DNA-Binding Chromosomal Non-Histone Proteins. Isolation, Characterization, and Tissue Specificity†

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ABSTRACT: A fractionation schedule is described which allows the isolation of a group of chromosomal non-histone proteins (NP) with affinity for DNA. In polyacrylamide gel electrophoresis these proteins isolated from rat liver are represented principally by a group of low molecular weight polypeptides. The NP fraction comprises about 2-4% of the total chromatin protein content in rat liver or Novikoff hepatoma. Experiments in vivo and in vitro revealed that the NP proteins do not incorporate significant amounts of ^{32}P Complexes of the chromosomal proteins NP with homologous DNA are immunologically tissue specific and the specificity can be transferred by reconstituting the NP proteins from one tissue to the residual chromatin from another.

Differential gene activation is of prime importance in determining cellular differentiation in eukaryotes. It appears that chromosomal proteins, both the histones and non-histones, play important roles in biochemical mechanisms by which specific genes are activated and regulated. While the histones are possibly involved in less specific regulations and in determining the structure of chromatin, the highly sophisticated activation of selected genes may be mediated by some of the chromosomal non-histone proteins (Paul, 1970, Baserga and Stein, 1971, Spelsberg et al., 1972, Hnilica, 1972, Stein et al., 1974). If chromatin of eukaryotic cells indeed contains gene regulatory proteins, it can be anticipated that such proteins will be present in small quantities and that they will exhibit strong affinity for the regulated substrate, i.e., DNA.

A search for biologically active chromosomal proteins is not possible without a suitable method for the fractionation of chromatin and its components. Irreversible denaturation of the components must be avoided, especially the use of extremes of pH or solvent environment. Because of the limited solubility and strong interactions of some of the chromosomal components, it is necessary to use urea or guanidine solutions in most fractionation schemes. It was shown by several investigators (Bekhor et al., 1969; Huang and Huang, 1969; Gilmour and Paul, 1969; Spelsberg et al. 1971) that the presence of urea in buffered salt solutions used for chromatin dissociation or fractionation not only facilitates the solubilization of its components but also does not destroy its transcriptional and immunological properties. We present here a method for the separation of chromosomal proteins into three main categories, each characteristic in its properties and composition.

Materials and Methods

Fractionation of Chromatin. Unless specified, all preparative work was performed at 2-4°C. The nuclei and chromatin were isolated by previously described procedures (Spelsberg and Hnilica, 1971; Wilhelm et al., 1972). After washing with 0.3 *M* NaCl and 1.5 m*M* NaCl-0.15 m*M* sodium citrate, the chromatin preparations were homogenized in 5.0 *M* urea-50 m*M* sodium phosphate buffer (pH 7.6) and stirred in an ice bath for 2 hr. The homogenate was centrifuged at 15,000*g* for 60 min. The pellets resulting from this centrifugation were resuspended in a fresh portion of buffered 5.0 *M* urea-50 m*M* sodium phosphate buffer (pH 7.6) by gentle homogenization and the mixture was centrifuged at 15,000*g* for 60 min. The combined supernatants, containing most of the chromatin non-histone proteins (fraction UP), were concentrated by ultrafiltration (Amicon PM 10 membrane). The pellets were gently rehomogenized in a solution of 5.0 *M* urea-2.5 *M* NaCl-50 m*M* sodium succinate buffer (pH 5.0). To assure complete dissociation of histones, the mixture was stirred in an ice bath for 2-3 hr. After this time, the DNA and small amount of non-histone proteins were pelleted by ultracentrifugation at 110,000*g* (max) for 36 hr. The histone-containing supernatants (fraction HP) were concentrated by ultrafiltration (Amicon UM-2 membrane). To obtain the last fraction containing the tissue-specific, DNA-binding non-histone proteins (fraction NP), the DNA pellets were suspended in 5.0 *M* urea-2.5 *M* NaCl-50 m*M* Tris-HCl buffer (pH 8.0), stirred for 2-3 hr on ice, and centrifuged at 110,000*g* (max) for 48 hr. The upper ¾ of the supernatants were gently decanted, concentrated (Amicon UM-2 membrane), and analyzed (fraction NP).

Immunoassay Procedure. The non-histone protein-DNA complexes for immunization were obtained by dehistonization of chromatin in 2.5 *M* NaCl-5.0 *M* urea-50 m*M* sodium succinate buffer (pH 5.0). Ultracentrifugation of this mixture at 110,000*g* for 42 hr produced pellets of DNA and non-histone proteins. The pellets were combined, homogenized in 1.5 m*M* sodium chloride-0.15 m*M* sodium citrate (pH 7.0), and dialyzed extensively against the same solution. After dialysis, the material (2 mg of DNA/ml) was mixed with an equal volume of complete Freund's adjuvant and injected into male New Zealand rabbits; 1 mg of DNA of the mixture was injected weekly into each rabbit for 7 weeks (Chytil and Spelsberg, 1971). The antisera collected 1 week after the last injection was purified by ammonium sulfate fractionation and chromatography on DEAE-cellulose (Rapp, 1964). The antigenicity of DNA-NP com-

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plexes, reconstituted materials, or chromatin was determined by the complement fixation method of Wasserman and Levine (1961). The antigens and antisera were reacted at 2-4°C overnight.

Iodination of Proteins. The concentrated protein fractions were dialyzed against 5.0 M urea in 50 mM Tris-HCl buffer (pH 8.0) and iodinated with ^{125}I. The chloramine-T method of Sonoda and Schlamowitz (1970) was followed.

Analytical Procedures. For polyacrylamide gel electrophoresis, protein samples were dialyzed against a buffer containing 0.1% sodium dodecyl sulfate-0.1% 2-mercaptoethanol-0.1 M sodium phosphate-8.0 M urea (pH 7.0) and electrophoresed in the same buffer without urea and mercaptoethanol. After electrophoresis the proteins were stained with Coomassie Brilliant Blue.

DNA was isolated from rat spleen or calf thymus by the modified procedure of Marmur (1961). The protein concentration was determined according to the method of Lowry et al. (1951), using unfractionated calf thymus histone or bovine serum albumin as standards. The DNA content was assayed by diphenylamine (Burton, 1956) and the amino acid composition of the hydrolyzed protein samples was determined with the aid of an automatic amino acid analyzer (Beckman).

Reconstitution Experiments. Reconstitution of the isolated chromatin proteins and DNA was accomplished by dissolving the interacting components in 5.0 M urea-2.5 M NaCl-50 mM Tris-HCl buffer (pH 8.0) and then removing the NaCl by slow gradient dialysis against 5.0 M urea-50 mM Tris-HCl buffer (pH 8.0). Finally, the urea was removed by dialysis against 10 mM Tris-HCl buffer (pH 8.0), or, if the complexes were to be used for complement fixation experiments, by dialysis against the immunoassay solvent [0.1 M NaCl-0.5 mM $MgCl_2$-0.15 mM $CaCl_2$-0.1% (w/v) bovine serum albumin-50 mM Tris-HCl buffer (pH 7.3)].

In Vitro RNA Synthesis. Aliquots of the individual DNA-containing pellets were suspended in 1.5 mM NaCl-0.15 mM sodium citrate (pH 7.0) and dialyzed against the same solvent to remove urea and salt. *Escherichia coli* RNA polymerase was prepared according to the method of Burgess (1969) and purified by gel filtration on a Bio-Gel A-5m column instead of by chromatography on phosphocellulose. The composition of the reaction mixture is described in the legend of Table III. The assays were incubated at 37° for 10 min and the reaction was terminated by adding 1 ml of cold 10% trichloroacetic acid. The ice-cold mixture was filtered through Whatman 3MM filter paper discs. The discs were washed four times with 10 ml of 5% trichloroacetic acid-1% sodium pyrophosphate solution and once with 1 ml of absolute ethanol; they were then dried and counted in a scintillation spectrometer.

RNA Synthesis without Reinitiation. The experiments of RNA synthesis without reinitiation followed the method of Cedar and Felsenfeld (1973). Various chromatin fractions were incubated with *E. coli* RNA polymerase at 37°C in 0.5 ml containing 10 mM Tris-HCl (pH 8.0), 1 mM $MnCl_2$, 80 μM each of ATP and GTP, and 22 μM [^{3}H]UTP (1000 cpm/pmol). The initiation was performed at 37°C for 15 min and was stopped by the addition of 0.16 ml of 1.6 M $(NH_4)_2SO_4$. RNA chain elongation was then started by the addition of CTP (80 μM) and $MgCl_2$ (final concentration 5 mM). After 20 min of incubation at 37°C, RNA synthesis was stopped by adding 2 ml of 10% trichloroacetic acid-1% sodium pyrophosphate. After 15 min at 0°, the precipitates were collected on Whatman 3MM filter paper discs. The discs were washed four times with 10 ml of 5% trichloroacetic acid-1% sodium pyrophosphate solution and once with 1 ml of absolute ethanol; after drying, the discs were counted in a liquid scintillation spectrometer. Since rat DNA contains 54.8% A + T (Woodhouse, 1954), the amount of total nucleoside triphosphate incorporated was assumed to be 3.65 times the incorporation of UTP. For sucrose gradient analysis, 0.2 ml of the final assay mixture was brought to 0.5% in respect to sodium dodecyl sulfate and 7.5 mM EDTA (pH 7.0) and kept at room temperature for 30 min before centrifugation.

Analysis of RNA Chain Length. The chain length of RNA transcribed from chromatin fractions was determined by sucrose density gradient centrifugation. Samples (0.2 ml) were layered on 5 ml of gradient from 5 to 20% sucrose in 10 mM Tris-HCl, 0.1 M NaCl, and 1 mM EDTA (pH 7.9), and centrifuged at 45,000 rpm in Beckman SW 50 rotor at 20°C for 4 hr. Twenty-five fractions (0.2 ml each) were collected and the acid-precipitable radioactivity in each fraction was determined. The average sedimentation coefficient of each fraction was determined by reference to the molecular weight markers (mammalian ribosomal RNAs). The chain length in nucleotides (N) was calculated using the following equation (Hyman and Davidson, 1970; Cedar and Felsenfeld, 1973): $\log N = 2.10 \log s_{20,w} + 0.644$. The number average chain length (N_{av}) of the synthesized RNA was determined from the following equation: $N_{av} = \Sigma_i n_i N_i / \Sigma_i n_i$ where n_i is the number of RNA chains and N_i is the length of RNA chain in nucleotides.

NP-DNA Binding Studies. NP proteins were iodinated and reconstituted by gradient dialysis to various samples of DNA. After reconstitution, [^{125}I]NP-DNA complexes were carefully layered over a buffered sucrose gradient (5-20% w/v sucrose in 10 mM Tris-HCl (pH 8.0)) and centrifuged for 6 hr in SW 40 at 38,000 rpm. The tubes were punctured at the bottom and 0.4-ml fractions were collected. A volume of 0.7 ml of distilled water was added to each sample. Aliquots were taken for the determination of OD at 260 nm and for the determination of radioactivity.

Phosphorylation of Chromatin Proteins. For the in vivo labeling of non-histone proteins, 2 mCi of [^{32}P]phosphoric acid (carrier-free, Schwarz/Mann, Orangeburg, N.Y.) neutralized in 0.5 ml of physiological saline was injected intraperitoneally (per 100 g rat weight) 60 min before removal of the livers. Nuclei and chromatin fractions were prepared as was described above. For in vitro labeling of non-histone proteins, isolated chromatin was incubated in a reaction mixture containing 80 mM Tris-HCl (pH 7.5), 16 μM [γ-^{32}P]ATP (0.23 Ci/mmol), 20 mM $MgCl_2$, and 100 mM NaCl at 37°C for 10 min (Chiu et al. 1973).

Results

Sequential extraction of chromatin with 5.0 M urea solutions containing various buffer or salt concentrations resulted in the separation of chromatin protein components into three principal fractions, designated as UP, HP, and NP. The DNA pelleted in each subsequent step contained proteins which were not removed by the previous solvent. For simplicity, these pellets were designated UC (urea-treated chromatin, devoid of urea-soluble proteins), HC (histone-depleted chromatin, devoid of urea-soluble proteins and histones), and finally, NC (protein-depleted chromatin or DNA).

As can be seen from the distribution of DNA and pro-

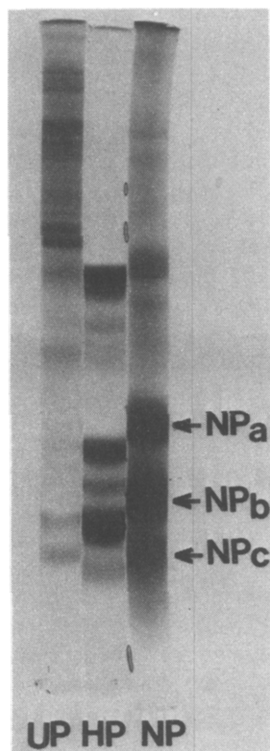

FIGURE 1: Polyacrylamide gel electrophoresis of the UP, HP, and NP fractions from rat liver chromatin. The electrophoresis was performed in the presence of sodium dodecyl sulfate (Wilhelm et al., 1972). The origin of migration is at the top of the gels.

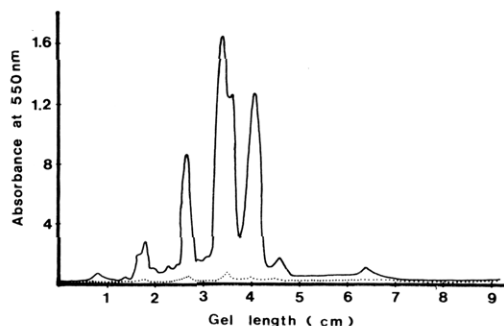

FIGURE 2: Optical scans of polyacrylamide gel electrophoregrams of HP fraction (—) and NP fraction (- - -) of rat liver chromatin. The electrophoresis was performed in acidic-urea condition as described by Panyim and Chalkley (1968). The origin of migration is on the left-hand side of the scans.

Table I: Distribution of DNA and Protein in Fractions Resulting from the Scheme in Figure 1.[a]

Fraction	% of Total DNA	% of Total Protein
Chromatin	100	100
UP	2–3	44–47
HP	1–2	50–52
NP	1–2	2–3
NC	93–96	3–5

[a] The values are averages of several preparations of rat liver chromatin with DNA/protein ratios 1.0:1.6–1.9.

Table II: Amino Acid Composition of the Protein Fractions Removed According to the Schedule Shown in Figure 1.[a]

	Fraction				
Amino Acid	UP	HP	NP	NP_a	NP_b
Lysine	5.5	14.1	7.8	7.5	8.8
Histidine	2.4	1.4	1.9	ND	ND
Arginine	5.8	7.4	8.0	8.4	9.9
Aspartic acid	9.9	4.9	7.5	5.2	6.0
Threonine	3.8	5.0	4.7	5.8	6.0
Serine	8.5	6.0	6.8	7.2	4.5
Glutamic acid	14.2	9.2	12.3	12.0	8.8
Proline	6.8	5.6	5.0	3.8	3.6
Glycine	12.5	9.5	10.7	9.6	13.8
Alanine	7.4	15.3	8.8	11.0	7.5
Valine	4.8	5.0	5.6	4.7	7.3
Methionine	1.8	0.6	1.4	ND	ND
Isoleucine	4.0	4.2	4.2	4.2	4.7
Leucine	7.6	8.0	8.8	7.4	7.3
Tyrosine	2.6	2.1	3.7	2.5	2.9
Phenylalanine	2.3	1.7	2.8	2.5	ND
Acidic/basic	1.7	0.6	1.1	1.08	0.82

[a] The amino acid concentration is expressed as mole percent of all amino acids recovered. Tryptophan was not determined. All serine values are corrected (10%) for hydrolytic losses. The figures are averages of 3–4 determinations. Samples NP_a and NP_b were obtained by polyacrylamide gel electrophoresis and it was not possible to obtain accurate values for histidine and methionine (ND). The amino acid analyses were kindly performed by Dr. D. N. Ward.

teins during the fractionation schedule (Table I), the NP proteins represent only a very small fraction of the total chromatin protein content. During the last extraction step, part of the DNA did not sediment in a firm pellet and formed a viscous layer over the pelleted material, perhaps because of the shearing during repeated resuspension of the chromatin. If minimal contamination of the protein fraction with DNA is desired, only the upper ¾ portion of supernatants should be used. The contamination of protein supernatants by DNA can be also decreased by placing on the bottom of each test tube a 1-cm layer of 0.8 *M* sucrose in the buffer used for the particular extraction step. The supernatant over this sucrose "shelf" is then used to recover the proteins.

Selectivity of the fractionation procedure employed for the isolation of DNA-binding, non-histone proteins is shown in Figure 1. The polyacrylamide gel electrophoresis performed in the presence of sodium dodecyl sulfate shows that the first step of the fractionation schedule removes essentially all the non-histone proteins, together with traces of histones, especially the H4 (F_{2a1} or IV) fraction. The second step of the fractionation removes essentially only histones. The NP protein fraction consists of only 2–3 major low molecular weight and several minor high molecular weight components.

The possibility that the NP fraction is contaminated by histones was also considered. Normal rat liver [^{125}I]NP or [^{125}I]HP were applied to DEAE-cellulose column preequilibrated with 5 *M* urea–10 m*M* Tris-HCl buffer (pH 8.0). The column was first washed with the same buffer and then eluted with 0–0.3 *M* KCl gradient in the same buffer.

The [^{125}I]HP was eluted with the first wash while the NP was eluted later with KCl. We have also examined the HP and NP fractions by polyacrylamide gel electrophoresis (Panyim and Chalkley, 1969). Only the HP, but not NP proteins, migrated under these conditions (Figure 2).

The UP, HP, NP, and two major components of the NP fractions were further characterized by their amino acid composition (Table II). From their ratios of acidic to basic

Table III: Templating Activity of Residual Pellets Resulting from the Scheme in the Figure 1.[a]

Fraction	[^{3}H]UTP (pmol/μg of DNA)	% Free DNA Activity
Chromatin	22.1	11.3
UC	50.0	25.6
HC	167.5	85.7
NC	176.8	90.5
DNA	195.4	100

[a] The results are averages of several preparations of rat liver chromatin. Free DNA was isolated from rat spleen. The reaction mixture (0.25 ml final volume) consisted of 40 m*M* Tris-HCl buffer (pH 8.0), 120 m*M* KCl, 0.1 m*M* EDTA, 2.5 m*M* $MnCl_2$, 1.0 m*M* dithiothreitol, 0.08 m*M* each ATP, GTP, and CTP, and 0.02 m*M* ^{3}H (specific activity 1 Ci/mol). The concentration of chromatin in each assay was 10–15 μg in respect to DNA, together with 20 units of *E. coli* RNA polymerase (specific activity 600 units/mg of protein). The assay mixtures were incubated at 37°C for 15 min and the reaction was terminated by adding 2 ml of 10% trichloroacetic acid–1% sodium pyrophosphate solution.

Table IV: Determination of the Number of Growing Chains by Sucrose Gradient Analysis.

Template	Nucleotides Incorporated (pmol)	Chain Length (Nucleotides)	Initiations (pmol)
DNA	1015	590	1.72
NC	918	570	1.61
HC	906	600	1.51
UC	308	540	0.57
Chromatin	155	500	0.31

[a] Assay conditions were as described under Materials and Methods. The average chain length was determined from 0.2 ml of each assay tube by sucrose gradient analysis. Each tube contained 1.5 μg of chromatin as indicated.

amino acids, the UP proteins are acidic. The composition of HP is similar to that reported for rat liver histones. The total NP protein fraction is not basic, the ratio being around 1.1. However, one of its components (component B) is slightly basic, but it is not a histone.

The efficiencies of the pellets from the individual fractionation steps to serve as a template for the in vitro RNA synthesis were determined (Table III). The initial extraction of chromatin with buffered 5.0 *M* urea (removal of most non-histone proteins and a small amount of the histones) more than doubled the templating efficiency of the residual chromatin. As can be expected, the removal of histones during the second extraction step derepressed the DNA, bringing its templating efficiency to about 80% of the control DNA isolated by the modified procedure of Marmur (1961). The templating efficiency of the NC pellets, which were devoid of essentially all the chromatin proteins, was slightly lower than that of the control DNA. A change in the templating efficiency of chromatin fractions may arise either from a change in the total number of available initiation sites or from a change in the rate of chain elongation.

To determine which change is the major factor contributing to the template efficiency, we have performed the kinetic studies of RNA initiation and elongation as described by Cedar and Felsenfeld (1973). The number of initiation sites available for RNA polymerase on DNA and chromatin is

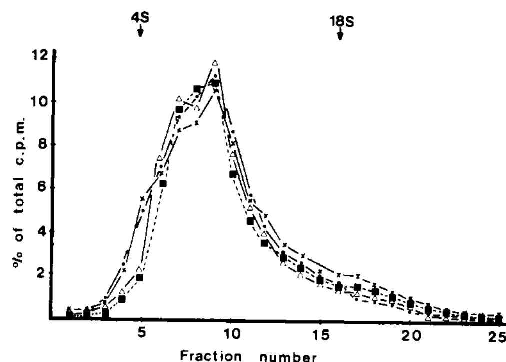

FIGURE 3: Sucrose gradient analysis of product made from DNA and chromatins. The assay conditions and sucrose gradient analysis were the same as described under Materials and Methods. The data are graphed by plotting the percentage of total counts as a function of RNA size. (■- - - -■) DNA; (●—●) NC; (△—△) HC; (×—×) chromatin.

assayed under conditions that allow only one RNA molecule to be made at each available initiation site. The experiment is carried out by allowing a large excess of enzyme to initiate synthesis in low salt with only three kinds of nucleotides present. The absence of the fourth nucleotide prevents extensive chain elongation and thus inhibits the formation of multiple initiations at one site. After 15 min the mixture is then brought to 0.4 *M* $(NH_4)_2SO_4$ which prevents further initiations, and the fourth nucleotide is added to permit the elongation of the started nucleotide chains. The number of growing chains is then calculated from sucrose gradient centrifugation data. As shown in Figure 3, the RNAs made from DNA and from chromatin fractions HC and NC had very similar molecular weight distributions. The number average size for these RNAs was between 400 and 600 nucleotides. Since these measurements are done under conditions where the number of RNA molecules is equal to the number of initiation sites, we can calculate the number of average chain length and the number of initiation sites (Table IV). Using 1.5 μg of template (2.2 nmol of base pairs), we observed 1.72 pmol of initiation sites on rat DNA and 0.31, 0.57, 1.51, and 1.61 pmol on rat liver chromatin, UC, HC, and NC fractions, respectively. This corresponds approximately to one initiation site for every 1850 base pairs on rat DNA and for every 13,400, 11,270, 3540, and 2780 base pairs on rat liver chromatin, UC, HC, and NC fractions, respectively. We have also determined the number of initiation sites on DNA or chromatin fractions by titration of a fixed concentration of RNA polymerase with varying amounts of template. As shown in Figure 4, one unit of *E. coli* RNA polymerase was titrated by various amounts of DNA or chromatin fractions. As the amount of template was increased, more initiations were produced until reaching a plateau. With DNA as a template, the plateau was obtained with about 2 μg of DNA for 1.65 pmol of enzyme. This corresponds to one initiation site for every 1700 base pairs for rat DNA. The titration point for chromatin UC, HC, and NC fractions, in an identical assay was 14, 12, 4, and 3.5 μg, respectively. This corresponds to one initiation site per 11,900, 10,200, 3400, and 2970 nucleotide base pairs, respectively.

The removal of UP fraction proteins only slightly changed the number of initiation sites. However, the subsequent removal of histones (HP) increased the initiation considerably. This indicates that histones may function as general repressors and the non-histone proteins (NP) may serve

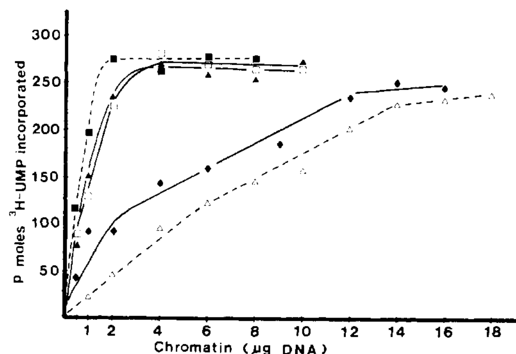

FIGURE 4: Titration of RNA polymerase with DNA and chromatin. Increasing amounts of DNA or chromatins were added to one unit of *E. coli* RNA polymerase. Incubation conditions were same as described under Materials and Methods. After 20-min propagation in high salt, the incorporation of UMP into trichloroacetic acid precipitable material was determined. The data are expressed as picomoles of UMP incorporated in 20 min.

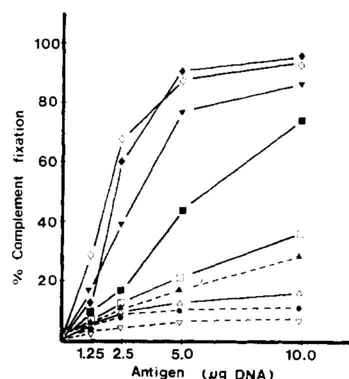

FIGURE 5: Complement fixation by chromatin preparations from Novikoff hepatoma (♦—♦), normal rat liver (●- - - -●), and reconstituted chromatins: NCT + UPT + HPT + NPT (◇—◇); NCT + UPL + HPL + NPT (▼—▼); NCL + UPL + HPL + NPT (■—■); NCT + UPT + HPT + NPL (□—□); NCT + UPL + HPL + NPL (▲- - -▲); NCL + UPT + HPL + NPL (△—△); NCL + UPL + HPL + NPL (▽- - -▽). The assays were performed in the presence of Novikoff hepatoma dehistonized chromatin antiserum. The reaction mixtures containing, in a total volume of 0.8 ml, 1.25-10 μg of DNA as chromatin, 0.1 ml of 200 times diluted rabbit antiserum, and 0.2 ml of 500 times diluted guinea pig complements were incubated at 4° for 17 hr. To each sample 0.2 ml of activated sheep erythrocytes was added and the mixture was incubated at 37° for 20 min. The extent of hemolysis was determined by reading the absorbance at 413 nm. All assays were corrected for anticomplementarity. NCT etc., NC pellet of Novikoff hepatoma; NCL etc., NC pellet of normal rat liver.

as specific regulators of either negative or positive transcriptional control. It was reported by Cedar and Felsenfeld (1973) and also by Tsai et al. (1975) that the number of binding sites for RNA polymerase on chromatin is much smaller than on free DNA, but that all the bound enzyme molecules are capable of chain elongation. The rate of chain elongation on chromatin is about one-third of that on DNA. Therefore, we conclude that the increase of template activity after the removal of UP, HP, and NP proteins is much more the result of the increasing numbers of initiation sites rather than of the increasing rate of elongation.

As was shown by Wakabayashi et al. (1973), the non-histone proteins of chromatin which could be identified by their immunological tissue specificity also contained proteins with affinity for native DNA. To show that the immunospecificity of chromatin non-histone proteins was not lost during the fractionation schedule, the immunoreactivity of the intact chromatin and of the UC, HC, and NC pellets was assayed. The dehistonized Novikoff hepatoma chromatin was used as antigen for the immunization of rabbits. The removal of the urea-soluble proteins (UP) and histone fraction (HP) changed the nature of the complement fixation very little. However, the complement fixing ability decreased considerably when the DNA-binding proteins (NP) were removed during the last fractionation step (NC pellet). The residual immunoactivity of the NC pellet may result from traces of NP proteins still present in the NC pellet. The complement fixation was also studied in reconstituted chromatins. Fractions UP and HP isolated from normal rat liver chromatin and reconstituted back to the UC or HC pellets of Novikoff hepatoma or vice versa did not change the immunospecificity of the reconstituted materials. However, the non-histone proteins of the last fraction (NP) were responsible for the transfer of immunological tissue specificity (Figure 5). As shown in this figure, the immunological tissue specificity was transferred by reconstituting non-histone proteins (NP) from Novikoff hepatoma to normal rat liver chromatin devoid of this fraction. Reconstituted chromatin which contained all components from Novikoff hepatoma except for the NP proteins (isolated from rat liver chromatin) behaved immunologically like normal rat liver chromatin.

Wakabayashi and Hnilica (1973) have shown that the immunological tissue specificity of DNA-binding, chromosomal non-histone proteins depends on their associations with homologous DNA. Therefore, the affinity of NP proteins to homologous and heterologous DNA was studied here by sucrose density gradient centrifugation. The NP fraction was iodinated and reconstituted to various DNA samples as was described under Materials and Methods. The reconstituted samples were layered over a 5-20% buffered sucrose gradient and centrifuged. The distribution of DNA in gradient was determined by its absorption at 260 nm, while the position of NP proteins was indicated by their ^{125}I radioactivity. As shown in Figure 6, the NP fraction prepared from Novikoff hepatoma binds well to rat DNA (Figure 6a) while only a very low binding to chicken erythrocyte DNA can be seen (Figure 6b). The electrophoretic identity of the DNA-binding proteins was determined by recovering the proteins from the DNA-protein complex on the gradients with 1% sodium dodecyl sulfate-1% 2-mercaptoethanol-50 m*M* Tris-HCl (pH 8.0). The extracted proteins were analyzed by polyacrylamide gel electrophoresis. The pattern of the DNA-binding proteins consisted of the three major polypeptide bands characteristic for the total NP fraction shown in Figure 1. The complement fixation assays in Figure 7 demonstrate that the part of protein fraction NP which binds selectively to homologous DNA is also tissue specific by immunological criteria.

Several investigators have recently reported the presence in chromatin of phosphorylated non-histone proteins which associated preferentially with homologous DNA (Kleinsmith, 1973; Teng et al. 1971; Patel and Thomas, 1973). The possibility of NP being a phosphorylated non-histone protein was therefore investigated. Chromatin non-histone proteins were phosphorylated either in vivo or in vitro (as described under Materials and Methods) and fractionated according to the described scheme. Only a very little amount of ^{32}P was incorporated into the NP fraction. And its electrophoretic analysis showed the presence of only high molecular weight material labeled with ^{32}P; the NP proteins were not phosphorylated.

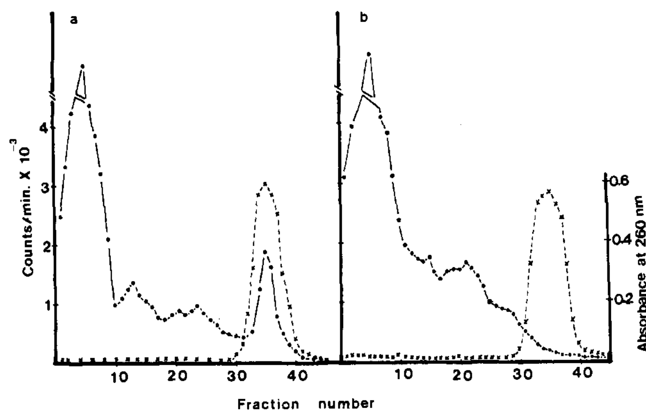

FIGURE 6: Separation of NP-DNA complexes after reconstitution in vitro. Rat Novikoff hepatoma chromosomal proteins NP fraction, labeled in vitro with ^{125}I, was reconstituted with (a) rat liver DNA or (b) chicken erythrocyte DNA under the conditions described under Materials and Methods. The DNA-protein mixture was layered over a 5-20% sucrose gradient and centrifuged at 38,000 rpm for 6 hr in Beckman SW 40 rotor. The position of DNA in the gradient is indicated by its absorption at 260 nm (X- - -X). The distribution of isotopically labeled ^{125}I is shown by ●—●.

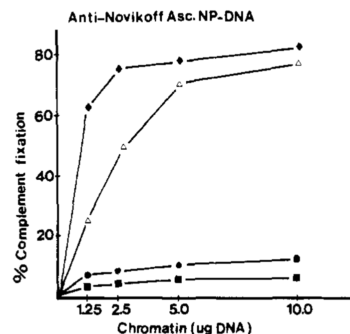

FIGURE 7: Complement fixation of Novikoff hepatoma chromatin, normal rat liver chromatin, and reconstituted NP-DNA complexes recovered from the gradients (Figure 6) in the presence of antiserum against Novikoff hepatoma NP-DNA. All experimental points were corrected for anticomplementarity. (♦—♦) Novikoff hepatoma chromatin; (△—△) Novikoff hepatoma NP-rat DNA complexes recovered from the gradients (Figure 6a); (●—●) Novikoff hepatoma NP-chicken DNA complexes recovered from the gradients (Figure 6b); (■—■) normal rat liver chromatin.

Discussion

By immunological analysis, nuclear non-histone proteins were found to be tissue specific (Henning et al. 1962; Chytil and Spelsberg, 1971, Wakabayashi and Hnilica, 1973) and it was shown that this specificity is the result of highly selective interactions between a group of non-histone proteins and homologous DNA (Wakabayashi et al., 1973, 1974). Using DNA column chromatography, it was possible to isolate a group of non-histone proteins which exhibited a high affinity for homologous DNA but did not interact with DNA isolated from other (heterologous) species. These proteins were represented by 3-4 bands in polyacrylamide gel electrophoresis and reconstituted with homologous DNA into immunologically tissue-specific complexes.

The fractionation scheme described here permits the separation of chromosomal non-histone proteins into three discrete fractions one of which represents the DNA-binding, immunologically tissue specific proteins. Gronow and Griffiths (1971) found that after removing about 70% of the total nuclear proteins with 8 *M* urea (pH 7.6) the non-histone proteins remaining with DNA migrated electrophoretically as two low molecular weight components. These two protein components are similar to the electrophoretic pattern of our major components of NP fraction and also resemble some of the proteins described by MacGillivray et al. (1972). However, these authors did not investigate the DNA-binding and immunological properties of their fractions and a direct comparison to our fraction NP is therefore difficult.

The presence in chromatin of non-histone proteins which can bind strongly to DNA was reported by several investigators. Kleinsmith et al. (1970) described a small fraction of chromosomal phosphoproteins which associated only with homologous DNA. In his more recent paper, Kleinsmith (1973) showed that about 1% of the phosphoprotein fraction of rat liver nuclei could bind selectively to homologous DNA. The binding ratio of about 1:100 (protein/DNA) suggested the formation of specific complexes. A lower, but still considerable binding to heterologous DNA preparations (mouse, calf, salmon, etc.) was also observed. According to the author, the molecular weights of the DNA binding proteins ranged from 30,000 to 70,000. Teng et al. (1971) described a fraction of nuclear phosphoproteins which were soluble in buffered phenol. These phosphorylated non-histone proteins also bound preferably to homologous DNA, but the binding ratios were considerably higher. The molecular weights of these proteins were similar to the DNA-binding proteins, described by Kleinsmith (1973).

Another group of non-histone proteins with affinity for DNA was isolated by Patel and Thomas (1973). According to their preliminary characterization, these proteins were phosphorylated and represented about 1.5-3.0% of the total nuclear proteins. In their electrophoretic heterogeneity, these DNA-binding non-histone proteins resembled the total phosphoprotein fraction of chromatin. Similar to the findings of Kleinsmith (1973), these proteins associated preferentially with homologous DNA but they also interacted strongly with heterologous DNA (calf, salmon, and *Flavobacterium*).

The tissue specificity of DNA binding by non-histone proteins described here resembles some of the properties of the protein preparations reported by Kleinsmith (1973) and by Patel and Thomas (1973). However, the tissue specific proteins, NP, are not phosphoproteins; they are also much more selective in their binding to homologous vs. heterologous DNA, and they are electrophoretically less heterogeneous (Wakabayshi et al. 1973).

Perhaps the closest chemical similarity of the tissue-specific, DNA-binding protein fraction NP can be drawn to the DNA-binding protein fraction of rat liver described by van den Broek et al. (1973) and Sevall et al. (1975). These authors isolated chromatin proteins binding specifically to rat liver DNA. The molecular weight range, amino acid composition, as well as DNA-binding properties of this fraction are similar to those described here for the tissue-specific, DNA-binding protein fraction NP.

Recently, Allfrey et al. (1975) described a whole spectrum of proteins in calf thymus nuclei which bind to the DNA. Some proteins were found to bind selectively to unique or to repetitive DNA sequences, others preferred native over denatured DNA. Several of the DNA-binding proteins interacted specifically with adenosine or guanosine 3′:5′-cyclic phosphates. It is evident that detailed studies of the nuclear DNA binding proteins may lead to the isolation of important macromolecules capable of regulating nuclear

metabolism and DNA transcription.

The complexes of the NP proteins with homologous DNA are highly immunospecific, as can be determined by the complement fixation method. The immunospecificity of the DNA-binding chromatin non-histone proteins appears to parallel the differentiated state of tissues. In chicken oviduct (Chytil and Spelsberg, 1971; Spelsberg et al. 1973) and in developing rat liver (Chytil et al., 1974) the immunochemical behavior of the chromatin non-histone proteins was shown to change with the differentiation of these tissues. Neoplastic growth also altered profoundly the immunospecificity of the DNA-binding non-histone proteins (Wakabayashi and Hnilica, 1973; Wakabayashi et al., 1974; Chiu et al., 1974, 1975). It can be speculated that if these proteins represent a part of the biochemical mechanism by which the cell maintains its phenotype, the fractionation schedule described here opens a way for their detailed characterization and biochemical study.

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