Exposure of Histone Antigenic Determinants in Chromatin†

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ABSTRACT: The exposure of antigenic determinants of histones present in "native" chromatin was studied by: (1) testing their ability to elicit anti-histone antibodies and (2) measuring their ability to interact with anti-histone sera. To this end, antisera specific to purified histone fractions and to purified rat liver chromatin were elicited in rabbits. The anti-chromatin sera did not react with pure histone fractions and pure histone fractions F_2b, F_3, F_2a_1, and F_2a_2 failed to inhibit the complement fixation resulting from the binding of anti-chromatin to chromatin. These results suggest that in native chromatin, determinants in these histones are not immunogenic. Histone F_1, however, inhibited the reaction between chromatin and anti-chromatin. Antisera elicited by histone fractions reacted weakly with "native" chromatin. The maximal complement fixations (obtained with 5–10 μg of chromatin DNA) were as follows: 60% with anti-F_2b, 20% with anti-F_1 and anti-F_3, and less than 5% with either anti-F_2a_1 or anti-F_2a_2. Studies of the interaction between anti-histone antibodies and chromatin in which chromatin was used as an immunoadsorbent indicated that antibodies against different histones were adsorbed to a different degree by the same amount of chromatin. Differences in the immunoadsorbing capacity between sonicated and nonsonicated chromatin were found. Quantitative adsorbtion studies revealed that in the "native" chromatin structure, antigenic determinants of F_1 and F_2b were more available to interact with homologous antibody than those of F_3 and F_2a_1 and that determinants in F_2a_2 were the least available. It could be calculated that the "equivalent antigenicity" of the histones in chromatin was 9.6% for F_1, 3.2% for F_2b, and 0.90% for F_3 and F_2a_1. Upon sonication these values did not change for F_1 but increased two-, three-, and fourfold for F_2b, F_3, and F_2a_1, respectively. Digestion of chromatin with trypsin totally abolished the ability of chromatin to adsorb anti-histone antibodies.

Chromatin is a macromolecular nucleoprotein complex composed of four main components: DNA, histones, acidic proteins, and RNA (for reviews see Comings, 1972; DeLange and Smith, 1971). The major protein components of chromatin are histones which are defined as basic proteins associated with DNA. The histones have been the subject of numerous studies and it has been established that there are five major histone fractions denoted as F_1, F_2b, F_2a_1, F_2a_2, and F_3.

While the structure and properties of free histone in solution are fairly well known, their state and location in native chromatin have not been well defined. Estimates on the amount of DNA covered by histone range from 50 to over 90% (Clark and Felsenfeld, 1971; Itzhaki, 1971). There are indications that the lysine-rich histones preferentially bind to DNA in regions rich in A and T, while the arginine-rich histones preferentially bind to the C,G regions (Clark and Felsenfeld, 1971). Some studies point out the possibility that the various histones are clustered in a definite arrangement rather than being randomly distributed along the DNA chain (Ziccardi and Shumaker, 1973; Varshavsky and Georgiev, 1972). There is evidence indicating that histones can "move" along the DNA chain (Clark and Felsenfeld, 1972) and that the specific manner in which the histones are associated with DNA changes during the cell cycle (Stein et al., 1974). Recent studies raise the possibility that interactions among histones may play an important role in maintaining the structure of chromatin (Sperling and Bustin, 1974; Kornberg, 1974; D'Anna and Isenberg, 1974).

Studies on the role of histones in maintaining the structure and regulating the function of chromatin are hampered by the fact that these proteins have no assayable function. Immunological techniques have been used to identify changes occurring in protein structures (Sela, 1973) and to locate a variety of molecules in cells. Stollar and Ward (1970) reported that antisera specific to histones can be elicited in rabbits. Furthermore, subsequent studies (Bustin and Stollar, 1973a; Sluyser and Bustin, 1974) have indicated that serological techniques can be used to study the phytogenetic relationship in F_1 histones and specific determinants in F_1 histones have been identified (Hekman and Sluyser, 1973; Bustin and Stollar, 1973b). Serological techniques can be potentially useful tools to study the state and location of histones in chromatin. Indeed, recently we have shown that anti-histone sera interacts specifically with chromatin (Bustin, 1973). In the present study we try to estimate the degree to which the antigenic determinants present in histones are also available in chromatin.

Materials and Methods

Rat livers were obtained from either Wistar or BN rats grown at the Weizmann Institute. Calf thymus was obtained from a local slaughterhouse and transported to the laboratory on ice. Amberlite IRC-50 (Biorex-70) was obtained from Bio-Rad; guanidinium chloride, ultrapure from Mann Research Co.; anti-sheep hemolysin from Difco. Guinea pig complement was prepared by using animal sera incubated 1 hr at room temperature, then centrifuged at 14,000*g* for 15 min and stored at −70°. Lysozyme, trypsin, and soya bean trypsin inhibitor were from Worthington Chemical Co.; *Escherichia coli* strain K-12 RNA polymerase was from Sigma.

Isolation of Chromatin. Triton nuclear chromatin was

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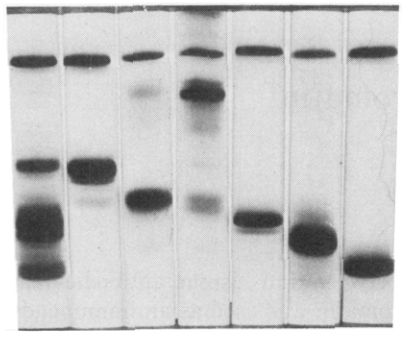

FIGURE 1: Purity of histone fractions used as immunogens and antigens. Electrophoresis according to Panyim and Chalkley (1969) in 2.5 *M* urea gels. Preelectrophoresis 16 hr at 1 mA/gel. Electrophoresis for 3.5 hr at 1.5 mA/gel and 130 V. From left to right whole histone 80 μg, F_1 30 μg; F_3 reduced 30 μg; F_3 oxidized 30 μg; F_2b 30 μg; F_2a_2 50 μg; F_2a_1 30 μg; Top band in all gels is bovine serum albumin.

prepared by a modification of the method described by Tata et al. (1972). Rats were anesthetized and bled through the abdominal vein and their livers perfused through the same vein with cold TKM (0.25 *M* sucrose in 0.05 *M* Tris (pH 7.4)–0.025 *M* KCl–0.005 *M* $MgCl_2$) solutions. All the following steps were done at 4°. Nuclei were obtained from the freshly perfused liver by the method of Blobel and Potter (1966). The nuclei were washed as recommended by Tata et al. (1972), once with 0.25 *M* sucrose in TKM, once with the same solution containing 1% Triton X-100, and finally again with 0.25 *M* sucrose in TKM. The washed nuclei obtained from about 15 g of tissue were suspended in a solution of 5 ml of 0.01 *M* Tris (pH 8.0), then 25 ml of 0.024 *M* EDTA (pH 8.0) was added and the suspension was allowed to stand at 4° for approximately 30 min. Nuclear disruption was checked by either phase contrast microscopy or by staining with a 5% Trypan Blue solution in 0.14 *M* NaCl–0.02 *M* sodium phosphate (pH 7.4). The nuclear suspension was then disrupted in a Potter-Elvejehm homogenizer (10 strokes, speed 5) to ensure complete breakage. The resulting suspension was centrifuged at 3000*g* for 10 min and the chromatin pellet washed once with saline–EDTA–Tris (Tata et al., 1972) and twice with 0.05 *M* Tris (pH 8.0). The washed chromatin was further purified by sedimentation through a 1.7 *M* sucrose gradient buffered to pH 8.0 with 0.05 *M* Tris (pH 8.0). The resulting pellet was suspended in 10 ml of 5 m*M* Tris (pH 8) and 0.02% NaN_3 by homogenization in a Potter-Elvejehm homogenizer (10 strokes, speed 10). The suspension was dialyzed at 4° against two changes (each 2 l.) of 5 m*M* Tris (pH 8) and 0.02% NaN_3. The solutions were sonicated at 4° for either 0.5 or 2.0 min in a Sonifier (Ultrasonics Model W185D) equipped with a Model L tip run at 60 W.

Preparation of Histones. Histones were prepared from washed calf thymus nucleoprotein by either method I or method II of Johns (1964). F_2a was separated into F_2a_1 and F_2a_2 fractions at 35° as recommended by Phillips (1968). These fractions were further purified by filtration through a Sephadex G-100 column equilibrated and eluted with 0.02 *M* HCl. F_1 was further purified by stepwise elution from an Amberlite IRC-50 column (Bustin, 1972). F_3 was obtained as a dimer after air oxidation in 6 *M* guanidinium chloride and filtration on a Sephadex G-100 column equilibrated and eluted with 0.02 *M* HCl. F_2b was purified by recycling (Oliver et al., 1972). The method of Bohm et al. (1973), which involves filtration on Bio-Gel P-60 equilibrated and eluted with 0.02 *M* HCl, 0.1 *M* NaCl, and 0.02% NaN_3 was also used to obtain histone fraction. In each case the final purity of the preparation was judged from its electrophoretic pattern in polyacrylamide gels and from amino acid analysis.

Immunological Procedures. Antisera against all histone fractions were prepared by immunizing rabbits with histone–RNA complexes (3:1 w/w) as recommended by Stollar and Ward (1970). Antisera against chromatin was elicited by injection of rabbits at multiple intradermal sites with 0.5 mg of chromatin DNA emulsified in a total of 1.5 ml of 60% complete Freund's adjuvant. Booster injections identical with the immunizing injections were given 14 days later and an intravenous injection containing 0.5 mg of chromatin DNA in 0.5 ml of 0.14 *M* NaCl; 0.02 *M* sodium phosphate (pH 7.4) was given 1 week after the booster. Animals were bled weekly starting 1 week after the intravenous booster.

Quantitative microcomplement fixation reactions were performed according to the method of Wasserman and Levine (1961) in a total volume of 1.4 ml as described by Stollar and Ward (1970). The serum dilutions noted are the final dilutions in the incubation mixtures.

Measurement of the Binding of Anti-Histone Antibodies to Chromatin. The principle of the method is that chromatin is used as an immunoadsorbant and that the adsorbed sera is always tested with the homologous antigen. A decrease in the complement fixation ability of the adsorbed antisera will be a measure of antibody absorption by the immunoadsorbant. Acid-washed tubes were used. Each tube contained the desired amount of chromatin, antisera at a 120-fold higher concentration than that used in the final assay mixture, and sufficient 1 m*M* Tris (pH 8.0) to bring the final volume to 0.25 ml. (If the chromatin solution is very diluted the volume can be scaled up.) The mixture was incubated at 37° for 1 hr and for 4 hr at 4° (in some cases the mixture was kept at room temperature without significantly changing the results). At this time the mixtures were made 0.14 *M* in NaCl and 0.02 *M* in sodium phosphate (pH 6.8), incubated at 4° for 15 min, and centrifuged to remove the chromatin with bound antibodies. The clear supernatant containing unadsorbed antibodies was diluted 100-fold with ISO-BSA (0.01 *M* Tris, 0.14 *M* NaCl, 0.5 m*M* $MgSO_4$, 0.15 m*M* $CaCl_2$, and 1 mg/ml of bovine serum albumin, pH 7.4) containing the necessary dilution of complement and 1 ml added to 0.2 ml of antigen solution and a micro complement fixation test was performed.

Tryptic Digestions. To the chromatin solution in 1 m*M* Tris (pH 8.0), 1 m*M* $CaCl_2$, trypsin, dissolved in 1 m*M* HCl, was added to a final ratio of chromatin protein to enzyme of 50:1 (w/w). Upon addition of $CaCl_2$ the solution turned turbid. After 10 min of incubation the solution became clear. The mixture was incubated for 4 hr at 37°. The reaction was terminated by adding soya bean trypsin inhibitor (2/3 weight to trypsin).

Analytical Procedures. Electrophoresis in polyacrylamide gels was performed according to Panyim and Chalkley (1969); DNA was determined by the diphenylamine reaction (Burton, 1956); RNA, by the Orcinol method (Dische, 1955); protein, by the method of Lowry et al. (1951). Amino acid analyses were performed according to Moore and Stein (1963).

Results

Antigens: Histones and Chromatin. In the present study we intended to use anti-histone sera as specific probes to

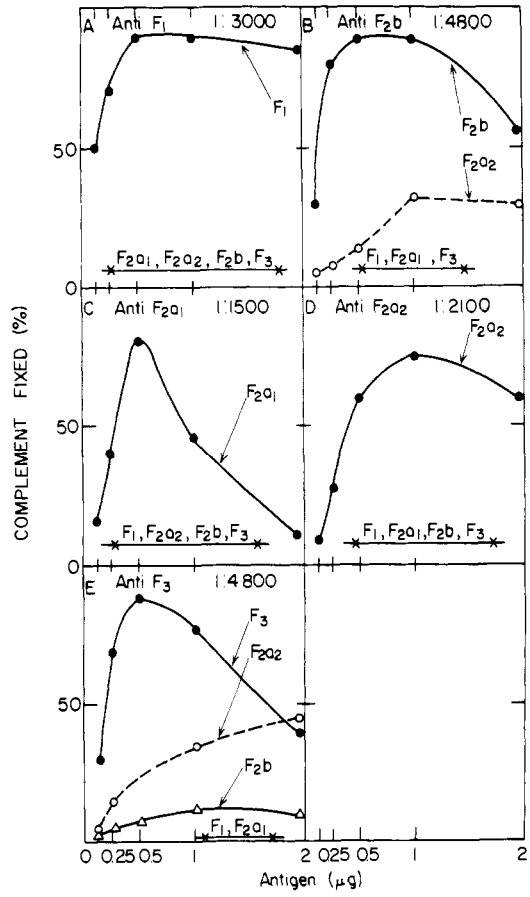

FIGURE 2: Complement fixation of antisera elicited by histone-RNA complexes with various histone fractions.

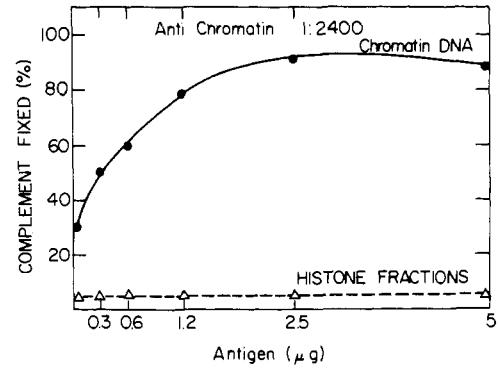

FIGURE 3: Lack of reaction between anti-chromatin sera and purified histone fractions.

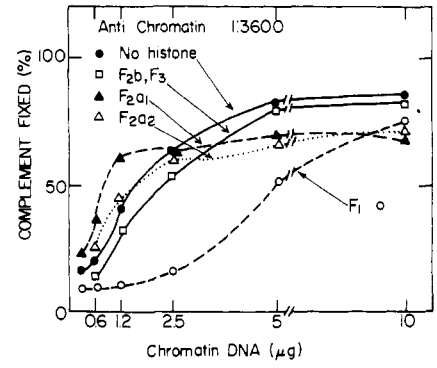

FIGURE 4: Inhibition of the serological reaction between chromatin and anti-chromatin sera by purified histone fractions. To 0.2 ml of anti-chromatin sera diluted 1:600 with ISO-BSA, 0.5 μg of purified histone dissolved in 0.2 ml of ISO-BSA was added. The mixture was incubated 1 hr at 37° and 4 hr at 4°. At the end of incubation 0.6 ml of ISO-BSA containing the appropriate dilution of complement and 0.2 ml of ISO-M containing appropriate dilutions of chromatin were added and the complement fixation assay was performed.

test the state of the various histones in chromatin. Such studies require sera strictly specific for the test antigen. To obtain specific antisera it is necessary to use pure antigens. We have assayed the purity of the histones by electrophoresis in in polyacrylamide gels and amino acid analysis. From the results presented in Figure 1, where the gels were overloaded to visualize possible contaminants, it can be seen that the histone fractions used were pure and not cross-contaminated.

Similarly, we looked for a method of chromatin isolation that will yield reasonably pure chromatin as free as possible of cytoplasmic and nucleophasmic contaminants. The method used by Tata et al. (1972) involves purification of nuclei by centrifugation through 2.3 *M* sucrose, extensive washing of nuclei and chromatin, and a final chromatin purification through sucrose centrifugation. The use of Trition X-100 and NaN_3 allows the obtaining of a fairly stable preparation as assayed by T_m analysis and RNA synthesis. The preparations obtained by us (13 different preparations) had a protein/DNA ratio of 1.8 ± 0.3 (w/w), a RNA/DNA ratio of 0.14 ± 0.03 (w/w), and A_{260}/A_{320} ratio of less than 0.05, and a uv spectrum typical of that reported by others for purified rat liver chromatin (Marushige and Bonner, 1966). The RNA synthesizing capacity per unit of DNA of the chromatin was 8–12% of commercial calf thymus DNA freed from proteins by phenol extraction (Saito and Miura, 1963).

Antisera. That the purified histone fractions elicited specific antisera can be seen from Figure 2. At the dilutions tested each of the sera reacted well only with the histone used as immunogen. F_2a_2 gave slight cross-reactivity with both anti-F_2b and anti-F_3.

Antibodies against chromatin were elicited by injecting four rabbits as described under Materials and Methods. Each of the sera obtained reacted with chromatin giving a maximum complement fixation of 90% at a serum dilution of 3000 with 2.5 μg of DNA. The complement fixation reaction between anti-chromatin sera and chromatin is presented in Figure 3.

Immunogenicity of Histone Antigenic Determinants in Chromatin. If histone antigenic determinants in chromatin are exposed to a similar degree as in histone-RNA complexes, then it could be expected that anti-chromatin sera would contain anti-histone antibodies. The results of studies designed to test this (Figure 3) indicated that at sera dilutions which have strong complement fixation with chromatin no reaction with histone fractions could be detected.

These results suggest that in native chromatin, the histone antigenic determinants are not immunogenic. That is to say, that upon injection of chromatin no, or very few, anti-histone antibodies are formed. The data presented in Figure 4 further support this observation. In this experiment the ability of 0.5 μg of histone to inhibit the reaction between chromatin and anti-chromatin was tested. It can be seen that, with the exception of F_1, free histones do not significantly affect the binding of anti-chromatin. F_1 histone, rather than causing a vertical shift in the amount of complement fixed, shifted the maximum reaction to higher antigen (chromatin) concentrations.

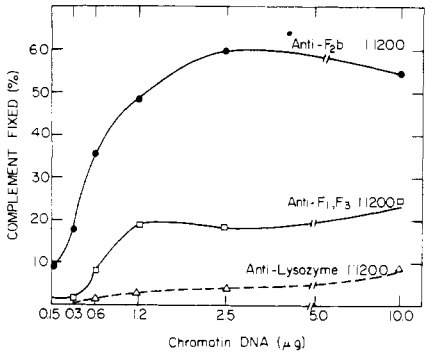

FIGURE 5: Reaction of anti-histone sera with chromatin. In this test chromatin was used as test antigen.

Antigenicity of Histone Determinants in Chromatin. The results presented so far suggest that with the possible exception of F_1, histone determinants in chromatin are not immunogenic in this state. It is, however, possible that the histones in chromatin are antigenic, and are capable of reacting with anti-histone sera. To test this, the various anti-histone sera were reacted with chromatin, and the complement fixing ability of the resulting complexes was measured. As seen by the data presented in Figure 5, the anti-histone sera displayed varying reactivities. At 1:1200 sera dilutions the maximal complement fixation was 60% with anti-F_2b sera, 20% with anti-F_1 and anti-F_3 sera, and less than 5% with either anti-F_2a_1, anti-F_2a_2, or anti-lysozyme sera.

These results suggest that some histone determinants are available in chromatin. We therefore looked for a different test system which would allow the use of higher chromatin concentrations which are prohibitive in the direct complement fixation test. As reported previously (Bustin, 1973) it was found that the interaction between anti-histone antibodies and chromatin can be better characterized when chromatin is used as an immunoadsorbant rather than test antigen. In such studies antisera was added to chromatin solutions, the chromatin and antibodies bound to it separated by centrifugation in 0.14 *M* NaCl (pH 6.8) solutions, and the remaining antibodies tested against homologous antigens. Loss of antibody due to their binding to histones in chromatin is detected as a vertical and, in some cases, also a lateral shift (compared to nonadsorbed serum) in the complement fixation curve. The shifts are dependent on the amount of chromatin used as immunoadsorbant. The finding that even 250 μg of chromatin DNA had no effect on anti-lysozyme sera (Bustin, 1973) is strong evidence that the loss of complement fixing ability is due to specific binding of anti-histone sera to chromatin rather than to nonspecific effects. Furthermore, treatment of chromatin with trypsin under conditions where most of the proteins in chromatin are digested (Simpson, 1972) totally abolished the ability of chromatin to adsorb anti-histone antibodies (Figure 6).

The magnitude of both the lateral and the vertical shifts resulting from depletion of antibody is highly dependent on the assay conditions used. Thus, when excess antisera is used the complement fixation obtained is strong because sufficient antibodies are left to give essentially maximal complement fixations. An example of such conditions is presented in Figure 7. It can be seen that in both anti-F_1-F_1 and anti-F_2a_1-F_2a_1 systems the drop in the percentage of complement fixed, due to adsorption of the antisera by the same amount of chromatin, is dependent on the particular sera dilutions used. Conversely, when high sera dilutions are used even small amounts of chromatin abolish all the apparent complement fixing ability.

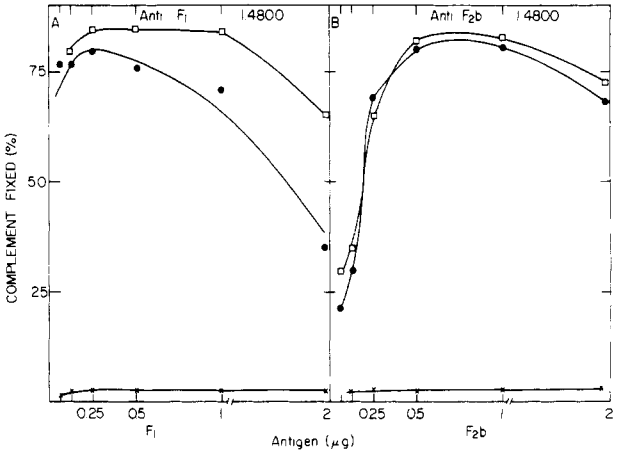

FIGURE 6: Effect of tryptic digestion of chromatin on its ability to bind anti-histone sera; 0.125 μg of chromatin DNA was used for adsorption of anti-histone sera. (□) Control, nonadsorbed sera; (×) control, sera adsorbed on undigested chromatin; (●) sera adsorbed on trypsin digested chromatin.

Therefore, to obtain reproducible results the sera dilutions have to be adjusted separately for each antigen-antibody system so that the maximum complement fixation obtained (without chromatin absorption) falls in the narrow range between 80 and 90%. Comparison of 12 different chromatin preparations obtained from rat liver indicated that the drop in complement fixation, due to adsorption of antibody by the same amount of DNA chromatin, varied by about 10% among the various preparations. Similar variability was found when the stability of the preparations was tested. One month old preparations bound practically the same amount of antibodies as fresh preparations. However, we did not use preparations that were stored for more than 3 weeks.

To obtain further insight into the question of the degree of exposure of histone determinants in chromatin, the capacity to adsorb antibody of nonsonicated chromatin was compared to that of chromatin preparations sonicated to various degrees. The results of such a study are presented in Figure 8. It can be seen that sonication of chromatin did not affect its ability to adsorb anti-F_1 antibodies (Figure 8A–C). Similarly, the ability of chromatin to adsorb anti-F_2b also was only slightly affected by sonication (Figure 8D and E) supporting our findings that in "native" chromatin determinants in F_1 and F_2b are relatively accessible to interact with antibody. In contrast, a significant effect of chromatin sonication was detected with both anti-F_2a_1 and anti-F_3 sera. A comparison of Figure 8J to 8L and 8M to 8O shows that for the same amount of DNA the sonicated chromatin was a significantly better immunoadsorbant, suggesting that determinants in these histones are buried inside the nucleohistone complex. For F_2a_2 the shifts were anomalous. When highly sonicated sera were used a significant lateral shift of the complement fixation curve was observed.

Discussion

So far two main approaches have been used in the study of chromatin structure (for reviews see Comings, 1972; Huberman, 1973; Simpson, 1973). One, a synthetic approach involves characterization of the whole macromolecular complex by a variety of physical techniques; the second, an

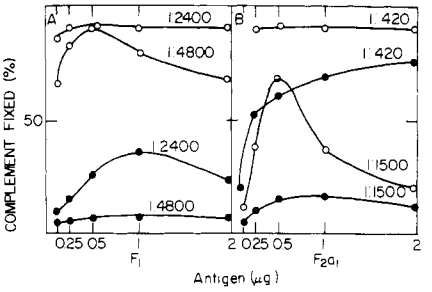

FIGURE 7: Detection of saturation of chromatin by anti-histone sera. (O) Control reactions between histones and anti-histone sera not adsorbed on chromatin. (●) Reaction between histones and anti-histone adsorbed on 125 μg of chromatin. (A) Anti-F_1 at 1:2400 and 1:4800 dilution; (B) anti-F_2a_1 at 1:420 and 1:1500 dilution.

analytical approach involves the study of the isolated chromatin components and their assembly to form structures resembling the native structure. Each of these approaches by themselves cannot provide all of the information necessary to understand the detailed structure of chromatin and chromosomes. Because most of the chromatin components have no assayable function the synthetic approach does not allow the study of individual components without an identifiable marker, while the analytical approach does not yield information on how the individual components are arranged in the "native" chromatin structure and their possible rearrangements with the changes that occur in the nucleoprotein structure during the cell cycle.

Immunological techniques can be used to bridge between the synthetic and analytic approach. The finding that antibodies against specific histones bind to chromatin (Bustin, 1973) raises the possibility that such antibodies may serve as reagents to identify specific histones and follow their fate during cell changes. It should be remembered that specific sera against other chromosomal components such as DNA (Stollar, 1970), non-histone proteins (Zardi et al., 1973), nucleosides (Schreck et al., 1973), and non-histone protein-DNA complexes (Chytil and Spelsberg, 1971) have already been elicited. Furthermore, it has been shown that such sera react with chromatin and that the fate of nuclear components during the cell cycle can be studied by immunological techniques (Tan and Lerner, 1972; Schreck et al., 1973).

That specific antisera directed against purified histone fractions can be elicited in rabbits immunized with RNA-histone complexes has been previously reported by Stollar and Ward (1970). Our results confirm their findings. Thus, it can be concluded that antisera can be elicited not only against species specific histone such as F_2c (Sotirov and Johns, 1972) but against nonspecies specific histones. Furthermore, we were able to elicit in rabbits antisera against rabbit F_1 histones.

We recently reported that in the case of F_1 histones specific antisera are potent tools to study the phylogenetic relationships in this class of histones. Furthermore, since it is possible to identify specific determinants in histones (Bustin and Stollar, 1973b; Hekman and Sluyser, 1973) and techniques for isolation of determinant specific antibodies are available (Arnon, 1971), it is conceivable that the state of specific regions of histones in chromatin can be studied by immunochemical techniques. In view of the nonrandom distribution of amino acid residues along the histone polypeptide chain such studies may be important.

Histone determinants seem not to be immunogenic in native chromatin. Our results provide conclusive evidence that

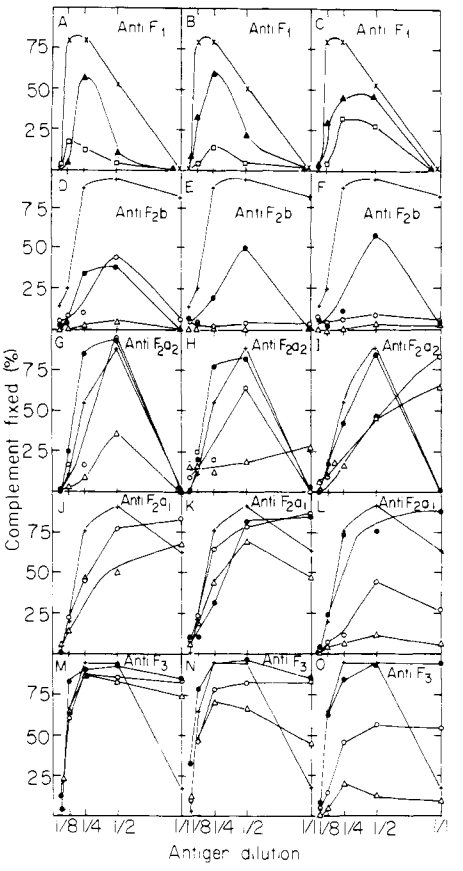

FIGURE 8: Effect of sonication of chromatin on its ability to bind anti-histone sera. (A,D,G,J,M) Nonsonicated chromatin; (B,E,H,K,N) chromatin sonicated for 30 sec; (C,F,I,L,O) chromatin sonicated for 120 sec. (A-C) anti-F_1 1:7500; (D-F) anti-F_2b 1:6000; (G-I) anti-F_2a_2 1:3000; (J-L) anti-F_2a_1 1:5000; (M-O) anti-F_3 1:6000. The amount of chromatin DNA used for adsorption was: (×) 0 μg; (▲) 7 μg; (□) 15 μg; (●) 25 μg; (O) 75 μg; (△) 125 μg. The amounts of antigen at 1:1 dilution were 2.0 μg for F_1, F_2b, F_2a_1, and F_3, and 4.0 μg for F_2a_2.

histone determinants in chromatin are antigenic, that is, they will react with anti-histone sera. So far the most efficient and sensitive way to study the interaction between histones complexed with DNA in the "native" chromatin and anti-histone sera is to use chromatin as an immunoadsorbant. The insolubility of chromatin at moderate salt concentration makes the separation of bound from unbound antibody a fairly easy task. Furthermore, this method enables the use of a relatively high concentration of chromatin (which in some case is anticomplementary) and allows the testing of each antisera with homologous antigen.

The loss of activity of adsorbed sera was shown to be specific since the test antiserum, anti-lysozyme, has not been adsorbed (Bustin, 1973) and digestion of chromatin with trypsin totally destroyed the binding activity of chromatin. Furthermore, in tests employing 12 different chromatin preparations reproducible results were obtained.

It was of interest to see whether the immunological reaction between anti-histone sera and chromatin could be used to study the state of histones in chromatin. The binding of anti-histone sera to histones in chromatin depends on two major factors: (a) the specificity, potency, and quantity of the various antibodies present in the test sera, and (b) the availability of antigenic sites in histones in "native" chromatin. The present study is mainly concerned with the second factor.

Table I: "Equivalent Antigenicity" of Chromatin-Bound Histones.[a]

Histone Fraction	Equivalent Antigenicity (%) "Native" Chromatin	Sonicated Chromatin
F_1	9.6	10.6
F_2b	3.2	6.4
F_2a_1	0.90	3.4
F_2a_2		3.4
F_3	0.90	3.4

[a] The data were calculated for adsorption with 75 μg of either native chromatin or chromatin sonicated for 2 min as described under Materials and Methods. With F_1 the adsorption was so strong that the data used were those obtained with 7 μg of chromatin. With F_2a_2 the adsorption was weak and the curve obtained with sonicated chromatin was anomalous. The value given is obtained for antisera absorbed with 75 μg of slightly sonicated chromatin. For calculation of "equivalent antigenicity" see text.

Since we wanted to compare different sera rather than use similar sera dilutions, the interactions of the various sera with their homologous antigens have been normalized to obtain optimal complement fixation curves with a maximum complement fixation in the range of 80–90%. We assumed, therefore, that the binding of anti-histone sera to chromatin would be a measure of the availability of various determinants. The availability of the various determinants is probably dependent on steric factors such as the extent to which the histone is buried inside the macromolecule nucleoprotein complex and upon conformational changes that occurred in the molecule upon binding to DNA or complexing with other chromosomal proteins.

Strictly for comparative purposes it is useful to quantitate the availability of the determinants in the histones complexed in chromatin. This value was defined as "equivalent antigenicity". It compares the ability of chromatin-bound histone to that of nonbound histone to adsorb antihistone antibodies. The amount of chromatin-bound histone can be calculated because it is known that in rat liver the weight ratio of histone/DNA is about 1.0 (Marushige and Bonner, 1966) and that each histone fraction comprises approximately 20% of the total histone complement. Thus, if the data in Figure 8J are taken as example, it can be seen that the amount of F_2a_1 histone present in 75 μg of chromatin DNA (i.e., 15 μg) lowered the maximum percent complement fixed by 1.0 μg of free F_2a_1 from 90 to 78%. Therefore the "equivalent antigenicity" of F_2a_1 in nonsonicated chromatin can be calculated to be (1.0 μg/15.0 μg) [(90% − 78%)/90%]100% = 0.90%. This means that under a defined set of experimental conditions (in our case 75 μg of chromatin adsorbing antibodies for 1 hr at 37° and 4 hr at 4°) the capacity of bound F_2a_1 to adsorb antibody is 0.90% of that of unbound F_2a_1. Because the ability of free histone to bind antibody has not been measured (i.e., it is not certain that under these conditions 15 μg of free F_2a_1 will bind 100% of the antibodies) the value of 0.90% cannot be taken as an absolute value. It is useful, however, for comparing sonicated to nonsonicated chromatin and because for each antigen–antibody system the complement fixation curve has been separately normalized, it can be used to compare the different histones.

The "equivalent antigenicity" of the various histones is presented in Table I. It can be seen that F_1 and F_2b have a higher value than the other histone fractions. Sonication had the greatest effect on F_2a_1 as it increased its value of "equivalent antigenicity" fourfold. The values for F_3, F_2b, and F_1 increased three-, two-, and 1.1-fold, respectively.

Our results suggest that determinants in F_1 histone are most available to interact with homologous antibody. The loss of activity in this serum due to adsorption (per unit of chromatin DNA) was greatest. Histone F_1 was also the only histone which inhibited the reaction between anti-chromatin and chromatin. Thus, it is possible that these molecules are located on the periphery of the chromosomal complex. Enzyme digestion (Chatterjee and Walker, 1973) and salt extraction (Ohlenbusch *et al.*, 1967) studies also imply that the F_1 histones are located on the outside of the chromatin. Similarly, determinants in F_2b were readily available and it should be remembered that anti-F_2b gave the best complement fixation curve when chromatin was used as antigen.

We have previously reported (Bustin, 1973) saturation experiments which suggested that determinants in the various histones are spatially separated enough for their determinants not to overlap. In view of the low equivalent antigenicity values of histones in chromatin, steric hindrance could be expected only if the histones would be placed in chromatin in a random, unorganized, overlapping fashion. Thus, the saturation experiments suggest that the histones in chromatin are organized in some ordered fashion. Several types of histone arrangements in chromatin have been suggested (Varshavsky and Georgiev, 1972; Zicchardi and Shumaker, 1973; Kornberg, 1974; Sperling and Bustin, 1974).

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Synthesis of Rhodopsin and Opsin in Vitro†

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ABSTRACT: Isolated bovine retinas have been used to study the synthesis of rhodopsin and the renewal of photoreceptor rod outer segments. Both leucine and glucosamine served as radioactive tracers to follow rhodopsin synthesis. In both cases the rod outer segment preparations contained large amounts of labeled macromolecules chromatographically distinct from rhodopsin, the latter representing only about 10% of the high molecular weight labeled material. However, electrophoresis on polyacrylamide gels with sodium dodecyl sulfate indicated that as much as 60-68% of the radioactivity coincided with opsin, the apoprotein of rhodopsin. Treatment of labeled rod outer segments with 9-*cis*-retinal caused much of the non-rhodopsin label to be converted to isorhodopsin. After such treatment the fraction of the label in visual pigment rose from about 10 to 51% with leucine as the radioactive tracer and to 78% with glucosamine. Similar treatment of bleached outer segments labeled with leucine gave identical results with complete regeneration of isorhodopsin (λ_{max} 487 nm) which then accounted for 56% of the labeled macromolecules. No such conversion occurred in controls lacking 9-*cis*-retinal. Both 9-*cis*- and 11-*cis*-retinal were effective but *all-trans*-retinal was ineffective in producing the conversion. Under in vitro conditions opsin appears to be accumulated in the outer segment prior to the addition of retinal.

Biochemical studies on the biosynthesis of rhodopsin were initially carried out in whole animals (Hall et al., 1968, 1969; Matsubara et al., 1968; Bargoot et al., 1969). Subsequent work in vitro has involved the use of isolated retinas (O'Brien et al., 1972; Basinger and Hall, 1973; O'Brien and Muellenberg, 1973, 1974; Bok et al., 1974). In working with bovine retina, we have consistently found in outer segment preparations highly labeled opsin-like proteins which we have recently succeeded in converting to visual pigment (O'Brien and Muellenberg, 1974). However, no such striking accumulations of opsin are observed in the

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