Fractionation of Chromatin by Thermal Chromatography†

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ABSTRACT: Chromatin isolated from avian erythrocytes was fractionated by absorbing to hydroxylapatite and elution by means of a temperature gradient. The elution profile paralleled the optical thermal denaturation curve. DNA was extracted from the chromatin fraction eluting at the lowest temperature and hybridized with erythrocyte RNA. The results demonstrate that this fraction contains those DNA base sequences complementary to red cell RNA. However, this fraction is not enriched for sequences transcribed in chicken liver.

An understanding of the mechanism of gene expression in eucaryotic cells would be greatly facilitated by the ability to separate active and inactive segments of chromatin. Such a separation can be achieved by shearing and differential centrifugation (Frenster *et al.*, 1963; Frenster, 1965). The same general approach has been used with success to demonstrate the localization of highly redundant satellite DNA sequences in condensed segments of chromatin (Yunis and Yasmineh, 1970; Yasmineh and Yunis, 1970; Duerksen and McCarthy, 1971).

The thermal denaturation profile of chromatin extends over a considerably larger range of temperature than does that for DNA. Furthermore, such melting profiles often show evidence of two or more resolvable components (Marushige and Ozaki, 1967; Paoletti and Huang, 1969). In fractionated chromatin the highly condensed portions display higher mean thermal denaturation temperatures than the extended fraction (Frenster, 1965; Duerksen and McCarthy, 1971). These and other observations imply a relationship between the thermal denaturation temperature and the template activity and suggest the possibility of fractionating chromatin on the basis of differential melting behavior. In the succeeding pages, we present a simple method based upon thermal denaturation and elution of chromatin from hydroxylapatite, together with evidence that the fractionation correlates with the *in vivo* template activity of chromatin segments (McCarthy *et al.*, 1971).

Materials and Methods

Preparation of Chromatin. The method employed for the preparation of chromatin was a combination of procedures described by Smith *et al.* (1969) and the glycine extraction procedure of Kongsvik and Messineo (1970). This method was preferable for these studies because it was rapid, very efficient, and did not require pelleting the chromatin which sometimes leads to aggregation and difficulty in resuspension. The criteria used for purity of the chromatin were spectral qualities (260/240 mμ = 1.4 ± 0.05 and 260/280 mμ = 1.7 ± 0.05) and consistent melting behavior. Chromatin prepared in this manner has spectral qualities, melting characteristics, and template activity almost identical with chromatin prepared by pelleting nuclei through sucrose, lysis of nuclei, and centrifugation of chromatin through sucrose.

Chromatin was extracted from red blood cells of embryonic chickens ranging in age from 9 to 15 days after fertilization. After cracking the eggs and exposing the amniotic membrane below the air sac, large blood vessels in the membrane were broken with forceps and the embryos were allowed to bleed for approximately 2 min. The fluid was withdrawn and centrifuged at 500*g* for 10 min to pellet red blood cells. The cells were resuspended in cold 0.1% Tween 80 and sheared in a Virtis homogenizer for 2 min at 40 V. The preparation was then checked microscopically for cell breakage and homogenization continued if breakage was incomplete. The homogenate was centrifuged at 1000*g* for 10 min. The crude nuclear pellet was washed several times in 0.1% Tween 80 and centrifuged again. The nuclei were then resuspended in 0.05 M Tris-HCl (pH 7.5) and washed in this buffer until the supernatant was clear. The nuclei were washed once in 0.01 M glycine (pH 6.0), centrifuged at 1000*g*, and resuspended in glycine. In general, lysis of the nuclei did not occur until the second addition of 0.01 M glycine. The preparation was maintained at 4° in 0.01 M glycine for at least 4 hr, and preferably overnight. The chromatin was then sheared at high speed in the Virtis homogenizer for 1 min to reduce the viscosity and solubilize the chromatin. The preparation was centrifuged 15 min at 10,000*g* to remove insoluble impurities. Less than 2% of the DNA present in the chromatin was insoluble under these conditions when a [^{3}H]thymidine-labeled chromatin preparation was monitored.

Radiolabeled red blood cell chromatin was prepared by making a small hole in the shell and placing a solution of [^{3}H]thymidine on the amniotic membrane. The eggs were incubated for varying periods of time at 37° and in some instances a second addition of label was made. Chromatin was later extracted from red blood cells of eggs treated in this manner, and specific activities of DNA in chromatin were obtained which varied between 400 and 1300 cpm per μg of DNA.

Chicken liver chromatin was extracted from freshly excised tissue of 19-day-old embryos. The livers were homogenized in 0.075 M NaCl–0.024 M EDTA (pH 8.0) at high speed in a Teflon motor-driven homogenizer. The homogenate was centrifuged for 10 min at 1000*g*. The nuclear pellet was washed three to five times by resuspension in the above buffer and centrifuged each time for 10 min at 1000*g*. The nuclei were then suspended in 0.05 M Tris (pH 7.5) and further extraction was then carried out in a manner analogous to that employed for red blood cell chromatin.

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Chromatin was always extracted from fresh cells or tissue and was used within 3 days of its preparation.

Preparation of DNA. Chicken DNA was prepared from 12-day whole embryos as described elsewhere (McCarthy and Hoyer, 1964) or extracted from red blood cells according to the Marmur (1961) procedure. DNA was prepared from fractionated chromatin by incubating the chromatin in 0.5% sodium dodecyl sulfate and 10 μg/ml of predigested Pronase for 16 hr at 37°. The SDS was then removed by precipitation with potassium phosphate (pH 7) and the DNA was precipitated with ethanol.

Radioactively labeled DNA was prepared from chromatin fractions by reduction with ^{3}H-labeled $NaBH_4$ according to a method described by Lee and Gordon (1971). The DNA was extracted from this fraction of the chromatin as described above and was then degraded to a single-strand molecular weight of 1.7×10^5 by limited depurination and alkaline hydrolysis (McConaughy and McCarthy, 1970; Grouse *et al.*, 1972). DNA (1.2 mg) in 0.5 ml of borate buffer was irradiated in the presence of 30 mCi of [^{3}H]$NaBH_4$ (specific activity 157 mCi/mg). This *in vitro* labeling procedure yielded DNA with a specific activity of 3000 cpm/μg.

Extraction of RNA. RNA was prepared from chicken embryo erythrocytes or adult liver according to a method described earlier by Shearer and McCarthy (1969).

The red blood cells were collected from 12- or 14-day-old embryos. They were washed once in 0.15 M NaCl and centrifuged at 500*g* for 10 min. The cells were resuspended in 0.15 M NaCl and one-fourth volume of a solution of 1.0 M LiCl–0.01 M sodium acetate (pH 5.2) and 2.5% sodium dodecyl sulfate was added. The solution was extracted with hot phenol (preequilibrated at 67° and saturated with a five-fold dilution of the above buffer). The water–phenol mixture was sonicated in a Branson Sonifier LS for 1 min at maximum output in order to reduce the viscosity of the aqueous phase and break up clumps. Sonication increased the efficiency of RNA extraction by severalfold. After centrifugation, RNA in the aqueous phase was precipitated with two volumes of cold ethanol for at least 4 hr at 0°. The RNA was purified by several ethanol precipitations, DNase digestion, and another phenol extraction at room temperature followed by additional ethanol precipitations. The chicken liver tissue was purchased at the Pike Place Market in Seattle. The tissue was minced with scissors and washed several times in saline. The soft tissue was homogenized two strokes with a motor-driven Teflon pestle at moderate speed. One-fourth volume of the LiCl–sodium acetate–sodium dodecyl sulfate solution was added and the RNA purification was then identical with that of the embryo erythrocytes. Two adult chicken livers yielded 100 mg of purified RNA.

Spectrophotometric Melting Curves. Absorbance changes during the thermal denaturation of DNA or chromatin were measured at 260 mμ in a Gilford-Beckman spectrophotometer fitted with an automatic recorder. The melting experiments were carried out in phosphate buffer, normally 0.025 M, because phosphate buffer is essential for hydroxylapatite chromatography. The percent hyperchromicity observed at a given temperature was plotted on linear graph paper or on normal probability paper according to a method proposed by Knittel *et al.* (1968).

Fractionation of DNA on Hydroxylapatite. Chicken erythrocyte DNA was sheared at 12,000 psi in a French pressure cell. This DNA was dialyzed against 0.12 M phosphate buffer (pH 6.8) and adsorbed to a Clarkson hydroxylapatite column at 60°. The column was consecutively washed at 90, 95, and 100° in 0.12 M phosphate buffer to obtain three fractions of DNA differing in average G + C content (Miyazawa and Thomas, 1965). These fractions represented 20, 51, and 29% of the total DNA, respectively. The DNA was concentrated, dialyzed against 0.01 × SSC (SSC is 0.15 M NaCl and 0.015 M sodium citrate), and used in reassociation experiments with DNA from fractionated chromatin to be described in the next section of this paper.

Fractionation of Chromatin on Hydroxylapatite. Since thermal dissociation can be used to fractionate DNA by chromatography on hydroxylapatite (Bernardi, 1965; Miyazawa and Thomas, 1965) this same methodology has been applied to DNA sequences in chromatin. The chromatin was sheared in the French pressure cell as 12,000 psi prior to adsorption to either Bio-Rad or Clarkson hydroxylapatite in 0.12 M phosphate buffer (pH 6.8) at 60°. The hydroxylapatite column was heated to increasing temperatures and single-stranded DNA eluted with 0.12 M phosphate. Elution of any DNA remaining on the column at 100° was accomplished by washing the column with 8 M urea–0.24 M phosphate buffer–0.01 M EDTA (Britten *et al.*, 1970). Although this technique can be used for fractionation of DNA sequences of either radioactively labeled or unlabeled chromatin, it is preferable to use chromatin in which the DNA is labeled since it is difficult to monitor the chromatography of the DNA in chromatin by absorbance due to light scattering produced by the denaturation and elution of some of the proteins associated with the chromatin. The radioactive DNA was precipitated with trichloroacetic acid and counted in a liquid scintillation spectrometer.

Reassociation of DNA in Solution. The kinetics of reassociation of sheared or depurinated denatured chicken DNA or DNA extracted from fractionated chromatin were assayed by hydroxylapatite chromatography. These reactions were carried out in 48% formamide and 5 × SSC at 37° as previously described (McConaughy *et al.*, 1969). Radioactively labeled DNA was incubated alone or with an excess of unlabeled total DNA or DNA fractionated on hydroxylapatite according to per cent G + C. The ratio of unlabeled DNA to radioactive DNA was 1000:1. At various times aliquots of the reaction mixture were removed, diluted 50-fold in 0.12 M phosphate buffer (pH 6.8), and adsorbed to Bio-Rad hydroxylapatite columns at 60°. The DNAs were then separated into single and double-stranded fractions and the radioactivity in each fraction monitored by precipitation with trichloroacetic acid and counted in a liquid scintillation spectrometer.

DNA–RNA Hybridization. DNA extracted from fractionated chicken erythrocyte chromatin was hybridized with RNA prepared from chicken red blood cells or chicken liver. Hybridization of the liver RNA to total chicken DNA or an isolated nonrepetitive fraction of chicken DNA was also studied. These unique sequences were separated from the redundant fraction by incubating the DNA in 48% formamide and 5 × SSC until the reassociation of the redundant fraction was complete (C_0t = 50). This results in a removal of most, although clearly not all redundant sequences. The resultant single- and double-stranded molecules were separated on hydroxylapatite (Britten and Kohne, 1968; McConaughy and McCarthy, 1970). Annealing was carried out at RNA concentrations of 20 mg/ml and DNA concentrations of 10 μg/ml in 0.4 M phosphate buffer at 70°. The DNA–RNA mixtures were sealed in capillary tubes, denatured at 100° for 10 min and incubated for periods up to 15 days. The solutions were diluted 50-fold in 0.14 M phosphate buffer and the amount of hybridization assayed by hydroxylapatite chromatography.

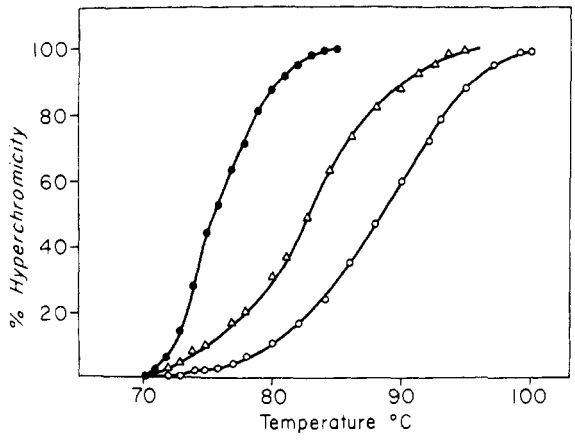

FIGURE 1: Thermal denaturation profiles of chicken DNA or chromatin in 0.025 M phosphate buffer (pH 6.8). Samples of DNA (●), liver chromatin (△), or erythrocyte chromatin (○) were heated to increasing temperatures in 1-cm path-length cuvets and the percent hyperchromicity monitored at 260 mμ as a function of temperature.

Results

Melting Profiles of Chromatin. It is well established that the thermal denaturation profiles of chromatin extend over a broader range of temperature than those for free DNA. Moreover, the mean thermal denaturation temperature is several degrees higher than for DNA (Huang and Bonner, 1962). This presumably results from the stabilization of the DNA double strand by the associated proteins. In accordance with the proposition that various segments of chromatin may be differentially stabilized by proteins in a manner connected with gene regulation, chromatin melting curves often show evidence of multiple components. In particular, it has been suggested that the lower melting components might be representative of genetically active segments (Huang *et al.*, 1964). Indeed, Frenster's (1965) experiments show a lower melting temperature in active than in repressed chromatin isolated from the same tissue.

Two typical chromatin melting curves for chicken erythrocyte and chicken liver chromatin are shown in Figure 1. In both cases most of the hyperchromic shift occurs at a higher temperature than is the case for chicken DNA. However, a fraction of each chromatin does melt between 70 and 80°, the proportion being much smaller for erythrocyte chromatin than for liver chromatin. Again, this finding is consistent with the proposition of differential low melting on the part of the active segments which might well be less abundant in highly differentiated red cells.

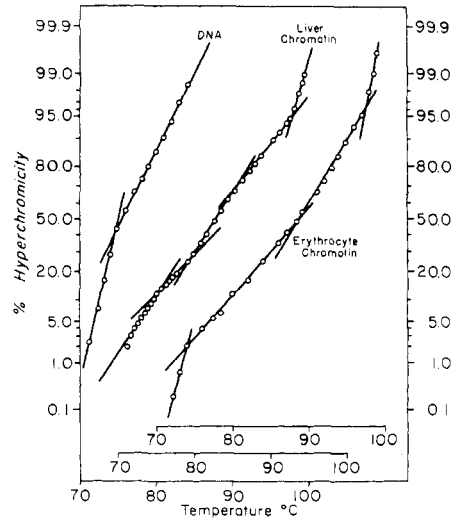

FIGURE 2: Thermal denaturation profiles of chicken DNA or chromatin extracted from liver or erythrocytes. Sheared DNA or chromatin was heated at 50 μg/ml in 0.025 M phosphate buffer (pH 6.8). The percent hyperchromicity at 260 mμ was monitored as a function of temperature and plotted on a probability scale.

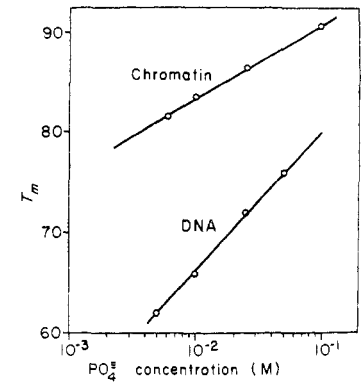

FIGURE 3: Dependence of the T_m of chicken erythrocyte DNA or chromatin on the phosphate concentration during the thermal dissociation.

An attempt to resolve these chromatin melting curves into separate components was made by transforming the data and plotting on probability coordinates (Duerksen and McCarthy, 1971). In principle, such a strategem might resolve overlapping components. This is certainly the case for DNA melting curves where satellites and nongaussian distributions of base composition are revealed (Knittel *et al.*, 1968; McConaughy and McCarthy, 1970). Although artifacts are possible, the method seems particularly appropriate for resolving small low-melting components. Both in liver and red cell chromatin a low-melting component is apparent (Figure 2). A 2.5% low-melting component is readily apparent in red cell chromatin, whereas the first clearly resolved component in liver chromatin amounts to some 15%. Although other components are apparently resolved, subsequent attention was focused on the lowest melting fraction, particularly that in red cell chromatin.

The purpose of the experiments described subsequently was to test the proposition that this small fraction was representative of DNA segments transcribed in red cells. In order to carry out this test, it was necessary to utilize the differential melting behavior as a preparative method for fractionating chromatin. Since thermal elution of DNA from hydroxylapatite is an efficient method for fractionating DNA based upon differential melting temperatures (Miyazawa and Thomas, 1965), the same principle was applied to chromatin.

Hydroxylapatite fractionation of DNA is most effective with about 0.12 M phosphate in terms of discrimination between double- and single-stranded DNA. Thus, in order to estimate an appropriate temperature range for chromatin fractionation, it was necessary to estimate the melting profile of chromatin in this higher phosphate concentration. The relationship of chromatin T_m to the logarithm of phosphate molarity is linear as is the case for DNA, but the slope is quite different (Figure 3). For an increase of phosphate molarity from 0.025 to 0.12 M, the increase in T_m is 5° whereas for DNA the increase is 10° (Figure 3).

Fractionation of Chromatin on Hydroxylapatite. Using the above correction factor, thermal elution of chick erythrocyte

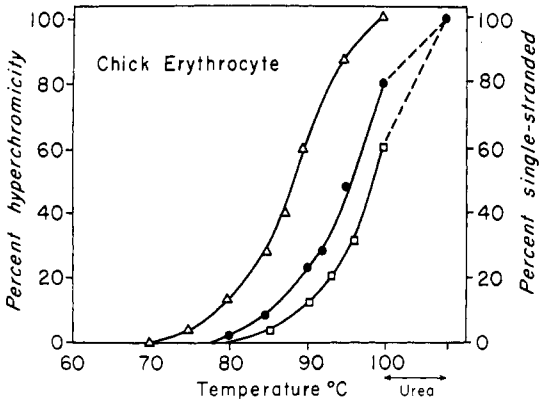

FIGURE 4: Thermal dissociation profiles of chick erythrocyte chromatin by spectrophotemetric measurements or hydroxylapatite chromatography. Sheared chromatin was heated at 50 μg/ml in 0.025 M phosphate buffer (pH 6.8) and the hyperchromicity measured as a function of temperature in a spectrophotometer at 260 mμ in 1-cm path-length cuvets (△). Sheared chromatin was also dissociated on a Bio-Rad (●) or Clarkson (□) hydroxylapatite column in 0.12 M phosphate buffer and the amount of single-stranded material was monitored as a function of temperature. Elution of any material remaining at 100° was accomplished by washing the column with 8 M urea–0.24 phosphate–0.01 M EDTA.

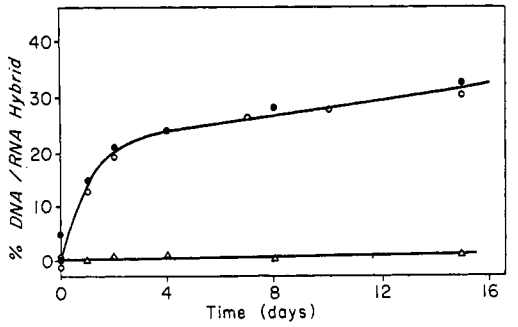

FIGURE 5: Formation of DNA–RNA hybrids between DNA extracted from chick erythrocyte chromatin fractions and chick erythrocyte RNA. 4 μg of DNA from *in vitro* ^{3}H-labeled (●) or *in vivo* ^{3}H-labeled (○) erythrocyte chromatin fraction A or *in vivo* ^{3}H-labeled fraction B (△) was incubated with 8 mg erythrocyte RNA. The incubations were carried out in 0.4 ml of 0.4 M phosphate buffer (pH 6.8) at 70°. At various times 50-μl aliquots were removed and diluted to 0.14 M phosphate buffer and the amount of DNA–RNA hybrid assayed by hydroxylapatite chromatography.

chromatin was followed after absorption to a hydroxylapatite column. The chromatin was absorbed at 60° with 100% efficiency and eluted in the range of 78–100° (Figure 4). A small fraction remaining at 100° was recovered after washing with 8 M urea. As is the case for DNA, the temperature of elution varied slightly according to the batch of hydroxylapatite and the supplier. Batches from Clarkson gave consistently higher T_m's than did those sold by Bio-Rad. Nevertheless, in both cases the elution profiles paralleled the optical denaturation profiles measured in the spectrophotometer.

The DNA eluted from the column appeared to be single-stranded associated with some protein. However, some chromosomal protein appears to be lost in the procedure.

Characterization of DNA in Fractionated Chromatin. If the fractionation based upon differential thermal elution has biological significance, then the DNA in the various fractions might differ in base sequence and complementarity with red cell RNA. In particular, we wished to test the possibility that the first few per cent eluted at the lowest temperature contained the DNA base sequences transcribed in chick erythrocytes. For this purpose chromatin was prepared from chick embryos exposed to [^{3}H]thymidine from the 8th to the 15th day of incubation. The red cell preparation was contaminated by less than 0.1% leucocytes which contained less than 1.5% of the ^{3}H-labeled DNA. The erythrocyte chromatin was prepared and fractionated as before to obtain a low-melting 3% fraction A and the remaining 97% fraction B. Unlabeled chromatin was fractionated in a parallel experiment to obtain two similar fractions. DNA was extracted and purified from each fraction. In the case of the unlabeled DNA, ^{3}H was introduced *in vitro* by photoreduction with [^{3}H]$NaBH_4$. The four labeled DNA samples were then fragmented to a size appropriate for hybridization, 1.7×10^5 single-stranded molecular weight, by limited depurination (McConaughy and McCarthy, 1970; Grouse *et al.*, 1972). The relationship of the base sequence of these four DNAs to red cell RNA was then investigated by hybridization assays.

Hybridization mixtures contained low concentrations of labeled single-stranded DNA and high concentrations of unlabeled erythrocyte RNA. The reaction is RNA driven and DNA–RNA hybrid formation proceeds with only very low levels of unique sequence DNA renaturation given a sufficiently long incubation period (Gelderman *et al.*, 1971; Hahn and Laird, 1971; Brown and Church, 1971; Hough and Davidson, 1971). The data presented in Figure 5 demonstrates that both low-melting fractions A reacted with RNA to an extent approaching 30%, while the remaining major fraction B exhibited little or no complementarity with red cell RNA. At each time point the data were corrected for the amount of double-stranded DNA formed in the absence of RNA, *i.e.*, renatured redundant DNA; 10% at 10 days.

This experiment shows that fraction A DNA is complementary to red cell RNA. However, it remains to be shown that this complementarity is specific for red cell RNA rather than RNA from any cell or tissue. For this reason, the experiment was repeated using chicken liver RNA. In this case, ^{3}H *in vitro* labeled fraction A reacts with liver RNA to only about 4% (Figure 6). This suggests that very little of the DNA transcribed in erythrocytes is represented as RNA molecules in the

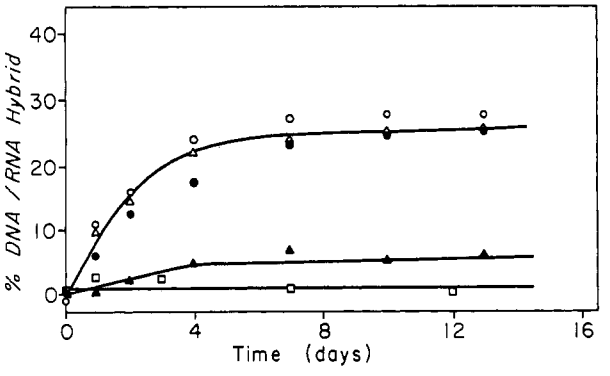

FIGURE 6: Formation of DNA–RNA hybrids between chicken DNA or DNA extracted from chicken erythrocyte chromatin fractions and chicken liver RNA. 4 μg of DNA from H-labeled fraction B (△) or ^{3}H-labeled fraction A (▲) was incubated with 8 mg of liver RNA in 0.4 ml of 0.4 M phosphate buffer at 70°. Incubations were similarly carried out with liver RNA and total DNA (○) or unique DNA sequences (●). The liver RNA–*in vivo* ^{3}H-labeled fraction B mixture was also treated with alkali to hydrolyze the RNA prior to incubation (□). The amount of DNA–RNA hybrid was assayed by hydroxylapatite chromatography.

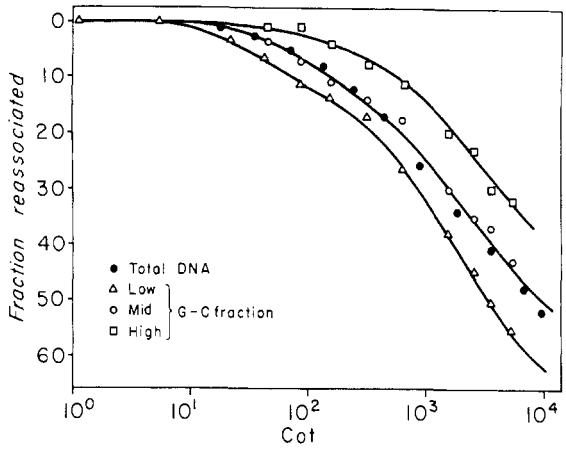

FIGURE 7: Reassociation in *in vitro* labeled DNA extracted from chicken chromatin fraction A with a 1000-fold excess of total chicken DNA or chicken DNA which has been fractionated according to G + C by hydroxylapatite chromatography. The reactions were run by incubating 3.3 μg of *in vitro* labeled fraction A with 3.3-mg total or fractionated DNA in 1.0 ml of 48% formamide 5 × SSC at 37° for various times. The reactions were carried out to a C_0t = 10,000 for the cold DNA and C_0t = 1 for the ^{3}H-labeled DNA; 100 μl of each reaction mixture was diluted to 5 ml with 0.14 M phosphate buffer and single- and double-stranded complexes were assayed by hydroxylapatite chromatography.

liver. Conversely, fraction B DNA did react to a sizeable extent with liver RNA.

Essentially the same saturation value was obtained for hybridization with liver RNA when total DNA or unique sequences purified from total DNA were used (Figure 6). This is to be expected if most of the sequences transcribed in liver are located in the major 97% fraction of erythrocyte chromatin.

One further control experiment is necessitated by the very high ratio of RNA to DNA used in these experiments. With such a large amount of RNA, it is possible that the reaction is really due to contaminating unlabeled DNA. That this is not the case is demonstrated by the absence of any reaction when the liver RNA preparation was treated with alkali (Figure 6).

The fact that the low-melting fraction A of chicken erythrocyte chromatin contains a special set of DNA base sequences can also be demonstrated by means of a G + C fractionation. Total chicken DNA was sheared and fractionated by thermal chromatography on hydroxylapatite according to Miyazawa and Thomas (1965). The various fractions were pooled to give a low G + C, a mid G + C fraction, and a high G + C fraction. Each was then mixed in 1000-fold excess with ^{3}H-labeled single-stranded DNA isolated from fraction A. The kinetics of renaturation, illustrated in Figure 7, show that the [^{3}H]DNA reacts most rapidly with the low G + C fraction, and more slowly with the high G + C fraction. Thus, fraction A must contain DNA of lower than average G + C content. It should be noted that the difference in the reaction rate cannot be attributed to the G + C difference itself, since high G + C DNA renatures more rapidly than low G + C DNA (Wetmur and Davidson, 1968).

Discussion

Several attempts to fractionate chromatin preparations have been made over the past few years. Methods based upon differential centrifugation of sheared chromatin have proved useful for separating active and inactive segments of thymocyte chromatin (Frenster *et al.*, 1963; Frenster, 1965) and more recently for isolating fractions containing highly redundant DNA (Yunis and Yasmineh, 1970; Yasmineh and Yunis, 1970; Duerksen and McCarthy, 1971). However, in our hands the resolution has proved to be limited. In this paper we present evidence for the efficacy of another method based upon differential melting behavior. Two conclusions are evident from this preliminary study: segments of chromatin active in RNA synthesis exhibit a lower thermal denaturation temperature and the DNA moiety of these segments may be purified by thermal elution gradients. It is apparent that the method is inappropriate for characterization of chromosomal protein in chromatin fractions since high temperature and the hydroxylapatite itself leads to denaturation and poor recovery. Furthermore the high temperature and relatively high ionic strength would tend to promote some exchange of chromosomal protein (Clark and Felsenfeld, 1971).

In the case of chick erythrocyte chromatin the fractionation appears to be quite efficient. The DNA of the major fraction, 97% of the total gives little or no reaction with red cell RNA. On the other hand, the smaller fraction contains DNA which reacts to almost 30%. Assuming one-strand transcription, this implies that about 60% of this DNA is complementary to RNA. Alternatively stated, the active DNA has been enriched some 20-fold as a result of this simple procedure. This part of the genome, although complementary to erythrocyte RNA, reacts only poorly with liver RNA. Indeed, the low reaction may result from the presence of red blood cells in the liver. Thus, one may conclude that the fractionation depends on the state of the genome in a particular cell rather than an intrinsic property of those DNA segments tending to make them low-melting. This conclusion must be qualified slightly however, since other experiments did indicate that active DNA in chicken red cells is of lower than average G + C. This could contribute to the low-melting behavior, although a G + C effect alone could not account for a T_m 15° below that of total chromatin. Unfortunately, we are as yet unable to ascribe the lower T_m to any difference in chemical composition of active chromatin. In accordance with currently popular models of chromatin structure, it could be due to the presence of acidic proteins neutralizing histone binding and stabilization, the complete absence of histones or modification of histones by methylation, acetylation, or phosphorylation. Resolution of this issue must await the application of other fractionation methods.

Several other features of these results attest to the validity of the approach. In the case of chick liver RNA, some 25% of the total DNA can be made double-stranded. These DNA base sequences are not enriched in the low-melting fraction of red cell chromatin. In fact, they are specifically excluded. Together with the fact that a much higher fraction of liver chromatin is low melting, these facts make it likely that quite different segments of the genome are low melting in liver chromatin. Parallel experiments with chicken liver chromatin suggest that the hydroxylapatite separation is applicable to other chromatin preparations since DNA isolated from low-melting liver chromatin again showed a higher capacity to hybridize with homologous RNA.

In summary, the method is simple and reproducible. Perhaps its applicability is limited by the inability to obtain a quantitative yield of undegraded chromatin proteins. For this purpose, a fractionation method based upon gel filtration appears to be much more promising (McCarthy *et al.*, 1971; M. Janowski and B. J. McCarthy, in preparation, 1971).

Nevertheless, the ability to enrich for active DNA segments, independently of the normal RNA–DNA hybridization procedure, adds to the repertoire of approaches to the mechanism of gene regulation in eucaryotes.

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Role of Zinc in Horse Liver Alcohol Dehydrogenase. Coenzyme and Substrate Binding†

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ABSTRACT: Formation of binary and ternary complexes of horse liver alcohol dehydrogenase (EC 1.1.1.1) and the zinc-free (apo) form of the enzyme has been investigated by fluorescence techniques. Both the native enzyme and the catalytically inactive apoenzyme bind NAD, NADH, and ADP-Rib, all of which quench the tryptophan's fluorescence emissions of the enzymes. The dissociation constants for the coenzymes are not significantly different for the two forms of the enzyme. In contrast to the native enzyme, the two sites in the apoenzyme do not appear to bind NADH independently. The sites are independent, however, in the binding of NAD in both apo- and native enzymes. The apoenzyme forms ternary complexes of coenzyme and substrate competitive inhibitors, such as isobutyramide and nitroethanol, with dissociation constants comparable to those of the native enzyme. The apoenzyme also forms an enzyme–NAD–butanol complex. The demonstration that the apoenzyme binds coenzymes and forms enzyme–coenzyme–substrate (or inhibitor) complexes with essentially the same dissociation constants as the native enzyme's rules out a role for zinc in binding coenzyme or substrate.

Zinc is known to be a constituent of many metalloproteins. Among the best-studied zinc enzymes are perhaps carboxypeptidase and carbonic anhydrase. It has been found that zinc in these two enzymes directly interacts with the substrates for the enzymes and presumably participates in catalysis (Lipscomb *et al.*, 1968; Wang and Riepe, 1968). Alcohol dehydrogenase (EC 1.1.1.1) is one of the few pyridine nucleotide dependent oxidoreductases that is a zinc-containing

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