Structure of Chromatin

DNA not Complexed with Protein

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Chromatinstructure, Poly-D-Lysine, Free DNA

Rat liver chromatin was titrated by poly-D-lysine of mol.wt. 140,000. Approximately 32% of the phosphate groups of the chromatin were found to react with the polycation. By submitting the chromatin-poly-D-lysine complex to pronase and DNase I digestion and by further submitting the obtained DNA-lysine complex to hydroxy-apatite-chromatography, "free" DNA was isolated. The mean S-value of this DNA was found to be 10 ± 0.8, corresponding to approximately 2100 nucleotide pairs, its renaturation kinetics similar to total DNA. On the basis of these results and on the findings that histone-saturated DNA-histone recombinates are digested with DNase I up to 65% a model of chromatin structure was proposed, taking into account protein covered- and free DNA regions.

Introduction

The arrangement and distribution of proteins on the DNA of chromatin is a major unsolved problem of current interest. Two main concepts have been proposed on the basis of existing data: The first assumes that the DNA is covered throughout by proteins [1–3] which inhibit transcription either by directly blocking access of the DNA dependent RNA polymerase to the DNA [4] or indirectly by virtue of conformation changes of the DNA [5, 6]. The second concept envisages the existence of protein-free stretches of DNA [7] on which regulatory proteins can be bound, positively or negatively affecting transcription of these regions. This model corresponds to the well studied regulatory mechanisms prevailing in bacteria [8, 9].

In the present communication, we describe the isolation and partial characterization of stretches of "free" DNA, a finding which lends support to the second of the proposed models, which has been further elaborated.

Materials

Pronase and pancreatic DNase I (RNase free) were purchased from Merck, Darmstadt; DNA from calf thymus, poly-D-lysine. HBr, mol.wt. 3000 ($\bar{P}$: 14) and mol.wt. 140 000 ($\bar{P}$: 670) from Serva, Heidelberg; hydroxyapatite from Biorad, Munich; Histone, prepared as described by Johns [10], was kindly donated by Prof. Gallwitz, Marburg. All other reagents were of analytical quality. Male Wistar BR II rats, weighing 140 – 180 g were used.

Methods

1. Starting from purified nuclei [11], chromatin was prepared according to Marushige and Bonner [12] as modified by Beato *et al.* [13]. Protein, RNA, DNA and phosphate were determined according to Lowry *et al.* [14], Ogur and Rosen [15], Burton [16] and Bartlett [17] respectively. DNA-histone (polylysine-)-complexes were prepared by adding varying amounts of the polycation to DNA in the presence of 2 M NaCl-5 M urea. After step dialysis according to Kleiman and Huang [18] the precipitated complex was sedimented, washed and then suspended for further use in appropriate buffers.

2. *Isolation of "free DNA"*

The isolation of the "free" DNA was accomplished by blocking the phosphate groups of the accessible DNA with poly-D-lysine [19], then digesting the chromosomal proteins with pronase, hydrolyzing the DNA with DNase I and finally separating the remaining DNA from the associated poly-D-lysine on hydroxyapatite.

a. Titration with poly-D-lysine

Chromatin was dispersed in 2.5 mM sodium phosphate buffer, pH 6.7 and treated for 3 min

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Enzymes: DNase I = EC 3.1.4.5; Pronase from *Streptomyces griseus.*

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with a Virtis homogenizer at 0 °C (81 Volt, graduation 8). The suspension was centrifuged for 20 min at 13 500 rpm (*g* max: 22 000). The clear supernatant was diluted with phosphate buffer to about 100 μg DNA/ml or 0.32 μmol nucleotides/ml. The chromatin solution was titrated under vigorous stirring with a polylysine-solution of 0.311 – 0.354 μmol monolysine per ml phosphate buffer. The binding of polylysine to chromatin results in precipitation of the chromatin. No more polylysine was added after the total chromatin was precipitated. This was detected by removing aliquots during the titration, centrifuging the precipitated chromatin at 10 000 rpm and measuring the OD (1/2) at 260 nm in the supernatant. Titration ended when no further decrease in the OD was established. In this way 32 ± 4% of the phosphate groups were found to be free to react with the polylysine (see Fig. 1). Further addition of polylysine results in displacement of proteins from the chromatin (see Fig. 2).

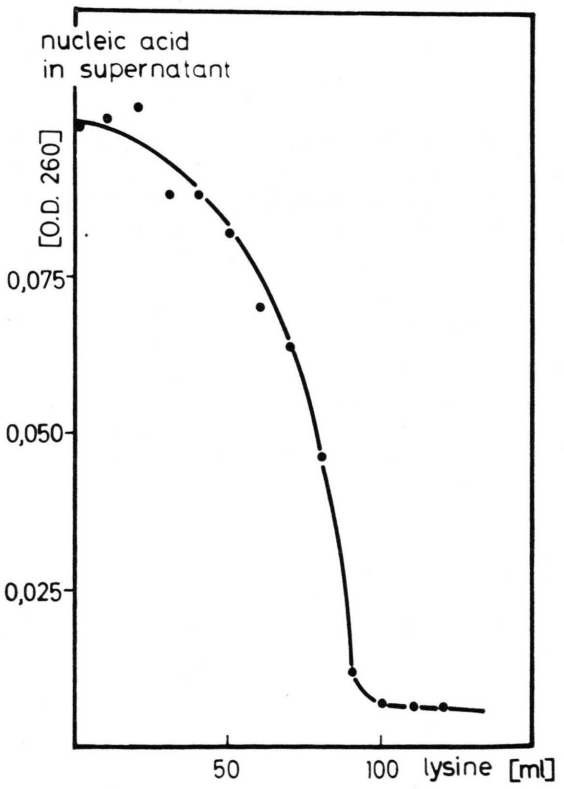

Fig. 1. Titration of chromatin with polylysine ($\overline{P}$: 670). Increasing amounts of polylysine (0.311 μmol mono-lysine per ml) were added under vigorous stirring to solubilized chromatin (0.263 μmol nucleotide/ml). Aliquots were taken, brought to a common end volume, centrifuged and OD 260 was determined in the supernatant.

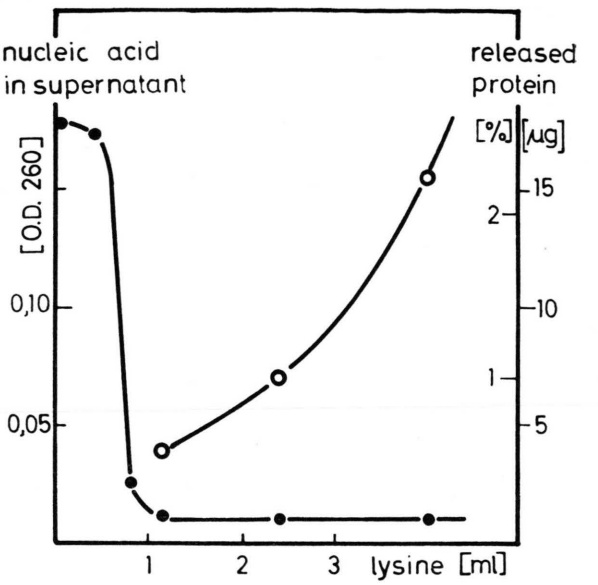

Fig. 2. Polylysine-titration and protein release. Taking account of the differing volumes, various amounts of polylysine (0.354 μmol mono-lysine/ml) were added to each 2 ml of solubilized chromatin (about 150 μg DNA/ml). After centrifugation, OD 260 and the protein content in the supernatant were determined.

b. Pronase treatment

The chromatin-polylysine complex was repeatedly washed with buffer and then finely suspended in 5 mM phosphate buffer, pH 6.7 at a concentration of about 1 mg protein/ml. An equal volume of a pronase solution in the same buffer (1 mg/ml), preincubated at 37 °C for 2 h, was added. The suspension was incubated for 3 h 37 °C and then submitted to centrifugation at 10 000 rpm for 10 min. The sediment obtained was washed five times with buffer.

c. DNase I digestion

80 units (40 μg) DNAase I in 1 ml of the same buffer were added to the same volume of a nucleoprotein suspension of about 600 μg DNA/ml buffer solution containing 1 mM $MgCl_2$. The suspension was incubated 3 h at 37 °C. These conditions are sufficient to completely digest the same amount of purified rat liver DNA, as seen in Table I. Further addition of DNase to the medium caused no renewed increase in DNA digestion. The suspension was centrifuged for 10 min at 10 000 rpm and the obtained sediment was washed repeatedly with buffer.

d. Hydroxyapatite chromatography

The DNA-polylysine complex was taken up in 2 M NaCl, buffered with 50 mM sodium phosphate, pH

Table I. Digestion of DNA complexes. Varying amounts from the polycation were mixed with DNA in 2 M NaCl—5 M urea, followed by step dialysis. The sedimented and washed complex was suspended in 1/15 M tris/HCl pH 7.9 2 mM $MgCl_2$ in a concentration of about 1 mg DNA per 3 ml and mixed with 60 μg (*+600 μg) DNase I per 3 ml of the same medium. After incubation of 2 h at 37 °C the sample was centrifuged at 10,000 × *g* and DNA was determined in the supernatant.

DNA [mg]	1	1	1	1	*1
Poly Lysine [mg]	—	1.5	—	—	—
Histone [mg]	—	—	4	8	8
digested DNA [%]	100	1—3	95	55	65

6.9 and passed through a column of hydroxapatite equilibrated with the same buffer. After washing the column with buffer, the DNA was eluted with 0.5 M sodium phosphate, pH 6.9.

3. *Characterization of the isolated DNA*

a. Determination of the molecular size

Molecular size was determined by sucrose gradient centrifugation in 5 – 18.5% (W/W) linear sucrose gradients in 10 mM tris/HCl buffer, pH 7.9 in a SW 56-Beckmann rotor at 39,000 rpm, for 15 h at 4 °C. The *S*-value was determined according to McEwen [20] using $r_0 = 5.84$ cm and $r_{max} = 11.64$ cm and a specific density for DNA in sucrose of 1.4 g/cm^3 [21]. The molecular weight for double-stranded DNA was determined according to van der Schans *et al.* [22] by the following relation: $S^{sucrose}_{20,w} = 0.047\ M^{0.38}$.

b. Renaturation kinetics of the DNA

The renaturation velocity of the DNA was followed according to Britten and Kohne [23]. The DNA was dissolved in 0.12 M sodium phosphate buffer, pH 6.9, and sonicated in an MSE-apparatus (output: 2 – 0.8 mA) for 1 h. Samples of 10 – 1000 μg/ml were denaturated at 100 °C for 10 min and furher incubated at 60 °C for varying time periods. Single- and double-stranded DNA were separated on small columns of hydroxyapapite (100 μg DNA/ml hydroxyapatite) and quantitated, taking into account the hyperchromicity of single-stranded DNA (OD 260 for single-stranded DNA = 1.49 × OD of double-stranded DNA).

4. *Digestion of DNA-histone complexes*

Complexes of DNA with histones (polylysine) were digested by DNase I, as described in 2 c.

Results and Discussion

The basis for the isolation of the "free" DNA stretches was the titration of chromatin with poly-D-lysine. The assumption that this polylysine (P = 670) covers only that part of the DNA not associated with proteins, is based on the following: the probable binding site of polylysine is the small groove of the DNA helix [24–26] whereas the chromosomal proteins are bound to the large groove [25, 27]. If the molecular size of the polycation is small, binding to protein-covered DNA stretches is possible, as already shown [27]. In the present experiments we have used a polylysine consisting of, on the average, 670 amino acids, which renders the binding of the very large molecule onto the small groove of protein-covered DNA regions very unlikely [28, 29]. Binding to such stretches is only possible if an excess of polylysine is used, accompanied by dissociation of proteins from the DNA (see Fig. 2).

By digesting the chromosomal proteins with pronase and hydrolyzing the DNA thus uncovered with DNase I, we could isolate free stretches of DNA, which were protected by the bound poly-D-lysine. On the basis of the polylysine titration, approx. 32% of the phosphate groups of the DNA in rat liver chromatin were found to be available for reaction with the polycation (see Fig. 1). This is less than values of 38% and 46% for titration with polylysine containing on the average 37 and 6 residues respectively as reported by Itzhaki for rat thymus [30], and the value of 50% for polylysine of 670 residues reported by Clark and Felsenfeld [7] for calf thymus chromatin. However, our results are in contrast to a report by Billing and Bonner [31] who concluded from experiments with excessive amounts of polylysine of only a few residues, that nearly all of the DNA in rat liver chromatin is accessible to macromolecules.

With regard to our experiments it should be mentioned that under conditions of tight packing of the chromatin, poly-D-lysine would not be able to interact with all the free phosphpate groups. However the low chromatin concentration and the ionic conditions of the buffer renders such tight packing very unlikely [32, 33]. On the other hand the amount of free DNA calculated by the polylysine titration could be an overestimation if some polylysine molecules do interact with protein covered DNA stretches [30] or with negative charged groups of protein [34].

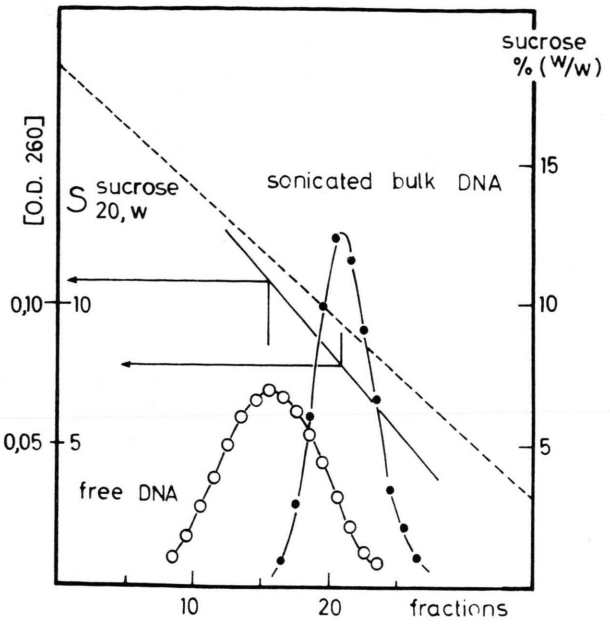

Fig. 3. Determination of molecular size by sucrose gradient centrifugation. Samples of 20 μl (1.7 mg DNA/ml) were applied on 4.7 ml 5–18.5% (w/w) linear sucrose gradients and centrifuged 15 h at 39 000 rpm.

The yield of isolated free DNA fluctates between 40 – 80% of the polylysine titrable DNA. On the basis of sucrose gradient centrifugation (see Fig. 3) a mean *S*-value of 10 ± 0.8 was found corresponding to a mean molecular weight for the isolated DNA of approx. 1.3×10^6 or 2100 nucleotide pairs. This is a minimum value, as polylysine-DNA complexes are not completely resistant to DNase I, but can be degraded up to 3%. The presence of DNA polymerase, other enzymes and/or regulatory proteins on the free stretches of DNA would also lead to cleavage by DNase at these sites.

The results of the renaturation experiments and of caesium chloride centrifugations demonstrated that the isolated DNA is representative of the bulk DNA, and that we have not enriched any special type of DNA in respect of base content or redundancy. It would be of value to confirm the finding of free DNA by using other methods; for instance by electron microscopic observations. Indeed Hennig [35] has demonstrated free DNA-stretches of several μ length on spread Y-chromosomes.

This observation is further supported by other studies on the structure of chromatin. Degradation of DNA by DNase I performed by Itzhaki [36] prove that about 75% of the DNA in chromatin is digestable by DNase, in a way as pure DNA. Similar values have been found by ourselves ($73 \pm 12\%$). Comparing the value of nuclease digestable DNA (approx. 75%) with that of polylysine titrable DNA (approx. 32%) we are led to the conclusion that the nuclease can also digest DNA regions not amenable to polylysine titration. One can assume that these regions are shielded by proteins in a way, that relatively small molecules, like the nuclease, can interact with the DNA whereas the polylysine can not. This assumption is supported by results of the digestion of DNA-histone complexes described in Table I. Depending on the DNA-histone ratio varying amounts of DNA were solubilized (Table I). With histone-saturated DNA a maximal value for digested DNA, at extremely high DNase I-concentration of 65% was reached, whereas 55% of DNA were solubilized under normal conditions.

Taking into consideration these findings and the percentage of DNase-digestable DNA in chromatin

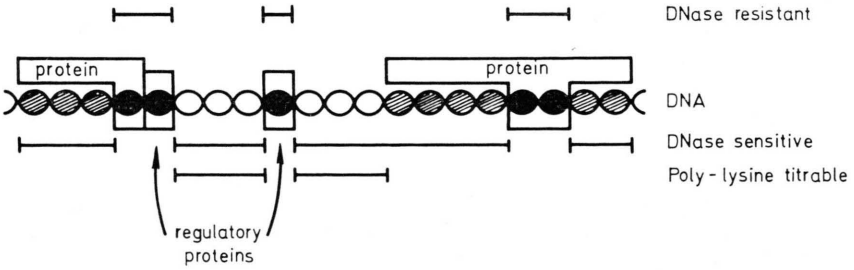

Fig. 4. Model of chromatin structure. a. Free DNA (○○), DNA not associated with proteins (approx. 32%). This DNA is sensitive to DNase and titrable with polylysine. Regulatory proteins may be attached to this DNA, possible as described in b. determining transcription and repression. b. DNA tightly complexed to proteins (◍◍). The tight association of the proteins to the DNA renders it DNase resistent and unaccessible to polylysine (approx. 25%). c. Protein-shielded DNA (●●). Regions of DNA, which are digestable by DNAse, due to the nature of the DNA-protein interaction, but nevertheless not accessible to polylysine (approx. 43%).

(approx. 75%) we can try to reach conclusions on the proportion of "free" DNA in chromatin af follows. Let us suppose that: 75% represents the proportion of nuclease digestable DNA, x% is the proportion of "free" DNA, $(75\text{-}x)$% the proportion of shielded DNA and $(100\text{-}x)$% the proportion of the covered DNA. Let us further assume, on the basis of the histone-DNA digestion experiments, that 65% of the protein-covered DNA is equal to the shielded DNA. Than we have: $(75\text{-}x) = 65/100(100\text{-}x)$ and thus $x = 28.5$, resembling the experimentally obtained value for "free" DNA.

Chromatin shows approximately 15% (± 5%) of the template activity of DNA [12, 37–41]. The difference between the values for "free" DNA obtained on the basis of transcription studies and of polylysine or actinomycin D binding studies [42] can be explained by specific repression of the template. This would signify that approximately $32\% - 15\ (\pm 5)\% = 17(\pm 5)\%$ of the free DNA is repressed. The inhibitory action of the non-histone proteins on transcription with rat liver RNA polymerase [43, 44] supports this hypothesis. The presented data and considerations lead to our concept of chromatin structure as is shown in Fig. 4. The model regards three states of DNA in chromatin in respect to its interaction with proteins: Free DNA (approx. 32%) and the protein-covered DNA (approx. 68%), consisting of stretches of DNA tightly complexed with protein and such, which are more loosely covered with proteins. These last DNA regions, protein shielded DNA (approx. 43%), are digestable with DNase, but not accessible to polylysine. The DNA tightly complexed with proteins (approx. 25%) is likewise not accessible to polylysine. Moreover these stretches are not digestable by DNase, in a way as pure DNA. The free DNA (approx. 32%) can interact with polylysine and is degraded by DNase. This DNA in chromatin, is thought to be transcribed.

The detection of free stretches of DNA in chromatin suggests that the state of part of the genome of higher organisms is similar to that of bacteria. If that were true, the basic control mechanisms prevailing in bacteria could be extrapolated to higher organisms, and models of control elaborated on the basis of the detailed knowledge of bacterial physiology.

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