chromatin. Satellite DNA is organized into nucleosomes and higher-order chromatin structures (Omori *et al.*, 1980; Igo-Kemenes *et al.*, 1980,1982; Singer, 1982; Musich *et al.*, 1982). Nucleosomes of (A+T)-rich satellite DNAs of *Drosophila* were recently shown to contain stoichiometric amounts of a tightly bound specific non-histone protein, D1 (Levinger & Varshavsky, 1982*a*,*b*); these results suggest that tandemly repetitive heterochromatins in other eukaryotic species may also contain heterochromatin-specific, DNA-binding non-histone proteins.

One extensively studied satellite DNA is the α-satellite in African green monkey cells (Rosenberg *et al.*, 1978; Fittler & Zachau, 1979; Brutlag, 1980; Maio *et al.*, 1981; Lee & Singer, 1982; Singer, 1982). It comprises approximately 13% of the genome, and has a repeat length of 172 bp (Rosenberg *et al.*, 1978). The predominant nucleotide sequence of the α-DNA repeat is known (Rosenberg *et al.*, 1978; McCutchan *et al.*, 1982).

† Abbreviations used: bp, base-pairs; DNP, deoxyribonucleoprotein; DBM, diazobenzyloxymethyl; SDS, sodium dodecyl sulfate; α-DNA, α-satellite DNA.

Recent studies on the nucleosome arrangement in α-chromatin have led to strikingly different conclusions, from an essentially random distribution of nucleosomes on α-DNA (Singer, 1979) to a precise phase relationship between nucleosomes and the entire α-DNA sequence (Musich *et al.*, 1982; see, however, Fittler & Zachau, 1979; Igo-Kemenes *et al.*, 1982). Although examples of an apparent nucleotide sequence specificity of nucleosome distributions in other defined subsets of chromatin have been reported (reviewed by Kornberg, 1981; Zachau & Igo-Kemenes, 1981), both interpretations of experimental data and biological significance of the observed patterns remain ambiguous. This is due in part to the insufficient directness and sensitivity of the methods currently in use and also to a recently suggested possibility that at least some of the apparently non-random patterns of nucleosome arrangement may be explained statistically, without invoking the notions of unique linker DNA lengths or sequence-specific interactions between the DNA and octameric histone cores (Kornberg, 1981). One important exception to the above ambiguity is the existence of nucleosome-free, nucleotide sequence-specific regions 300 to 500 bp long around active control regions in both simian virus 40, polyoma and cellular chromosomes (Varshavsky *et al.*, 1978,1979; Scott & Wigmore, 1978; Jacobovitz *et al.*, 1980; Saragosti *et al.*, 1980; Wu, 1980; McGhee *et al.*, 1981; Gerard *et al.*, 1982; reviewed by Elgin, 1982; Weisbrod, 1982; see also Wittig & Wittig, 1982 and Bloom & Carbon, 1982 for other recent studies on the problem of nucleosome arrangement in chromatin).

Our data on α-chromatin strongly suggest that *both* non-random and apparently random structural motifs contribute to the pattern of nucleosome arrangement on the α-DNA in isolated chromatin. We discuss the possible significance of these and other structural features of the α-chromatin discovered in the course of the present work.

2. Materials and Methods

(a) *Preparation of CV-1 chromatin*

African green monkey cells (CV-1 line) were maintained as monolayers in Eagle's MEM medium supplemented with penicillin/streptomycin and 10% calf serum (GIBCO). In a typical experiment, cells in ten 15-cm plastic plates (Lux) at about 30% confluency were labeled with [*methyl*-^{3}H]thymidine (20 Ci/mmol, New England Nuclear) at 10 μCi/ml for ~ 20 h. Cell monolayers were rinsed with cold 0·14 M-NaCl, 1 mM-Tris · HCl (pH 7·5) followed by addition of ~6 ml per plate of 0·25% Triton X-100, 0·5 mM-phenylmethylsulphonyl fluoride (PMSF; freshly added from 0·5 M stock in absolute ethanol), 10 mM-NaEDTA, 5 mM-Na butyrate, 10 mM-Tris · HCl (pH 8·0). The lysate was scraped with a rubber policeman and centrifuged at 1000 *g* for 5 min. The nuclear pellet was resuspended and pelleted once more in the lysis buffer. Nuclei were then washed twice with 0·14 M-NaCl, 5 mM-Na butyrate, 0·1 mM-PMSF, 5 mM-Tris · HCl (pH 8·0) for a total time of about 1 h at 4°C. The pellet obtained was washed twice with 0·1 mM-PMSF, 1 mM-NaEDTA, 10 mM-NaHEPES (pH 7·5) followed by gentle resuspension of the chromatin in the same buffer to about 0·5 mg of DNA/ml, using a loosely fitted Dounce homogenizer.

(b) *Digestion of CV-1 chromatin with restriction endonucleases and fractionation of digests*

A restriction endonuclease (*Eco*RI, *Hin*dIII or *Hae*III) was added to the chromatin suspended in 0·1 mM-PMSF, 1 mM-NaEDTA, 10 mM-NaHEPES (pH 7·5) (see above) and

incubated at 4°C for 5 min followed by addition of 0·5 vol. 3× digestion buffer so that the final composition of the medium was ~ 5% glycerol, 80 mM-NaCl, 1 mM-NaEDTA, 8 mM-$MgCl_2$, 0·5 mM-dithiothreitol, 0·1 mM-PMSF, 10 mM-NaHEPES (pH 7·5). The samples were incubated at 37°C for 40 min, with gentle shaking of chromatin suspensions.

All restriction endonucleases used in this work (*Eco*RI, *Hin*dIII, *Mbo*II, *Hae*III, *Pvu*II and *Bam*H1) were obtained from New England Biolabs. The amount of restriction endonuclease used for direct digestions of chromatin (*Eco*RI, *Hin*dIII or *Hae*III) was 1000 units (as defined by N. E. Biolabs) per ~ 100 μg of DNA in 1 ml of the digestion mix. This amount corresponded to approx. 8-fold excess over the minimum amount required to completely digest an equal quantity of purified CV-1 DNA under identical conditions.

Digested chromatin samples were cooled to 4°C and centrifuged at 12,000 ***g*** for 5 min. The supernatant containing a subset of the 172-bp α-*monomer* DNP particles soluble in the digestion buffer but virtually no oligonucleosomal particles (see Results) was removed, and the pellet was washed once with the cold digestion buffer, followed by a brief rinse with 5 mM-NaHEPES (pH 7·5). Virtually no [^{3}H]DNA was released during the latter wash. The carefully drained pellet (~ 0·2 ml) was resuspended in ~ 0·6 ml of 0·5 mM-NaEGTA, 1 mM-NaEDTA, 1 mM-NaHEPES (pH 7·5), and incubated for 1 h at 4°C, with gentle shaking, followed by centrifugation at 12,000 ***g*** for 5 min. The supernatant containing solubilized α-satellite chromatin fragments, was layered over 16·6 ml of a linear 5% to 40% (w/v) sucrose gradient containing 0·5 mM-NaEGTA, 1 mM-NaEDTA, 1 mM-NaHEPES (pH 7·5) and centrifuged in the SW 27·1 rotor (Beckman) at 23,000 revs/min for 16 h at 4°C. Fractions were collected from the bottom of the nitrocellulose tube and portions were counted with Aquasol (New England Nuclear). Fractions were processed further either immediately or after storage at −70°C for up to a month. A single freezing and thawing did not influence any of the results.

Yields of the total chromatin DNA solubilized by either *Eco*RI or *Hin*dIII were determined by comparing amounts of the total ^{3}H in DNA from the supernatants and pellets (the latter solubilized with SDS). In calculating yields of the solubilized α-DNA, it was assumed to constitute 13% of the total DNA in isolated CV-1 chromatin. Relative contents of the α-DNA in the total solubilized DNA *versus* total DNA in the unfractionated CV-1 chromatin were determined by dot blot hybridization (Kafatos *et al.*, 1979; Varshavsky, 1981) with a ^{32}P-labeled cloned α-DNA probe (see section (g), below).

(c) *Gel electrophoresis of DNA*

Sucrose gradient fractions (see Fig. 1) or unfractionated α-satellite chromatin samples were made 1% in SDS and 0·15 M in NaCl followed by addition of carrier yeast transfer RNA (Sigma) to 25 μg/ml, 2·7 vol. cold 95% ethanol and centrifugation at 12,000 ***g*** for 15 min; the pellets were air-dried, dissolved in an SDS-containing sample buffer, heated at 55°C for 20 min and then subjected to electrophoresis in 30 cm long, 2·5 mm thick vertical slab gels containing, unless stated otherwise, 1·5% (w/v) agarose (Sigma, type I), 0·1% SDS, 1 mM-NaEDTA, 5 mM-Na acetate, 40 mM-Tris · HCl (pH 8·0). In some of the experiments horizontal agarose gels were used as described previously (Sundin & Varshavsky, 1981). For additional details of electrophoresis and fluorography with presensitized X-ray films see Varshavsky *et al.* (1979). Electrophoresis of higher molecular weight DNAs was carried out in horizontal, SDS-containing, 0·4% agarose gels. Ethanol precipitation of DNA was omitted in these experiments; the samples were loaded onto the gel directly after addition of SDS and heating at 55°C for 20 min.

(d) *Preparation of CV-1 nuclear DNA*

Purified CV-1 DNA was obtained by 2 cycles of phenol deproteinization of Pronase/SDS-digested CV-1 chromatin followed by treatment with RNase A, additional 2 cycles of deproteinization, precipitation of high molecular weight DNA with ethanol, solubilization of the pellet in 1 mM-NaEDTA, 1 mM-NaHEPES (pH 7·5) and extensive dialysis against

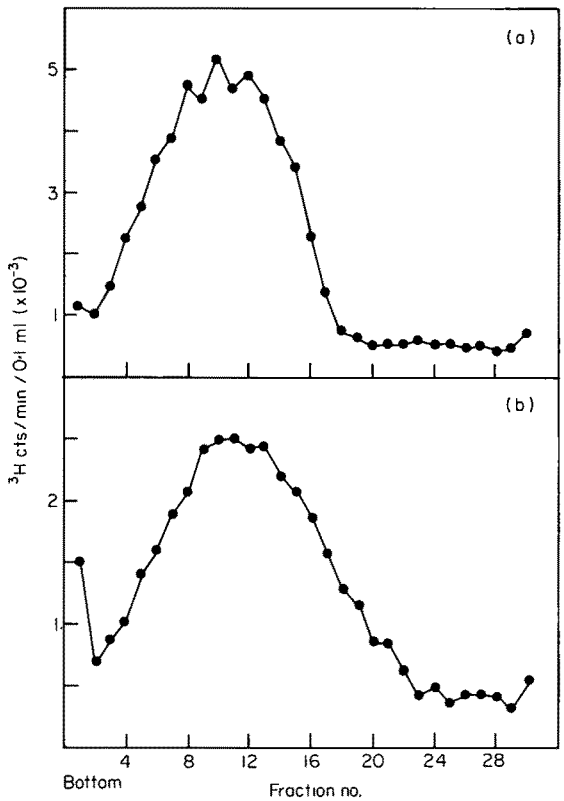

FIG. 1. Sedimentation patterns of *Eco*RI- and *Hin*dIII-solubilized CV-1 chromatin. (a) *Hin*dIII-solubilized chromatin (see Materials and Methods). (b) *Eco*RI-solubilized chromatin.

the same buffer at 4°C. The specific radioactivity of the [^{3}H]DNA was 1×10^5 to 2×10^5 ^{3}H cts/min per μg.

(e) *Two-dimensional DNP→DNA electrophoresis of α-satellite chromatin fragments*

α-Satellite oligonucleosomes from sucrose gradient fractions (see Fig. 1) were electrophoresed in the first (DNP) dimensions at 4°C through a vertical 0·8% agarose gel (3 mm thick, 30 cm long) containing 0·5 mM-NaEGTA, 1 mM-NaEDTA, 5 mM-Tris·HCl (pH 8·0). Electrode buffer (the same as in the gel) was stirred in both compartments and recirculated between the compartments. The first-dimension strip was cut and then soaked in ~10 vol. 1% SDS, 0·01% bromophenol blue, 1 mM-NaEDTA, 10 mM-Na acetate, 80 mM-Tris·HCl (pH 8·0) at 40°C for 1 h. It was then cast into a wide slot in the horizontal second-dimension gel (30 cm long) containing 1·5% agarose in 0·1% SDS, 1 mM-NaEDTA, 10 mM-Na acetate 80 mM-Tris·HCl (pH 8·0) as described by Sundin & Varshavsky (1981).

(f) *Redigestion of* Eco*RI-solubilized, sucrose gradient-purified α-chromatin particles with restriction endonucleases*

Digestions were carried out directly in fractions from preparative sucrose gradients (see Fig. 1). A chosen fraction was mixed with 0·5 vol. of the 3 × digestion buffer so that the final composition was 10 to 25% sucrose, 80 mM-NaCl, 1 mM-NaEDTA, 0·3 mM-NaEGTA, 0·5 mM-dithiothreitol, 8 mM-$MgCl_2$, 0·1 mM-PMSF, 10 mM-NaHEPES (pH 7·5). A restriction

endonuclease (*Hin*dIII, *Eco*RI, *Mbo*II or *Hae*III) was then added followed by incubation at 37°C for 40 min. The reaction was stopped by adding EDTA and SDS and the sample was processed for DNA electrophoresis as described above. The amount of restriction endonuclease used for redigestion was 20 N.E. BioLabs units per μg of DNA in 50 μl of the digestion mix. This amount corresponded to approx. 10-fold excess over the minimum amount required to completely digest an equal quantity of the purified DNA from the same fraction under identical conditions. In some experiments redigestion of α-chromatin fragments was preceded by selective removal of histone H1 by treatment with tRNA (Ilyin *et al.*, 1971). A sucrose gradient fraction (see Fig. 1) was made 40 mM in NaCl by adding 0·3 M-NaCl followed by addition of the purified total yeast tRNA (10 mg/ml; Sigma) to a final tRNA concentration of 1 mg/ml. The stock tRNA solution was dialysed before use against 40 mM-NaCl, 1 mM-NaEDTA, 1 mM-NaHEPES (pH 7·5). The sample was incubated at 4°C with gentle shaking for 1 h followed by gel chromatography on Sepharose 4B equilibrated with 40 mM-NaCl, 1 mM-NaEDTA, 1 mM-NaHEPES (pH 7·5), to remove both free tRNA and tRNA–protein complexes. The void-volume, DNP-containing fractions were concentrated by ultrafiltration using PM30 membranes and an Amicon 3-ml stirred cell. Redigestion of the tRNA-treated α-DNP with restriction endonucleases was carried out as described above.

(g) *Two-dimensional hybridization mapping of α-satellite nucleosomes*

Isolated CV-1 chromatin (see above) was digested with staphylococcal nuclease under conditions described previously for the HeLa and *Drosophila* chromatin (Levinger *et al.*, 1981; Levinger & Varshavsky, 1982*a*,*b*). The digest was fractionated in the first (DNP) dimension in a 4% low ionic strength polyacrylamide gel (Strauss & Prunell, 1982) followed by a second-dimension (DNA) electrophoresis in 9% polyacrylamide/SDS gel (Levinger *et al.*, 1981). Fractionated DNA was denatured *in situ* by heating the gel at 100°C followed by electrophoretic transfer of DNA to DBM paper as described previously (Levinger *et al.*, 1981). The transferred DNA was hybridized with the ^{32}P-labeled cloned α-DNA probe (pHG20A, Graf *et al.*, 1979; a gift from Dr H. Zachau, University of Munich) under conditions described previously (Levinger & Varshavsky, 1982*a*). Second-dimension electrophoresis of protein components of nucleosomes resolved in the first (DNP) dimension was carried out in 18% polyacrylamide/SDS gels as described previously (Levinger & Varshavsky, 1980).

3. Results

(a) *Solubilization and fractionation of α-satellite chromatin fragments produced by restriction endonucleases*

While most of the 172-bp α-DNA repeats have a *Hin*dIII site (Singer, 1982; see also Fig. 3(a)), considerably fewer α-DNA repeats have an *Eco*RI site (Fig. 2(b)) and those that do have it appear to be strongly clustered within the genome (see below, and also Lee & Singer, 1982). The high proportion of the α-DNA in the total CV-1 DNA (~ 13%) and the relatively infrequent cutting of non-α-DNA with "six-letter" enzymes, such as *Hin*dIII and *Eco*RI, allow straightforward fluorographic detection of ^{3}H-labelled α-DNA fragments in chromatin digests after electrophoresis (Figs 2 and 3). With two exceptions, all discrete bands seen in fluorograms of [^{3}H]thymidine-labeled DNA shown in this paper correspond to α-DNA fragments, as has been confirmed by Southern hybridizations with a cloned α-DNA probe (data not shown, see Materials and Methods). One exception

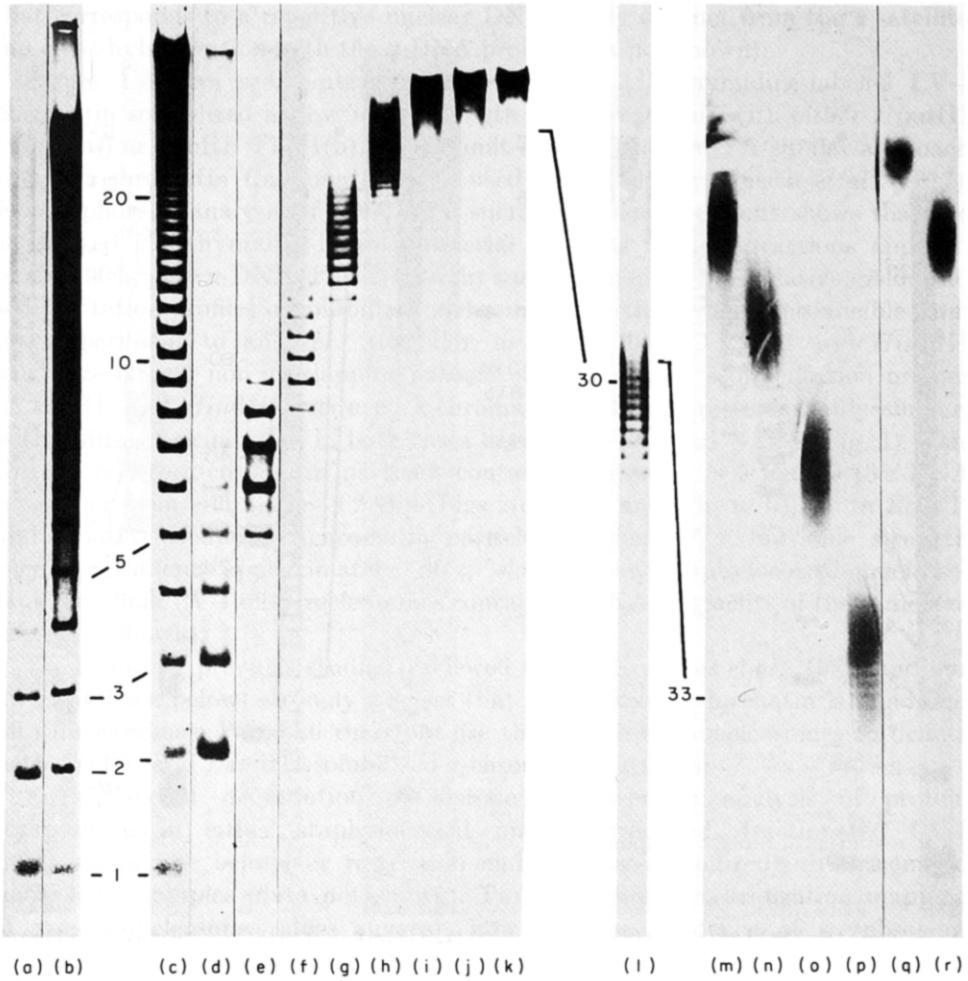

FIG. 2. Electrophoretic analysis of DNA from *Eco*RI-solubilized, fractionated CV-1 chromatin. Electrophoresis in 1·5% ((a) to (l)) and 0·4% ((m) to (r)) agarose/SDS gels (see Materials and Methods). (a) *Eco*RI limit digest of the DNA purified from the *Eco*RI-solubilized, unfractionated CV-1 chromatin (compare with (c)). (b) *Eco*RI digest of the purified total CV-1 nuclear DNA (compare with Fig. 3(a)). (c) DNA from *Eco*RI-solubilized, unfractionated CV-1 chromatin. (d) Same as in (c) but after sedimentation in sucrose gradient (Fig. 1(b), fraction 18). (e) to (k) Same as in (d), but fractions 16, 14, 12, 10, 8, 6 and 4, respectively. (l) Same as in (h) but electrophoresis for a longer time to resolve α-DNA bands. (m) Fraction 3 of the sucrose gradient in Fig. (1b) electrophoresed in 0·4% agarose. (n), (o) and (p) Same as in (m) but fractions 4, 6 and 8, respectively. (q) DNA from *Hin*dIII-solubilized, sucrose gradient-fractionated CV-1 chromatin (Fig. 1(a), fraction 2), electrophoresed in 0·4% agarose. (r) Same as in (q) but fraction 4. Numbers from 1 to 33 indicate oligomers of the 172-bp α-DNA repeat.

is the ~ 900-bp band designated "C" (cytoplasmic) in Figure 3(a). This DNA band does not hybridize to the α-DNA probe and was positively identified as a *Hin*dIII-produced DNA fragment derived from traces of mitochondrial DNA present in our nuclear preparations (data not shown). The other exception is a prominent *Hin*dIII-produced ~ 2·4 kbp band (indicated by an arrow in Fig. 3(l))

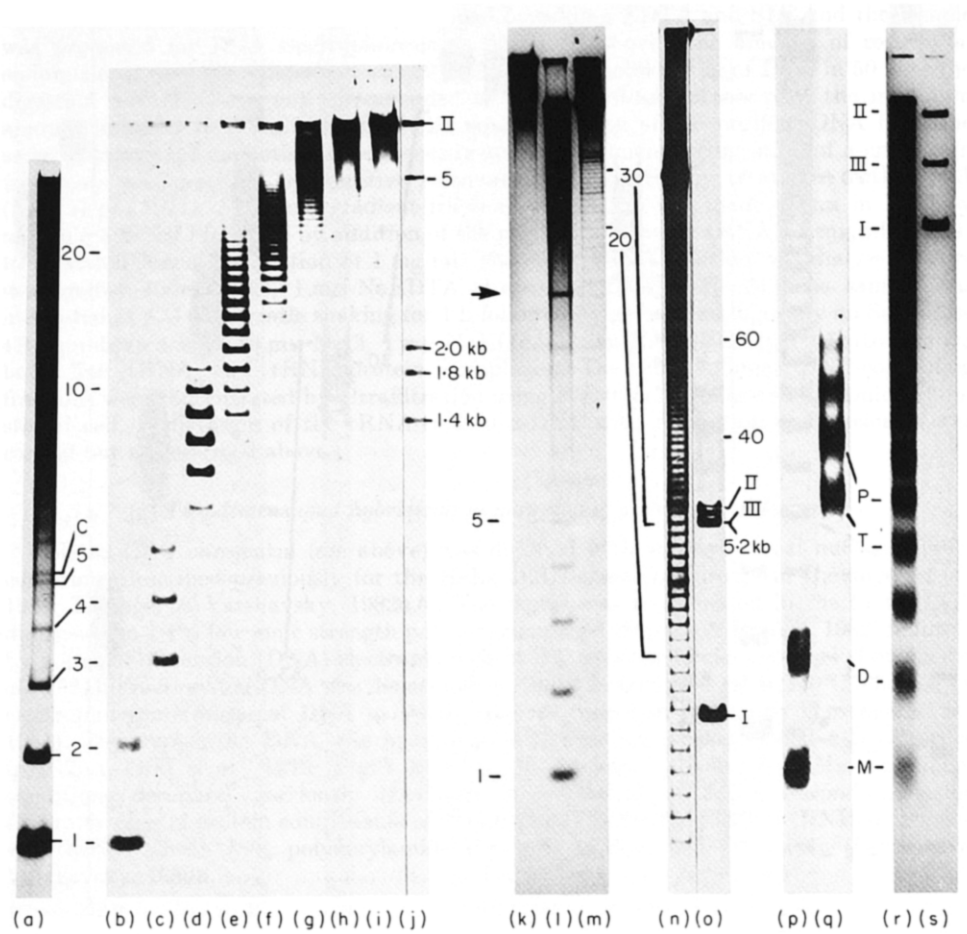

FIG. 3. Electrophoretic analysis of DNA from *Hin*dIII- and *Hae*III-solubilized, fractionated CV-1 chromatin. Electrophoresis in 1·5% ((a) to (m), (p) to (s)) and 0·6% ((n), (o)) agarose/SDS gels, respectively (see Materials and Methods). (a) *Hin*dIII limit digest of the purified total CV-1 nuclear DNA; (b) to (i) DNA from *Hin*dIII-solubilized, sucrose gradient-fractionated CV-1 chromatin (see Fig. 1(a)); fractions 19, 17, 16, 14, 12, 10, 8 and 6, respectively. (j) A mixture of partial *Bam*HI and complete *Pvu*II digests of the ^{3}H-labeled SV40 DNA (a marker). (k) Total DNA from undigested CV-1 chromatin (control). (l) Partial *Hin*dIII digest of purified total CV-1 nuclear DNA. (m) *Hin*dIII limit digest of CV-1 chromatin (total DNA pattern *before* DNP solubilization; relatively short fluorographic exposure). (n) Same as in (m) but electrophoresed in 0·6% agarose. (o) Partial *Bam*HI digest of the ^{3}H-labeled SV40 DNA (a marker for (n)). (p) DNA from *Hae*III-solubilized, sucrose gradient-fractionated CV-1 chromatin (pooled mono- and dinucleosomal peaks). (q) Same as in (p) but pooled fractions corresponding to tetra-, penta- and hexanucleosomes produced by *Hae*III. (r) *Hae*III limit digest of CV-1 chromatin (total DNA pattern *before* DNP solubilization). (s) Same as in (o) but run in 1·5% agarose (a marker). Numbers from 1 to 60 designate oligomers of the 172-bp α-DNA repeat. The ~900-bp DNA band designated C in lane (a) is cytoplasmic (mitochondrial) origin (see the main text). Roman numbers I to III designate closed circular, nicked circular and linear SV40 DNA, respectively. Letters M,D,T and P in lanes (p) to (r) designate DNA fragments corresponding to *Hae*III-produced mono-, di-, tetra- and pentanucleosomes, respectively. An arrow indicates a prominent *Hin*dIII-produced DNA band (~ 2·4 kb) in lane (l) that corresponds to a repetitive DNA family distinct from the α-satellite (no cross-hybridization with the α-DNA probe; data not shown).

that corresponds to a repetitive nuclear DNA family distinct from the α-satellite (no cross-hybridization with the α-DNA probe; data not shown).

Figure 1 shows sedimentation profiles of the [^{3}H]thymidine-labeled CV-1 chromatin solubilized at low ionic strength after treatment with either *Hin*dIII (Fig. 1(a)) or *Eco*RI (Fig. 1(b)) under limit-digest conditions. A similar approach to the α-chromatin fractionation was used previously by Musich *et al.* (1977). Electrophoretic analysis of DNA from sucrose gradient fractions shows that the solubilized [^{3}H]thymidine-labeled material contains in peak fractions approximately 90% pure α-DNA (Figs 2(c) to (h) and 3(b) to (g)). Both relative yields and sedimentation profiles of solubilized α-chromatin particles were reproducible from one experiment to another. Although, as shown below, *Eco*RI and *Hin*dIII solubilize largely non-overlapping subsets of α-chromatin, sedimentation profiles of *Eco*RI- and *Hin*dIII-produced α-chromatin particles are remarkably similar, with diffuse peak position in both cases between ~17 S and ~22 S (Fig. 1). This corresponds to α-chromatin particles containing between ~ 7 and ~ 13 α-DNA repeats or from ~ 1·2 to ~ 2·2 kbp (Figs 2(c) to (i) and 3(b) to (i)). Both *Eco*RI- and *Hin*dIII-produced α-chromatin particles sediment in a low-ionic strength sucrose gradient approximately 20% *slower* than staphylococcal nuclease-produced bulk CV-1 oligonucleosomes containing DNA fragments of the same size (data not shown).

The results of previous studies (reviewed by Igo-Kemenes *et al.*, 1982) and our own data (see below) strongly suggest that the bulk of α-chromatin is organized into nucleosomes. We shall therefore use the term α-oligonucleosomes to denote both *Eco*RI- and *Hin*dIII-solubilized α-chromatin particles.

No significant degradation of histone was seen in analysis of protein composition in either staphylococcal nuclease-produced, fractionated CV-1 nucleosomes (see below) or restriction endonuclease-solubilized, unfractionated nucleosome samples (data not shown). Two-dimensional hybridization mapping of α-mononucleosomes does suggest, however, the existence of a subset of α-nucleosomes associated with a specific non-histone protein (Fig. 7 and discussion below).

Approximately 3 and 6% of the *total* chromatin [^{3}H]DNA was solubilized by *Hin*dIII and *Eco*RI, respectively, under the conditions used (see Materials and Methods). Although at least 80% of all 172-bp α-DNA repeats have a *Hin*dIII site, and less than 15% of the α-DNA repeats have an *Eco*RI site (Figs 2(b) and 3(a); see also Singer, 1982; Lee & Singer, 1982), *Eco*RI is much more effective than *Hin*dIII in solubilizing α-oligonucleosomes (Fig. 1). Specifically, while approximately 2% of the α-DNA is solubilized from CV-1 chromatin by *Hin*dIII under limit-digest conditions, approximately 8% of the α-DNA is solubilized by *Eco*RI under the same conditions (see Materials and Methods). Thus digestion of chromatin with *Eco*RI is approximately 30-fold more effective in producing soluble α-oligonucleosomes than digestion with *Hin*dIII, with relation to the respective cutting frequences of *Hin*dIII and *Eco*RI (see above). Most of the experiments described below were carried out with the *Eco*RI-solubilized α-oligonucleosomes.

(b) Eco*RI-solubilized α-oligonucleosomes lack "hidden"* Eco*RI cuts*

"Hidden" DNA cuts, that is sites of double-stranded DNA cleavage present in a DNP particle whose integrity is maintained through protein–protein and/or protein–DNA interactions, are a frequent feature of staphylococcal nuclease-produced oligo- and mononucleosomes (Levinger & Varshavsky, 1980; Igo-Kemenes *et al.*, 1982). To see whether any hidden *Eco*RI cuts are present in the *Eco*RI-solubilized, fractionated α-oligonucleosomes, fraction number 13 from the sucrose gradient shown in Figure 1(b) was subjected to further fractionation by a low ionic strength electrophoresis in 0·8% agarose (Fig. 4). Similar results were obtained with several other sucrose gradient fractions from Figure 1(b) (data not shown). Fraction 13 (Fig. 1(b)) contained α-oligonucleosomes in the size range of ~ 13 to ~ 17 α-DNA repeats. Although a single diffuse nucleoprotein band is observed in the first-dimension low ionic strength gel (Fig. 4(b)), α-oligonucleosomes of different DNA sizes with the DNP band are partially resolved from each other, as shown by the second-dimension electrophoresis of DNA in a 1·5% agarose/SDS gel (Fig. 4(a)). It is also clear from the pattern in Figure 4(a) that no hidden DNA cuts are present in these *Eco*RI-solubilized oligonucleosomes.

An unexplained feature of the second-dimension DNA pattern, revealed only at much higher fluorographic exposures (Fig. 4(c)), is the presence of a second, minor "arc" of the α-DNA fragments, that *intersects* the major α-DNA "arc" at a point corresponding to ~ 15 α-DNA repeats.

(c) Eco*RI- and* Hind*III-solubilized α-oligonucleosomes are derived largely from non-overlapping regions of α-chromatin*

Redigestion of the α-DNA purified from *Eco*RI-solubilized α-oligonucleosomes with *Eco*RI converts most of the DNA to α-monomers and α-dimers (Fig. 2(a)). In striking contrast, few cuts are produced by *Eco*RI in the α-DNA purified from *Hind*III-solubilized α-oligonucleosomes (Fig. 6(n)). On the other hand, *Hind*III digestion of the DNA purified from either *Eco*RI- or *Hind*III-solubilized oligonucleosomes, converts it to monomers (Fig. 6(k) and data not shown). One conclusion from these data is that *Hind*III and *Eco*RI solubilize largely non-overlapping regions of the α-chromatin. Another conclusion is that *Eco*RI restriction sites are strongly clustered in a subset of the α-chromatin, which we shall call below an "*Eco*RI subset" (see also Lee & Singer, 1982).

(d) Eco*RI-solubilized α-oligonucleosomes are resistant to redigestion with* Eco*RI*

Although DNA of the *Eco*RI-solubilized α-oligonucleosomes contains close to one *Eco*RI site per every 172-bp α-DNA repeat (see above), these α-oligonucleosomes are true limit-digested products, since no further DNA cuts are produced by redigestion of the *Eco*RI-solubilized, purified α-oligonucleosomes with *Eco*RI (Fig. 6(l)). We conclude that "accessible" *Eco*RI sites at the ends of every *Eco*RI-solubilized α-oligonucleosome either occurred in the internucleosomal (linker) regions (in contrast to the *internal Eco*RI sites within the same α-oligonucleosome) or were a part of a non-nucleosomal structure (see Discussion).

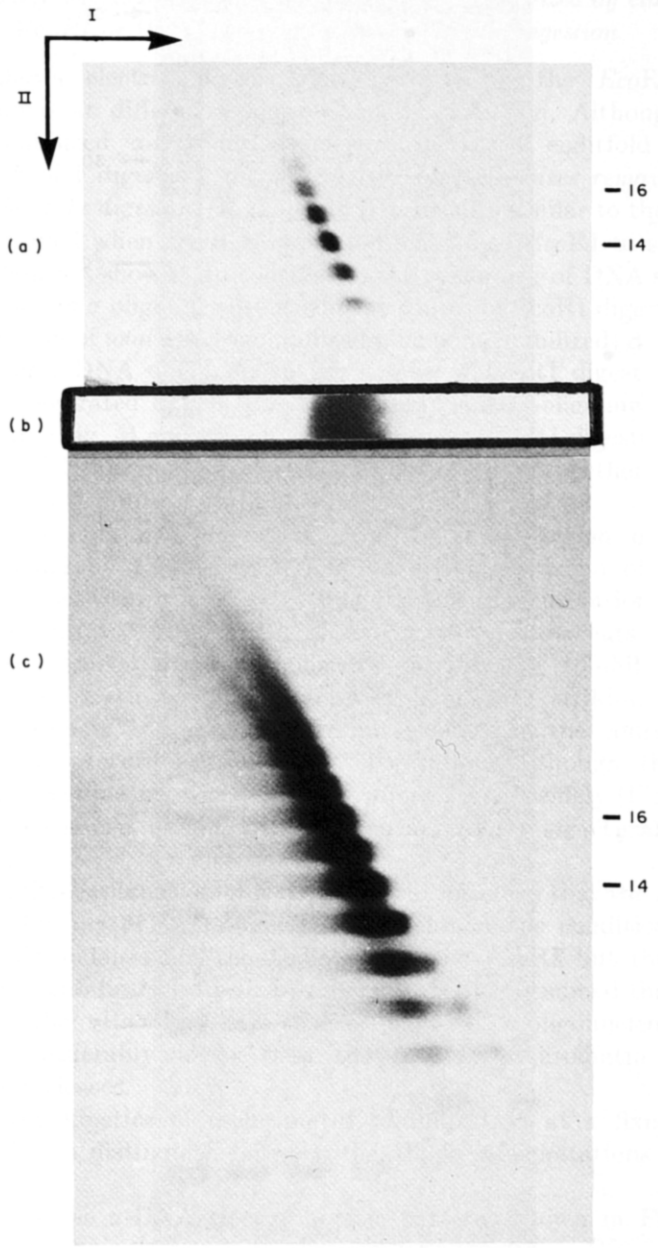

FIG. 4. Two-dimensional electrophoretic fractionation of *Eco*RI-solubilized α-chromatin fragments: evidence that there are no hidden DNA cuts. α-Chromatin fragments from fraction 13 of the sucrose gradient shown in Fig. 1(b) were fractionated in the first-dimension low ionic strength 0·8% agarose gel followed by second-dimension DNA electrophoresis in a 1·5% agarose/SDS gel (see Materials and Methods). (a) Fluorogram of the second-dimension (DNA) pattern. (b) First-dimension (DNP) pattern of *Eco*RI-solubilized, sucrose gradient-fractionated α-chromatin fragments (fraction 13 in Fig. 1(b)). (c) Same as in (a) but a 5-fold longer fluorographic exposure. Numbers on the right designate oligomers of the 172-bp α-DNA repeat.

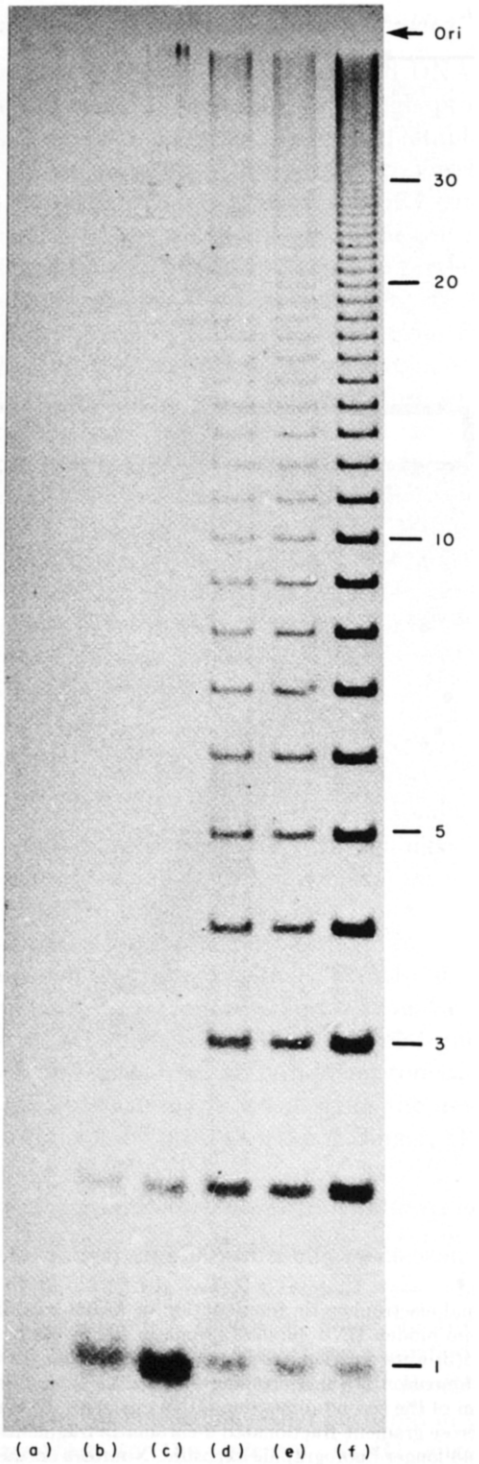

FIG. 5.

(e) *DNA distributions of α-oligonucleosomes solubilized by either* Eco*RI or* Hind*III are established early in digestion*

Figure 5 shows electrophoretic DNA patterns of the *Eco*RI-solubilized α-oligonucleosomes at different stages of *Eco*RI digestion. Although the total amount of solubilized α-oligonucleosomes is increased eightfold from 5 to 40 minutes of *Eco*RI digestion, *the distribution of DNA sizes remains strikingly constant throughout the digestion* (Fig. 5(d) to (f)). Results similar to those in Figure 5 were also obtained when *Hin*dIII was used instead of *Eco*RI to solubilize the α-chromatin (data not shown). In contrast to the constancy of DNA size distribution of the *solubilized* α-oligonucleosomes in the course of *Eco*RI digestion (Fig. 5), the size distribution of *total* DNA (solubilized plus non-solubilized) does shift from longer to shorter α-DNA oligomers in the course of *Eco*RI digestion (data not shown). As demonstrated above, the *Eco*RI-solubilized α-oligonucleosomes lack hidden DNA cuts (Fig. 4) and are resistant to further *Eco*RI digestion in spite of abundance of *Eco*RI sites in their α-DNA (Fig. 6(l)). Taken together, these results are consistent with two interpretations.

(1) Two *Eco*RI cuts in α-chromatin lead to solubilization of an α-oligonucleosome flanked by these cuts if and only if there is no other *accessible* *Eco*RI site(s) in between. In other words, in this interpretation a structure containing an uncut, accessible *Eco*RI site(s) in between the cuts made at two other accessible *Eco*RI sites is sufficient to prevent solubilization of a corresponding α-chromatin particle, thereby explaining the striking constancy of the size distribution of solubilized α-oligonucleosomes in the course of *Eco*RI digestion (Fig. 5). According to this interpretation, although the distances between *closest* accessible *Eco*RI sites in α-chromatin vary widely (Fig. 2(c)), each pair of *closest* accessible *Eco*RI sites defines a distinct structural domain in α-chromatin.

(2) An alternative explanation of the same set of results is that the rate-limiting step in the solubilizing of an α-oligonucleosome under the conditions used (see Materials and Methods) is not the DNA cutting by *Eco*RI but the process of oligonucleosome solubilization itself. In other words, it is assumed that under the conditions used the rate of release of the "excised" α-oligonucleosomes from chromatin is considerably lower than the rate of α-chromatin cutting by restriction endonucleases.

Analysis of the kinetics of α-chromatin solubilization at a fixed degree of cutting by *Eco*RI to distinguish between the above interpretations is currently underway.

Quantitation of the α-DNA electrophoretic patterns shown in Figure 5 was

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FIG. 5. Electrophoretic DNA patterns in *Eco*RI-solubilized α-chromatin as a function of digestion time. After digesting CV-1 chromatin with *Eco*RI, the samples were quickly chilled to 0°C and centrifuged at 12,000 *g* for 3 min *before* the addition of EDTA. Lanes (a) to (c) show total DNA from the supernatants (Mg^{2+}-supernatants) obtained after digesting CV-1 chromatin for 3, 15 and 40 min, respectively. The pellets (see above) were resuspended in a low ionic strength, EDTA-containing buffer to solubilize α-oligonucleosomes (see Materials and Methods). Lanes (d) to (f) show the corresponding DNA patterns. Equal volumes of the Mg^{2+} supernatants were applied in (a) to (c). Equal total ^3H cts/min were applied in (d) and (e) and twice as many ^3H cts/min in (f).

carried out by densitometry. The molar yield is highest for α-monomers (~ 25% of all *Eco*RI-solubilized α-particles are α-monomers) and decreases with an increase of DNA size of solubilized α-chromatin particles (data not shown). This type of distribution can be readily explained by assuming that the rate-limiting step in the solubilization of an α-oligonucleosome under the conditions used (see Materials and Methods) is not the DNA cutting by *Eco*RI or *Hin*dIII but the process of α-oligonucleosome solubilization itself (α-monomers are solubilized faster than α-dimers and so forth). Other interpretations are still possible, however (see Discussion). It should also be noted that in most of the experiments the molar yield of either *Eco*RI- or *Hin*dIII-solubilized α-chromatin fragments peaks at *both* α-monomers and α-octamers, the latter maximum being much smaller than the former (data not shown). Detailed analysis of the distribution of *accessible Eco*RI sites within α-chromatin, and of the kinetics of solubilization of α-chromatin fragments by *Eco*RI should distinguish between possible interpretations of these complex patterns and is currently underway.

(f) α-*Monomers solubilized by either* Eco*RI or* Hind*III are soluble in* Mg^{2+}*-containing buffer*

One striking property of virtually all of the α-monomers (and a small proportion of α-dimers as well) is that unlike larger α-chromatin particles, they are solubilized by *Eco*RI or *Hin*dIII directly in the digestion buffer, that is in the presence of 6 mM-$MgCl_2$ and 80 mM-NaCl (Fig. 5(a) to (c)). Since most of the staphylococcal nuclease-produced, histone H1-containing mononucleosomes are insoluble under these conditions (McGee & Felsenfeld, 1980), this result suggests that the α-monomer particles either lacked histone H1 or have lost it upon nuclease digestion. Further analysis of the nucleoprotein structure of Mg^{2+}-soluble α-monomers is underway.

(g) *Redigestion of* Eco*RI-solubilized α-oligonucleosomes with restriction endonucleases: evidence for non-random arrangement of nucleosomal cores*

Restriction endonucleases under appropriate conditions cut oligonucleosomes virtually exclusively within linker DNA and not within intranucleosomal core DNA, as demonstrated previously in a number of systems (e.g. Igo-Kemenes *et al.*, 1980; see also Fig. 3(p) to (r)). This follows also from the complete resistance of the *Eco*RI-solubilized α-oligonucleosomes to redigestion with *Eco*RI (Fig. 6(l)).

*Hin*dIII, *Mbo*II and *Hae*III each cut once per 172-bp α-DNA repeat (Rosenberg *et al.*, 1978); positions of the corresponding sites relative to a single *Eco*RI site are shown in Figure 8. While virtually all of the α-DNA repeats in the *Eco*RI-solubilized α-oligonucleosomes have sites for *Eco*RI, *Hin*dIII and *Mbo*II (Figs 2, 3 and 6), only some of the α-DNA repeats in the "*Eco*RI subset" of the α-chromatin have a site for *Hae*III (Figs 6 and 8 and data not shown; see also Lee & Singer, 1982).

We have redigested the *Eco*RI-solubilized, sucrose gradient-purified α-oligonucleosomes of specific size classes (from 5-mers to 10-mers) with the above

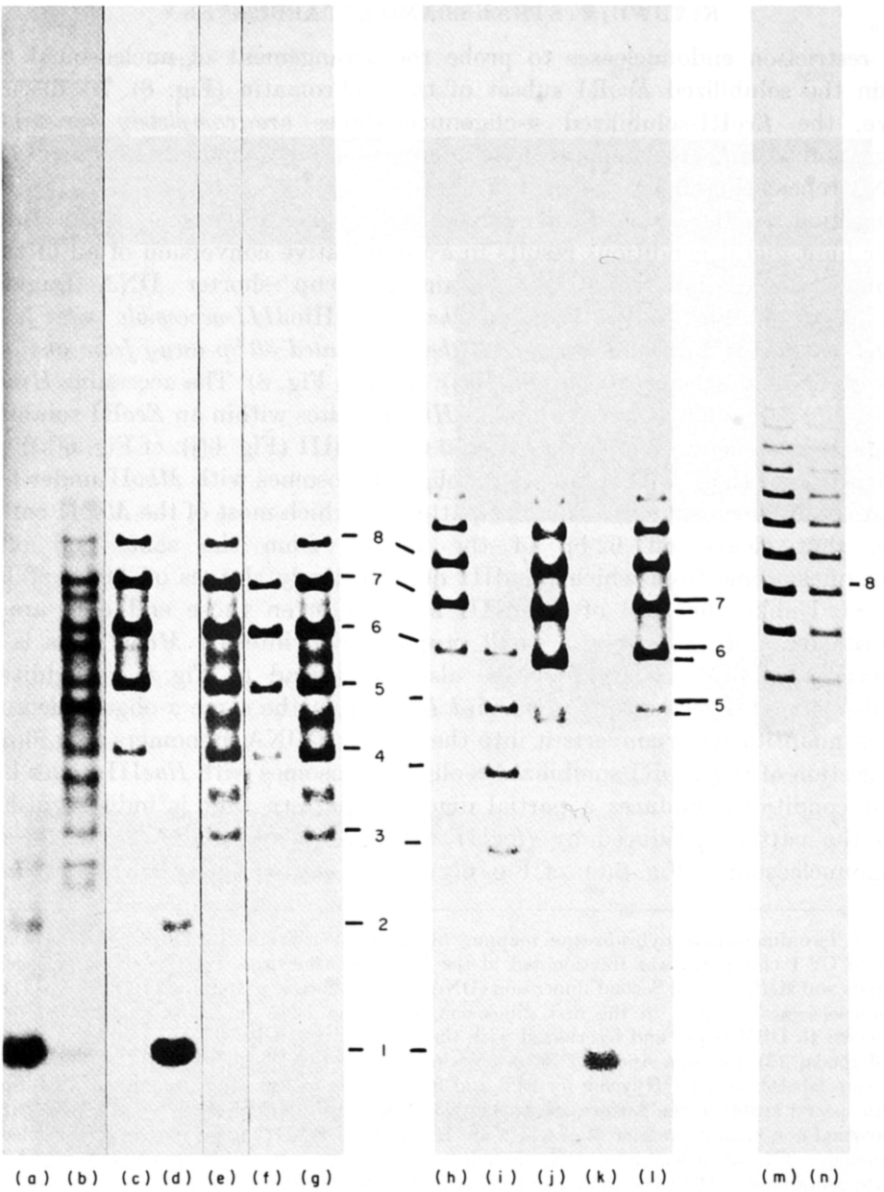

FIG. 6. Redigestion of *Eco*RI- or *Hin*dIII-solubilized α-oligonucleosomes with restriction endonucleases. DNA was electrophoresed in a 1·5% agarose/SDS gel. (a) DNA was purified from *Eco*RI-solubilized, sucrose-gradient-fractionated α-oligonucleosomes (fraction 16 in Fig. 1(b)) and treated with *Mbo*II under limit-digest conditions. (b) Same as in (a) but digestion with *Mbo*II was carried out *before* deproteinization (see Materials and Methods). (c) Same as in (a) but no digestion with *Mbo*II. (d) Same as in (a) but digestion with *Eco*RI under conditions for *Eco*RI* activity. (e) Same as in (a) but digestion with *Hae*III instead of *Mbo*II. (f) Same as in (c) (a shorter fluorographic exposure). (g) Same as in (b) but digestion with *Hae*III instead of *Mbo*II. (h) DNA from *Eco*RI-solubilized, sucrose gradient-fractionated α-oligonucleosomes (fraction 15 in Fig. (1b)). (i) Same as in (h) but a different sucrose gradient fraction (no. 17 in Fig. 1(a)). (j) Same as in (h) but digestion with *Hin*dIII *before* deproteinization (note a shift in mobility of every α-DNA band due to a *Hin*dIII cut). (k) Same as in (j) but digestion with *Hin*dIII after deproteinization. (l) Same as in (h) but digestion with *Eco*RI *before* deproteinization. (m) DNA from *Hin*dIII-solubilized, sucrose gradient-fractionated α-oligonucleosomes (fraction 16 in Fig. 1(a)). (n) Same as in (m) but digestion with *Eco*RI *after* deproteinization.

four restriction endonucleases to probe the arrangement of nucleosomal cores within the solubilized *Eco*RI subset of the α-chromatin (Fig. 6). As discussed above, the *Eco*RI-solubilized α-oligonucleosomes are *completely resistant* to redigestion with *Eco*RI, in spite of the presence of one *Eco*RI site in every 172-bp α-DNA repeat (Fig. 6(1)).

Digestion of the same *Eco*RI-solubilized α-oligonucleosomes with *Hin*dIII under limit-digest conditions results in a quantitative conversion of all of the α-oligonucleosomes into particles containing 30 bp shorter DNA fragments (Fig. 6(j); cf. Fig. 6(l)). That is, *the only* Hind*III-accessible site in the* Eco*RI-solubilized α-oligonucleosomes is the one located 30 bp away from one of the two ends of an α-oligonucleosome* (Fig. 6(j); see also Fig. 8). The accessible *Hin*dIII site is *fully* accessible, whereas all other *Hin*dIII sites within an *Eco*RI-solubilized α-oligonucleosome are *completely resistant* to *Hin*dIII (Fig. 6(j); cf Fig. 6(k)).

Digestion of the *Eco*RI-solubilized α-oligonucleosomes with *Mbo*II under limit-digest conditions results in a complex pattern in which most of the *Mbo*II cuts are those that cleave off 62 bp of the α-DNA from the same end of an α-oligonucleosome from which *Hin*dIII quantitatively cleaves off 30 bp of DNA (Fig. 8). Unlike the case of *Hin*dIII, however, even those end cuts are not quantitative and, moreover, a small proportion of internal *Mbo*II cuts is also observed (Fig. 6(b); cf Fig. 6(c); see also the legend to Fig. 8 for additional details). *Mbo*II digestion of the *purified DNA* from the same α-oligonucleosomes almost quantitatively converts it into the 172-bp α-DNA monomers (Fig. 6(a)).

Digestion of the *Eco*RI-solubilized α-oligonucleosomes with *Hae*III under limit-digest conditions produces a partial digestion pattern that is indistinguishable from the pattern produced by *Hae*III with the purified DNA from the same α-oligonucleosomes (Fig. 6(e); cf Fig. 6(g)). Thus all or almost all of the *Hae*III

FIG. 7. Two-dimensional hybridization mapping of α-mononucleosomes. (a) Staphyloccoccal nuclease digest of CV-1 chromatin was fractionated in the first dimension in a 4% polyacrylamide gel (see Materials and Methods). (b) Second-dimension (DNA) electrophoretic pattern (ethidium-stained) of the mononucleosomes resolved in the first dimension in (a). (c) DNA in (b) was denatured *in situ*, transferred to DBM paper and hybridized with the cloned, ^{32}P-labeled α-DNA probe (see Materials and Methods). (d) Two-dimensional DNP → protein pattern of [^{3}H]lysine-labeled CV-1 nucleosomes. Cells were labeled with L-[^{3}H]lysine for 24 h and fractionated identically to unlabeled cells. Specific protein species visible in the pattern are designated on the right; NH, denotes a group of apparently nucleosomal non-histone proteins, 2 of which are HMG14 and HMG17 (data not shown). Nucleosome terminology: TN, trinucleosomes; DN, dinucleosomes; MN2, metastable mononucleosomal intermediate containing ~ 165 to ~ 185 bp long DNA fragment, core histone octamer and one molecule of histone H1; MN1, core mononucleosome containing ~ 146-bp DNA fragment and core histone octamer but lacking histone H1 and HMG proteins. MN1 nucleosomes in which either one or both H2A histone molecules are substituted with ubiquitin-H2A (uH2A) semihistone (Levinger & Varshavsky, 1980, 1982*a*), are only partially separated from uH2A-lacking MN1 mononucleosomes in this electrophoretic system (Strauss & Prunell, 1982; compare with Fig. 1 of Levinger *et al.*, 1981). An apparent over-representation of the uH2A spot in (d) is due to the fact that labelling with [^{3}H]lysine in this experiment was carried out in a CV-1 cell population with a relatively low proportion of cycling cells. Therefore the ubiquitin moiety of uH2A attained a much higher specific radioactivity than its H2A moiety in the course of labeling (ubiquitin moiety of chromosomal uH2A undergoes exchange with free ubiquitin *in vivo* (Wu *et al.*, 1981)).

An apparent under-representation of the H1 spot in the MN2 region of (d) is due to a preferential retention of H1 from mononucleosomes (but not from oligonucleosomes) at the boundary between stacking and separating SDS/gels (data not shown). The reason for this consistently observed artifact is not understood.

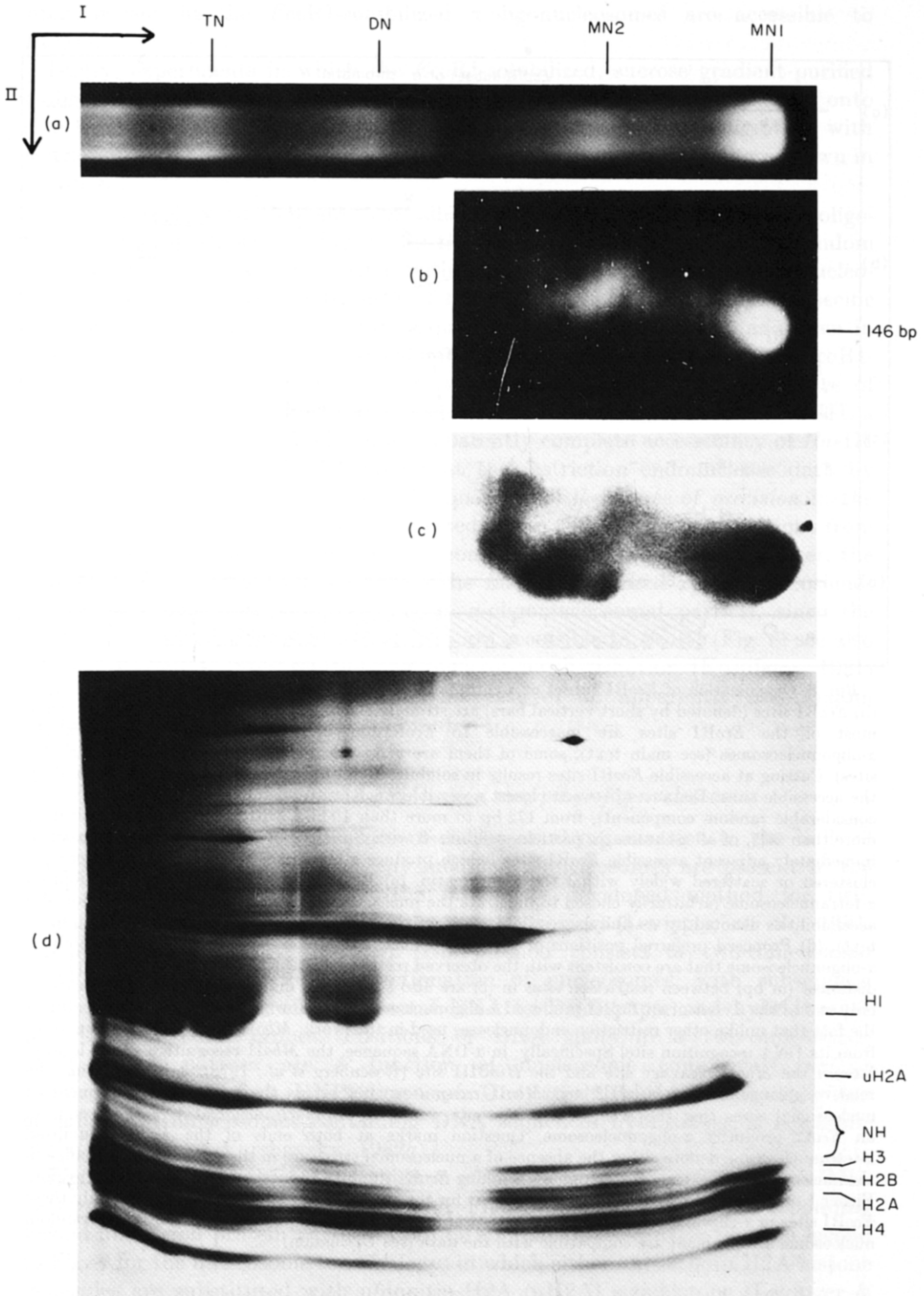

FIG. 7.

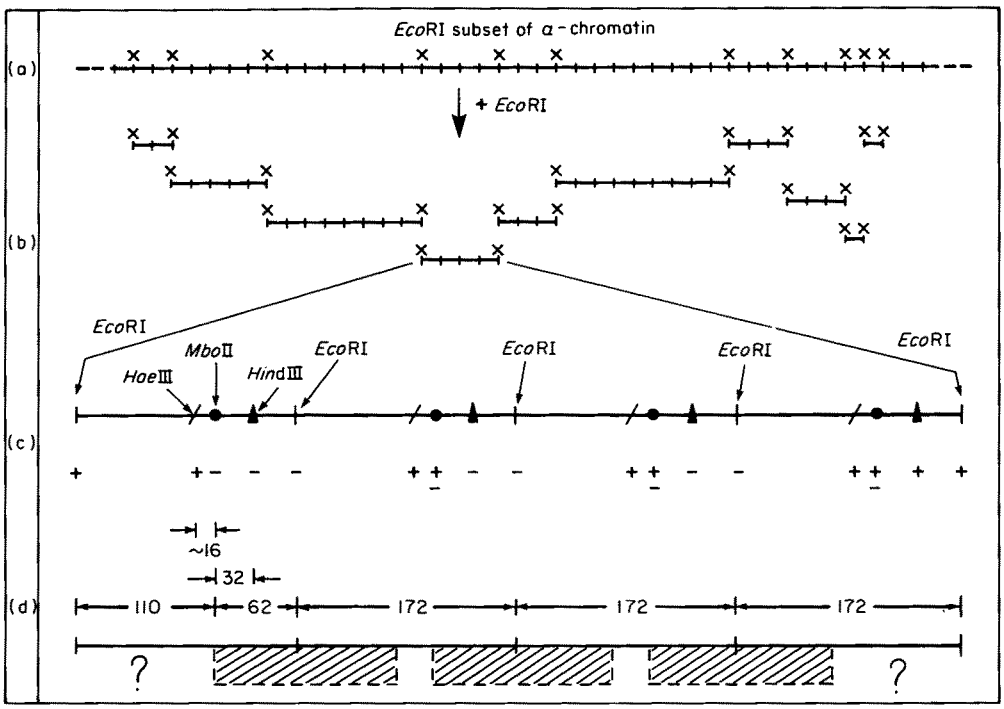

FIG. 8. Organization of *Eco*RI subset of α-chromatin as probed by restriction endonucleases. (a) to (b) *Eco*RI sites (denoted by short vertical bars) are strongly clustered in α-DNA (see main text); while most of the *Eco*RI sites are inaccessible to *Eco*RI in both α-chromatin and solubilized α-oligonucleosomes (see main text), some of them are accessible (denoted by crosses above *Eco*RI sites). Cutting at accessible *Eco*RI sites results in solubilization of an α-chromatin particle flanked by the accessible sites. Distances between closest accessible *Eco*RI sites vary widely (apparently with a considerable random component), from 172 bp to more than 10 kbp, with α-monomers constituting more than 25% of all α-chromatin particles solubilized with *Eco*RI. It is not known whether pairs of immediately adjacent accessible *Eco*RI sites (which produce α-monomers upon *Eco*RI digestion) are clustered or scattered widely within the α-chromatin. (c) Restriction map of an *Eco*RI-solubilized α-tetranucleosome (arbitrarily chosen to illustrate the nuclease cutting patterns), with restriction site accessibilities denoted by + (fully accessible), ± (partially accessible) and − (inaccessible) (see main text). (d) Proposed preferred positions of the 146-bp nucleosomal cores within an *Eco*RI-solubilized α-oligonucleosome that are consistent with the observed restriction site accessibilities shown in (c). The distances (in bp) between restriction sites in (c) are also shown (see main text). That *Mbo*II cleaves only at 1 of the 2 ends of an *Eco*RI-produced α-oligonucleosome, as shown (see Results), may be due to the fact that unlike other restriction endonucleases used in this work, *Mbo*II cleaves DNA 12 bp away from its DNA recognition site. Specifically, in α-DNA sequence, the *Mbo*II recognition site is located *between* the *Mbo*II cleavage site and the *Hin*dIII site (Rosenberg *et al.*, 1978); it is clear from the relative arrangement of *Hin*dIII and *Mbo*II *recognition* sites *versus* the proposed arrangement of nucleosomal cores that the *Mbo*II *recognition* site would be *intranucleosomal* at 1 of the 2 ends of an *Eco*RI-produced α-oligonucleosome. Question marks at both ends of the *Eco*RI-solubilized α-tetranucleosome denote either the absence of a nucleosomal structure in these regions or a change in the nucleosome arrangement which puts a flanking *Eco*RI site into an internucleosomal (linker) region. Either 1 of these 2 interpretations would account for the observed accessibility of these and only these *Eco*RI sites in α-chromatin (see Results). Statistical interpretations of such an apparently non-random nucleosome arrangement are compatible with the data (see Discussion).

sites present in the *Eco*RI-solubilized α-oligonucleosomes are accessible to *Hae*III.

Lastly, experiments in which the *Eco*RI-solubilized, sucrose gradient-purified α-oligonucleosomes were selectively stripped of H1 histone by an exchange onto tRNA (Ilyin *et al.*, 1971; see Materials and Methods) before redigestion with restriction endonucleases produced results (not shown) identical to those shown in Figure 6.

The map of restriction site accessibilities in the *Eco*RI-solubilized α-oligonucleosomes is shown in Figure 8; the results clearly preclude a random arrangement of nucleosomal cores within the *Eco*RI-solubilized α-oligonucleosomes and are consistent with most of the nucleosomal cores occupying specific positions relative to the nucleotide sequence of the α-DNA repeat as shown in Figure 8. The proposed preferred nucleosome arrangement in the *Eco*RI-solubilized α-oligonucleosomes (Fig. 8) accounts for the complete resistance of these α-oligonucleosomes to *Eco*RI, a specific end-cleavage pattern for *Hin*dIII, a greater accessibility to *Mbo*II and an apparently complete accessibility of *Hae*III (Figs 6 and 8). It should be noted that the restriction endonuclease data by themselves (Fig. 6) do not address the question of *the degree of precision* in the nucleosome alignment within the suggested frame (Fig. 8); small deviations from the proposed arrangement may be still compatible with the data. Moreover, the above nucleosome arrangement within the *Eco*RI-solubilized α-oligonucleosomes (Fig. 8) is violated *at the ends* of *every* α-oligonucleosomal particle, since the flanking, but not the internal *Eco*RI sites are accessible to *Eco*RI (Fig. 8; see also above). Statistical interpretations of nucleosome distribution (Kornberg, 1981) may therefore be relevant even in this case of an apparently non-random nucleosome arrangement (see Discussion).

(h) *Two-dimensional hybridization mapping of α-nucleosomes*

To directly confirm the presence of nucleosomal organization in the bulk of α-DNA and also to see whether any modified α-nucleosomes are present in the CV-1 chromatin, we have used the previously developed method for two-dimensional hybridization mapping of nucleosomes (Levinger *et al.*, 1981; Levinger & Varshavsky, 1982*a*,*b*). The method consists of two-dimensional (DNP → DNA) electrophoretic fractionation of nucleosomes, with subsequent electrophoretic transfer of nucleosomal DNA to DBM paper and hybridization with specific DNA probes. Positions of DNA spots in a two-dimensional DNP → DNA pattern are a function of nucleosome composition and/or conformation in the first (DNP) dimension. One can thus deduce the composition of subsets of nucleosomes containing DNA sequences hybridizing to a specific probe (Levinger & Varshavsky, 1982*a*).

Figure 7(b) shows the total (ethidium-stained) two-dimensional DNP → DNA pattern of CV-1 mononucleosomes and Figure 7(d) shows the corresponding second-dimension protein pattern. Core (MN1) mononucleosomes (see the legend to Fig. 7 for the nucleosome terminology) in which either one or both H2A histone molecules are substituted with ubiquitin-H2A (uH2A) semihistone (Levinger &

Varshavsky, 1980,1982*a*,*b*), are only partially separated from the core (MN1) mononucleosomes lacking uH2A in this electrophoretic system (Fig. 7(b) to (d)). The histone H1-containing mononucleosome MN2 migrates as a diffuse band in the first (DNP) dimension (Fig. 7(a)) and displays a range of DNA sizes ~ 20 to ~ 35 bp longer than core-length (~ 146 bp) DNA in the second (DNA) dimension (Fig. 7(b)).

Figure 7(c) shows the α-DNA-specific subset of the total mononucleosomal DNA pattern seen in Figure 7(b). Strikingly, the cloned α-DNA probe (a gift from Dr H. Zachau; see Materials and Methods), in addition to hybridizing to the MN1 DNA spot, lights up a non-MN1, non-MN2 area that contains very little DNA in the total (ethidium-stained) DNA pattern (Fig. 7(c); cf. Fig. 7(b)).

One interpretation of this α-DNA-specific pattern (Fig. 7(c)) is that a non-histone protein (presumably specific for α-DNA) is bound to the α-mononucleosomes in addition to the core histones, resulting in more slowly migrating nucleosomal particles. A strikingly analogous set of the two-dimensional hybridization mapping data has recently led to a direct identification of a ~50,000 M_r non-histone protein D1 as a specific component of *Drosophila melanogaster* nucleosomes containing (A+T)-rich satellite DNAs (Levinger & Varshavsky, 1982*a*,*b*).

Although first-dimension (DNP) positions of several non-histone protein bands in Figure 7(d) (two of which are HMG14 and HMG17; data not shown) approximately coincide with the first dimension (DNP) position of the α-DNA-specific hybridization pattern (Fig. 7(c)), an unambiguous identification of the putative α-DNA-specific protein was not possible using the two-dimensional hybridization mapping approach alone. Therefore we have recently attempted a direct isolation of this putative α-DNA-binding protein from CV-1 chromatin using more conventional extraction and chromatographic procedures and a direct *in vitro* α-DNA–protein binding assay. Preliminary results indicate the existence in CV-1 chromatin of a non-histone protein with an apparent molecular weight of approximately 18,000 which highly preferentially binds α-DNA *in vitro* (F. Strauss and A. Varshavsky, unpublished data).

4. Discussion

The three major results of the present work are as follows.

(1) Mapping of α-nucleosome positions with restriction endonucleases as *primary* probes reveals a single "preferred" frame for nucleosome arrangement for most but not necessarily all of the α-nucleosomes within at least one specific minor subset of α-chromatin defined by the presence of *Eco*RI sites (Fig. 8). This arrangement is close to the one suggested recently by Musich *et al.* (1982) from their data on initial staphylococcal nuclease cleavages with α-chromatin. Our data, however, in contrast to the interpretation by Musich *et al.* (1982), preclude the existence of a single type of nucleosome arrangement throughout α-chromatin, in agreement with the results of earlier studies using other methods (Fittler & Zachau, 1979).

(2) A unique preferred arrangement of nucleosomes (detectable *without* the use of staphylococcal nuclease) within the *Eco*RI-solubilized α-oligonucleosomes is violated *at the ends* of these same α-oligonucleosomal particles (*flanking Eco*RI sites in these α-oligonucleosomes are accessible to *Eco*RI, whereas the *internal Eco*RI sites are not). This striking pattern (Fig. 8) can be explained by accessible *flanking Eco*RI sites either being in the internucleosomal (linker) regions (in contrast to the *internal Eco*RI sites within the same α-oligonucleosome) or being a part of a non-nucleosomal structure. Both interpretations assume a local disruption in regularity of nucleosome arrangement and suggest therefore a role for statistical factors (Kornberg, 1981) in formation of such patterns, as discussed below.

(3) The distance between two *closest* accessible *Eco*RI (or *Hin*dIII) sites in α-chromatin varies widely from 172 bp to more than 10 kb, *with 172 bp being the most frequent distance* (see Results). Such wide and relatively "smooth" distribution of distances between *closest* accessible *Eco*RI (or *Hin*dIII) sites in α-chromatin strongly suggests at least a partially statistical origin of this and analogous patterns (see below).

(a) *Factors that determine nucleosome arrangement in chromatin*

The following factors (among others) may together influence the pattern of nucleosome arrangement in any given subset of chromatin.

(i) Octameric histone cores themselves may have intrinsic binding preferences to specific nucleotide sequences in DNA. Depending on the relative strength of such preferences for any given stretch of DNA, the resulting arrangement of nucleosomes would be *deterministically* more or less non-random with regard to the corresponding DNA sequence either within the same cell (for a repetitive sequence) or between different cells (for both unique and repetitive sequences). Although the evidence on this point is still fragmentary, both theoretical arguments based on bendability of DNA (Trifonov, 1981) and the results of *in vitro* nucleosome reconstitution experiments (Wasylyk *et al.*, 1979; Chao *et al.*, 1980) suggest that intrinsic preferences in histone–DNA binding do exist but that such preferences are generally below two orders of magnitude; that is, much smaller than differences in specific *versus* non-specific DNA-binding affinities of *lac* repressor-type proteins (see, however, Simpson & Stafford, 1983).

(ii) Assuming that for a nucleosome to be formed, a minimum length of DNA (e.g. ~ 160 bp) is required, and that the centers of adjacent nucleosomes do not come closer along the fiber than some minimum distance (e.g. ~ 160 bp), one can show by direct statistical computations that a nucleotide sequence-specific start point for nucleosome assembly should generate a *statistically* non-random arrangement of nucleosomes relative to the nucleotide sequences flanking the start point (Kornberg, 1981, and a personal communication). Significantly, this conclusion can be derived in the absence of any additional assumptions, such as the degree of constancy of the linker length. The sequence-specific start point for nucleosome assembly (called below a "singularity" (Weintraub, 1980)), may be served *a priori* by a wide variety of structures, such as a sequence-specific DNA-

binding non-histone protein, a stretch of single-stranded DNA generated, for instance, in the course of DNA replication or repair (Wittig & Wittig, 1982; Zolan *et al.*, 1982), an event of nucleotide sequence-specific removal of a nucleosome that generates a stretch of naked DNA and so forth. The degree of non-randomness in this type of nucleosome arrangement, being in the absence of other factors purely statistical in nature, would decrease upon an increase of the distance from a "singularity". The rate of such decrease would be a function of the nucleosome packing density, the latter parameter defining a mean nucleosomal DNA repeat (Kornberg, 1981). Some lineages of somatic cells within the same organism are known to have different mean nucleosomal DNA repeats (Spadafora *et al.*, 1976; Thomas & Thompson, 1977; Sperling & Weiss, 1980). These results alone preclude the existence of a fixed, unique ("phased") nucleosome arrangement in relation to bulk single-copy nucleotide sequences in cells of different lineages within the same organism.

(iii) The degree of variability of the linker DNA length is expected to strongly influence the patterns of nucleosome arrangement. For example, a unique linker length in combination with a sequence-specific nucleosome assembly start point (see item (2) above) would generate a *unique* (sometimes called "phased") nucleosome arrangement in relation to the corresponding DNA sequence. In a more general case, a non-random variability in the linker length would modify statistically derived non-random arrangement of nucleosomes discussed in the item (ii) above into a different type of non-random arrangement. Although the linker length is definitely variable when measured in the bulk chromatin (Lohr *et al.*, 1977; Prunell & Kornberg, 1982; Strauss & Prunell, 1982), it may be non-random in specific subsets of chromatin.

(iv) It is possible that nucleosomes may slide *in vivo*; such sliding has been observed so far only *in vitro* under nucleosome-destabilizing solvent conditions (reviewed by McGhee & Felsenfeld, 1980; Igo-Kemenes *et al.*, 1982). Analogous *in vivo* sliding of nucleosomes, if it occurs, may be physiologically relevant. The possibility of nucleosome sliding, by introducing a dynamic aspect into the problem of nucleosome arrangement, does not necessarily trivialize the problem, since sliding, if it occurs, may be at least partially nucleotide sequence-specific and may be also limited to relatively short DNA distances, specific subsets of chromatin and specific stages of the cell cycle.

The functional significance, if any, of preferred nucleosome arrangements and their changes *in vivo* remains unknown, with an important exception that nucleosomes appear to be absent from active control regions in both cellular and viral chromosomes (see Introduction).

(b) *Deterministic and apparently statistical aspects of the α-chromatin organization*

Our data on α-nucleosome arrangements (see above) are clearly compatible with the existence of intrinsic preferences of histone octamers for binding to specific sequences with the 172-bp α-DNA repeat (Fig. 8(d); the data are also compatible with a non-random distribution of linker DNA lengths in at least some subsets of α-chromatin. However, the same data can be explained without invoking these

notions (see below). The question of nucleosome sliding is not addressed by the design of our experiments ; it should be emphasized, however, that the use of restriction endonucleases as *primary* probes apparently precludes artifactual nucleosome sliding *in vitro*, since in this approach relatively infrequent *endonucleolytic* cuts are made within α-oligonucleosomes ; moreover, a preliminary removal of histone H1 from α-oligonucleosomes by exchange of H1 onto added tRNA in a low ionic strength buffer does not change any of the mapping data (see Results).

Two striking features of the α-chromatin organization revealed in the present work are first, the existence of apparent "singularities" in the arrangement of α-nucleosomes within the *Eco*RI subset of α-chromatin (see item (ii) in the Discussion above) and second, an extremely wide distribution of *closest accessible Eco*RI sites in the *Eco*RI subset of α-chromatin (see item (3) in the Discussion above). These results strongly suggest a contribution of statistical factors of the type discussed in item (ii) above to the arrangement of nucleosomes in α-chromatin. For clarity we defer a discussion in detail of the many possibilities formally compatible with the data above; instead, we present two alternative, but not mutually exclusive hypotheses sufficient to account for our results.

First, one notes that a set of the *Eco*RI-solubilized α-oligonucleosomes is by definition a *non-random* subset of α-chromatin; in this subset *sequence-specific Eco*RI cuts at accessible *flanking Eco*RI sites can be themselves formally viewed as *sequence-specific singularities* of the type considered in item (ii) above. Thus, according to this interpretation, an apparently non-random arrangement of nucleosomes within the *Eco*RI-solubilized α-oligonucleosomes *flanked* by the *accessible Eco*RI sites (Fig. 8) may be viewed as being at least partially statistical in origin, with an accessible flanking *Eco*RI site formally interpreted as a singularity. That such singularities *themselves* are arranged in an at least partially random manner throughout the *Eco*RI subset of α-chromatin (see Results) is an additional, though still indirect argument for the statistical hypothesis.

Another way of interpreting the same data is to suggest that there is a sequence-specific DNA-binding non-histone protein(s) in CV-1 cells that recognizes a specific nucleotide sequence within the 172-bp α-DNA repeat and by binding there creates a sequence-specific singularity (either nucleosomal or non-nucleosomal) for nucleosome assembly in the flanking DNA regions. *Eco*RI-accessible sites in α-chromatin (and possibly also *Hin*dIII-accessible sites; see Results) would be then viewed as manifestations of such singularities, the distribution and relative content of the latter reflecting the distribution and the titer of a putative α-DNA-specific protein in relation to the number of the 172-bp α-DNA repeats per cell. This simple idea accounts for all of our major observations but clearly does not follow from them. We consider it at the present time a useful working hypothesis in so far as: first, there is a precedent for satellite DNA-specific non-histone protein; in *Drosophila melanogaster*, a $\sim$50,000 M_r protein called D1 was shown recently to be a specific, stoichiometric component of isolated *D. melanogaster* nucleosomes containing (A+T)-rich satellite DNA (Levinger & Varshavsky, 1982*a*,*b*). Second, in our recent application of the method for two-dimensional hybridization mapping of nucleosomes to the CV-1

chromatin we have detected what appears to be an α-satellite-specific non-histone protein (F. Strauss and A. Varshavsky, unpublished data; see also Fig. 7 and Results). Our current work is directed toward isolation and characterization of this protein; determination of its binding specificity on α-DNA and α-nucleosomes should increase our understanding of the α-chromatin organization.

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Note added in proof: More recently, the putative α-DNA-specific non-histone protein (see Results) was purified to homogeneity from an 0·35 M-NaCl extract of CV-1 nuclei by chromatography on phosphocellulose followed by double-stranded DNA-cellulose chromatography. A novel gel electrophoretic assay was used to detect fractions with specific α-DNA-binding activity. Although the purified α-DNA-binding protein (M_r of about 18,000) is distinguishable from both core histones and major species of HMG14/17 proteins, its electrophoretic properties and solubility in 10% CCl_3 COOH define it operationally as an HMG protein (F. Strauss & A. Varshavsky, unpublished results).