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Solubility and Structure of Domains of Chicken Erythrocyte Chromatin Containing Transcriptionally Competent and Inactive Genes

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ABSTRACT: Chromatin generated by micrococcal nuclease digestion of erythrocyte nuclei can be fractionated into two pools of differing solubility in solvents containing 0.15–0.25 M NaCl. A fixed percentage of the chromatin is soluble under these conditions, independent of the average size of the DNA in the unfractionated chromatin. Chromatin containing particular gene sequences is also distributed between soluble and insoluble fractions in a way that is independent of the average size of the starting material. However, the actual percentage of gene copies present in each fraction is not necessarily the same as for bulk chromatin. The transcriptionally active chicken erythrocyte adult β-globin gene is more soluble than the bulk, while the ovalbumin gene in the same tissue is less soluble. These differences do not appear to be related to variations in content of RNA, core histones, or two classes of non-histone proteins. Instead, we find that the soluble chromatin pool is somewhat depleted in histones H1 and H5 and contains lower molecular weight DNA than precipitable chromatin. The soluble fraction can be made insoluble by addition of H1. If the precipitable chromatin fraction is redigested to reduce its size and then recombined with the soluble fraction and reprecipitated, the distribution of globin gene is randomized. The results suggest that the partitioning of chromatin into soluble and insoluble pools in 0.15–0.25 M NaCl arises from redistribution of a limiting amount of histones H1 and H5 to the chromatin fractions containing the longest DNA.

Numerous attempts have been made to fragment chromatin and to separate the product into fractions differing in physical properties in a way that might be correlated with gene activity (Gottesfeld, 1977; Pederson, 1978; Nasser & McCarthy, 1975; Bonner et al., 1975). A number of procedures have made use of mild digestion with nucleases that preferentially attack and release transcriptionally active chromatin. Subsequent fractionation then depends upon some combination of differences in the size and solubility of the active and inactive material.

Recent studies of the enhanced nuclease sensitivity of chromatin near transcriptionally active genes suggest that perturbations in chromatin structure can involve not only the genes themselves but also domains at least several kilobases in length surrounding them (Stalder et al., 1980; Flint & Weintraub, 1977; Wood & Felsenfeld, 1982; Weintraub & Groudine, 1976). this raises the question whether there may be some distinguishable compositional modification of these domains in vivo that would also affect their solubility. The earlier nuclease digestion studies mentioned above unfortunately do not address this question directly, since no attempt was made to separate the size-dependent effects on solubility and yield from effects that are independent of size. A recent exception is the work of Fulmer & Bloomfield (1981), who found that gentle micrococcal nuclease digestion of chicken erythrocyte nuclei yields two populations of chromatin differing in their solubility in 0.15 M NaCl. They deduced that the two populations were apparently not in a precursor–product relationship to each other, since they were released and digested at characteristically different rates at two different temperatures.

In this paper, an attempt is made to determine the physical basis of these solubility properties by studying the solubility behavior of chicken erythrocyte chromatin as a function of salt concentration, molecular weight, and concentration. For all of the chromatin preparations examined, the salting-out behavior does not depend upon the average molecular weight of the starting material or the starting concentration: At salt concentrations below 80 mM, most (80–95%) of the chromatin is soluble; at salt concentrations between 175 and 250 mM, for chromatin preparations varying widely in concentration and starting DNA molecular weight, a fixed fraction (about 65%) of this soluble material precipitates. When fractions are tested for the abundance of the adult β-globin gene and ovalbumin gene, it is found that the former is concentrated in the soluble fraction and the latter in the insoluble fraction. Again, these distributions are quantitatively independent of the molecular weight of DNA in the starting chromatin preparation.

These differences in solubility properties are not correlated with the content of RNA, core histones, or two distinct classes of non-histone proteins. However, the average molecular

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weight distribution of DNA is always smaller in the soluble fraction than in the insoluble fraction from the same experiment. Furthermore, the soluble pool is slightly depleted in histones H1 and H5 relative to the insoluble pool. Experiments in which the relative sizes of the two pools were varied by further nuclease digestion show that the determining factor in the solubility behavior is the size of the chromatin: The highest molecular weight fraction in a given preparation is always precipitated. This could be mediated through rearrangement of histones H1 and H5. The fractionation of "active" genes into the soluble pool is presumably an indirect result of the greater nuclease sensitivity, and thus smaller size, of chromatin containing such genes. These results show that although the solubility properties of active chromatin fractions may indirectly reflect structural differences within the nucleus, the salt precipitation procedure cannot be used to deduce the histone composition of active chromatin in vivo.

Materials and Methods

Preparation of Chromatin. Chromatin was prepared by a low ionic strength Triton X-100 extraction from chicken erythrocyte nuclei. Adult white leghorn chickens were bled into 50 mM ethylenediaminetetraacetic acid (EDTA); the blood was washed 3 times with phosphate-buffered saline, incubated 10 min in reticulocyte suspension buffer [RSB = 10 mM NaCl, 10 mM tris(hydroxymethyl)aminomethane (Tris) at pH 7.4, and 3 mM $MgCl_2$] containing 0.5% Triton X-100, and then washed twice more in this same buffer followed by four washes in this buffer omitting Triton and, finally, twice in this buffer modified to contain 1 mM $CaCl_2$ instead of $MgCl_2$, and at pH 8. The material was adjusted to 5 mg/mL, preheated to 37 °C, and then digested for 0.5 h by addition of an appropriate amount of micrococcal nuclease (Worthington). The digestion was terminated by addition of EDTA to 10 mM, and the nuclei were opened by dialysis overnight against 0.2 mM EDTA, pH 8. The sample was clarified by centrifugation at 8000*g* for 10 min and stored at 2 mg/mL DNA and 4 °C until use. All solutions contained 5 mM sodium butyrate and 0.1 mM PMSF (phenylmethanesulfonyl fluoride). All procedures were performed at 4 °C unless otherwise stated.

Solubility Assay. Chromatin samples at 2 mg/mL in 0.2 mM EDTA at pH 8.0 were diluted with 1 volume of water and then adjusted to 0.5 mg/mL by addition of 2× AG buffer (1× AG buffer = 25 mM sodium phosphate and 40 mM NaCl at pH 7.0). To 120-μL aliquots of this solution was rapidly added 40 μL of a NaCl solution with vortexing to jump the ionic strength to the desired level. The samples were cooled on ice 15 min and centrifuged at 10000*g* for 10 min. The A_{260} of the clear supernatant was then determined, and the globin gene content was assayed by a dot blotting procedure.

Gel Electrophoresis. DNA samples were prepared from chromatin by standard Proteinase K–phenol–chloroform treatment followed by ethanol precipitation and were then subjected to electrophoresis in gels containing 0.7% agarose and Tris–acetate–EDTA buffer. Chromatin samples to be analyzed for protein were dialyzed at equal volumes and DNA concentrations against 50 mM ammonium acetate, pH 7.5, and 0.1 mM PMSF. The concentration was rechecked after dialysis, and several amounts were analyzed in polyacrylamide gels run as described (Laemmli, 1970). In cases where large amounts of non-histone were present, DNA concentration was also determined chemically (Thomas & Farquhar, 1978).

Dot Blot Procedure. Supernatants derived from solubility determinations were examined for globin gene content by a modification of a procedure of Kafatos et al. (1979) to allow direct dot blotting of chromatin samples on nitrocellulose membranes without prior DNA purification. Sixty microliters of a sample containing 20 μg of DNA as chromatin was adjusted to 190 μL with water. Ten microliters of 1 mg/mL Proteinase K was added, and the sample was incubated at 37 °C for 30 min and then brought to 500 μL and made 100 mM in NaOH and 1.2 mM in EDTA. After 30 min at 25 °C, equal volumes of 4 M NaCl and 2 M ammonium acetate were added, and the samples were chilled at 4 °C. Two hundred microliter aliquots were spotted in quadruplicate on nitrocellulose filters (BA-85, Schleicher & Schuell, Inc.) enclosed in a minifold apparatus (Schleicher & Schuell, Inc.). The filters were hybridized (Southern, 1975) with the probe pCAβG1 (Wood & Felsenfeld, 1982), which carries a 6.2 kilobase (kb) *Eco*RI fragment containing the chicken β-globin region, or with the 2.4 kb *Eco*RI fragment of the chicken ovalbumin gene (Lai et al., 1978). The pCAβG1 probe under stringent washing conditions still gives a 5–10% signal due to repeated sequence content, which controls have shown to be mainly in precipitable sequences elsewhere in the genome. Treatment of samples with RNase did not affect the signal strength. The filters hybridized to the β-globin and ovalbumin probes were washed at 65 and 50 °C, respectively, in 0.1× SSPE (1× SSPE = 0.18 M NaCl, 10 mM sodium phosphate, and 1 mM EDTA, pH 7.7) and then exposed to X-O-MAT film (Kodak) using a Cronex (Du Pont) intensifying screen, and the spot densities were measured with a Joyce Loebl scanning densitometer. The linear range of the assay is up to 6.0 μg of input DNA.

RNase Digestion. tRNA (yeast tRNA, Schwarz/Mann) and samples of chromatin at 2 mg/mL were diluted as above for solubility assays. To 120 μL of substrate solution was added 10 μL of freshly boiled RNase (Calbiochem) solution. The mixture was incubated at 37 °C for 30 min. Samples of chromatin were then removed for solubility assays followed by optical density and globin gene determinations as above. The tRNA samples were diluted 1:2 with ice-cold 20% perchloric acid, kept on ice 10 min, and then centrifuged at 10000*g* for 5 min at 4 °C. The supernatants were diluted to measure OD_{260}. Corrections are included in Figure 7 for background and hyperchromicity.

H1 Purification. Milligram quantities of electrophoretically pure H1 were prepared from adult chicken erythrocyte nuclei by using a 5% perchloric acid extract of the nuclei, which was further fractionated by molecular sieve chromatography on Bio-Gel P-60 in 10 mM HCl (Sung, 1977). The fractions containing H1 were lyophilized for 2 days and then stored at 2 mg/mL in water at −20 °C until use.

H1 Standardization. Approximately 100 μg of H1 was hydrolyzed in 6 M HCl for 24 h at 107 °C and run in two dilutions on a Beckman 121 MB amino acid analyzer. The amount of H1 present was determined from these results by utilizing its known molecular weight of 21 000 and known amino acid composition (Murray et al., 1968).

H1 Titration Procedure. Chromatin samples to be titrated with H1 were prepared as described above under Solubility Assay. Four microliters of H1, diluted into 1× AG buffer, was added to each chromatin sample of 120 μL to give the desired stoichiometry, based upon the unfractionated bulk DNA present. A mole fraction of 1 refers to one H1 molecule per nucleosome. We used a molecular weight of 21 000, an extinction coefficient of 2.45 mL/(cm·mg) for H1, and a nucleosome DNA molecular weight of 120 000.

The titrated chromatin remained at room temperature for 15 min and was then precipitated and analyzed for bulk and

specific gene solubility as usual.

In Vitro Length Variation. Chromatin at 2 mg/mL in 0.2 mM EDTA was diluted to 0.5 mg/mL with water. Calcium was introduced by addition with vortexing of 2.4 μL of 100× digest buffer to 240 μL of chromatin (1× digest buffer = 5 mM Tris and 1 mM $CaCl_2$ at pH 8.0). Graded amounts of micrococcal nuclease in 10-μL volumes were added, and the samples were incubated at 37 °C for 30 min. Digestion was stopped by addition of 2 μL of EDTA to a final concentration of 2 mM. Twenty-eight microliters of 10× AG buffer was added, the samples were pipetted into 120-μL volumes in duplicate, and solubility analyses were performed. DNA from the remainder was used for DNA gel electrophoresis on a Bio-Rad mini-sub cell. Enzyme concentrations varied from 0 to 0.1 unit/mL.

Sucrose Gradients. Isokinetic sucrose gradients from 5% to 26% were buffered with 1× AG buffer (I = 80 mM) or 1× AG buffer with additional NaCl (I = 175 mM). Chromatin (405 μL at an A_{260} of 40) was buffered with 45 μL of 10× AG buffer and received 150 μL of either 1× AG (low-salt gradients) or sodium chloride solution to reach I = 175 mM (high-salt gradients) as in solubility assays described above. The sample was immediately layered on 11.75-mL gradients which were run for 2 h at 35000 rpm in SW40 rotors at 4 °C. Gradients were then eluted in 13 1-mL fractions which were tested for A_{260}, A_{230}, and β-globin gene content. After being dialyzed against 1 L of 50 mM ammonium acetate at pH 7 and 0.1 mM PMSF, one-third of each sample was lyophilized and analyzed by protein electrophoresis.

Two-Stage Precipitations with Digested Precipitates. An initial preparative precipitation was performed on a 1-mL scale (see above methods) starting at an A_{260} of 40. This generated a supernatant, S1, which was diluted with water to an ionic strength of 80 mM, and a precipitate, Pr1, which was resuspended in water to reach an A_{260} of 11.8. Aliquots (240 μL) of the precipitate were buffered with 2.4 μL of 100× digest buffer and digested for 30 min at 37 °C by additions of 10 μL of micrococcal nuclease solution to 0–4 units/mL final concentration. Reactions were terminated by addition of 2 μL of an EDTA solution, to reach 10 mM final concentration, and 28 μL of 10× AG buffer.

The treated precipitates and the nonredigested supernatant were recombined at a final A_{260} of 5 at a 3:1 DNA mass ratio to mimic the predetermined starting solubility of the native chromatin at this concentration, using 1× AG buffer as diluent. The mixtures were then reexamined in a routine solubility test on a 150-μL scale and the second supernatants (S2) tested for A_{260} and adult β-globin gene content. Chromatin containing 0.5 μg of DNA from the supernatant and precipitate (Pr2) from each reaction was also analyzed by DNA gel electrophoresis in 0.7% agarose in the presence of 0.03% sodium dodecyl sulfate (SDS). Mock redigested precipitates which contain only buffered, chelated micrococcal nuclease but no chromatin were also added to supernatants and precipitates under identical conditions to determine that digestion was indeed terminated before recombination in this two-stage procedure.

RESULTS

Solubility Assay. Chromatin samples in 80 mM ionic strength solvent were rapidly brought to final ionic strengths ranging from 60 to 250 mM, cooled, and pelleted, and the A_{260} of the supernatants was measured (Figure 1). The graph contains the following three segments: plateaus at low and high salt concentrations and a symmetric transition region centered at 125 mM NaCl. Two parameters describe this

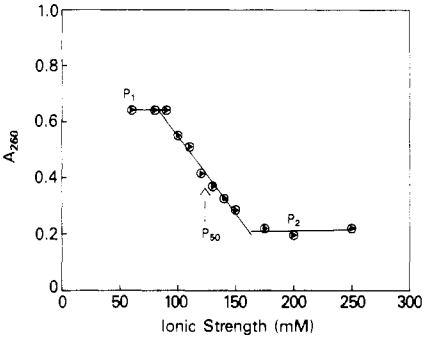

FIGURE 1: Standard assay for determination of P_{50} and S. Chromatin at an A_{260} of 10 was rapidly brought to the ionic strength given on the abscissa, cooled, and pelleted. The absorbance of the supernatant is given for diluted fractions. An absorbance of 0.65 corresponds to an absorbance of 9 (90% solubility) in the undiluted solution. In this experiment and those shown in Figures 2 and 3, digestions were carried out under standard conditions (see Materials and Methods) with 1 unit/mL micrococcal nuclease for 30 min.

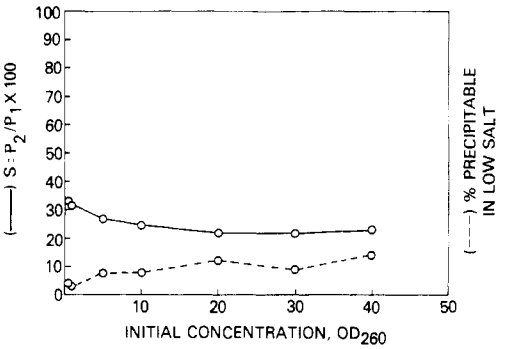

FIGURE 2: Dependence of S and recovery upon initial concentration. Chromatin samples at a series of concentrations were treated as in Figure 1 (solid line). Unspun duplicate tubes from the low-salt plateau were used to find by difference the size of the low-salt precipitate (dashed line, low-salt precipitate in percent).

curve. The first of these is P_{50}, the salt concentration at the midpoint of the solubility transition. The second is the relative solubility, S, the percentage of the material soluble in the low ionic strength limit (about 90% of the total) that remains soluble in the high ionic strength plateau region [S = 100-(P_2/P_1)]. We found that the breadth of the transition was affected to some extent by the initial concentration of the sample and its DNA length, by the cell of origin of the chromatin, and by the details of how the experiment is performed. However, a chromatin made as above from a given cell source had values of S and P_{50} which were nearly invariant. We obtained values of S from 33% to 40% for fresh adult chicken erythrocyte chromatin. Within 3–6 days, some preparations had declining S values. We did not find the source of this variation. P_{50} values for adult chicken erythrocyte chromatin were 125–135 mM.

Soluble and Precipitable Pools. In Figure 2 is shown the dependence of S on the initial concentration of chromatin before the salt jump. At values of A_{260} below 2, there was a rapid rise in S, while in the range A_{260} = 5–40, the S value fell to a limit which was about 25% in most assays. The lower curve in Figure 2 accounts for the 5–15% of the chromatin that was precipitated even in the low-salt plateau region. Below, we study the behavior of the 80–95% of material that was soluble at low salt concentrations. The parameter S thus measures the fractional solubility in the upper plateau region of most of the chromatin.

Properties of the Fractions. We examined the physical and chemical properties of the fractions that were soluble and

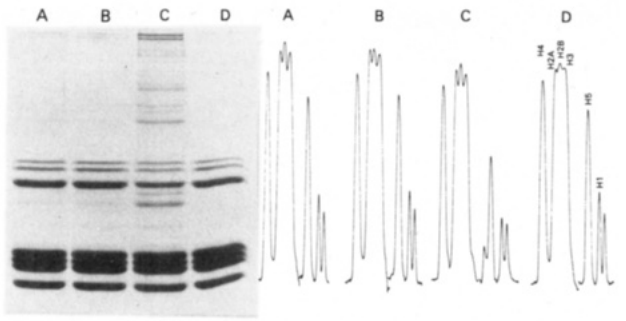

FIGURE 3: Protein gel analysis of the precipitation reaction. (Left panel) Twenty micrograms of DNA as chromatin (see text) was run from (A) whole chromatin (dialyzed from 0 mM ionic strength), (B) low-plateau supernatant (from 11 mM), (C) high-plateau supernatant (from 150 mM), and (D) high-plateau precipitate resuspended in water (from 0 mM) in SDS gel electrophoresis. (Right panel) Densitometer tracings of core histone and H1/H5 regions of the gel only. HMG proteins migrate between the fast-moving core histones and the slow-moving H1 and H5 (three strong upper bands).

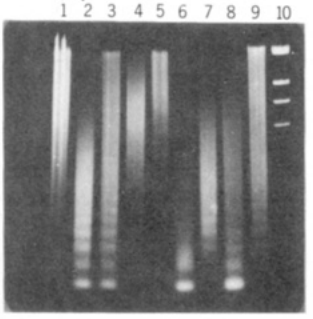

FIGURE 4: DNA gel analysis of products of solubility assays. Five microliters of purified DNA was run in DNA gel electrophoresis in 1% agarose. Samples of total chromatin in lanes 1–3 were derived from the following digests: (lane 1) 0.25 unit/mL digest; (lane 2) 4 units/mL digest; (lane 3) equal mixture of 0.25 and 4 units/mL digests. Samples of lanes 4–9 show the supernatant and precipitate fractions, respectively, from reactions using (lanes 4 and 5) 0.25 unit/mL digest, (lanes 6 and 7) 4 units/mL digest, (lanes 8 and 9) mixed 0.25 and 4 units/mL digests, and (lane 10) λ *Hin*dIII marker (23.5, 9.7, 6.6, 4.3, and 2.2 kilobase pair bands are visible).

insoluble in the high-salt plateau region. No reproducible differences in core histone content were seen in several fractions (Figure 3). However, it is apparent that high-mobility group (HMG) proteins, as well as a wide spectrum of other high molecular weight non-histone proteins, were highly enriched in the supernatant material. Detailed analysis of the gels showed that the abundance of H5 in the soluble fraction was only 70% of that in the unfractionated chromatin, and there was also a reproducible 15% reduction in the fast component of H1.

It is unlikely that the quantitatively reproducible depletions in histones H1 and H5 result from progressive degradation or loss by diffusion during the precipitation. The same electrophoretic profile was obtained when aliquots of supernatant and precipitate were flash-frozen in 0.2% SDS at −20 °C immediately after the procedure, or were exposed to prolonged dialysis against 0.05 M ammonium acetate at pH 7.0 (data not shown).

We also compared the DNA length distributions of the two pools with that of an unfractionated sample. An equal amount of purified DNA from each was analyzed by gel electrophoresis. Figure 4 shows that the soluble and insoluble fractions contained DNA of unequal lengths. The supernate was enriched in shorter DNA, with some overlap in size (scans showed 34% of the soluble fraction was longer than 52% of the insoluble fraction; Figure 4, lanes 4 and 5).

Table I: DNA Lengths of Micrococcal Nuclease Digests before Fractionation

micrococcal nuclease (units/mL)	% yield	$L_{mass,av}$[a]	peak mass[a]	L_{median}[a]
0.25	76	8.2	26.9	5.4
0.5	83	4.6	4.3	3.1
1	86	3.6	2.6	1.9
2	76	3.0	1.9	1.7
4	81	2.4	0.26	1.3
		3.4[b]	103.4[b]	4.2[b]

[a] All lengths (L) are in kilobase pairs. [b] Ratio of parameters for least digested chromatin relative to most digested chromatin (L_{max}/L_{min}).

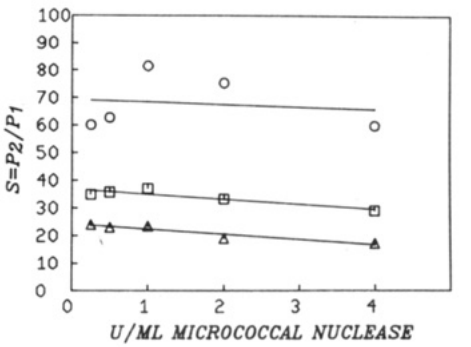

FIGURE 5: Solubility of chromatin pools as a function of length. Chromatin from several digests was analyzed as in Figure 1: bulk chromatin (squares, by optical density); globin and ovalbumin chromatin (circles and triangles, respectively, by dot blot analysis).

We varied the extent of nuclear digestion with micrococcal nuclease to change the overall size distributions. If fiber length per se were the principal determinant of chromatin solubility, then such a variation of size could be expected to alter radically the relative solubility (S) of the population, while a solubility intrinsic to the fiber would be unaffected. Micrococcal nuclease digestions at enzyme concentrations from 0.25 to 4 units/mL yielded samples with a progressive variation of peak mass lengths from 26.9 to 0.26 kb of DNA, with equivalent yields (Table I). The full precipitation curves of five such samples were obtained, and the result (Figure 5, squares) supports the idea that average population length is not the determinant of fiber solubility. This does not exclude the possibility that relative length within a population determines which fibers are soluble (see below).

Effect of Population Length on Solubility of the Globin and Ovalbumin Gene Regions. These results led us to extend our studies to the adult β-globin gene, which has a history of recent transcription in the adult chicken erythrocyte. Globin gene abundance was measured by a dot blotting procedure in soluble fractions (see Materials and Methods), and values of S were obtained for the globin gene chromatin analogous to those for bulk chromatin. For material of intermediate length, a solubility of 65–80% was found for globin gene chromatin, while bulk and ovalbumin chromatin solubilities were 33% and 20%, respectively. With a 10% error for the specific gene determinations, these are significant differences. The inactive chromatin carrying the ovalbumin gene sequence is somewhat more precipitable than bulk chromatin, while the globin gene is much more soluble.

It should be noted that almost all the adult β-globin gene copies of the nucleus (>80%) are typically present in the chromatin fractions (soluble and insoluble) that we are studying. Only a small proportion of the globin gene (at genomic abundance or lower) is contained in the nuclear pellet, insoluble at low ionic strength, that is removed during the initial preparation of chromatin. This is not in any sense contra-

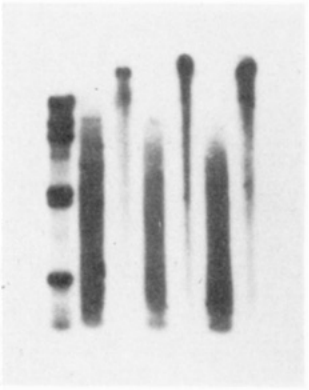

FIGURE 6: Nuclei were digested under standard conditions with micrococcal nuclease. Soluble and insoluble fractions were prepared as described, except that the material was not subjected to an initial centrifugation at low ionic strength. DNA was isolated, and equal amounts were electrophoresed on a 1% agarose gel, transferred to Gene Screen Plus (New England Nuclear) paper, and probed with nick-translated plasmid pCAβG1. Discrete bands in some of the lanes probably reflect nuclease cleavage at hypersensitive sites in the β-globin domain [see McGhee et al. (1981)]. The lane at the extreme left contains the *Hind*III digest of λ-phage DNA. Other lanes, reading from left to right, alternately contain DNA from the soluble and insoluble fractions obtained after digestion with 1, 2.5, or 10 units of micrococcal nuclease.

dictory to the observations of Hentzen et al. (1984), who found that globin DNA of chicken erythrocyte appeared to be attached at higher than genomic abundance to nuclear matrix material insoluble in 2 M NaCl. In our experiments, unlike those of Hentzen et al., the chromatin was obtained from nuclei that had first been digested extensively with micrococcal nuclease. The digestion results in preferential cleavage of chromatin at hypersensitive sites bordering the adult β-globin domain, and these fragments are soluble in low ionic strength buffers.

We next examined the dependence of globin gene solubility on overall chromatin length. This was done by again widely varying the extent of digestion of the chromatin (Table I), titrating with salt, and assaying the supernatant solutions for specific gene abundance by the dot blot method. We emphasize that S measured by this method is a ratio of experimental values, which removes possible systematic errors that might have arisen from choice of probe or details of hybridization conditions. We found that neither the globin nor the ovalbumin gene showed significant dependence of solubility on the average length of the sample (Figure 5) in repeated assays using chromatin from several preparations. There was some scatter in the values of S obtained, but we did not see any significant monotonic trend in the value of S as a function of the average fiber length.

It was shown in Figure 4 that the average size of total DNA in the soluble fraction is smaller than the average size in the insoluble fraction. The same is true of the size distribution of the adult β-globin gene in the two fractions. Figure 6 shows the results of an experiment in which the DNA of the two fractions was transferred to Gene Screen Plus (New England Nuclear) and probed with pCAβG1. At each point in the digestion, the soluble fraction clearly contains globin DNA of smaller size.

The results obtained so far pertain to chromatin released from nuclei with yields that are largely unaffected by the extent of digestion (Table I). However, the incomplete yield leaves a possibility that we actually observe two compensating effects, such as a marked length-dependent solubility that is obscured by a shift in the chromatin pool that is released. This possibility was eliminated by obtaining long chromatin free

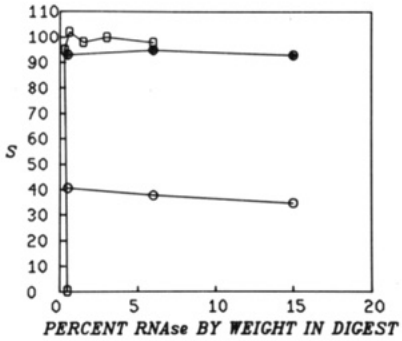

FIGURE 7: RNase effect upon chromatin solubility. Digestion was carried out with large excesses of RNase. Effects are shown on the relative solubility of bulk chromatin (circles) and globin chromatin (inscribed triangles) and on the acid solubility of tRNA (squares) as a control.

from nuclei and then performing the graded nuclease digestion in vitro; this material also showed no length dependence of solubility.

Chemical Differences. We considered several properties of soluble chromatin that might distinguish it from bulk and precipitable chromatin: content of RNA, HMG protein, high molecular weight proteins, and histones H1 and H5; and relative length of DNA.

There are instances where special physical properties of chromatin samples have been traced to RNA content (Gottesfeld & Butler, 1977). We investigated the possibility that this could be the cause of the high solubility levels we detected for active genes. We digested chromatin or an equal mass of RNA with a series of RNase concentrations ranging from 0% to 15% of the nucleic acid mass. In Figure 7, it is shown that the gentlest of these treatments digested the RNA to acid solubility, while the harshest treatment caused only a slight decrease in bulk chromatin solubility that could easily be attributed to stoichiometric binding of RNase rather than digestion. Dot blot analysis of these samples revealed no attenuation of globin solubility after RNase treatment. (RNase does not interfere with the dot blotting procedure.) There was also no RNase-induced alteration in the size of the soluble fraction of bulk chromatin from 9-day embryonic chicken erythrocytes, in which transcriptional levels are thought to be much higher than in adult erythrocytes.

We have tried to determine whether the HMG protein content of the soluble pool affects its solubility properties. We attempted to redistribute HMG proteins by dialyzing a chromatin sample into 0.35 M NaCl and then back to 80 mM ionic strength prior to salt jumping. Another approach was to remove HMG protein by washing nuclei twice in 0.35 M NaCl prior to extracting chromatin. These controls showed only an occasional 5–10% effect of HMG protein mobilization upon solubility of the globin gene, with about 50% of these proteins removed.

Adult chicken erythrocyte chromatin also contains in small quantities a class of high molecular weight proteins that are of unknown function and may in fact not be bound to DNA. We wished to determine whether this class of proteins might be responsible for the features of chromatin solubility that have been described. Whole chromatin of average length 40 nucleosomes was sedimented in isokinetic sucrose gradients at 20, 80, and 175 mM ionic strength. In each of these conditions, the high molecular weight class of proteins sedimented without variation at the top of the gradient, clearly separated from the peaks for the globin gene and bulk chromatin. However, the A_{260} and globin gene lost from the gradient by pelleting between 80 and 175 mM salt were that expected from

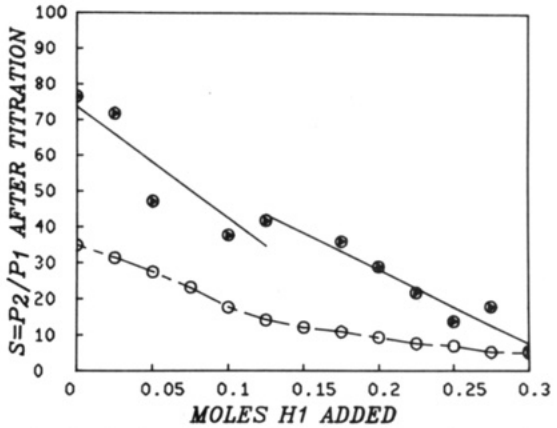

FIGURE 8: H1 titration of whole chromatin. Chromatin samples were combined with excess H1 (see text). The effect on bulk solubility (circles) and globin gene chromatin solubility (inscribed triangles) is shown.

the reactions on unfractionated chromatin, showing that the precipitation phenomenon does not depend upon high molecular weight protein binding to chromatin (data not shown).

H1 Titration Experiment. We reasoned that the apparent depletion of H1 and H5 histones in the supernatant material could play a role in its solubility. The depletion of H5 relative to whole chromatin is 30%, and that of H1 is 15%. A fraction of electrophoretically pure H1 was prepared from adult chicken erythrocyte nuclei. We were able to convert all of the chromatin to a precipitable form by adding to bulk chromatin one-third of a mole of H1 histone per nucleosome. A complete titration curve was also performed, and measurements were made of the globin gene concentration in the resulting supernates (Figure 8). H1 clearly interacts with the globin gene. In addition, the decline of bulk chromatin solubility is superimposable upon the globin gene curve if the data are normalized for untitrated solubility. This suggests that the globin chromatin does not represent a special class within the bulk soluble chromatin pool. The amount of H1 required for complete titration is consistent with the assumption that precipitable chromatin also binds, on a mass basis, the same number of H1 molecules as does soluble chromatin; however, the precipitable fraction is unobservable in the present assay.

Two-Stage Precipitations with Digested Precipitates. As suggested above, it is possible that chromatin sorts into soluble and precipitable fractions based upon *relative* length within the overall population. To test this idea, a preparative precipitation was done at high concentration to generate soluble and precipitable chromatin with the usual length disparity. The precipitable chromatin was rehydrated, and aliquots were redigested to different extents with micrococcal nuclease so that the most redigested material would actually be shorter than the soluble pool. Each member of this family of treated precipitates (Pr1) was then recombined with an aliquot of soluble chromatin (S1) at a concentration for which one could achieve a return through dilution to the initial ionic strength for reassay. The mole fractions of Pr1 and S1 in the mixture were set to match the native solubility of the chromatin preparation at an A_{260} of 5, and a second precipitation was performed. (A control experiment was carried out which showed that the nuclease had been inactivated by chelation prior to exposure to the supernatant.) The second-stage precipitates (Pr2) and supernatants (S2) for each family of reactions were analyzed by DNA gel electrophoresis (Figure 9). As in the case of the first precipitation, the precipitable fractions, Pr2, were longer than the corresponding soluble fractions, S2. (The percent of chromatin soluble also remained

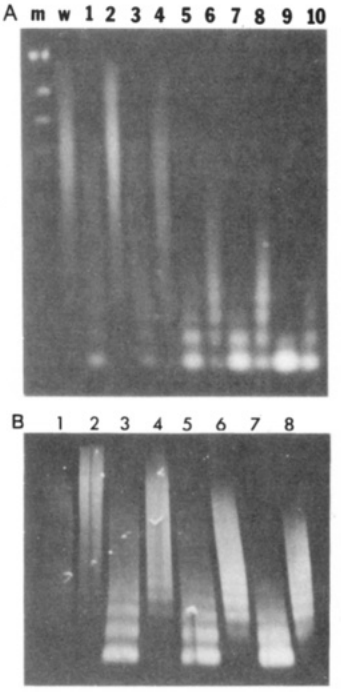

FIGURE 9: Two methods of analyzing DNA from reprecipitation experiments. Chromatin was digested with micrococcal nuclease and fractionated by salt precipitation. The insoluble fraction was redigested and recombined with the soluble fraction as described in the text. The mixture was then reprecipitated. 0.5 μg of DNA from the products of this second-stage precipitation was electrophoresed on agarose in the presence of SDS (Materials and Methods). (A) Chromatin soluble at low salt (most of starting material) and high salt. Lanes 3–10, odd-numbered lane and even-numbered lane to its right are high-salt- and low-salt-soluble material, respectively, from second-stage precipitation using first-stage precipitates that had been redigested with 0, 1, 2, and 4 units/mL micrococcal nuclease. Lane m, λ *Hind*III digest markers; lane w, DNA from whole chromatin. Lanes 1 and 2, supernate and precipitate, respectively, from the first-stage digestion. (B) Comparison of supernates and precipitates from the second-stage precipitation in high salt. Odd-numbered lane and even-numbered lane to its right represent, respectively, soluble and insoluble material from second-stage reactions using first-stage precipitates redigested with 0, 0.5, 1, and 2 units/mL micrococcal nuclease, respectively.

the same.) If precipitability were an intrinsic property of the Pr fractions, then redigestion of Pr1, followed by mixing with S1 and reprecipitation, should have resulted in shortening only of the Pr2 fractions, and not the S2 fractions. In fact, S2 also decreases in length with increasing digestion and contains material that could only have derived from the redigested Pr1 fractions. Thus, the solubility behavior is not an intrinsic property of the fraction. Dot blot analysis showed that the abundance of globin gene in the soluble fraction also declined when redigestion reduced the size of Pr1 so that it was comparable to that of S1 (Figure 10).

We conclude that the identity of the soluble pool is in fact determined by its relative length within the population. In any given chromatin preparation, a fixed fraction of the chromatin is precipitable, and the longest chromatin is contained in this fraction.

Discussion

It has often been noted that chromatin containing transcriptionally active genes is released from chromatin gels or digested nuclei to a greater extent than bulk chromatin (Bloom & Anderson, 1978; Levy et al., 1979; Goldsmith, 1981). In addition, the active chromatin has been shown to be more soluble than bulk chromatin in solubility assays depending upon titration with monovalent and divalent cations. It has never been clear whether these findings are independent

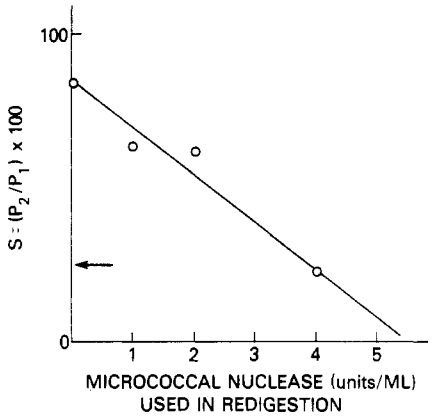

FIGURE 10: Solubility properties of chromatin containing the adult β gene from supernates of the second stage of precipitation following redigestion, as in Figure 9, with varying amounts of nuclease. The adult β-gene concentration was determined by dot blot analysis. The arrow shows the solubility of total chromatin material after the first digestion (no redigestion).

phenomena. In addition, the studies referred to have not been standardized for chromatin length, and so the question remains as to whether enhanced solubility is a reflection of an intrinsic modification found in certain subsets of chromatin. Since Fulmer & Bloomfield (1981) have observed that soluble chromatin is a well-defined pool, even after consideration of length, it seemed important to study this question from the point of view of specific genes and to search for a chemical explanation. We confirm the observation that soluble chromatin and precipitable chromatin are produced from chicken erythrocyte nuclei in a proportion that is independent of the average length of the population in the preparation. [It should be noted, however, that the conditions of digestion and preparation of fractions used in this paper differ considerably from those reported by Fulmer & Bloomfield (1981).] In addition, we find that the active gene is enriched in the soluble fraction, while the inactive ovalbumin gene is enriched in the insoluble fraction. The enrichment, like the pool size itself, is not altered by progressive reduction of the population length. The case for an intrinsic modification in active chromatin rests on the idea that length dependence has been eliminated as an issue by these studies.

Missing from these considerations, however, is the fact that certain histones, H1 and H5, are known to affect chromatin solubility (Muyldermans et al., 1980), that they exchange rapidly between chromatins even at rather low salt concentrations (Caron & Thomas, 1981; Thomas & Rees, 1983), and that such a transfer has been observed to be chromatin length dependent in certain cases (Thomas & Rees, 1983; Renz et al., 1977). We studied chromatin of the soluble fraction and found it to be somewhat depleted in H5 and H1 relative to the insoluble fraction. We found that the abundance of the soluble fraction could be reduced by adding H1 or H5 to the chromatin before precipitation: The soluble fraction was completely eliminated at the point where 0.3 mol of H1 per mol of nucleosome had been added. The simplest interpretation of this result is that the H1 and H5 missing from the soluble fraction are responsible for its solubility properties and that restoration of H1 is therefore sufficient to render the soluble and insoluble fractions indistinguishable. Of course, this is not conclusive proof: The added H1 may not bind correctly when added in this way, or depletion of some other basic protein might be responsible for the properties of the soluble fraction.

Our results suggest that when the salt concentration is raised into the range where precipitation occurs, a rearrangement of histones H1 and H5 can take place that will result in formation of two distinct phases: a soluble phase, somewhat depleted of H1 and H5, and an insoluble phase, in which sufficient H1 and H5 are bound to reduce the overall charge on the chromatin below some critical value. The favorable free-energy change associated with this phase separation would presumably provide the driving force for the unequal distribution of H1 and H5 in the two phases.

It is not surprising that such a phase separation partitions the chromatin according to molecular weight; many polymer systems behave in this way. The experiments described above demonstrate that the insoluble chromatin fraction always contains the higher molecular weight components of the starting population. Furthermore, the experiments show that relative size alone determines solubility properties, since redigestion of an "insoluble" fraction to the point where it is smaller than the "soluble" fraction results in an interchange of attributes. This is true not only for bulk chromatin but also for chromatin containing the β-globin gene as well.

It seems likely that the unusual solubility properties of chromatin containing transcriptionally active genes may be related simply to their relatively greater accessibility to nucleases. If such genes tend to appear in the lower molecular weight fraction, they will tend to be more soluble. This does not mean that there are no differences between the properties and composition of active and inactive chromatin within the nucleus. The high nuclease sensitivity of active chromatin is in itself evidence of such differences, and the results of Kimura et al. (1983) and of Smith et al. (1984) indicate that active chromatin is probably depleted of histone H1 (and/or H5) in vivo. Our results show, however, that studies of chromatin fractions that differ in solubility do not provide a simple way to assess differences between active and inactive chromatin. It should be clear that caution is necessary in identifying the chemical components of such fractions when those components are capable of exchange under the conditions of isolation, and when there are driving forces that can affect their distribution.

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Effects of DNA Binding Proteins on DNA Methylation in Vitro†

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ABSTRACT: The inheritance of DNA methylation patterns may play an important role in the stability of the differentiated state. We have therefore studied the inhibitory effects of DNA binding proteins on DNA methylation in vitro. Mouse L1210 cells grown in the presence of 5-azacytidine acquire hemimethylated sites in their DNA. Purified hemimethylated DNA accepted methyl groups from *S*-adenosyl-L-methionine in the presence of a crude maintenance methylase more readily than purified DNA isolated from cells not exposed to 5-azacytidine. On the other hand, chromatin fractions isolated from cells grown in the presence or absence of 5-azacytidine were poor substrates for the maintenance methylase irrespective of the number of hemimethylated sites present in the DNA. Inhibition of DNA methylation was shown to be associated primarily with chromatin proteins bound to DNA, and trypsinization of nuclei increased their methyl accepting abilities. Methyl acceptance was increased by salt extraction of chromosomal proteins. These data suggest that association of histones with DNA may play a role in the modulation of methylation patterns.

A eukaryotic cell must accurately replicate both its genotype and its phenotype when traversing S phase. Newly replicated DNA is rapidly organized into nucleosomes by the association of proteins with DNA (DePamphilis & Wassarman, 1980; McGhee & Felsenfeld, 1980; Igo-Kemenes et al., 1982), and nucleic acid–protein interactions are likely to be important in controlling gene expression. However, the processes by which cellular phenotypes are maintained or altered are still unclear. Evidence indicating that the 5-methylcytosine (5-MeCyt)[1] pattern present in DNA is a component of the multilevel control of gene expression has accumulated in many systems (Razin & Riggs, 1980; Ehrlich & Wang, 1981; Doerfler, 1983). The correlation between hypomethylation of specific gene sequences and the ability of these genes to be expressed has been shown (Busslinger et al., 1983; Lieberman et al., 1983; Stein et al., 1983), and agents which induce DNA hypomethylation can induce changes in gene expression (Jones & Taylor, 1980; Venolia et al., 1982). Thus, there may be a cause and effect relationship between losses of 5-MeCyt residues at specific sites and cellular differentiation. The copying of 5-MeCyt patterns in DNA may depend upon a "maintenance methylase" (Riggs, 1975; Holliday & Pugh, 1975). This enzyme would be responsible for maintaining the methylation pattern over many generations (Stein et al., 1982) and would transfer methyl groups from SAM to hemimethylated sites, consisting of a cytosine residue opposite a methylated CG doublet in duplex DNA.

Since interactions between the enzyme and the DNA substrate are necessary for methylation to occur, we have studied the effects of DNA binding proteins on the ability of hemimethylated DNA to accept methyl groups from SAM in a test

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[1] Abbreviations: 5-MeCyt, 5-methylcytosine; SAM, *S*-adenosyl-L-methionine; 5-aza-C, 5-azacytidine; Tris-HCl, tris(hydroxymethyl)-aminomethane hydrochloride; EDTA, ethylenediaminetetraacetic acid; Cl_3CCOOH, trichloroacetic acid; SSC, 0.15 M NaCl and 0.015 M sodium citrate; SDS, sodium dodecyl sulfate.

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