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Immunospecificity of Nonhistone Proteins in Chromatin†

K. Wakabayashi, Sophia Wang, and L. S. Hnilica*

ABSTRACT: A fraction of nonhistone proteins of chromatin (NP fraction) was found to contain protein species which can form immunochemically tissue-specific complexes with the DNA isolated from the same animal species (homologous DNA). When alone, complexed with heterologous DNA, or other polyanionic molecules, the NP fraction lost its tissue specificity detectable by the complement fixation method. The NP fraction from rat liver was found to contain proteins which were firmly retained on polyacrylamide–agarose columns containing rat-spleen DNA; there was no measurable retention of these proteins by calf-thymus DNA. The affinity of rat-liver NP proteins for homologous DNA was very strong. While all the rat-liver histones and other nonhistone proteins could be eluted from rat-spleen DNA columns with 0.2 M KCl, a concentration of 0.4 M KCl was necessary for the elution of NP proteins. When analyzed by polyacrylamide gel electrophoresis in the presence of sodium dodecyl sulfate, the DNA-binding NP proteins eluted from rat-spleen DNA column with 0.4 M KCl consisted mainly of two major and one minor polypeptide bands with mobilities corresponding approximately to 12,000–15,000 daltons.

The biochemistry and morphology of a cellular phenotype are determined by the process of cytodifferentiation and generally do not change during the lifetime of differentiated cells. Parental phenotype is passed onto the new cells produced by each division and a major change in the differentiated cellular state occurs only rarely. Perhaps the best known example of self-perpetuating change in cellular differentiation is the neoplastic growth.

Experiments performed with isolated chromatin indicate that a definite restrictional pattern is imposed on the DNA genome during differentiation and that certain nonhistone proteins together with the histones determine the tissue specificity of DNA transcription in chromatin (Paul and Gilmour, 1968; Gilmour and Paul, 1969, 1970; Spelsberg and Hnilica, 1970; Spelsberg *et al.*, 1971; Bekhor *et al.*, 1969; Smith *et al.*, 1969; Kamiyama and Wang, 1971). It was shown by Henning *et al.* (1962) and more recently by Chytil and Spelsberg (1971) that a fraction of chromatin nonhistone proteins can elicit the formation of tissue-specific antibodies when injected together with DNA into the rabbits. A preliminary report from this laboratory (Wakabayashi and Hnilica, 1973) showed that the tissue-specific antibodies are formed against the complexes of chromosomal nonhistone proteins with homologous DNA. Here we report that a nonhistone protein fraction of a limited heterogeneity interacts with the DNA in chromatin in a highly specific manner.

Materials and Methods

Unless specific, all preparative work was performed at 2–4°.

Preparation of Chromatin and Nonhistone Protein–DNA Complexes. Male albino rats (Sprague-Dawley) fed *ad libitum* were sacrificed by cervical dislocation and their livers were excised and placed immediately into an ice-cold solution of 0.25 M sucrose. The rat-liver nuclei were solated according to the method of Blobel and Potter (1966).

Thymus glands of young calves were obtained from a local slaughter house. The excised glands were placed into crushed ice, transported to the laboratory, and processed as was described for the livers.

Transplantable rat neoplasms (Walker carcinosarcoma, Novikoff hepatoma, rat 30D hepatoma) were maintained by weekly transplantations. The cells of both hepatomas (ascites) were collected by centrifugation and washed 2–3 times with several volumes of isotonic sucrose solution and centrifuged. This treatment removed most of the contaminating erythrocytes. The method described by Wilhelm *et al.* (1972) was used to prepare hepatoma nuclei; Walker tumor nuclei were obtained by the procedure of Busch *et al.* (1959). All nuclear preparations received a final wash with 0.25 M sucrose–5.0 mM $MgCl_2$ in 10 mM Tris-HCl buffer (pH 7.5). Chromatin was prepared from the isolated nuclei by the method of Spelsberg and Hnilica (1971a) as modified by Wilhelm *et al.* (1972). After washing with 0.3 M NaCl, chromatin preparations were dissociated in buffered 2.5 M NaCl–5.0 M urea solutions.

Bekhor *et al.* (1969) as well as Gilmour and Paul (1969) have shown that essentially all the chromatin proteins can be separated from DNA by dissociation in 2.0 M NaCl–5.0 M urea–10 mM Tris-HCl buffer (pH 8.3) and prolonged ultracentrifugation. If this procedure is performed at pH 6.0, all the histones and about 20–30% of the chromatin nonhistone proteins remain in the supernatant while the DNA and associated nonhistone proteins form a gelatinous pellet (Spelsberg *et al.*, 1971).

In our experiments, the viscous solution containing about 0.2–0.3 mg/ml of chromatin DNA in 2.5 M NaCl–5.0 M urea–

† From the Department of Biochemistry, The University of Texas System Cancer Center, M. D. Anderson Hospital and Tumor Institute at Houston, Houston, Texas 77025. *Received October 23, 1973.* Supported by U. S. Public Health Service Contract NIH-NCI-E-72-3269 and Grant CA-07746 and Robert A. Welch Foundation Grant G-138.

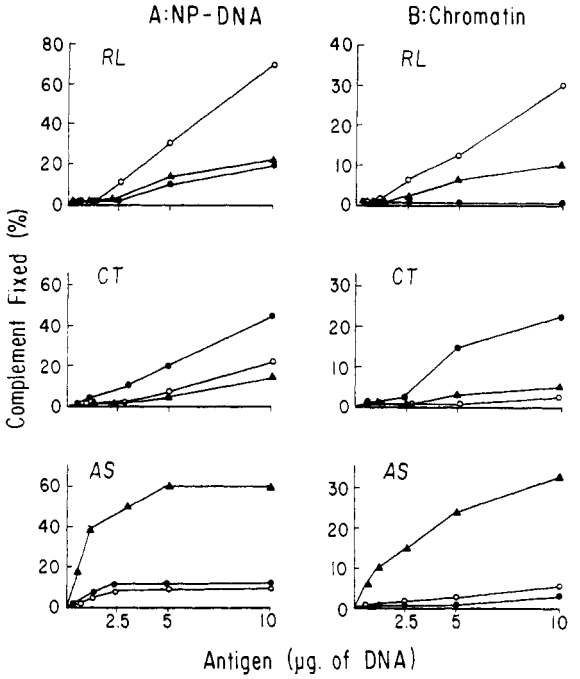

FIGURE 1: Complement fixation of NP-DNA complexes (A) and chromatin (B) from rat liver, calf thymus and Novikoff hepatoma in the presence of antiserum against the individual NP-DNA complexes. The reaction mixtures, each containing in a total volume of 0.8 ml various amounts of NP-DNA complexes or chromatin, antiserum (0.1 ml of 200× diluted rabbit antiserum), and complement (0.2 ml of 50× diluted guinea pig serum), were incubated at 37° for 90 min. The samples were then chilled in an ice bath for 5 min after which time 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 measuring the absorbancy at 413 nm in a spectrophotometer. All experimental points were corrected for anticomplementarity: RL, CT, and AS, antisera against NP-DNA pellets from rat liver, calf thymus, and Novikoff hepatoma, respectively. The NP-DNA complexes (A) or chromatin (B) were prepared from rat liver (○), calf thymus (●), and Novikoff hepatoma (▲).

50 mM sodium phosphate buffer (pH 6.0) was stirred on ice for several hours and then centrifuged at 105,000*g* (max) for 36 hr to produce the nonhistone protein–DNA pellet (NP-DNA).

To recover the nonhistone proteins, the NP-DNA pellets were dissociated in 2.5 M NaCl–5.0 M urea–50 mM Tris-HCl (pH 8.0), stirred for 3–4 hr, and centrifuged at 105,000*g* (max) for 36 hr. The nonhistone protein fraction (NP) was obtained from the supernatant by ultrafiltration (Amicon UM-2 filter). The remaining DNA pellets contained less than 2% of associated proteins.

Complexes of DNA or various polyanions with isolated chromatin protein fractions were reconstituted by dissolving and mixing the interacting components in 2.5 M NaCl–5.0 M urea–50 mM Tris-HCl buffer (pH 8.0) and decreasing the NaCl concentration by overnight dialysis against 5.0 M urea in 50 mM Tris-HCl buffer (pH 8.0). The volume of urea solution was calculated to give a final NaCl concentration of approximately 0.1 M. After this time, the urea was removed by rapid and extensive dialysis against 0.1 M NaCl, 0.5 mM $MgCl_2$, 0.15 mM $CaCl_2$, and 0.1% (w/v) bovine serum albumin solution in 50 mM Tris-HCl buffer (pH 7.3). This latter solution was the principal solvent for the microcomplement fixation assay. It was used to make all the appropriate dilutions of antigens.

Immunoassay Procedures. The NP-DNA pellets, resulting from centrifugation at pH 6.0, were homogenized in 1.5 mM NaCl–0.15 mM sodium citrate (pH 7.0) and dialyzed extensively against the same solution. After dialysis, the NP-DNA complexes, or other materials used for immunization, were mixed with an equal volume of complete Freund's adjuvant and injected into male New Zealand rabbits; 1 mg of DNA of the NP-DNA complexes was injected weekly into each rabbit for 7 weeks. The blood was collected by cardiac puncture one week after the last injection and the antisera were purified by chromatography on DEAE-cellulose (Rapp, 1964).

The antigenicity of individual NP-DNA complexes, chromatin, and other materials was tested by the microcomplement fixation method of Wasserman and Levine (1961). The antigens and antisera were allowed to interact at 37° for 90 min instead of 2–4° for 18 hr as described in the original procedure.

DNase Treatment. DNase I (Worthington Biochemical Co., sp act. 2500 U/mg) was added to samples dissolved in 0.15 M NaCl–15 mM sodium citrate–5.0 mM $MgCl_2$ in the ratio of 250 U/mg of DNA. The mixture was incubated at 37° for 30 min and subsequently chilled on ice. The DNase-digested samples (NPd) were used in the complement fixation assays as well as for the immunization of rabbits.

Iodination of Proteins. The nonhistone proteins obtained by centrifugation at pH 8.0 were concentrated by ultrafiltration, dialyzed against 5.0 M urea in 50 mM Tris-HCl buffer (pH 8.0), and iodinated with ^{125}I in the presence of chloramine-T. The method of Sonoda and Schlamowitz (1970) was used for the iodination of nonhistone proteins.

Analytical Procedures. For polyacrylamide gel electrophoresis, protein samples were dialyzed against a buffer containing 0.1% sodium dodecyl sulfate, 0.1% 2-mercaptoethanol, and 10 mM sodium phosphate (pH 7.0), and electrophoresed in the same buffer (Wilhelm *et al.*, 1972). After electrophoresis, the proteins were stained with Coomassie Blue, scanned at 600 mμ, cut into 1-mm thick slices, and dissolved in H_2O_2 followed by NCS (Amersham-Searle), a toluene-based scintillation fluid, and their radioactivity was determined in a scintillation spectrometer (β emission). Several experimental series were counted directly in a γ counter.

DNA was isolated from rat spleen and other tissues by the procedure of Marmur (1961) as modified by Spelsberg and Hnilica (1971b). The DNA-containing columns were prepared according to Cavalieri and Carroll (1970).

The protein concentration was determined by the Lowry *et al.* (1951) procedure using unfractionated calf-thymus histone or bovine serum albumin for standards. The DNA was assayed by the diphenylamine method of Burton (1956).

Results

Experiments with the nonhistone protein–DNA pellets (NP-DNA) confirmed the tissue specificity of these proteins as described by Chytil and Spelsberg (1971). The NP-DNA from rat liver, Novikoff hepatoma, and calf thymus was assayed. As a rule, the immunoreactivity with antiserum against homologous NP-DNA was much higher than with the antiserum against heterologous NP-DNA (Figure 1A). The use of intact chromatin preparations instead of NP-DNA complexes in the complement fixation assays decreased the complement fixation significantly. Nevertheless, the immunospecificity of chromatins isolated from individual tissues was well expressed (Figure 1B).

To determine the possible species differences, the complement fixation of NP-DNA complexes prepared from mouse or rat livers and calf or rat thymus was compared using antisera against rat liver NP-DNA or calf thymus NP-DNA,

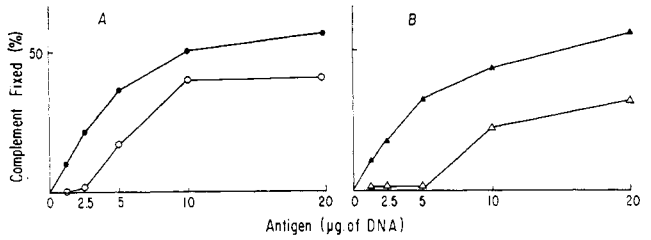

FIGURE 2: Complement fixation of NP-DNA complexes from rat or mouse liver and calf or rat thymus. The complement fixation was performed (A) in the presence of NP-DNA complexes from rat (●) or mouse (○) liver, using antiserum against rat liver NP-DNA, and (B) in the presence of NP-DNA complexes from calf (▲) or rat (△) thymus, using antiserum against calf-thymus NP-DNA.

respectively. Additionally, several transplantable tumors were assayed with the antiserum against Novikoff hepatoma NP-DNA. The results are presented in Figures 2 and 3. The NP-DNA complexes are both tissue and species specific. However, there is essentially no tissue specificity between the NP-DNA pellet from Novikoff and 30D ascites hepatoma or Walker carcinosarcoma as tested in the presence of antiserum against Novikoff hepatoma NP-DNA (Figure 3A). On the other hand, these three tumor chromatins exhibited only marginal reactivity with the antiserum against rat liver NP-DNA (Figure 3B). It appears as if the neoplastic process changed the specificity of the original tissue into an immunochemically new type, common to at least all the three tumors compared in Figure 3.

It was reported recently by Wakabayashi and Hnilica (1973) that the immunospecificity of the NP-DNA material depends on the presence of tissue-specific complexes between DNA and some nonhistone proteins in chromatin. Only intact chromatin or samples obtained by the reconstitution of nonhistone proteins with the DNA isolated from the same species (homologous) were immunoreactive. Free rat DNA or nonhistone proteins, as well as reconstituted complexes of rat liver nonhistone proteins with sea urchin DNA, several synthetic polyanions, or RNA were completely inactive (Figure 4).

To investigate the immunospecificity of their protein components, the NP-DNA complexes from rat liver and Novikoff hepatoma were digested with deoxyribonuclease I and antisera were obtained by injecting rabbits with the digested samples of nonhistone proteins (NPd). In the presence of an antiserum against rat-liver NPd, both NPd samples prepared from rat liver or Novikoff hepatoma fixed the complement essentially to the same extent (Figure 5A). Identical results were obtained when antiserum against Novikoff hepatoma NPd was allowed to react in the presence of NPd samples

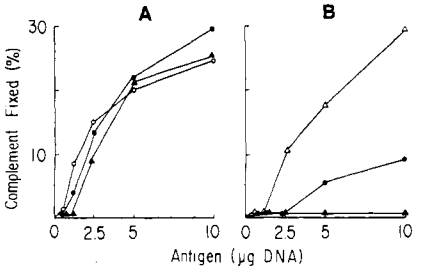

FIGURE 3: Complement fixation of chromatin from various tumors. The assay was performed in the presence of antisera against (A) Novikoff hepatoma and (B) rat-liver NP-DNA complexes: rat liver chromatin (△); 30D Ascites hepatoma chromatin (●); Walker carcinosarcoma chromatin (▲); Novikoff hepatoma chromatin (○).

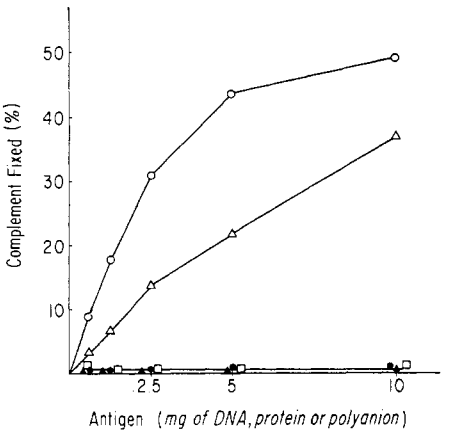

FIGURE 4: Complement fixation of reconstituted nucleoproteins and synthetic complexes. The complement fixation was performed with various reconstituted products in the presence of antiserum against the rat-liver NP-DNA complex (pellet): (○) rat-liver NP-DNA pellet (pH 6.0) reconstituted by slow dialysis of the NP and DNA components; (△) rat-liver NP protein (pH 8.0 supernatant of NP-DNA pellet) reconstituted with purified rat-spleen DNA and the histone-containing pH 6.0 supernatant of rat-liver chromatin; (●) rat-liver NP protein (pH 8.0 supernatant of NP-DNA pellet) reconstituted with sea urchin (*Strongylocentrotus purpuratus*) DNA and the histone-containing pH 6.0 supernatant of rat liver chromatin; (□) rat-liver NP protein (pH 8.0 supernatant) or rat-spleen DNA alone; (▲) rat-liver NP protein (pH 8.0 supernatant) reconstituted with yeast RNA, poly(glutamic acid), polyethylene sulfonate, or dextran sulfate.

from rat liver or Novikoff hepatoma (Figure 5B). Removal of the DNA from the NP-DNA complexes resulted in a complete loss of their immunospecificity. The DNase I alone did not exhibit any detectable antigenicity when assayed in the amounts present in the individual NPd samples (*i.e.*, 1.25 U or 0.5 μg when 20 μg of rat-liver NPd was used as antigen).

To confirm the absence of immunospecificity in NP-DNA complexes treated with DNase I, immunoabsorption experiments were performed. Antiserum against NPd from Novikoff hepatoma (10 ml of 50× diluted sample) was incubated with rat-liver NPd (6–4 μg of protein) at 4° for 24 and 48 hr. After incubation the reaction mixture was centrifuged at 105,000*g* for 60 min and the supernatant (antiserum) was titrated with NPd from rat liver and Novikoff hepatoma. This antiserum did not fix any complement in the presence of either rat-liver or Novikoff hepatoma NPd.

The deletion of immunochemical tissue specificity by DNase treatment of the NP-DNA complexes raises a question whether the original NP-DNA or intact chromatin prepara-

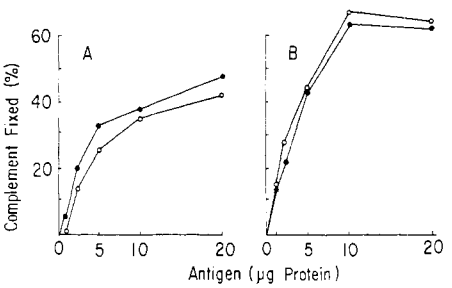

FIGURE 5: Complement fixation of DNase-treated NP-DNA complexes. The assay was performed in the presence of antisera against DNase-treated NP-DNA complexes (pellets) from (A) rat liver and (B) Novikoff hepatoma: (○) rat-liver DNase-treated NP-DNA complex; (●) Novikoff hepatoma DNase-treated NP-DNA complex.

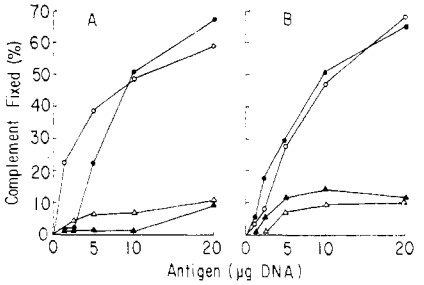

FIGURE 6: Complement fixation of chromatins or NP–DNA complexes in the presence of antisera against DNase-treated NP–DNA pellets. The complement fixation was performed with antisera against (A) DNase-treated rat liver NP–DNA and (B) the DNase-treated Novikoff hepatoma NP–DNA complex: (○) NP–DNA from rat liver; (●) NP–DNA from Novikoff hepatoma; (△) chromatin from rat liver; (▲) chromatin from Novikoff hepatoma.

tions can manifest their specificity in the presence of antisera against the corresponding NPd samples. As shown in Figure 6A, both the rat liver or Novikoff hepatoma NP–DNA complexes reacted with the rat liver NPd antiserum in the same manner. Intact chromatin exhibited only low complement fixation without appreciable tissue specificity. Essentially the same results were obtained with antiserum against Novikoff hepatoma NPd (Figure 6B).

In their recent report, Wakabayashi *et al.* (1973) pointed out that the nonhistone protein fraction (NP) of chromatin which can be identified with the immunochemical tissue specificity also contained proteins with affinity for native DNA. In an attempt to correlate these two features of the proteins present in the NP–DNA pellets, the NP–DNA fractions from liver and Novikoff hepatoma were further dissociated in 2.5 M NaCl–5.0 M urea–50 mM Tris-HCl buffer (pH 8.0) and centrifuged at 105,000*g* for 36 hr. The nonhistone protein containing supernatants were concentrated by ultrafiltration (Amicon UM-2) and iodinated with carrier-free ^{125}I (Sonoda and Schlamowitz, 1970) to facilitate the detection of minute protein quantities. Affinity chromatography, using native DNA trapped into agarose–polyacrylamide gels (Cavalieri and Carroll, 1970), was employed for the isolation of DNA-binding NP. The labeled samples dissolved in 5.0 M urea–50 mM Tris-HCl buffer (pH 8.0) were applied to the columns equilibrated with the same solvent. Calf-thymus or rat-spleen DNA was used for the separation. Individual protein fractions were eluted by applying a stepwise gradient of KCl in 5.0 M urea–50 mM Tris-HCl buffer (pH 8.0). As documented by the elution patterns shown in Figure 7, a fraction of the rat liver chromatin in NP was retained only on the column containing homologous (rat spleen) DNA. There was no detectable retention of the same protein on calf-thymus DNA-containing columns. The rather high (0.4 M) KCl concentration necessary for the elution of this DNA binding part of the nonhistone protein fraction from its binding to homologous DNA indicates an exceptionally strong interaction between these two macromolecular species.

When the ^{125}I-labeled peak eluted from rat-spleen DNA columns was concentrated and subjected to polyacrylamide electrophoresis in the presence of sodium dodecyl sulfate (Wilhelm *et al.*, 1972) it was found that it consists of three major polypeptide bands of a relatively low molecular weight (12,000–15,000 daltons). The photograph of a stained gel with superimposed ^{125}I radioactivity pattern is shown in Figure 8. Similar NP fractions isolated from several other rat and calf tissues also exhibited a limited heterogeneity. Although it

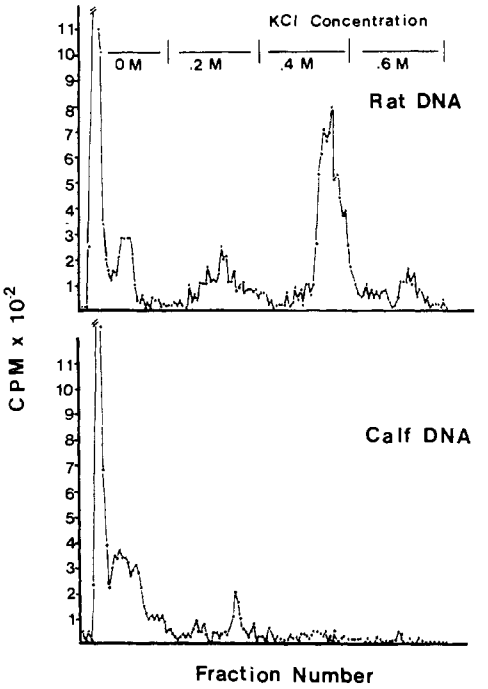

FIGURE 7: Affinity chromatography of rat-liver nonhistone proteins on DNA columns. Rat-liver nonhistone proteins (pH 8.0 supernatant) were labeled with ^{125}I *in vitro* and applied in 5.0 M urea–50 mM Tris-HCl buffer (pH 8.0) to a chromatographic column containing rat-spleen or calf-thymus DNA. The DNA columns were previously equilibrated with the same solvent. The labeled proteins were eluted with a stepwise gradient of 0.2, 0.4, and 0.6 M KCl.

cannot be excluded that each of the three stained polypeptide bands in Figure 8 may represent more than one polypeptide, it is unlikely that there would be a large number of polypeptides with nearly identical molecular weights binding selectively to homologous DNA.

Molecular weights of the three DNA-binding polypeptide bands from rat liver (Figure 8) are relatively low and resemble those of histone fractions KAS (F2b or IIb2), a ARE (F3 or III), and LAK (F2a2 or II2b1). To ascertain that the proteins represented here by the three stained bands are not histones, the amino acid composition of the DNA-binding rat-liver chromatin NP was determined. It is shown in Table I. The DNA-binding nonhistone proteins of rat-liver chromatin are not basic, although they contain slightly more lysine and arginine than was reported for most of the nonhistone chromatin protein preparations (Hnilica, 1972). When the rat-liver DNA-binding nonhistone proteins isolated by affinity chromatography were reconstituted to rat-spleen DNA, a NP–DNA complex was obtained with the immunochemical specificity of the reconstitution product of the original rat-liver NP fraction with rat-spleen DNA. It can be concluded from the experiments described here that the nonhistone chromatin fraction contains protein species with affinity for homologous DNA. The complexes of such proteins with homologous DNA are immunochemically tissue specific.

Discussion

Nuclear nonhistone proteins of higher animals were found to be tissue specific by various criteria (Hnilica, 1972). Many of these proteins can be phosphorylated *in vivo* or *in vitro* and the phosphorus content of some of the fractions could be correlated to quantitative and qualitative changes in the tran-

TABLE I: Amino Acid Composition of NP from Rat Liver.[a]

AA	Mol/100 mol of Total AA
Lys	7.9
His	1.9
Arg	8.1
Asp	7.3
Thre	4.7
Ser	6.8
Glu	12.2
Pro	5.1
Gly	10.8
Ala	9.0
Half-Cys	ND
Val	5.6
Met	1.3
Ile	4.2
Leu	8.8
Tyr	3.6
Phe	2.8
Acidic/basic	1.1

[a] Serine values were corrected (10%) for hydrolytic losses. ND = not determined. Although this protein contains tryptophan, this amino acid was not determined quantitatively.

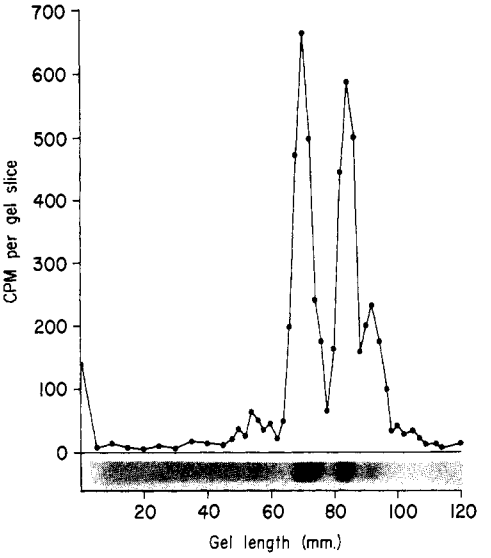

FIGURE 8: Electrophoresis in polyacrylamide gel of the radioactive material eluted with 0.4 M KCl from a rat-spleen DNA containing column (Figure 7). The test tubes comprising the 0.4 M KCl peak in Figure 7 were pooled, concentrated, and analyzed electrophoretically in the presence of sodium dodecyl sulfate. The gel was stained with Coomassie Blue and cut into 1-mm thick slices which were counted in the scintillation spectrometer. The radioactivity curve is superimposed over the photograph of stained gel.

scriptional activity of isolated chromatin (Kamiyama and Wang, 1971; Teng *et al.*, 1971; Shea and Kleinsmith, 1973).

Paul and Gilmour (1968) and Gilmour and Paul (1969) pointed out that the tissue-specific DNA restriction in chromatin, measurable by DNA–RNA hybridization, depends on the presence of nonhistone proteins in chromatin used as a template for the *in vitro* RNA synthesis. It was also shown (Gilmour and Paul, 1970; Spelsberg and Hnilica, 1970; Spelsberg *et al.*, 1971) that the transcriptional specificity characteristic for chromatin samples isolated from individual tissues can be transferred with a fraction of chromatin nonhistone proteins. These proteins, together with the histones, appear to associate with the DNA in a selective pattern, necessary for certain genes to be transcribed and others to remain silent. Additionally, specific binding of certain hormones could also be correlated to the nonhistone–DNA pellets of their target tissue chromatin preparations (Spelsberg *et al.*, 1972).

Using the nonhistone–DNA pellets which were shown to contain the macromolecular species necessary for the transcription of tissue-specific RNA species (Spelsberg and Hnilica, 1970; Spelsberg *et al.*, 1971), Chytil and Spelsberg (1971) demonstrated that these proteins are immunochemically tissue specific. Their findings were confirmed by Wakabayashi and Hnilica (1973) who showed that the tissue specificity of chromatin nonhistone proteins contained in the nonhistone–DNA pellets can be attributed to the presence of specific complexes between a fraction of these proteins and homologous DNA.

Chromatin preparations from the tissues of higher vertebrates contain proteins which can bind to the DNA with various degrees of specificity (Kleinsmith *et al.*, 1970; Teng *et al.*, 1971; Patel, 1972; Wakabayashi *et al.*, 1973). We have shown here that chromatin of several tissues contains proteins which bind only to the DNA from the same species (homologous DNA). Complexes of these proteins with homologous but not with heterologous DNA are tissue specific by immunochemical criteria. Their immunospecificity is similar to that exhibited by intact chromatin from the same tissue. This indicates that natural complexes between specific nonhistone proteins and DNA exist in native chromatin. Additional experiments are necessary to determine whether these tissue-specific complexes can be correlated with the transcriptionally specific DNA restriction in chromatin.

Acknowledgments

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Protein–Ribonucleic Acid Interactions in *Escherichia coli* Ribosomes. Solution Studies on S4–16S Ribonucleic Acid and L24–23S Ribonucleic Acid Binding†

C. Schulte, C. A. Morrison, and R. A. Garrett*

ABSTRACT: A study was made of the interaction of ribosomal proteins S4 and L24 with 16S and 23S RNAs, respectively, of *Escherichia coli*. The optimal temperature and solution conditions of binding were compared and shown to be almost identical. They were heating at 35–45°, 10^{-2}–10^{-1} M Mg^{2+}, 0–0.4 M K^{+}, and pH 7.4–7.9. A critical level of magnesium, associated with a structural change in both RNAs, was required for binding. The RNA and protein binding sites were both shown to be very stable at optimal ionic conditions over a wide temperature range. Only a slight decrease in binding affinity occurred between 0 and 42–48°. Kinetic and sedimentation evidence indicated that under optimal binding conditions conformational changes in a least a fraction of both 16S and 23S RNA populations occurred.

Several ribosomal proteins bind directly to 16S RNA (Mizushima and Nomura, 1970; Schaup *et al.*, 1970a, 1971a; Garrett *et al.*, 1971; Zimmermann *et al.*, 1972) and 23S RNA (Stöffler *et al.*, 1971a,b). Currently the nucleotide sequences of the protein binding sites on both 16S RNA (Schaup *et al.*, 1971b; Zimmermann *et al.*, 1972, 1974) and 23S RNA (Branlant *et al.*, 1973) and the amino acid sequences of many of the RNA-binding proteins are being determined (Wittmann-Liebold, 1971; Reinbolt and Schiltz, 1973). Very little is yet known, however, about either the structural chemistry or the mechanism of the protein–RNA interactions, except for some preliminary work on the latter for the S8–16S RNA interaction (Schulte and Garrett, 1972).

Two proteins, namely S4 and L24, were selected for detailed investigation. S4 binds specifically to 16S RNA (Mizushima and Nomura, 1970) and L24 binds to 23S RNA (Stöffler *et al.*, 1971a,b). For both proteins the nucleotide sequences of the RNA binding sites, which are protected against nuclease digestion by the protein, are almost completely known. They occur at the 5′ ends of their respective RNA molecules and are both about 400 nucleotides long (Schaup *et al.*, 1971b; Zimmermann *et al.*, 1972, 1974; Nanninga *et al.*, 1972; Branlant *et al.*, 1973). Moreover, the amino acid sequence of protein S4 is known (Reinbolt and Schiltz, 1973) and that of L24 is almost completed (R. R. Crichton and B. Wittmann-Liebold, unpublished work).

The results presented here are an attempt to elucidate the factors that influence the specificity of protein–RNA interactions with a view, later, to correlate and coordinate them with the structures of the RNA and protein binding sites. First, the binding conditions of the two proteins were characterized and compared. It was shown that these conditions were almost identical for the two proteins. Second, the stability of the protein and RNA binding sites was examined. They were both shown to be stable over a wide temperature range. Third, the possible occurrence of structural changes in the RNA and proteins was investigated and evidence for conformational changes occurring in the RNAs prior to binding was found.

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