Loosened nucleosome linker folding in transcriptionally active chromatin of chicken embryo erythrocyte nuclei

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ABSTRACT

We have investigated the mechanism of the electrophoresis-driven chromatin aggregation which had been described by Weintraub (1984, *Cell* 38, 17–27) as a putative mean for propagation of genetic repression in eukaryotes. We show that the oligonucleosome aggregates are assembled *de novo* at the starting zone of DNP electrophoresis. A new system of native two-dimensional DNP electrophoresis has been worked out to separate the oligonucleosome aggregates ('A' particles) and the freely-migrating oligonucleosomes ('B' particles). The 'B' particle fraction which is derived from transcriptionally-active chromatin regions undergoes an extensive nuclease degradation of its DNA termini during the nuclease digestion. This fraction is partially depleted of histones H1 and H5 and is enriched in HMG nonhistone proteins. 'A' particles comprise the repressed chromatin DNA fragments which are about 60 b.p. longer than the corresponding DNA oligomers of 'B' particles. An oligonucleosome preparation containing the elongated DNA oligomers has been also isolated by means of sucrose gradient ultracentrifugation. Exonuclease III mapping reveals that the two chromatin fractions differ by an extent of terminal linker DNA trimming during the *Micrococcus* nuclease digestion rather than by the nucleosome repeat length. The complex character of nuclease digestion is not observed when the chromatin is digested in solution after the nuclear lysis. We argue that the protection of terminal oligonucleosome linkers is due to selective condensation of inactive chromatin in chicken erythrocyte nuclei and that the terminal DNA tails together with linker histones bound to them mediate the aggregation of repressed chromatin fragments.

INTRODUCTION

Studies of eukaryotic chromatin structure are intimately allied to the pursuit of molecular mechanism(s) for control of gene activity during cell differentiation. Transcriptionally-active chromatin is marked by an enhanced overall DNase I sensitivity which is inherent in the domains of an altered chromatin

organization extending for several dozens of kilo-base pairs (1,2). This active chromatin feature has been shown to be propagated independent of transcription (3,4) and to be associated rather with gene expression than with transcription *per se* (5). The structural basis of general chromatin activation marked by the DNase-I sensitivity remains obscure. It has been variously attributed to a loss of linker histones (6,7), to an association with nonhistone proteins HMG 14/17 (8), to an altered supranucleosome chromatin folding (9,10), to a rearrangement of nucleosome core structure (11,12), and even to a preferred location at the nuclear periphery (13,14).

As it has been originally demonstrated by Weintraub (15), the soluble chromatin fragments originating from DNase I-sensitive chromatin domains of chicken embryo erythrocyte nuclei ('B' particles) migrate during DNP electrophoresis as free oligonucleosomes while transcriptionally-inert chromatin fragments migrate as complex self-associated units ('A' particles). The author has proposed that linker histones H1 and H5 hold together the 'A' particle oligonucleosomes and thus determine the repressed state of inactive chromatin domains (15,16). However, it remained obscure whether the self-associated oligonucleosomes retained their native chromatin organization or appeared as an artifact of the experimental procedures.

Here we show that the 'A' particle assembly is promoted by DNP electrophoresis and that the DNA oligomers of the association-prone and of the resistant chromatin particles differ in their length. The transcriptionally- inactive oligonucleosomes maintain their terminal linker regions intact during the nuclease treatment of the nuclei whereas the terminal DNA regions of the active chromatin-derived oligonucleosomes are extensively trimmed by the nuclease. We attribute the observed property of transcriptionally-active chromatin to a loosened higher-order folding which renders the nucleosome linkers exposed to nucleases and, possibly, to various factors supporting gene expression.

METHODS

Isolation of cells and nuclei

The blood from 20 fourteen-day old white leghorn chicken embryos (10 ml) was collected by vein puncture, added to 30 ml of solution containing 140 mM NaCl, 3 mM KCl, 10 mM

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Na-citrate, 10 mM Na-phosphate, pH = 7.4 and centrifuged at 5000 rpm for 5 min. in a Beckman JA-20 rotor. The cells were washed twice with RSB (3 mM MgCl₂, 10 mM NaCl, 10 mM Tris-HCl pH = 7.6) containing 0.5 mM PMSF, the final pellet was suspended in 20 ml of RSB containing 0.5% NP-40 and 0.5 mM PMSF and homogenized in a Dounce homogenizer for 30 min. The nuclei were pelleted into a RSB underlayer containing 1 M sucrose by centrifugation at 10000 rpm for 10 min. in JA-20 rotor and resuspended in 10 ml of RSB by several strokes of Dounce homogenizer. An equal volume of cold glycerol was added and the nuclear preparation was stored at -20°C up to 30 days. All other procedures were performed at +4°C.

Nuclease digestion and chromatin isolation

An aliquot of the nuclear preparation was pelleted from the glycerol solution for 5 min. at 5000 rpm. in JA-20 rotor and resuspended in 5 ml of RSB containing 0.5 mM PMSF (A_{260} = 20). 5 μ l of 1 M CaCl₂ and 15 units of Micrococcal nuclease (Boehringer) were added and the reaction was carried out with stirring at +37°C. Aliquots were taken periodically and the DNA was analyzed by an agarose gel electrophoresis in order to estimate the time interval needed to achieve the desired extent of digestion. To obtain a 'standard' chromatin preparation (the mean number of nucleosomes in a chain N = 4) the reaction was terminated after 30 min. by adding 0.5 ml of 0.1 M Na₂-EDTA and 5 ml of ice-cold TE (10 mM Tris-HCl, 1 mM EDTA pH = 7.6). The digested nuclei were pelleted for 10 min. at 10000 rpm. in JA-20 rotor, the pellet resuspended in 5 ml TE and pelleted under the same conditions. The supernatant S2 was collected and stored at +4°C up to 14 days. For a solution-digestion experiment an S2 sample was obtained from the nuclei digested for 3 min. and redigested in TE or in TE + 100 mM NaCl solutions by adding of 1 mM CaCl₂ and 1 U/ml of Micrococcal nuclease.

DNP electrophoresis

A one-dimensional native DNP electrophoresis was routinely performed in a horizontal slab of 1% Sigma type IV agarose in TAEW electrophoresis buffer (25 mM Na-acetate, 2 mM Na₂-EDTA, 20 mM Tris-acetate pH = 7.4, (15)) for 12 hours at 1 V/cm. To obtain a DNP/DNA two dimensional separation the gel was stained with Ethidium bromide, an appropriate gel stripe was cut out and incubated at +37°C for 1 hour in 5 ml of TAEW electrophoresis buffer (2 mM Na₂-EDTA, 20 mM Na-acetate, 40 mM Tris-acetate pH = 7.8 (17)) containing 1% SDS and 50 μ g/ml proteinase K. The protease-treated gel slices were applied onto a start line of a horizontal slab of 1.1% Sigma type I agarose and electrophoresed in TAEW for 4 hours at 3 V/cm. One of the DNP electrophoresis was carried out in the first direction in TE buffer. To obtain a native DNP/DNP two-dimensional separation a gel taken after a one-dimension run in TAEW buffer was dialyzed for 2 hours against two changes of the low-salt buffer EA (1.5 mM Na₂-EDTA, 1 mM Na-acetate pH = 5.6) and electrophoresed in perpendicular direction at 3 V/cm for 5 hours. In one case EA buffer was substituted for TE in the second-direction run. All of the DNP runs were performed at +4°C with a permanent buffer recirculation.

When the electrophoresis was over the slabs were stained with Ethidium bromide, photographed on a UV-transilluminator, treated with 0.4 N HCl, denatured with alkali, neutralized with Tris-HCl and blotted on nylon sheets Hybond-N ('Amersham') as described (18).

Ultracentrifugation

For a preparative separation 1 ml of standard S2 sample (A_{260} = 40) was loaded on a 5–30% sucrose gradient (11 ml) containing TE or TAEW buffer. Ultracentrifugation was carried out in SW-41 rotor on a Beckman L5-65 centrifuge for 2 hours at 39000 rpm. 15 fractions were collected from the bottom of the tubes through Uvicord S (LKB) ultraviolet optical unit and aliquotes were taken for DNA electrophoresis. Fraction 13, 12 and 8, 9 of TAEW gradient were combined to give the 'Slow' and the 'Fast' oligonucleosome preparations respectively.

Exonuclease III digestion

Aliquotes (2 ml, A_{260} = 4) of the intact S2 sample and the 'Slow' and the 'Fast' oligonucleosomes separated on a sucrose gradient were taken and digested with 0.25 U/ml of trypsin for 20 min. at +37°C. The protease digestion was terminated with 1 mM PMSF. 5 mM MgCl₂ and 1 μ l of Exonuclease III enzyme solution (180 u/ μ l, Pharmacia) were added and the reaction continued for 15 min. at +37°C with permanent stirring. Aliquots were taken in the course of digestion and the reaction was halted with 10 mM EDTA. The DNA from the samples was isolated as described below.

DNA isolation, 5'-labelling, and electrophoresis

The intact and the nuclease-digested chromatin samples were treated with 50 μ g/ml of proteinase K in 1% SDS and twice extracted with phenol, once with phenol-chloroform, twice with chloroform, and twice precipitated with ethanol. The final material was readily cleaved with restrictases into appropriate bands detected by hybridization and radioautography. DNA from agarose gel slices was isolated by the electroelution onto dialysis membranes as described (19) and electrophoresed once more according to Loening (17). Otherwise, the DNA samples were labelled with [γ -³²P]-ATP by T4 polynucleotide kinase, denatured and electrophoresed in a 40 cm-long 'sequencing gel' containing 6% polyacrylamide or in a 20 cm-long gel containing 3% polyacrylamide as described (19). The high molecular weight DNA standards were obtained by the cleavage of phage lambda DNA with Hind III and Eco RI endonucleases. The low molecular weight DNA markers were obtained by cleavage of pBR 322 DNA with Msp I endonuclease.

DNA probes and hybridization

Here we used a 1364 b.p. Msp I fragment from the plasmid pCA β G1 (20) as a probe for the adult beta-globin gene coding region (probe β) and a 1340 b.p. Msp I fragment from the plasmid pCBG4 (21) as a probe for the embryonic rho-globin gene (probe ρ). DNA of the total rho-globin gene Hind III fragment from the plasmid pCBG4 was used for detecting the repeated DNA sequences (probe Rep). A DNA probe for the transcriptionally-inert ovalbumin gene (probe Ov) is a 2.4 kbp Eco RI fragment from a plasmid pOV 12 containing a part of the coding region of the chicken ovalbumin gene (2). The DNA probes were labelled by nick-translation to about 5×10^8 cpm/ μ g DNA, heat-denatured, reassociated with the genomic DNA attached to nylon filters and washed as described (18). The washing conditions were set by monitoring of the purity of the genome DNA restriction bands. The most stringent washing was carried out at +65°C in 1 $\times$ SSC for the ovalbumin gene and in 0.5 $\times$ SSC for the beta- and the rho-globin gene probes. The washed Hybond sheets were autoradiographed at -70°C with intensifying screens. The distribution of a particular gene DNA

among different chromatin fractions was estimated by the densitometry of the autoradiographs with a LKB Ultrascan laser densitometer.

Protein isolation and electrophoresis

To identify the proteins associated with the oligonucleosome fractions separated by native DNP/DNP electrophoresis the appropriate agarose gel fragments were cut out from the Ethidium-stained gel slabs and placed in an Eppendorf tube. The gel slices were minced with a razor and twice frozen at -70°C . One part of $5\times$ Laemmli sample buffer was added to four parts of agarose, the tubes were shaken for 30 minutes at room temperature and the agarose was pelleted in an Eppendorf microcentrifuge for 5 minutes. The aliquotes of the resulting supernatants were boiled for 2 minutes and applied on a 18% polyacrilamide SDS electrophoresis (22). The gels were fixed and stained with AgNO_3 as described (23). The protein samples were also labelled with ^{125}I by ICI method as described (24), and electrophoresed as above. The densitograms of the autoradiographs were obtained and integrated to give the protein peak areas with a LKB Ultrascan laser densitometer.

RESULTS

Oligonucleosome reassociation during DNP electrophoresis and ultracentrifugation

To obtain a preparation of soluble chromatin, the nuclei from 14-day old chicken embryos were digested with Micrococcal nuclease and extracted with TE buffer. Most of the soluble oligonucleosomes were recovered in the supernatant S2 obtained after the second TE extraction. This sample contained about 90% of the total nuclear DNA. The 30 minute digested preparation with a mean DNA size of 800 b.p. (Fig 1a) is employed in most of the experiments throughout this work. When subjected to a two-dimensional DNP/DNA electrophoresis, this sample is

separated into a lagging and a leading oligonucleosome diagonals termed by Weintraub (15) as 'A' and 'B' particles respectively (Fig. 1). In the first direction (DNP) 'A' particles migrate as a discrete band slightly ahead of xylene cyanol marker dye. The apparent size of the 'A' particle band is about 5000 b.p. Upon deproteinization it gives a set of oligomer DNA fragments showing a DNA size distribution close to that of total S2 sample (800 b.p.). Therefore an average 'A' particles appear to be a composite of several separate oligonucleosomes linked together.

When the DNP electrophoresis is carried out in TE buffer (the media for the chromatin solubilization and storage) all of the oligonucleosomes migrate as free particles within one diagonal (Fig. 1b). This effect has been previously attributed to a putative dissociation of 'A' particles during the low-salt electrophoresis (15). To check up this possibility we subjected the oligonucleosomes separated in TAEW to a second dimension electrophoresis in TE. In course of this experiment we did not observe any dissociation of the 'A' particles (Fig. 1c). Their partial dissociation was observed in a second-dimension electrophoresis buffer having a much lower salt concentration (Fig. 7). It can be deduced from this experiment that the oligonucleosome self-association occurs at the starting zone of the DNP electrophoresis and is promoted by the elevated salt concentration.

Previously Weintraub (15) had illustrated his idea of the integrity of 'A' particles by centrifugation in a 100 mM NaCl solution. Here we found out that the S2 sample sediments in TE buffer as free particles while in TAEW it is partitioned into the 'Slow' oligonucleosomes which sediment according to their molecular weights and the 'Fast' fraction sedimenting in a broad zone of the gradient (Fig. 2). The data obtained here confirm the results of an ultracentrifugation study carried out by Jorcano et al. (25) who have shown that in the presence of 30 mM NaCl chicken erythrocyte oligonucleosomes (trimers to 20-mers) form spontaneously heterogeneous particles containing about 25

Figure 1. Oligonucleosome self-association during DNP-electrophoresis. (a) — DNA electrophoresis and DNP/DNA two-dimensional electrophoresis (DNP from left to right, DNA from top to bottom) of S2 sample obtained from chicken embryo erythrocyte nuclei digested with Micrococcal nuclease for 30 minutes. The molecular weight markers (M) is the DNA of phage lambda cut with Hind III restrictase. (b) — Two-dimensional DNP/DNA electrophoresis of S2 sample in TE buffer. (c) Two-dimensional DNP/DNP electrophoresis of standard S2 sample in TAEW buffer (left to right) and in TE buffer (top to bottom). A lane of a parallel one-dimensional DNP electrophoresis in TAEW is shown at the top. The location of xylene cyanol marker dye after the first-direction run is shown by a black arrow.

nucleosomes. The salt-dependent oligonucleosome aggregation has been also observed by Thomas et al. (26). The latter authors, however, did not observe the aggregation of oligonucleosomes smaller than hexamers. This discrepancy may reflect a more extensive redistribution of linker histones from small oligonucleosomes in the preparation of Thomas et al. (26).

The aggregation tendency of an oligonucleosome is correlated with the length of its oligomer DNA molecule

Owing to a better resolution provided by our full-size agarose slabs as compared with Weintraub's minigels we noticed that the mobility of the 'A' and 'B' particle DNA bands in the second dimension was uneven (Fig 1 a) and that the 'A' particle oligonucleosomes were longer than the 'B' particle oligomers of the same number. To rule out the possibility of an artificial distortion of the DNA mobility in the DNP/DNA gels we extracted the DNA from electrophoretic zones representing 'A' particles, 'B' particles and a foremost Ethidium-stained zone containing the nucleosome cores ('C' particles). Electrophoresis of DNA extracted from these gel slices (Fig. 3) shows that the 'A' particle DNA fragments are indeed longer than their 'B'

particle counterparts. The length of the 'A' particle DNA fragments is close to the multiples of 210 b.p., the length of the chicken erythrocyte nucleosome repeat (27). The difference between the two sets of DNA fragments rises in parallel with the increase of the oligonucleosome band number reaching the value of 70 b.p. (Table 1). The 'B' particle' DNA bands are much more prominent than those of 'A' particle DNA. As it was estimated by densitometry of the negatives the relative DNA concentration in the 6–8-mer size class (where the trimming of 'A' particles is negligible) is close to 7 : 2 : 1 for 'A', 'B'₁, and 'B'₂ fractions respectively. Most of the smaller oligomers (mono-trimers) are extensively trimmed by the nuclease and migrate as aggregation-free particles.

The DNA samples extracted from the agarose gels were also labelled with ³²P, heat-denatured, and applied on denaturing polyacrilamide gels. The result showing that the DNA length difference is about 50–60 b.p. in the tri-pentanucleosome size range (Fig 3b, Table 1) is consistent with that obtained by the agarose DNA electrophoresis. When we electrophoresed the ³²P-labelled DNA isolated from the combined fractions 8–9 ('Fast') and 12–13 ('Slow') of the high-salt sucrose gradient we

Figure 2. Oligonucleosome self-association in course of ultracentrifugation. Electrophoretic separation of DNA samples obtained after the ultra-centrifugation of a standard S2 preparation in a sucrose gradient containing TE buffer (left) and TAEW (right). The fractions are numbered from the bottom to the top of the gradients. Corresponding sedimentation profiles are shown above.

Table 1. DNA fragment sizes of oligonucleosome fractions separated by DNP electrophoresis and by ultracentrifugation.

band	agarose gel electrophoresis					denaturing electrophoresis				
	S2	'A'	'B' ₁	'B' ₂	'C'	S2	'A'	'B' ₁	'Fast'	'Slow'
1	175	—	—	180	155	175	—	—	—	170
2	376	—	376	375	—	400	—	380	—	402
3	584	603	571	570	—	605	613	560	640	610
4	805	820	778	786	—	809	826	771	877	813
5	1012	1019	980	996	—	1010	1021	955	1070	1012
6	1216	1221	1173	1197	—	1160	1175	1123	1297	—
7	1411	1425	1369	1396	—	1390	1410	ND	1502	—
8	1622	1629	1550	1582	—	ND	ND	ND	ND	—

The DNA peak positions on the gels were estimated by densitometry of agarose gel negatives and of autoradiographs obtained after electrophoresis of ³²P-DNA on 6% (bands 1–3) and 3% (bands 4–7) polyacrylamide gels. To obtain the DNA size values (b.p.) the DNA size marker peaks were plotted against the logs of their molecular weights and the DNA fragment sizes were taken from the calibration curves. (—): the corresponding band is absent from the nuclease digest. (ND): the band is not detected owing to a poor resolution in this size range. Sample standard deviation varies from ±1 b.p. for band 1 to ±18 b.p. for band 8.

Figure 3. The aggregation-prone and the resistant oligonucleosomes differ in the length of their DNA molecules. The scheme for the dissection of agarose DNP/DNA gel is drawn in the upper left corner. The native DNA samples isolated from the agarose and from a total S2 sample were electrophoresed on a new agarose gel and stained with Ethidium bromide (a), and also labelled with ³²P, electrophoresed on a denaturing 6% polyacrylamide gel and autoradiographed (b). The molecular weight markers (M) are the DNA of phage lambda cut with Hind III restrictase (a) and the DNA of pBR 322 plasmid cut with Msp I restrictase (b).

observed that the DNA molecules from the 'Fast' preparation are even longer than the corresponding oligomers of 'A' particles (Table 1, see also Fig. 5). In contrast to the electrophoretically-separated 'A' particles, 'Fast' oligonucleosomes constitute only a minor fraction (less than 10%) of the total S2 sample. It looks like the centrifugation-driven aggregation process has a more strong preference for the longest oligomer DNA than the electrophoresis. 'Slow' DNA oligomers contain the bulk of the soluble chromatin preparation and their DNA size distribution is close to that of the total S2 sample.

Exonuclease III mapping of oligonucleosome DNA tails

The observed DNA length variation between two sets of oligonucleosome particles could be attributed either to a longer nucleosome repeat of the 'A' type chromatin or to a differential higher-order folding of the nucleosome chain. To distinguish between these two possibilities we undertook an exo III mapping of DNA tails in the oligonucleosome fractions separated by ultracentrifugation. This enzyme is known to digest nucleosome linkers and to hesitate at the beginning of core DNA (28). Therefore one can expect that the exo III pauses would mark the true positions of nucleosome cores.

Furthermore, the exo III mapping of Micrococcal nuclease cutting sites may be used to discriminate between different models for nucleosome chain folding.

For example, the cross-linker model (29) predicts a protection of linker DNA from Micrococcal nuclease while the folded zig-zag ribbon model (30) and the solenoid model (31) with an outer orientation of linker DNA are consistent with a linker accessibility for nucleases. Exo III shows a selectivity for one DNA strand (3'-terminal) and if at least one DNA end in a oligonucleosome is protected by a nucleosome core, it would generate a DNA strand inaccessible for exo III which could be observed by means of denaturing electrophoresis.

To overcome the problem of magnesium-induced precipitation of the oligonucleosomes and also to induce the 'A' particle dissociation we subjected the centrifugation-separated oligonucleosomes to a mild treatment with trypsin. Under these conditions histones H1, H5, and H3 are cleaved resulting in the unfolding of the higher order structure (32). The trypsin-digested polynucleosomes were soluble in the exo III-digestion buffer and sedimented as free particles but retained the nucleosome repeat indistinguishable from that obtained with the intact polynucleosomes. As it is shown in Fig. 4 'Slow' oligonucleosomes demonstrate a rapid progression of the exonuclease digestion at the initial stage of reaction which is

Figure 4. Exonuclease III digestion of 'Fast' and 'Slow' chromatin samples. Autoradiograph (a) and its densitogram (b) obtained after a denaturing electrophoresis of ^{32}P -labelled DNA from 'Fast' oligonucleosomes and 'Slow' oligonucleosomes digested with exo III for 0–8 min. as indicated below the tracks. M—the ^{32}P -labelled DNA of phage lambda DNA digested with Eco RI and Hind III restrictases.

hampered after elimination of about 30 b.p. from the oligonucleosome termini. 'Fast' particles are digested by the nuclease much more extensively. The enzyme slows down after 8 minutes of digestion when about 75 nucleotides are deleted from the 3' end of 'Fast' particles—twice as much as from 'Slow' DNA (Fig. 4). At this stage of digestion there still remains a size difference between the 'Fast' and the 'Slow' DNA oligomers which is eliminated when the residual single-stranded DNA (5'-terminal) is degraded by nuclease S1. This result demonstrates that the maintenance of the cleaved nucleosome linkers rather than nucleosome repeat discriminates between the two types of oligonucleosome particles. No exo III-resistant DNA bands were observed on the electrophoretic pattern (Fig. 4)—in accordance with an idea that the *Micrococcus* nuclease cutting sites of both chromatin fractions lie within their nucleosome linkers. The appearance of a fraction containing two linkers at both ends of the oligonucleosome ('Fast' particles) as well as of oligonucleosomes deprived of both terminal linkers (monotrimers) is consistent with the results of mononucleosome termini mapping by chemical crosslinking (35) and with a variant of solenoid model considered therein.

Three levels of chromatin activity are recognized by *Micrococcus* nuclease within nuclei

Since we have found out that the process of oligonucleosome self-association is dependent upon the length of their DNA molecules a question arises, whether the repressed and the transcriptionally-active chromatin fragments are selectively distributed between 'A' and 'B' particles according to the same rule. The recombinant DNA probes for a chicken gene being actively transcribed (beta-globin), a silent but retaining the DNase

I-sensitivity (rho-globin) gene, and a transcriptionally-inert (ovalbumin) gene in 14-day old embryo erythrocytes were taken for hybridization experiments.

The reassociation with EcoRI restrictase-cleaved DNA gives the unique bands corresponding to the appropriate gene regions. A two-dimensional DNP/DNA electrophoresis was carried out in parallel with a one-dimensional DNA electrophoresis of S2 sample and blott-hybridized with appropriate gene probes (Fig. 5). On the autoradiographs of the one-dimensional DNA separation one can see that the DNA of actively transcribed beta-globin gene is more sensitive to the nuclease treatment and gives less distinct bands in the range of mono-tetranucleosomes. The superposition of the autoradiographs reveals that the DNA oligomer bands of ovalbumin and rho-globin genes closely follow the nucleosome patterns of 'A' and 'B' particles respectively.

The autoradiographs of a two-dimensional DNP/DNA separation show that 'A' particles are heavily enriched in ovalbumin gene DNA. The smallest ovalbumin oligonucleosomes are gradually trimmed by the nuclease and are respectively redistributed between the two diagonals—in accordance with the idea that the maintenance of the terminal linkers determines the electrophoretic mobility of the soluble oligonucleosomes. The DNA of actively transcribed beta-globin gene and of non-transcribed embryonic rho-globin gene which resides within the DNase I-sensitive domain encompassing the chicken beta-globin cluster (1) is found exclusively within the 'B' diagonal. A DNP/DNA blot was also reassociated with DNA of the total Hind III fragment of plasmid pCBG4 which is known to contain the DNA region lying at the 3' flank of the rho-globin gene which is repeated several dozen times in the chicken genome (21). These sequences (Rep) appear to be distributed between both 'A' and 'B' diagonals.

Figure 5. Blott-hybridization analysis of electrophoretically separated oligonucleosomes. Agarose gel after a two-dimensional DNP/DNA electrophoresis of a standard S2 was stained with Ethidium bromide. An aliquot of the S2 DNA was electrophoresed in parallel with the second-direction run and blott-hybridized with probes β , ρ , and Ov. The 2-d DNP/DNA agarose gel was blotted onto nylon and reassociated with the probes β , ρ , Ov, and with DNA of total Hind III fragment of plasmid pCBG4 (Rep).

The results reported here are in a good agreement with the original hybridization study of Weintraub (15) where he had shown that the partition into 'A' and 'B' particles corresponding to the transcriptional activity is inherent in the whole DNase I-sensitive beta-globin domain and in several other inert and active

genes in chicken erythrocyte nuclei. The combined data give a solid ground for an ascribing of the structural features typical of 'A' and 'B' particles for most of the repressed and the transcriptionally active chromatin domains in chicken erythrocyte nuclei.

Chromatin solubilization results in a loss of the specific nuclease-recognized structural features

The variations in chromatin structure reported here could be attributed to a differential nucleosome chain folding (higher-order chromatin organization) of transcriptionally-active and repressed chromatin domains. To verify this proposition we redigested an aliquot of '3 minute' S2 sample in a low-salt (TE) solution where the higher order structures are known to undergo a gradual unfolding (34). The nucleosome bands of the solution-digested material are shown to occupy an intermediate position between the 'A' and the 'B' particle bands of the nucleus-derived oligonucleosomes (Fig 6a). The nucleosome pattern of the solution-digested chromatin is much more distinct than that of the nuclear chromatin digest being the composite of 'A' and 'B' particle oligonucleosomes. The 'A' particle diagonal is not observed on a DNP/DNA gel of the chromatin digested in solution (Fig. 6a).

In solutions of moderate ionic strength (50–100 mM monovalent cations) a formation of 250–300 Å chromatin fiber is observed (34). When we compared the soluble chromatin digests obtained in a 10 mM and in a 100 mM ionic strength solutions we observed only a slight increase in the DNA fragment length in the range of mono-trinucleosomes (Fig. 6b). Neither the elongated DNA oligomers nor the 'A' particle diagonal were restored under these conditions. Upon a further increase in the salt concentration in the digestion buffer the chromatin precipitated and gave a smeared nuclease-cutting pattern.

When we blot-hybridized the oligonucleosome DNA digested in solution we found out that the nucleosome patterns of beta-globin and ovalbumin genes were identical with that of the total solubilized chromatin independent of the salt concentration in the 10–100 mM range. Taking into account that histone rearrangement and nucleosome sliding do not occur in low salt solution (35), our results indicate that the biphasic mode of nuclease trimming of transcriptionally-active and repressed chromatin is associated with a particular level of nucleosome chain folding which is supported within nuclei and is lost after the nuclear lysis.

Two-dimensional electrophoretic separation of native DNP and protein composition of the oligonucleosome fractions

The main shortcoming of the two-dimensional DNP/DNA electrophoretic system is the necessity to get rid of the chromatin proteins before the second separation. We decided to separate the chromatin fractions by inducing their dissociation before the second-dimension run. A partial separation of 'A' particles from the main diagonal (Fig 7) was achieved by carrying out the electrophoresis in a two times diluted DNP-electrophoresis buffer described elsewhere (36). The 'A' particles separated by the DNP/DNP gel show a weaker Ethidium staining than the corresponding zone of the DNP/DNA separation. However, when we analyzed the DNA and the proteins extracted from the DNP/DNP gel, the concentration ratios of the two oligonucleosome fractions were equal to that estimated for the DNA obtained from the DNP/DNA gel. Some kind of protection of the native 'A' particles from the Ethidium stain may account for this observation. The hybridization experiments (Fig. 7) reveal

Figure 6. DNA and DNP-electrophoresis of chromatin digested with Micrococcal nuclease in solution. a) DNA electrophoresis of 'A' and 'B₁' particles, total S2 sample, '3 minute' S2 sample redigested in TE solution with Micrococcal nuclease for 7 minutes (TE). A 2-d DNP/DNA electrophoresis pattern of the latter sample is shown on the right. M—the DNA of phage lambda DNA digested with Eco RI + Hind III restrictases (see Fig. 5 for the DNA fragment sizes). b) Ethidium-stained agarose gel and the autoradiographs obtained after electrophoresis of a '3 minute' S2 sample redigested in TE (1, 3, 5) and in TE + 100 mM NaCl (2, 4, 6) solution with Micrococcal nuclease for 3 (1), 7 (2,3), 15 (4,5) and 30 (6) minutes. Slots 3 and 4 were blott-hybridized with DNA of ovalbumin (Ov) and beta-globin (β) probes. M—the ³²P-labelled DNA of phage DNA digested with Hind III restrictase (see Fig. 1a for the size of DNA fragments).

that most of the ovalbumin gene DNA is found within the 'A' particle area which leaves the main diagonal during the low-salt run. Both the rho-globin and the beta-globin genes are distributed along the main diagonal corresponding to the 'B' particle diagonal observed on the DNP/DNA autoradiographs.

Figure 7. Native two-dimensional electrophoretic separation of transcriptionally active and inert chromatin. An agarose slab after a two-dimensional DNP/DNP electrophoresis of a standard S2 sample (TAEW from left to right, EA from top to bottom) was stained with Ethidium bromide and blott-hybridized with the probes β , ρ , and Ov. The location of xylenecyanol marker dye after the first-direction run is shown by an arrow. The location of bromophenol marker dye after the second direction run is marked with a triangle.

A DNP/DNP agarose gel slab was dissected into the 'A', 'B', and 'C' zones corresponding to the gel areas enriched with the specific gene DNA (Fig. 8). The agarose pieces were cut out, proteins were extracted, electrophoresed on a 18% polyacrylamide gel and stained with silver. The agarose gel and the remaining DNA produced a significant background which spoiled the upper half of the pattern. For this reason only the protein bands smaller than 40 kD are shown here. Within this size range the most abundant proteins of soluble mono- and oligonucleosomes from chicken erythrocyte nuclei are well characterized (see e.g. 37,38). In addition to the histones and their ubiquitinated variants they include the High Mobility Group (HMG) chromosome proteins (Fig. 8, lane 1). The electrophoretic positions of the HMG proteins were verified by a protein isolation and a acid-urea/SDS two-dimensional electrophoresis (not shown). In Fig. 8 one can see that the 'A' particles are enriched in linker histones H1 and H5 while the zone 'B' contains a lesser amount of these proteins than the total S2 sample. 'B' particles are enriched with non-histone proteins HMG 2, E and with HMG 14. The largest protein of this class, HMG 1, is not observed owing to its comigration with H5, while HMG 17 is not seen for its low concentration. No other protein bands are seen on a electrophoretic pattern of the total S2 sample containing about ten times more material than the samples eluted from the gel. Therefore one should not expect to observe any more protein bands in the fractions extracted from the agarose gel, none of which contains less than 20% of the starting material.

The Ag-staining does not allow the quantitative estimation of the protein content owing to its non-linear dependance upon the protein concentration. For instance, histone H5 and ubiquitinated histone H2A (uH2A) are underrepresented in diluted probes as compared with more concentrated sample. To overcome this problem, the proteins were labelled with ¹²⁵I and electrophoresed in the presence of an excess of carrier proteins.

Figure 8. Chromatin proteins from 'A', 'B', and 'C'-particles. A scheme for the dissection of a native DNP/DNP agarose gel is drawn at the left. The gel after a protein SDS-electrophoresis of a standard S2 preparation (1) and of the samples extracted from the agarose gel zones 'A' (2), 'B' (3), and 'C' (4) was stained with silver. The autoradiographs were obtained after the electrophoresis of ^{125}I -labelled proteins extracted from the zones 'A' (5), 'B' (6), and 'C' (7).

The autoradiograph of the protein electrophoresis is shown in Fig. 8, lanes 5–7. The non-histone proteins were not detected owing to their poor reactivity with ICl and to the high background imposed by the agarose. However the histones including uH2A variant are well seen and can be monitored by a densitometry scanning. The protein concentration ratios of the ^{125}I -labelled histones obtained from the DNP/DNP agarose gel to those of the starting S2 sample were calculated from the densitograms. Core histones are equally well distributed among all of the chromatin fractions. The amount of linker histones is increased by 18% in the 'A' fraction and decreased by 25% in the 'B' fraction as compared with the bulk S2 preparation. The concentration of the ubiquitinated form of H2A is increased by 64% in the 'B' preparation. An analogous observation has been made previously by a selective salt-dependent precipitation of transcriptionally-inert chromatin (38).

DISCUSSION

Now we know that the two types of oligonucleosomes separated by the DNP electrophoresis differ by their DNA length as well as by linker histone content. Both of these structural features may contribute to the chromatin aggregation. Several indirect evidences stress the role of terminal linkers in this process. The nuclease trimming of DNA termini makes small ovalbumin gene-containing oligonucleosomes to leave the 'A'-particle diagonal (Fig. 5). When total chromatin is cleaved by the nuclease in solution where both the transcriptionally-active and the repressed genes undergo a moderate terminal trimming no 'A'-particles are observed on the DNP/DNA gel (Fig. 6). In both cases the nuclease digestion was carried out under conditions preventing the redistribution of linker histones which could interfere in the 'A'-particle stability. Furthermore, the aggregation of the oligonucleosomes in a sucrose gradient requires even more elongated DNA oligomers (compare 'fast' and 'A'-particles, Table 1). Basing on these observations we suppose that the terminal DNA tails (probably together with linker histones bound

to them) determine whether a given oligonucleosome particle would be involved in the aggregation process.

The of nucleosome linker DNA from the nuclease trimming could be attributed to a more compact higher-order organization of repressed chromatin. Indeed, this structural feature vanishes upon the chromatin unfolding in a low salt solution. However, under the conditions known to support the refolding of the solenoid level of chromatin organization neither the linker length variation nor the A-particle diagonal were restored. The latter finding is not quite surprising since it has been stated by Widom (39) that the solenoid filaments are either loosely formed in solution or are transiently fluctuating between the open and the closed states. Within nuclei chromatin solenoids are further condensed into thick coiled-coil fibers (40). It would be reasonable to assume that the selective condensation of transcriptionally-inactive chromatin is responsible for the observed differences between the transcriptionally-active and the repressed genes.

Mature erythrocyte nuclei are characterized by a very tight chromatin condensation as well as by a high content of histone H5 (41). Histone H5 is known to induce a higher compaction or stability of chromatin (42). Therefore it looks like the selective condensation of repressed chromatin in chicken embryo erythrocyte nuclei is supported by an excess of linker histones present in this fraction. When we assayed under similar conditions the nuclei of several other cell types lacking H5 (liver, oviduct) as well as the nuclei of Avian erythroleukemia virus-transformed erythroblasts characterized by a lesser content of this histone (close to that found in 'B' particles) we observed neither the DNA length variation nor the oligonucleosome self-association. Nevertheless, a length difference between the oligonucleosomes of beta-globin (inactive) and ovalbumin (active) genes in oviduct cells has been observed by Bellard et al. (43) who used a higher salt concentration and a lower temperature in their nuclease digestion experiments than were used here. It thus appears that various condensation states of nuclear chromatin require appropriate assay conditions to visualize the nuclease-recognized alterations of transcriptionally-active chromatin.

The absence of nuclease-recognized differences in decondensed chromatin is in a good agreement with the results of the sedimentation study performed by Caplan et al. (44) who have demonstrated that in solution the chromatin organization of the repressed and the active polynucleosomes (with the exception of nuclease-hypersensitive sites where nucleosomes are deleted) is identical. Taken together this and our results argue against the statement of Weintraub (15,16) that the repressed state of a gene is imprinted in its nucleosome structure. At the same time the results reported here show that the transcriptionally-active chromatin maintains a loosened configuration of its nucleosome linkers in spite of an overall chromatin condensation in mature erythrocyte nuclei. Previously we have considered that the opening of the nucleosome linkers is a minimal topological requirement for RNA polymerase movement along a nucleosome chain (45). By an analogy with nucleases, one can also propose that the open nucleosome chain configuration is recognized by protein factors regulating the genetic activity. Indeed, Hannon et al. (46) have found out that the exposed linkers are needed for a proper RNA polymerase binding. Furthermore, our experiments on the nuclei reconstituted with radioactively labelled soluble non-histone proteins have shown that these proteins (in contrast to linker histones) have a clear preference for the 'B'-particle chromatin (results to be published elsewhere). Future studies should clarify whether the tight higher-order folding of chromatin domains participates in genetic repression or is just a mean for temporal withdrawal of silent genes from the active biochemical processes.

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