Electrophoresis of Chromatin on Nondenaturing Agarose Gels Containing Mg\textsuperscript{2+}

SELF-ASSEMBLY OF SMALL CHROMATIN FRAGMENTS AND FOLDING OF THE 30-nm FIBER*

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Salvador Bartolomé, Antonio Bermúdez, and Joan-Ramon Daban

From the Departament de Bioquímica i Biologia Molecular, Facultat de Ciències, Universitat Autònoma de Barcelona, 08193-Bellaterra, Barcelona, Spain

We show that nondenaturing agarose gels can be used for the study of the structure and dynamic properties of native (uncross-linked) chromatin. In gels containing 1.7 mm Mg\textsuperscript{2+}, chicken erythrocyte chromatin fragments having from about 6 to 50 nucleosomes produce well defined bands. These bands have an electrophoretic mobility that decreases only slightly with molecular weight. This surprising behavior is not observed in low ionic strength gels. Fragments with less than 6 nucleosomes and low content of histones H1–H5 give rise to broad bands in gels with Mg\textsuperscript{2+}. In contrast, fragments containing only 3–4 nucleosomes but with the normal H1–H5 content are able to form associated structures with a mobility similar to that observed for high molecular weight chromatin. Electron microscopy results indicate that the associated fragments and the fragments of higher molecular weight show similar electrophoretic properties because they become very compact in the presence of Mg\textsuperscript{2+} and form cylindrical structures with a diameter of ~33 nm. Our results suggest that the interactions involved in the self-assembly of small fragments are the same that direct the folding of larger fragments; in both cases, the resulting compact chromatin structure is formed from a basic element containing 5–7 nucleosomes.

In chromatin an octamer of histones H2A, H2B, H3, and H4 associated with 146 bp\textsuperscript{1} of DNA forms the core of the nucleosome (1, 2). The two turns of DNA (about 165 bp) around these core histones are sealed by histone H1 (H5 in avian erythrocytes) (3–5). Nucleosomes connected by linker DNA form a filament that can fold into a condensed chromatin fiber of about 30 nm in diameter (reviewed in Ref. 6).

The mechanism of chromatin folding and the structure of the 30-nm fiber have been studied by sedimentation methods (7–9), enzymatic and chemical digestions (10–12), electric, flow, and photochemical dichroism (13–15), x-ray diffraction (16, 17), small angle x-ray scattering (18–20), neutron scattering (21, 22), transmission electron microscopy (23–32), and scanning force microscopy (33). The results obtained in these studies have suggested different models for the folding and structural organization of the 30-nm chromatin fiber. These models differ essentially in the location of the linker DNA. In the one-start helix models, the linker DNA is folded and connects laterally consecutive nucleosomes (8, 13, 17, 23, 32). In the twisted-ribbon models, a two-start helix is formed by pairs of nucleosomes with the linker DNA parallel to the fiber axis (24, 26). Finally, in several continuous (11, 18, 28) and discontinuous (27) crossed-linker models, and in the variable zigzag nucleosomal ribbon model (30, 31), the linker DNA is extended in the fiber interior.

In this work we show that nondenaturing agarose gel electrophoresis can be used for the study of the folding of chromatin. We have found that, when electrophoresis is performed in the presence of Mg\textsuperscript{2+}, chicken erythrocyte chromatin fragments of a relatively high molecular weight change dramatically their mobility due to the formation of very compact structures. Furthermore, we have found that under these conditions small oligonucleosomes are able to associate forming structures with the same structural characteristics as larger chromatin fragments. The results obtained using gel electrophoresis have been interpreted taking into account the analysis by electron microscopy of the same samples. Our electrophoretic method does not require the cross-linking of the chromatin samples, but the structures produced by the association of uncross-linked oligonucleosome fragments are stable enough to form the well defined bands detected in nondenaturing gels at the end of the electrophoresis. This suggests that the forces that allow the self-assembly of oligonucleosomes are the same that direct the folding of chromatin of higher molecular weight. We have also investigated the role of histones H1–H5 in these structural transitions.

EXPERIMENTAL PROCEDURES

Preparation of Chromatin Fragments—Chicken erythrocyte nuclei were obtained in 90 mm KCl, 30 mm NaCl, 0.5 mm spermidine, 0.15 mm spermine, 0.4 mm phenylmethylsulfonyl fluoride (PMSF), and 10 mm triethanolamine-HCl (pH 7.4) and digested with micrococcal nuclease (Sigma, 1 unit/mg of DNA) for 30–90 min in the presence of 1 mm CaCl\textsubscript{2} at 37 °C (32, 34). Digested nuclei were extracted overnight at 4 °C with 40 mm NaCl, 1 mm EDTA, 0.4 mm PMSF, and 10 mm triethanolamine-HCl (pH 7.4), and finally centrifuged at 2,500 × g for 10 min. The soluble chromatin in the supernatant was concentrated (Diaflo PM10, Amicon) to 5–6 mg of DNA/ml and fractionated by sedimentation on a 5–20% linear sucrose gradient containing 5 mm EDTA, 0.4 mm PMSF, and 10 mm triethanolamine-HCl (pH 7.4). When necessary, prior to density gradient centrifugation, the digested chromatin was depleted of histones H1–H5 following the method of Ruiz-Carrillo et al. (35). Selected fractions from the sucrose gradients were dialyzed against the indicated buffers (see “Results”); other fractions were concentrated (Centricon 10, Amicon) to 2–5 mg/ml and further fractionated by nondenaturing gel electrophoresis (see below). Chromatin of high molecular density...
weight was obtained as described elsewhere (32) and further purified by sucrose gradient sedimentation followed by nondenaturating gel electrophoresis. The length of the isolated DNA corresponding to the different chromatin fragments was analyzed on agarose gels stained with ethidium bromide. The histone composition of the different samples was analyzed on SDS-polyacrylamide gels stained with the fluorescent dye Nile red (36). Photographic negatives were scanned with a Shimadzu CS-9000 densitometer.

Nondenaturing Gel Electrophoresis—Electrophoresis of chromatin fragments on gels containing 0.1 × TB (9 mM Tris borate, pH 8.3) was performed following essentially the procedure previously described for the analysis of core particle DNA-histone complexes (37) but using 0.5% agarose gels instead of polyacrylamide gels. These gels were pre-electrophoresed, and the buffer was recirculated between the electrode compartments. Nondenaturing 0.5% agarose gels (10 cm long) of higher ionic strength contained TB and the concentrations of MgCl₂ indicated in the figure legends. In this case, pre-electrophoresis and buffer recirculation were omitted. Chromatin samples directly obtained from the sucrose gradients or in the buffers indicated in the figure legends (in all cases without Mg₂⁺) were mixed with 0.25 volume of 5 × TB containing 12.5% Ficoll and loaded onto the TB-Mg₂⁺ gel. Generally, electrophoresis was carried out at 100 V for about 1.7 h. The apparent equilibrium constant for the reaction of association of small chromatin fragments (see “Results”) was calculated from the densitometric analyses of the electrophoretic bands corresponding to the small and associated fragments of chromatin samples having different concentrations (ranging from 0.19 to 1.7 mg of DNA/ml).

Chemical Cross-linking—After electrophoresis the selected bands were cut out of the gel, washed twice with 1.5 ml of TEAB buffer (90 mM triethanolamine-borate, pH 8.6) containing 1.7 mM MgCl₂, for 2 h, and treated with 1.5 ml of 0.1% glutaraldehyde for about 15 h at 4°C in the same buffer. Finally, the agarose block containing fixed chromatin fragments was loaded onto an Ultafree-CL tube (0.45 μm Durapore membrane, Millipore), and centrifuged at 2,500 × g for 30 min. The chromatin eluted from each band was used in electron microscopy experiments. The chromatin samples prepared without gel electrophoresis were fixed with 0.1% glutaraldehyde for about 15 h at 4°C in the indicated buffers before electron microscopy. The specific cross-linking conditions of the chromatin fragments used for second-dimension electrophoretic analyses are described (see “Results”).

Electron Microscopy—Spread preparations of the resulting samples (final concentration 2–10 μg of DNA/ml) were obtained as described previously in carbon-coated copper grids pretreated with Aloeian blue (36). The grids were rotary-shadowed with platinum-carbon at an angle of about 7°. Micrographs were obtained at a magnification of ×30,000 using a Hitachi H7000 transmission electron microscope. Images from the original negatives were acquired either directly using a transfilm minimator and a Hamamatsu C2400 camera or at a higher magnification with the same camera attached to a modified Zeiss Axioskop microscope equipped with a ×5 objective. Additional experimental details have been described elsewhere (32).

RESULTS

Behavior of Chromatin Fragments of Different Molecular Weights in Electrophoretic Gels of Low Ionic Strength—The electrophoretic mobility of chromatin samples in 0.5% agarose gels containing 0.1 × TB is lower than that found for the DNA extracted from the same samples (Fig. 1a). The relative retardation of the mononucleosome band produced by this nondenaturing agarose gel is lower than that observed in low ionic strength polyacrylamide gels (37). Nevertheless, whereas oligonucleosomes cannot enter the polyacrylamide gels (38), the agarose gels described in this work can be used even with chromatin samples of high molecular weight. The results presented in Fig. 1b show that in 0.1 × TB agarose gels the electrophoretic mobility of native chromatin samples having a wide range of molecular weights is in all cases lower than that of the corresponding free DNA obtained from these samples.

Chromatin Electrophoresis on Nondenaturing Gels Containing Mg²⁺—In contrast with the results presented in the preceding section, when the electrophoresis is performed using buffers of higher ionic strength (see Fig. 2), the mobility of chromatin fragments of increasing molecular weight shows a complex behavior. In gels containing 1 × TB, the plot of the log bp of the DNA corresponding to a given chromatin sample versus electrophoretic mobility (Fig. 2d) has two regions: one with a small negative slope (corresponding to low molecular weight chromatin samples) and the other with a high negative slope (corresponding to chromatin of higher molecular weight). These differences in electrophoretic behavior are more pronounced using electrophoretic buffers containing Mg²⁺ (Fig. 2d). We have obtained essentially the same results with gels containing 1.7 and 2.5 mM MgCl₂. The transition between the two regions of these plots is observed when the chromatin fragments contain 5–6 nucleosomes.

With chromatin samples containing more than 6 nucleosomes, the changes in electrophoretic mobility produced by the increase of the molecular weight of the chromatin are very small. Note that in gels containing Mg²⁺, the negative slope of the plots shown in Fig. 2d corresponding to samples with more than 6 nucleosomes has a very high value. This surprising behavior of native chromatin produces an apparent increase in the electrophoretic mobility of the high molecular weight chromatin fragments relative to the mobility of free DNA. In fact, whereas the mobility of naked DNA corresponding to chromatin fragments containing 6 nucleosomes is roughly 2-fold higher than that observed for the native fragments, chromatin samples containing about 50 nucleosomes (i.e. DNA of ~10 kb) show approximately the same mobility than the naked DNA extracted from these samples (see the intersection of the chromatin and DNA curves in Fig. 2d).

Electrophoretic Mobility of Chromatin Fragments Depleted of Histones H1–H5—In agarose gels of low ionic strength, the electrophoretic bands corresponding to chromatin without H1–H5 migrate faster than the equivalent bands of chromatin

FIG. 1. Electrophoresis of chromatin fragments on a low ionic strength nondenaturing gel. a, chromatin fragments of different length (lanes with •) and the DNA isolated from these fragments (lanes with —) were electrophoresed on a 0.1 × TB, 0.5% agarose gel. The size of some DNA markers (in kb) is indicated. b, plot of log bp versus electrophoretic mobility (relative to the migration of free DNA of 1 kb) for native chromatin (●) and chromatin depleted of H1–H5 (□). The mobility of free DNA (○) is shown as reference.
containing all the histones, but slower than the corresponding free DNA obtained from these samples (see Fig. 1b).

In gels of higher ionic strength in absence or in presence of Mg$^{2+}$, the slope of the plots of log bp versus relative mobility of chromatin depleted of histones H1–H5 (see Fig. 2e) are different from that obtained in gels of low ionic strength. However, in contrast with the results obtained in the case of native chromatin fragments with all the histones, the plots of log bp versus mobility of samples without H1–H5 in gels of relatively high ionic strength cannot be divided in two regions (compare the plots presented in Fig. 2, d and e). This indicates that histones H1–H5 are presumably responsible for the complex electrophoretic behavior of native chromatin in gels containing Mg$^{2+}$.

Furthermore, as can be seen in Fig. 3a, when chromatin is treated with increasing NaCl concentrations and then electrophoresed on agarose gels with Mg$^{2+}$, a remarkable structural transition is observed at about 0.6 M NaCl. This transition is not observed with chromatin depleted of histones H1–H5 (Fig. 3b). Taking into account that histones H1–H5 are dissociated from chromatin when the NaCl concentration is 0.6 M (34), these results indicate that the presence of histones H1–H5 is necessary in order to maintain the integrity of the structures that produce the typical electrophoretic bands of native chromatin.

**Fig. 2.** Electrophoresis of chromatin fragments on nondenaturing gels containing Mg$^{2+}$. a and b, chromatin fragments of different length were electrophoresed on a 0.5% agarose gel containing TB and 1.7 mM MgCl$_2$. The average length of the DNA of the chromatin fragments analyzed in a is indicated in the bottom of this panel. c, the DNA isolated from the chromatin fragments shown in Panel b was analyzed on TB, 1.7 mM MgCl$_2$ gels. The size of some DNA markers (in kb) is indicated. d and e, relationship between the length of DNA in chromatin fragments and mobility (relative to the migration of free DNA of 1 kb) in gels containing TB and 0 (○), 1.7 (▲), and 2.5 (●) mM MgCl$_2$. In Panel d the analyzed chromatin fragments contained histones H1–H5; in Panel e chromatin fragments were depleted of H1–H5. Some chromatin samples produce additional bands (indicated by stars in Panels a and b) with a relatively low mobility; in Panel d these slow bands are indicated by small stars (gels with 1.7 mM MgCl$_2$) and large stars (gels with 2.5 mM MgCl$_2$). The mobility of free DNA in TB gels containing 0 (●), 1.7 (▲), and 2.5 (●) mM MgCl$_2$ is shown as reference.
Well Defined Chromatin Bands in Gels Containing Mg$^{2+}$: Self-assembly of Chromatin Fragments with 3–4 Nucleosomes—Native chromatin fragments containing the four core histones and histones H1–H5 produce broad bands in low ionic strength gels (Fig. 1a). In contrast, fragments with 6 or more nucleosomes form well defined bands in gels with Mg$^{2+}$ (Fig. 2, a and b). This observation is probably related with the almost invariable mobility in gels with Mg$^{2+}$ observed for native chromatin samples of increasing molecular weight (see above). Nevertheless, note that, even in gels containing Mg$^{2+}$, chromatin fragments with less than 6 nucleosomes produce broad bands (Fig. 2b, lanes 1–5). Furthermore, in samples corresponding to fragments containing 3–4 (Fig. 2, b and c, lane 4) to 5–7 (Fig. 2, b and c, lane 7) nucleosomes, these broad bands are accompanied with a slower narrow band (indicated by a star) that shows the same mobility as the fragments of higher molecular weight. The relative mobility of the additional slower bands is also indicated by stars in Fig. 2d. These bands are not observed in gels without Mg$^{2+}$.

The slow and rapid bands of each lane shown in Fig. 2b contain the same number of nucleosomes. For instance, the analysis of a sample similar to that of lane 4 of Fig. 2a on a second-dimension denaturing gel shows that both the slow and rapid bands contain the DNA corresponding to 3–4 nucleosomes (see Fig. 4, lanes 2 and 3). One possible interpretation of this surprising observation is that the slow band is due to the association of the oligonucleosome fragments that produce the rapid band. This possibility is demonstrated in the cross-linking experiment shown in Fig. 4. It can be seen that whereas the slow band produces cross-linked structures of low mobility in second-dimension denaturing gels (lane 4), the rapid band does not give rise to cross-linked material (lane 5). This result indicates that small fragments of chromatin containing 3–4 nucleosomes can produce associated structures having the same electrophoretic properties as chromatin fragments of higher molecular weight. The association of small chromatin fragments is confirmed in the electron microscopy studies presented below. We have assumed that two small fragments with few nucleosomes associate to form a structure containing about 6 nucleosomes, according to the equation $f + f \rightleftharpoons F$, where $f$ and $F$ correspond, respectively, to the small and associated fragments. The densitometric analysis of gels loaded with samples containing fragments with about 4 nucleosomes has allowed us to estimate that the apparent association constant for this assembly reaction determined at four different concentrations is $1.4 \pm 0.7 \times 10^5$ M$^{-1}$.

The histone composition of the chromatin samples containing more than 6 nucleosomes is the same as that of whole nuclei samples, but the chromatin samples containing few nucleosomes have a low H1–H5 content (see Fig. 5b). Nevertheless, second-dimension electrophoresis shows that the slow band detected in chromatin samples containing less than 6 nucleosomes has approximately the same H1–H5 content as normal chromatin (see Fig. 5a, lane 2 and legend), indicating that these...
Histones are necessary for the association of small chromatin fragments to form the band that has the same mobility as the fragments of higher molecular weight.

Cross-linking of Chromatin Bands in the Gel and Electron Microscopy Analysis: The Folding of Chromatin Fragments Is Responsible for the Changes of Electrophoretic Mobility Observed in Gels Containing Mg$^{2+}$—The similar mobility, in gels with Mg$^{2+}$, observed for chromatin structures produced by the assembly of small fragments and by native chromatin fragments having a wide range of molecular weights (see above) suggests that all these chromatin samples adopt a compact conformation that make them indistinguishable in the gel. In order to test this possibility, chromatin samples containing 2–3, 3–4, and 5–6 nucleosomes were electrophoresed under nondenaturing conditions in presence of Mg$^{2+}$ and, after electrophoresis, the slow band was cross-linked in the gel with glutaraldehyde; then the cross-linked material was eluted from the gel and finally analyzed in the electron microscope. We have observed that, in all cases, in the presence of 1.7 mM Mg$^{2+}$, the slow band produces circular structures (see Fig. 6e) similar to those obtained with fragments containing from about 6 to 35 nucleosomes (see Fig. 6g and Ref. 32).
The measurements presented in Fig. 7 show that the diameter of the structures extracted from the slow bands corresponding to samples containing from 2–3 to 5–6 nucleosomes (Panels b–d) is about 33 nm, i.e., the same diameter found for chromatins containing a larger number of nucleosomes (see Panel a). In contrast, the rapid band of samples containing few nucleosomes does not produce the typical circular structure (see Fig. 6d). Before electrophoresis, in presence of 1.7 mM Mg\(^{2+}\), the samples containing few nucleosomes are mixtures of the circular structures and small particles (Fig. 6c). Electrophoresis causes the separation of these two components. Control experiments show that at low ionic strength (Fig. 6a) or in absence of Mg\(^{2+}\) (Fig. 6b) small fragments cannot associate. Without Mg\(^{2+}\), folding of very large fragments cannot take place (Fig. 6f).

As can be seen in Fig. 8, the highly magnified images obtained from the associated small chromatin fragments present in the slow electrophoretic bands (indicated by a star in Fig. 2) are equivalent to the typical images of larger chromatin fragments that we have described previously (32). The structures assembled from small chromatin fragments, and the fragments of higher molecular weight have approximately the same electrophoretic mobility because they become very compact in presence of Mg\(^{2+}\). In agreement with this interpretation, we have found previously (32) that fragments containing from about 6 to 35 nucleosomes show approximately the same diameter (33 nm) in the electron microscope because in presence of Mg\(^{2+}\) they are highly packed, favoring the vertical placement of the resulting short cylindrical structures on the grid. This gives rise to images corresponding to the top view of folded chromatin (see Fig. 8 and Figs. 4 and 5 of Ref. 32).

**DISCUSSION**

The remarkable changes in the electrophoretic behavior of chromatin fragments observed when electrophoresis is performed in the presence of Mg\(^{2+}\) has allowed us to detect the assembly of oligonucleosomes and the folding of larger fragments. Note that the experiments with the nondenaturing agarose gels have been performed using uncross-linked chromatin samples. While this investigation was in progress, Krajewski et al. (39) reported that current agarose gels without Mg\(^{2+}\) can be used for the structural analysis of chromatin cross-linked with glutaraldehyde under different conditions before electrophoresis. We have used glutaraldehyde cross-linking exclusively after electrophoresis in order to stabilize samples for electron micros-
copy analysis. In this case, before spreading, it is necessary to make a large dilution of the sample that causes nucleosome dissociation in the uncross-linked chromatin (not shown).

The main conclusions of this work can be summarized in the scheme presented in Fig. 9. Our results show that about 6 nucleosomes are enough to produce the compact structures having approximately the same electrophoretic mobility as the folded chromatin fragments of higher molecular weights. Early sedimentation studies (8) showed a marked structural transition when the chromatin fragments analyzed contained 6 nucleosomes. We have found that even small fragments (containing 3–4 nucleosomes) can associate giving rise to compact particles with the same mobility as the folded fragments of higher molecular weight. When observed in the electron microscope all these structures are apparently equivalent. According to our previous observations (32), this is due to the fact that the images obtained correspond to the top view of the folded chromatin fragments (see above). These findings suggest that the structure of folded chromatin fibres must be very compact and simple enough to be started and stabilized with a basic element containing about 6 nucleosomes. Such a structure is compatible with the compact one-start helix model proposed from the detailed analysis of the electron microscope images of small chromatin fragments in presence of 1.7 mM Mg²⁺ (32).

In agreement with our findings about the self-assembly of small chromatin fragments, other laboratories using different techniques have reported results about the association of oligonucleosomes to form higher order structures (35, 40–42). Taken together, these observations indicate that chromatin fibers are able to self-organize even when there is no covalent continuity in the DNA of the fiber. This stresses the fundamental role of histones in organizing the chromatin structure. Histones H1–H5 are necessary to produce the folding of chromatin fragments. The characteristic electrophoretic behavior of the chromatin fragments analyzed in this study is not observed in samples depleted of these histones. Moreover, the association of small fragments does not take place in the oligonucleosome fraction that has a low H1–H5 content; only the fraction having the normal content of these histones can form self-assembled structures. These results are consistent with previous findings indicating the essential role of histones H1–H5 for chromatin assembly (43) and folding (44). Recent results (45) have shown that histones H1 and H5 suppress the mobility of the histone octamers positioned on constructs of 5 S rRNA. Furthermore, neutron scattering analysis of chromatin containing deuterated histone H1 has indicated that this histone is located in the interior of the folded chromatin at a distance from the fiber axis of about 6 nm (46). This internal location of H1 (and presumably H5) is probably responsible for the stabilization of the folded structure.

It is very likely that the observed association of small chromatin fragments is originated by the same interactions that cause the spontaneous folding of the chromatin fiber. Since the association constant corresponding to this assembly (~10⁵ M⁻¹, see “Results”) is about 10⁴-fold lower than the association constant of histone octamers with DNA (~10⁻⁴ M⁻¹ as estimated from the dissociation analysis of core particles in 0.2 M NaCl) (38), it can be suggested that DNA in chromatin is packaged following a sequence of compaction steps (core particle formation → H1–H5 binding → folding of the 30 nm fiber) involving lower amounts of free energy release in each consecutive level. Although the binding of histone H1 and similar proteins to naked DNA is very cooperative and the corresponding association constant is apparently very high (47–49), the binding energy of H1 to chromatin must be relatively low in order to favor the remarkable dynamic and functional properties of this histone (50–55). Furthermore, it has to be taken into account that even compact chromatin containing histones H1–H5 is not necessarily inert from a functional point of view. It has been found recently that chromatin with the normal H1–H5 content in the presence of 1.7 mM Mg²⁺ can bound reversibly excess core histones, suggesting that folded chromatin may be involved in the transient association of the core histones released during transcription (56).

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