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Fractionation of Chick Oviduct Chromatin

NUCLEASE-RESISTANT DEOXYRIBONUCLEIC ACID

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Chromatin isolated from several chick tissues was treated with micrococcal nuclease. A limited degree of tissue specificity of chromatin DNA resistance to nuclease digestion was observed. No difference in the extent of nuclease resistance of chromatin DNA was detected during oestrogen-induced oviduct differentiation. This suggested that the amount of non-histone chromosomal protein does not play an important role in the sensitivity of chromatin DNA to nuclease digestion. Studies of nuclease resistance of chromatin DNA after dissociation and reconstitution of chromatin proteins and ethanol extraction of chromatin indicate that the histones protect the DNA from nuclease attack. Slow thermal denaturation of nuclease-resistant DNA suggests that the protected DNA sequences may be (A+T)-rich, and the (G+C)-rich satellites present in total chick DNA are sensitive to nuclease.

The genetic expression of much of the DNA in eukaryotic cells is effectively suppressed by chromosomal proteins which restrict the accessibility of certain DNA sequences to RNA polymerase (Bonner *et al.*, 1968; Spelsberg & Hnilica, 1971). These chromosomal proteins block not only the binding of RNA polymerase to DNA in chromatin, but also other enzymes which utilize DNA as a substrate, e.g. a variety of nucleases (Clark & Felsenfeld, 1971; Gottesfeld *et al.*, 1974).

The significance of the nuclease-resistant fraction of chromatin DNA is presently unknown. The demonstration that nuclease-resistant DNA is enriched for certain nucleotide sequences (Clark & Felsenfeld, 1972) and that repressed DNA or heterochromatin is more resistant to nucleolytic attack than actively transcribed regions (Marushige & Bonner, 1971; Namiki, 1973) makes it possible, however, that these resistant sequences may be of fundamental importance in chromatin structure and function. In view of this possibility we undertook the characterization of nuclease-resistant DNA fragments obtained from chick oviduct chromatin.

The chick oviduct is a steroid-hormone-target organ which is stimulated to differentiate and grow when oestrogen is administered to immature pullets (O'Malley *et al.*, 1969; O'Malley & Means, 1974). Oviduct chromatin has previously been shown to undergo alterations in template activity and protein content during hormone-dependent development (Spelsberg *et al.*, 1973). Therefore by using the chick oviduct it is possible to study nuclease resistance in chromatin from a steroid-hormone-target tissue which undergoes compositional variation during growth and differentiation.

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Materials and Methods

Chromatin preparation

Chromatin was prepared from purified nuclei isolated from oviducts or brains of diethylstilboestrol-treated chicks, hen erythrocytes, or calf thymus by the method of Spelsberg & Hnilica (1971). This method gave chromatin with a histone/DNA ratio of approx. 1.0 (w/w) and a non-histone protein/DNA ratio of 0.2–1.6:1 (w/w) depending on the tissue and stage of development.

Nuclease digestion

Incubation of chromatin with nuclease was carried out by the procedure of Clark & Felsenfeld (1971). Freshly prepared chromatin was dialysed against two changes of 0.005M-sodium phosphate buffer (pH 6.7) with 25μM-$CaCl_2$. The dialysed chromatin was diluted with the same buffer to a DNA concentration of 50–100μg/ml, divided into 1ml portions, and incubated with micrococcal nuclease (Schwarz/Mann, Orangeburg, N.Y., U.S.A.) at 37°C. At intervals, a portion was removed and 0.1vol. of ice-cold 4M-$HClO_4$ added. After 30min, the acid-insoluble precipitate was pelleted by centrifugation at 5000*g* for 15min. Pellets were washed twice by centrifugation

with 0.4M-$HClO_3$, and the DNA content was determined by the diphenylamine reaction (Burton, 1956).

Purification of nuclease-resistant DNA

Chick oviduct chromatin was incubated with 10μg of micrococcal nuclease/ml for 2h at 37°C. The resulting insoluble aggregate was pelleted by centrifugation at 5000*g* for 15min. After centrifugation, no acid-insoluble material remained in the supernatant fraction. The pellet was suspended by gentle homogenization with a Dounce homogenizer in 0.01M-Tris–HCl (pH8.0)–0.01M-EDTA (disodium salt)–1.0% sodium dodecyl sulphate. DNA was extracted by shaking the suspension with phenol equilibrated with buffer (0.01M-Tris–HCl, pH8.0) plus an equal volume of chloroform. Extraction of the aqueous phase was continued until no interphase materials were detectable. The final aqueous phase was made 0.1M with NaCl and DNA was precipitated with 2vol. of ethanol at −20°C.

After overnight storage at −20°C, the precipitate was collected by centrifugation, washed briefly with ethanol, air-dried and dissolved in 0.01M-Tris–HCl (pH7.2)–0.003M-$MgCl_2$. The DNA solution was incubated with electrophoretically pure and heat-denatured pancreatic ribonuclease (Worthington, Freehold, N.J., U.S.A.; 50μg/ml) at 37°C for 30min, then with pre-digested Pronase (Worthington; 250μg/ml) for 60min. The enzyme-treated DNA was extracted three times with buffer-equilibrated phenol (pH8.0) plus an equal volume of chloroform and the DNA precipitated with ethanol as described above.

The DNA was pelleted by centrifugation, dissolved in 0.01M-Tris–HCl (pH8.0) with 0.1M-EDTA (disodium salt) and desalted on a Sephadex G-50 column equilibrated with water. DNA in the excluded volume was termed 'nuclease-resistant DNA' and used in the subsequent characterizations.

Sedimentation analysis

The sedimentation of DNA was performed in a Spinco model E analytical centrifuge equipped with u.v. optics. Band sedimentation in 1M-NaCl–0.05M-Na_2HPO_4 (pH7) and in 0.9M-NaCl–0.1M-NaOH was used to obtain the $s_{20,w}$ for the double-stranded and the single-stranded forms respectively. The viscosity and the specific gravity of these buffers were determined by classical viscosimetry and pycnometry. Molecular weights of the DNA were calculated by using the Studier (1965) equations.

Thermal denaturation

Thermal denaturation of DNA was followed at 260nm in an automatic temperature-programmed digital recording spectrophotometer similar to that described by Ansevin & Brown (1971). High-resolution 'melting' of DNA used the modifications of the derivative 'melting' procedures of Ansevin & Brown (1971) devised by Vizard & Ansevin (1974). Briefly, DNA in 5mM-sodium cacodylate (pH7.0)–0.1mM-EDTA (disodium salt) was heated at a constant rate of temperature increase, 0.5°C/min. At 10s intervals alternate temperature and absorbance readings were recorded on paper and simultaneously encoded on to punch tape. The result was over 550 sets of data entries for each of three DNA samples plus a blank containing only buffer which were analysed simultaneously.

Hyperchromicity and its derivative with respect to temperature were calculated and plotted by computer which used each of the data entries to calculate and draw the curves, but printed only every fifth data point on the graph.

Dissociation and reconstitution of chromatin

The dissociation and reconstitution of chromatin was carried out at both high and low pH with high concentrations of salt and urea. Under low-pH conditions (pH6.0), the histones and a small proportion of non-histone proteins are dissociated from DNA (Paul & Gilmour, 1966; Spelsberg & Hnilica, 1971; Spelsberg *et al.*, 1971*a*); under high-pH conditions (pH8.5), 95% of the chromosomal protein is dissociated from the DNA.

Chick oviduct chromatin was dissociated and reconstituted under defined salt, urea and pH conditions as described by Spelsberg *et al.* (1972). Chromatin at a DNA concentration of 0.2mg/ml was dissociated in 2M-NaCl–5M-urea—0.001M-$NaHSO_3$–0.01M-potassium phosphate buffer (pH6.0) or 0.01M-Tris–HCl (pH8.5). The dissociated chromatin was reconstituted by lowering the salt concentration by stepwise dialysis against 1.0M-, 0.75M-, 0.5M-, 0.25M-, 0.15M- and finally 0.0M-NaCl containing 5.0M-urea, 0.001M-$NaHSO_3$ and 0.01M-potassium phosphate buffer (pH6.0) or 0.01M-Tris–HCl (pH8.5). Urea was finally removed by overnight dialysis against 0.01M-potassium phosphate (pH6.0). The reconstituted chromatin was then purified by the same protocol described above for the preparation of chromatin from isolated nuclei (Spelsberg & Hnilica, 1971).

Ethanol extraction of chromatin

Chromatin, at a DNA concentration of 1mg/ml, was placed in 50% (v/v) glycerol. Ethanol (6vol.) was slowly added to the chromatin with moderate stirring. The chromatin appeared as small gel-like beads which were almost transparent. The alcohol, containing extracted non-histone protein, was decanted and the small amount of alcohol which remained was evaporated under moderate vacuum [1.3kPa (10mmHg)].

Ethanol-extracted chromatin was analysed by gel electrophoresis and by acid extractability of proteins. This procedure removes approx. 50% of the non-histone proteins but does not significantly alter the histone protein population (N. T. Van, unpublished work).

Results

Calf thymus chromatin digested with micrococcal nuclease by the procedure of Clark & Felsenfeld (1971) gave reproducible results of marked similarity to theirs, i.e. 43% of chromatin DNA was resistant to nucleolytic attack (Fig. 1). The amount of DNA resistant to nuclease remained constant and independent of incubation time for periods up to 2h or enzyme concentration up to 50μg/ml (Fig. 2). We therefore adopted the procedure of Clark & Felsenfeld (1971) for our incubations, in which an enzyme concentration of 10μg/ml is used and chromatin is digested for 120min. During the course of incubation, individual time-points were taken, and all values expressed represent plateau values obtained after 2h of digestion.

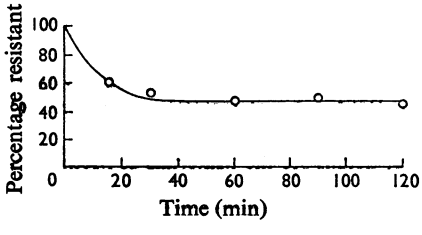

Fig. 1. *Effect of micrococcal nuclease on calf thymus chromatin DNA*

Chromatin digestion (100μg of micrococcal nuclease/ml at 37°C) was stopped at the times indicated and acid-insoluble DNA content determined as described in the Materials and Methods section.

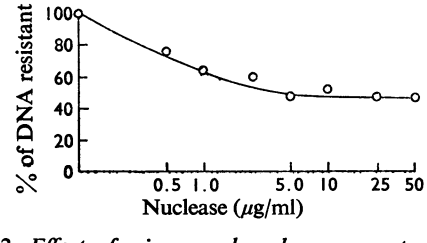

Fig. 2. *Effect of micrococcal nuclease concentration on DNA content of calf thymus chromatin*

Chromatin (100μg/ml) was incubated with the indicated concentration of nuclease at 37°C for 30min. Digestion was stopped and acid-insoluble DNA content determined as described in the Materials and Methods section.

Chick oviduct chromatin showed no endogenous nucleolytic activity (defined as acid insolubility), whereas about 34% of chromatin DNA was resistant to nuclease digestion (Fig. 3). Under these conditions, purified chicken DNA was totally acid-soluble after 5min. Chicken chromatin from different organs displayed some tissue specificity of resistance to digestion with nuclease (Table 1). The 20% difference in nuclease resistance observed between erythrocyte, brain and oviduct suggest that a correlation existed between nuclease resistance and non-histone chromosomal protein content which unlike histone shows organ specificity (Stein *et al.*, 1974) and is lower in erythrocyte than in oviduct chromatin (Spelsberg *et al.*, 1971*b*). To investigate these possible correlations, we determined the nuclease resistance of chromatin DNA isolated from oviducts at different stages of oestrogen-induced differentiation. Oviduct chromatin after 4 days of stimulation has been shown to have a higher non-histone protein content than that found in 18-day-stimulated oviduct, untreated

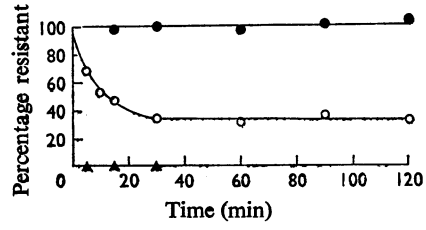

Fig. 3. *Endogenous nucleolytic activity of chick oviduct chromatin*

Oviduct chromatin was incubated with no (●) or 10g (○) of micrococcal nuclease/ml for the indicated times. ▲, Purified chick DNA.

Table 1. *Effect of micrococcal nuclease on chromatin DNA from different organs of the chicken*

Chromatin (50–100μg) was digested with 10μg of micrococcal nuclease by the procedure described in the Materials and Methods section. Values represent plateau values of individual time-points taken over a period of 120min.

Organ	Percentage of DNA that is nuclease resistant
Chick oviduct, unstimulated	32
Chick oviduct, 4 days of diethylstilboestrol treatment	34
Chick oviduct, 18 days of diethylstilboestrol treatment	34
Chick oviduct, 18 days of diethylstilboestrol+10 day withdrawal	32
Chick brain	44
Hen erythrocyte	54

oviduct chromatin, or chromatin isolated from chicks withdrawn from oestrogen treatment (Spelsberg *et al.*, 1971*a*). Despite the differences between these preparations, nuclease resistance was the same in chromatin isolated from untreated oviducts, from oviducts treated with diethylstilboestrol for 4 days or for 18 days and from oviducts withdrawn from diethylstilboestrol treatment (Table 1).

We further investigated the role of histone and non-histone chromosomal proteins in nuclease resistance by comparing results obtained from oviduct chromatins from which total proteins were dissociated and then reconstituted by gradient dialysis. Nuclease resistance of chromatin DNA appears to correspond most closely to histone content. With nucleoprotein from which all histone but only 33% of the non-histone protein has been extracted, no DNA was resistant to nuclease; if 98% of the histone removed was restored and essentially all of the non-histone protein, nuclease resistance of chromatin DNA returned to that of unextracted chromatin (Table 2).

Similarly, calf thymus chromatin from which 50% of non-histone protein but little histone has been extracted with ethanol showed little change in nuclease resistance before and after extraction (Table 3).

We next characterized the nuclease-resistant oviduct chromatin DNA. Molecular weight of the resistant DNA was determined from the slope of the line obtained by plotting the log of the distance sedimented under neutral or alkaline conditions against time (Studier, 1965; Fig. 4). The results show the nuclease-resistant DNA to have a mean length of only

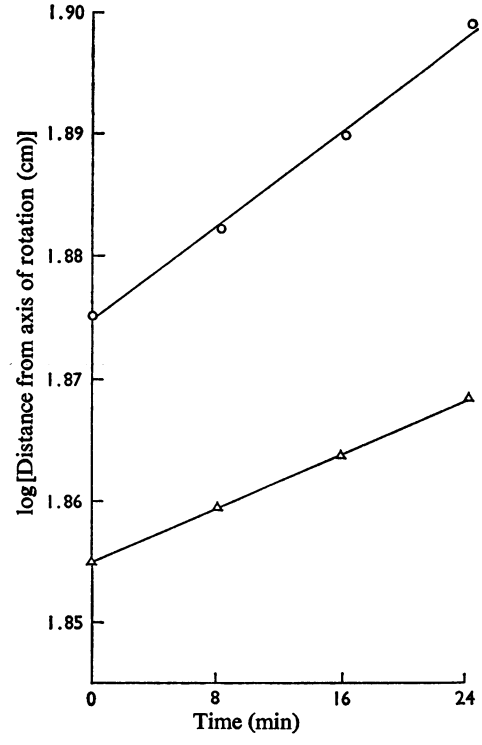

Fig. 4. *Sedimentation of nuclease-resistant chick oviduct chromatin DNA under neutral* (○) *and alkaline* (△) *conditions*

For further details see the Materials & Methods section.

Table 2. *Effect of extraction and restoration of chromatin proteins on the nuclease resistance of chick oviduct chromatin DNA*

Nuclease digestion and chromatin reconstitution were performed as described in the text. Percentage of nuclease-resistant DNA was obtained as in Table 1.

Treatment	Histone DNA	Non-histone protein DNA	Percentage of DNA that is nuclease-resistant
None	1.056	1.064	33.8
Extracted	0.000	0.600	0.0
Reconstitution I	0.824	0.884	20.0
Reconstitution II	0.973	0.988	28.0
Reconstitution III	1.029	1.216	34.8

Table 3. *Effect of ethanol extraction of non-histone chromosomal proteins on the nuclease resistance of calf thymus chromatin DNA*

Non-histone protein was extracted by the procedure described in the Materials and Methods section. Percentage of nuclease-resistant DNA was obtained as described in Table 1.

Treatment	Histone DNA	Non-histone protein DNA	Percentage of DNA that is nuclease-resistant
None	1.020	0.750	43
Ethanol extraction	0.965	0.359	41

Table 4. *Size and molecular weight of nuclease-resistant chick oviduct DNA as determined by sedimentation analysis*

DNA was extracted from micrococcal nuclease-treated chromatin with phenol–sodium dodecyl sulphate and purified by ribonuclease and Pronase digestion as described in the Materials and Methods section. Sedimentation coefficients were obtained by band sedimentation under neutral and alkaline conditions.

Conditions of sedimentation	$s_{20,w}$	Mol.wt.	Average no. of nucleotides
1.0M-NaCl–0.05M-Na_2HPO_4	4.786	1×10^5	312
0.9M-NaCl–0.1M-NaOH	2.945	1.7×10^4	53

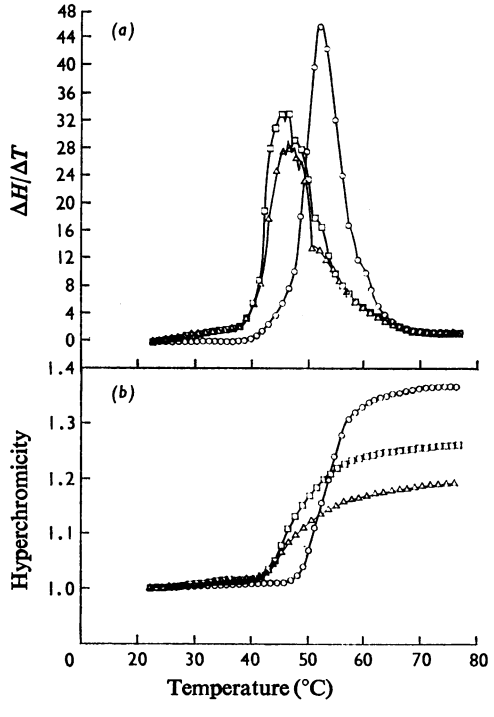

Fig. 5. *Derivative (a) and integral (b) plots of the thermal denaturation of chick DNA*

Native chick DNA (○); DNA from nuclease-treated oviduct chromatin (□); DNA from nuclease-treated oviduct chromatin from which histones and non-histone proteins were dissociated and then restored (△).

50 nucleotides when centrifuged under denaturing conditions (Table 4).

For analysis of base composition of the DNA fragments from nuclease-resistant chromatin, we used controlled slow thermal denaturation in a programmed 'melting' spectrophotometer with subsequent calculation of the integral and derivative 'melting' curves performed and plotted by digital computer. Under the defined salt conditions used, total chick DNA 'melted' with a T_m ('melting' temperature) of 52°C (Fig. 5). Plotted as the derivative of the hyperchromicity with respect to temperature, total chick DNA 'melts' as a nearly homogeneous peak where the maximum equals the T_m (Fig. 5). Under our salt conditions, 52°C equals a G+C content of 44% for total chick DNA (Sueoka, 1961). Slight shoulders at 58°C and 61°C indicate the presence within the chicken genome of a limited number of sequences (satellites) with a G+C content of greater than 44% (Vizard & Ansevin, 1974).

Nuclease-resistant DNA shows about 70% of the hyperchromicity of total chick DNA and a T_m lower by about 6°C. The derivative 'melting' curve (Fig. 5) shows at least three components which 'melt' as discrete families of sequences. The major peak is at 46°C, with some indication that it may demonstrate more than one 'melting' component. A second fraction 'melts' with a T_m of 48°C. Finally, there is a small component with a T_m near that of total chick DNA.

Since many of the properties of oviduct chromatin, including nuclease resistance, can be restored when chicken DNA is reconstituted with oviduct chromatin proteins, we examined the thermal denaturation of DNA fragments obtained from nuclease-treated reconstituted oviduct chromatin. DNA from nuclease-treated reconstituted chromatin has only about 50% of the hyperchromicity of total chick DNA. The derivative curve, however, shows most of the salient features of that obtained with nuclease-resistant DNA from control oviduct chromatin (Fig. 5).

Discussion

The results that we have obtained with micrococcal nuclease digestion of chromatin under low-salt conditions are markedly similar to those of Clark & Felsenfeld (1971). In our laboratory, calf thymus chromatin shows nearly the same resistance to nucleolytic attack as reported by them. Moreover, the size of the double-stranded DNA fragments obtained from digested oviduct chromatin is nearly identical with that which they obtained from calf thymus chromatin (100000 daltons compared with 105000 daltons). Subsequent investigations of the size of nuclease-resistant chromatin DNA have demonstrated the presence of higher-molecular-weight DNA fragments with less extensive digestion (Axel *et al.*, 1974; Oosterhof *et al.*, 1975). The larger fragments appear to be multiples of the shorter fragment size or are directly convertible into the smaller size, which has resulted in a model of chromatin organization based on a subunit DNA fragment of about 150

base pairs (Axel *et al.*, 1974; Oosterhof *et al.*, 1975; Hewish & Burgoyne, 1973; Noll, 1974). It appears, therefore, that the nuclease-resistant DNA fragment that we describe may represent that subunit.

Sufficient evidence exists to conclude that it is primarily histones which protect the DNA in chromatin from nuclease digestion (Murray, 1969; Clark & Felsenfeld, 1971; Mirsky & Silverman, 1972). Our results with reconstituted chick oviduct chromatin and ethanol-extracted calf thymus chromatin indicate that non-histone chromosomal proteins contribute little if anything to chromatin nuclease resistance. Since there is little difference in the histone content of chromatin among different tissues (Panyim & Chalkley, 1969) and since the more heterogeneous non-histone chromosomal proteins do not appear to contribute to nuclease resistance, the basis for differences observed in nuclease resistance between chromatin from oviduct, brain and erythrocyte remains unresolved. Use of limited nuclease digestion by Marushige & Bonner (1971) and Gottesfeld *et al.* (1974) to free the more diffuse euchromatic regions of chromatin from more compact heterochromatin suggests, however, that structural differences influenced by histones may play a major role in the differential sensitivity to nuclease.

The disparity in the size of nuclease-resistant DNA fragments centrifuged under neutral as compared with alkaline conditions suggests that numerous single-strand breaks must have occurred during digestion. Since a DNA duplex of 300 nucleotides should yield two single strands of 150 nucleotides on denaturation, we estimate an average of four nuclease-induced scissions per double-stranded fragment. The observation that the nuclease-resistant DNA fragments show approx. 30% less hyperchromicity during 'melting' than does total DNA suggests that these points of scission include appreciable single-stranded regions. These may well represent regions of the DNA duplex where only one strand is protected by histone, and therefore indicate that the protective histones are unequally distributed about the DNA helix. The spatial separation or unequal distribution of histones on DNA has previously been deduced both from studies on the nuclease resistance of chromatin (Clark & Felsenfeld, 1971), and from the interaction of antibodies directed against histones with chromatin (Bustin, 1973).

Slow thermal denaturation of DNA under the proper salt conditions has been shown to resolve 'thermal satellites' in some DNA species when the derivative of the hyperchromicity with respect to temperature is calculated and plotted (Pivec *et al.*, 1974; Vizard & Ansevin, 1974). These satellites are detected when there are families of DNA sequences in the genome of similar base composition so they denature co-operatively at characteristic temperatures. If the histones that protect DNA from nucleolytic attack have real sequence specificity in their association with DNA, we reasoned that unique thermal satellites might be expected to appear in the nuclease-resistant DNA isolated from chick chromatin. The lower T_m of nuclease-resistant DNA suggests that most of the protected sequences are (A+T)-rich. This result is similar to that reported for calf thymus chromatin by Oliver & Chalkley (1974). It is difficult to estimate the precise amount of enrichment, however, since the low molecular weight of the duplexes which result from chain scissions tends to decrease the T_m (Walker, 1969).

Clark & Felsenfeld (1971) detected no difference in the base composition between total bovine DNA and that of nuclease-resistant calf thymus chromatin. They subsequently showed, however, that within the nuclease fragments, arginine-rich histones protected smaller DNA sequences of enriched G+C content (Clark & Felsenfeld, 1972). We detected no sequences within the nuclease-resistant oviduct DNA with a G+C content greater than that of total chick DNA. Indeed, the (G+C)-rich satellites which are present in total chick DNA are lost after nuclease digestion of chromatin.

Previous investigations of possible sequence specificity of histones have indicated that lysine-rich histones show a preference for (A+T)-rich regions of DNA, whereas arginine-rich histones show only a slight preference for G+C regions (Leng & Felsenfeld, 1966; Olins *et al.*, 1968). Mirsky & Silverman (1972) compared the nuclease resistance of DNA in control and dehistonized calf thymus nuclei. They found that although both lysine-rich and arginine-rich histones make a contribution to the protection of DNA, lysine-rich histone is decidedly the most effective. Axel *et al.* (1974) have reported that the selective extraction of lysine-rich histone decreases the mean of the DNA length that can be isolated from nuclease-resistant chromatin. The relative (A+T)-richness of our nuclease-resistant oviduct chromatin DNA compared with total chick DNA is consistent with the notion that these fragments represent regions preferentially enriched for lysine-rich histone.

The heterogeneity observed in the derivative 'melting' profile suggests that there is some diversity in the kinds of sequences protected by histones. The similarity between the derivative 'melting' profiles of nuclease-resistant DNA from control and reconstituted oviduct chromatin suggests that whatever histones act in protecting DNA, they bind back to DNA with similar sequence composition. Although non-histone chromosomal proteins do not appear to contribute to nuclease resistance directly, they may somehow contribute to the specificity with which histones bind to DNA. The importance of the non-histone proteins in restoring at least partial transcription fidelity to reconstituted chromatin has been previously demonstrated (Paul & Gilmour, 1966;

Gilmour & Paul, 1969; Spelsberg *et al.*, 1971*a*), and they may function similarly in restoring nuclease resistance.

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