The Arrangement of Proteins on the Deoxyribonucleic Acid in Chromatin

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The arrangement of the protein component on the DNA of the chromatin complex was studied by comparing the rate of release of oligonucleotides and of protein after addition of deoxyribonuclease I and deoxyribonuclease II to rat thymus chromatin. Also the action of deoxyribonuclease I on normal chromatin and on chromatin depleted of non-histone protein was compared, to elucidate the role of the latter protein in chromatin structure. As a preliminary to the above, the rate of action of deoxyribonuclease I on DNA and on chromatin at the same DNA concentration, and the dependence of the action of this enzyme on the Mg^{2+} concentration, were studied. It was found that: (1) little if any DNA in chromatin is present in extensive, truly 'free' zones, i.e. completely uncovered by protein; (2) at relatively low concentrations of added Mg^{2+} , deoxyribonuclease I degrades chromatin more rapidly than DNA; (3) the non-histone protein is not attached directly to the DNA in chromatin.

In chromatin, the arrangement of histone and non-histone proteins on the DNA is unknown but is obviously of interest in relation to chromatin structure and function. Recent studies on the binding to chromatin of Toluidine Blue and of polylysine of various molecular weights have shown that about 48% of the DNA phosphate groups in chromatin are 'free' and that about 38% of the 'free' phosphate groups are present in fairly long stretches (Itzhaki, 1970, 1971). Are these stretches extensive, truly 'free' zones between successive histones on some or all DNA molecules (model A), or are they 'covered' by, but not bound to, associated histones, lying perhaps beneath stretches of non-basic residues (model B)? If the latter model were correct there would be few, if any, extensive, truly 'free' DNA zones.

An attempt has been made to distinguish between these possibilities by comparing the rate of release of oligodeoxyribonucleotides and of protein from chromatin after the action of endonucleases. In model A, the bare DNA zones should be preferentially attacked: thus oligonucleotide release should precede any release of protein. In model B, breakage of the DNA would lead to concurrent release of the surrounding protein, i.e. equal proportions of the two components would be released in a given time.

The protein component of the chromatin consists of both histones and non-histones, but the present assay method detected only histone release. How-

ever, endonuclease action on 'normal' chromatin and on chromatin from which some of the non-histone protein had been removed (R-chromatin)* was compared. As a preliminary, the overall effect of protein was studied by comparing the degradation rate of DNA and chromatin. The main finding was that there are few extensive 'free' DNA zones in chromatin.

MATERIALS AND METHODS

Chromatin was prepared from rat thymus glands as described by Itzhaki (1966). The final product was a gel and this was diluted usually with water to the required concentration (0.003–0.03% DNA). The chromatin contained about 37% nucleic acid, 49% histone (see below) and 14% non-histone protein.

R-chromatin was prepared by adding NaCl to chromatin to give a final concentration of 0.15M. The mixture was kept at 0°C for 15 min and was then centrifuged at 3000g for 15 min. The precipitate was dissolved in water. About 90–92% of the DNA and 76–87% of the protein was recovered in R-chromatin. About 94% (range 87–104%) of the protein was soluble in 0.2M-HCl compared with about 77% (range 72–84%) for normal chromatin, i.e. R-chromatin was depleted of non-histone protein.

DNA (highly polymerized, from calf thymus) was obtained from Worthington Biochemical Corp., Freehold, N.J., U.S.A. Pronase was obtained from Calbiochem,

* Abbreviation: R-chromatin, reprecipitated chromatin.

Los Angeles, Calif., U.S.A. and deoxyribonuclease I (EC 3.1.4.5) and deoxyribonuclease II (EC 3.1.4.6) from Worthington Biochemical Corp. Urea was recrystallized from ethanol, dissolved in water to give a 10M solution and centrifuged to remove traces of suspended material.

DNA concentrations were measured either from the $E_{260}^{1\%}$ ($E_{1\text{cm}}^{1\%}$ at 260 nm = 210), or by the diphenylamine method (Burton, 1956). Protein was measured by a microbiuret method (Itzhaki & Gill, 1964). Histones were measured, after extracting the chromatin twice in ice-cold 0.2M-HCl, by micro biuret analyses of the extracts. Na^+ concentrations were determined by flame photometry.

To assay nuclease action, the amount of material soluble in 0.15M-NaCl or in 0.67M- HClO_4 was determined. Comparison of oligonucleotide release from deoxyribonuclease I-treated DNA and chromatin was made by measurement of the changes in solubility in HClO_4 rather than in NaCl, as DNA is soluble in the latter. Release of nucleotides and protein from deoxyribonuclease-treated normal chromatin or R-chromatin was followed by measurements of solubility in 0.15M-NaCl, since HClO_4 would presumably have dissociated the two components.

Mixtures of chromatin and nuclease were incubated at the required temperature, together with controls of chromatin alone. At known times, samples were precipitated by addition of NaCl to 0.15M, and after 15 min at 0°C were centrifuged for 15 min at 3000g. The supernatants were then analysed for nucleotide and protein content. This assay is based on the solubility of oligonucleotides and insolubility of chromatin in 0.15M-NaCl. A similar procedure was used for determining the acid-soluble fraction, 2M- HClO_4 , being added to 2 vol. of chromatin or DNA solution.

RESULTS

General. Chromatin-deoxyribonuclease mixtures become progressively less gelatinous and more turbid with time. The dependence on Mg^{2+} of the rate of degradation caused by deoxyribonuclease I was studied by using dilute chromatin at 0.0036% DNA and deoxyribonuclease at about 0.12 $\mu\text{g}/\text{ml}$. The rate increased as the magnesium chloride concentration was increased from 0.1 μM to about 1–3 μM and then remained constant. At a high chromatin concentration (0.017% DNA) with deoxyribonuclease at about 0.6 $\mu\text{g}/\text{ml}$, the degradation rate increased as the Mg^{2+} concentration was increased from 2 to 100 μM . Mg^{2+} concentrations above 100 μM precipitated the chromatin.

Comparison of the degradation rate of DNA and chromatin. Fig. 1(a) shows the changes in acid-solubility of dilute DNA and chromatin in 100 μM - Mg^{2+} during deoxyribonuclease I action. The degradation of chromatin is slower than that of DNA. With 1 μM - Mg^{2+} the rate of degradation of chromatin was the same as that in 100 μM - Mg^{2+} but there was no degradation of DNA. Fig. 1(b) shows changes in acid-solubility of concentrated DNA and chromatin in 100 μM - Mg^{2+} during deoxyribonuclease action. The action on DNA is initially

Fig. 1. (a) Release of nucleotides from deoxyribonuclease I-treated DNA (○) and chromatin (◇) at a low DNA concentration. The DNA and chromatin, both at 0.0036% DNA, were in 1mM-NaCl-100 μM - MgCl_2 ; the concentration of deoxyribonuclease I was about 0.13 $\mu\text{g}/\text{ml}$. (b) Release of nucleotides from deoxyribonuclease I-treated DNA (○) and chromatin (◇) at a high DNA concentration. The concentration of DNA was 0.0197%, of chromatin 0.0192% and of deoxyribonuclease I about 0.25 $\mu\text{g}/\text{ml}$. The DNA and chromatin were in 50 μM -tris-HCl buffer, pH 7.1, containing 100 μM - MgCl_2 . Incubation was at room temperature. ●, ◆, Control values.

slower than that on chromatin. Thus at both high and low concentrations of DNA, a relatively low $\text{Mg}^{2+}/\text{DNA}$ ratio causes a more rapid degradation of chromatin than of DNA by deoxyribonuclease I. This finding and the partial lack of dependence on added Mg^{2+} suggests that further studies on the degradation of chromatin, as opposed to DNA, may be relevant to the control of DNA synthesis, as nucleases have been implicated in this process (Shugar & Sierakowska, 1967).

Release of nucleotides and of protein by deoxyribonuclease I and deoxyribonuclease II. The rates of release of nucleotides and of protein from the chromatin after deoxyribonuclease I action, as

Fig. 2. Release of nucleotides (a) and of protein (b) from deoxyribonuclease I-treated chromatin. The chromatin (concn. 0.0226% DNA, 0.0394% protein) was in water. The concentration of Mg^{2+} was $10\mu M$ and of deoxyribonuclease I about $1\mu g/ml$. Incubation was at $37^{\circ}C$. Curves (1) show the solubility in $0.15M$ -NaCl ($\square$), curves (2) in $5M$ -urea- $0.15M$ -NaCl (∇) and curves (3), the total solubility ($\triangle$).

measured by the solubility of chromatin in $0.15M$ -sodium chloride, were studied and Figs. 2(a) and 2(b), curves (1), show the results. Values are corrected for the solubility of controls in $0.15M$ -sodium chloride (about 4% for DNA and about 20% for protein). The release of nucleotides is apparently not accompanied by release of protein. However, it was suspected that the turbidity of the original chromatin-enzyme mixtures might be due to aggregation of released protein; such aggregates might then be precipitated in $0.15M$ -sodium chloride. Centrifugation of the mixtures without added salt was found to separate a small amount of precipitate and the protein/DNA ratio in the supernatants decreased progressively during the incubation (Table 1). After prolonged digestion, there was also

an appreciable loss of DNA from the supernatants, presumably owing to aggregation of protein and chromatin.

The chromatin-salt precipitates were extracted with $5M$ -urea- $0.15M$ -sodium chloride, so that the urea could disperse any protein aggregates. With the control chromatin only 1-2% of the original DNA and 7-8% of the protein were extracted. Figs. 2(a) and 2(b), curves (2), show the release of nucleotides and protein in the urea-salt extract after deoxyribonuclease I action. Curves (3) show the sum of values of the salt extract plus the urea-salt extract. The rate of (total) release of nucleotides is similar to that of protein, at least during the first 2h. The higher percentage of nucleotides than of protein released subsequently could be caused by the inability of urea to dissociate highly aggregated protein.

Similarly, after deoxyribonuclease II treatment of chromatin, nucleotides but not protein were released into $0.15M$ -sodium chloride. After urea-sodium chloride extraction of the salt precipitates, the rates of total release of the two components were found to be approximately equal.

A possible artifact could arise from contamination of the nucleases by proteases that would degrade and release proteins, but this is unlikely, as addition of trypsin to chromatin caused progressive release of both DNA and protein into salt solution in the absence of urea, in contrast with deoxyribonuclease action. Also, treatment of chromatin with a mixture of deoxyribonuclease II and Pronase ($20\mu g/ml$ and $1\mu g/ml$ respectively) caused release of all the protein and 80% of the DNA after 2h at $20^{\circ}C$.

Degradation of R-chromatin. The action of deoxyribonuclease I on normal chromatin and on R-chromatin at two concentrations was compared after adjusting the Na^{+} concentration of the normal chromatin solution to that of R-chromatin (usually about $0.5mM$), since the rate of action of deoxyribonuclease I on chromatin was found to depend on salt concentration. No significant difference in the rate of release of nucleotides from the two materials was found at either high or low DNA concentrations. This suggests that non-histone proteins are not bound directly to the DNA in chromatin; if they were, their absence from R-chromatin would lead to an increased rate of degradation of the DNA.

DISCUSSION

The concurrent release of nucleotides and of protein after treatment of chromatin with deoxyribonuclease I or deoxyribonuclease II supports model B, suggesting that the DNA in chromatin is extensively 'covered' by the associated histones (though up to about 10% could be truly 'free' of histones without being detectable in the present

Table 1. *Precipitation of protein from chromatin by deoxyribonuclease I action*

Mixtures of chromatin and deoxyribonuclease I were incubated at 37°C for the times listed. Samples were then centrifuged at 10000g in Expts. 1 and 3, and at 3000g in Expt. 2, for 15 min and the nucleotide and protein content of the supernatants was determined. All mixtures contained 10 μ M-MgCl₂. Different experiments refer to different chromatin preparations.

Expt. no.	Sample	Incubation time (h)	Nucleotide concentration (mg/100 ml)	Protein concentration (mg/100 ml)	Protein/nucleotide ratio in supernatant
1	Control chromatin	0	25.5	44.5	1.7
		2	24.3	44.7	1.8
		5	21.0	23.1	1.1
		>6	12.3	3.3	0.3
2	Control chromatin	0	23.5	41.2	1.8
	Chromatin + deoxyribonuclease (2 μ g/ml)	4.5	23.3	28.7	1.2
	Chromatin + deoxyribonuclease (1.5 μ g/ml)	6	13.5	9.6	0.7
3	Control chromatin	0	22.6	39.4	1.7
	Chromatin + deoxyribonuclease (1 μ g/ml)	5	20.3	31.2	1.5
	Chromatin + deoxyribonuclease (1 μ g/ml)	6	19.4	23.0	1.2

system). The high percentage of 'free' phosphate groups in chromatin (Itzhaki, 1970, 1971) could therefore arise from zones of DNA lying beneath, but not bound to, histone.

After the present work was completed, Clark & Felsenfeld (1971) reported similar studies on calf thymus chromatin. They found that nuclease treatment of the chromatin released up to 50% of the DNA into the supernatant obtained by high-speed centrifugation and that this DNA was accompanied by only a small percentage of the protein. In the present study, at least 75% of the DNA was found to be degradable (as assayed by acid- or salt-solubility), the percentage finally obtained being dependent on the amount of deoxyribonuclease added. Clark & Felsenfeld (1971) concluded that about half of the DNA was present in zones completely free of protein. However, they mentioned that the chromatin-enzyme mixtures became turbid; by analogy with the present results it is likely that protein was released but was sedimented during centrifugation because of its aggregated state. Their value of 50% maximum degradation may then have arisen from loss of chromatin through aggregation with histone, as was observed in the present study after extensive nuclease action.

In agreement with the present results, Lloyd & Peacocke (1968) found that after γ -irradiation of nucleohistone, the histone/DNA ratio in the supernatants obtained by high-speed centrifugation and

by precipitation in 0.15M-sodium chloride was much lower than in the unirradiated material. They concluded that irradiation caused dissociation of the histone from the DNA with subsequent aggregation to other histone or nucleohistone molecules.

The finding that there are few, if any, truly 'free' DNA zones in chromatin relates to the control of gene transcription and it remains to be found whether any truly 'free' DNA zones are the sole active gene sites or whether DNA can in fact be transcribed when covered by protein.

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