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Antibodies to Histones and Histone-Histone Complexes: Immunochemical Evidence for Secondary Structure in Histone 1

Abstract. *Highly specific antibodies were raised to histone 1 (H1) and the histone complexes $H3_2$–$H4_2$ and H2A–H2B, isolated by salt extraction. Antibody to H1 could detect irreversible conformational changes in acid- or urea-treated H1. The antibodies showed different reactivities with chromosomes as compared to antibodies in acid-extracted histones and should be useful in studies of native chromatin and chromosome structure.*

Evidence for the existence of secondary structure in histones has been obtained from studies of circular dichroism, infrared and nuclear magnetic resonance (*1*), fluorescence anisotropy (*2*), and optical rotatory dispersion (*3*). The consensus is that histone fractions exist largely as random coils in solutions of low ionic strength. However, small increases in ionic strength or the addition of DNA gives rise to considerable helicity in all histones with the exception of histone 1 (H1), which is thought to assume an extended β-conformation (*4*). All these studies have been carried out with histones isolated from chromatin by acid extraction procedures, the assumption being that acid-induced conformational changes are largely reversible. Although acid-treated histones do assume α-helical conformation in salt solutions, there is no evidence that this is the same α-helical structure found in native histones. Moreover, evidence is growing (*5*, *6*) that the conformational changes of histones exposed to acids are irreversible. The question of whether histones revert to their respective native configurations after exposure to denaturing conditions must be answered if meaningful interpretations are to be derived from studies in vitro relating to nucleohistone function and structure and to histone-histone interactions (*7*).

Problems dealing with secondary structure are amenable to investigation by immunochemical methods (*8*). Although antibodies have been produced to calf thymus histone fractions (*9*–*11*), here, too, the histones used as antigens were isolated by procedures that included denaturing agents such as urea, guanidinium chloride, and dilute mineral acids. Inasmuch as the H1 monomer, the $H3_2$–$H4_2$ tetramer and the H2A–H2B oligomers can now be isolated by mild salt extraction procedures (*5*, *12*), we attempted to produce antibodies to such preparations and to determine whether they can be used to provide insight on the conformational characteristics of histones.

For the preparation of the histones, 30 g of frozen calf thymus was homogenized at 700 rev/min for 4 minutes (*13*) with the use of the grinding medium of Panyim *et al.* (*14*). Nuclei were washed once with grinding medium containing 0.5 percent (weight to volume) Triton X-100 and sedimented (SW 25.1 rotor) through a grinding medium that was 1.62*M* in sucrose (*15*). The pellets were washed again as above. Electron microscopy revealed mostly intact nuclei stripped of their outer nuclear membrane and attached ribosomes. Purified chromatin was obtained from two such preparations of nuclei by freeze-thawing and Dounce homogenization in hypotonic buffer containing ethylenediaminetetraacetate (EDTA) (*14*), followed by three washings with 0.25*M* NaCl–0.01*M* tris-HCl (*p*H 8.0). The H1 was extracted with 310 ml of 0.6*M* NaCl–0.05*M* sodium acetate buffer (*p*H 5.0), and the deoxyribonucleoprotein, from which most of the H1 had been removed, was sedimented by ultracentrifugation (36 hours at 2°C) in polycarbonate tubes (*16*). The deoxyribonucleoprotein was extracted further with 2.0*M* NaCl–0.05*M* sodium acetate (*p*H 5.0), sedimented and reextracted with 2.0*M* NaCl to give a mixture of $H3_2$–$H4_2$ tetramer and H2A–H2B oligomer. Histone solutions were desalted at 4°C by ultrafiltration through a UM10 membrane (Amicon) and lyophilized. About 1.4 g of total histone was recovered from 60 g of calf thymus, a yield that compared favorably with yields from direct extraction of washed calf thymus homogenates with acid (*17*). Histone 1 was purified by chromatography through a Sephadex G-100 column (2.5 by 95 cm) at 5°C; the other histones, present as complexes, were also purified (*12*). All solutions contained 0.05*M* $NaHSO_3$ as protease inhibitor (*18*).

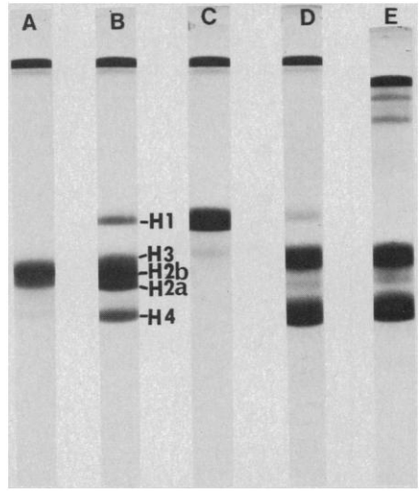

Fig. 1. Salt-extracted histones were subjected to electrophoresis for 3.5 hours in 15 percent acrylamide, 2.5*M* urea, 0.9*N* acetic acid, *p*H 2.7 (*24*). Histone 1 (25 μg) was added as a marker in each gel 1 hour before termination of electrophoresis (top band of each gel is the marker). Gel A, 25 μg of H2A–H2B; gel B, 50 μg of total histone; gel C, 50 μg of H1; gel D, 50 μg of $H3_2$–$H4_2$; gel E, 50 μg of $H3_2$–$H4_2$, from the second extraction with 2.0*M* NaCl. (Gel E was not processed at the same time as gels A, B, C, and D. The two extra bands immediately below the H1 marker in gel E represent degradation products of H1 resulting from storage at 4°C for 2 weeks. Freshly dissolved H1 was used as marker for gels A to D.) For immunization and complement fixation, histone antigens represented by gels A, C, and E were used. Histone complexes dissociate into their respective monomers under the electrophoretic conditions used.

Histone 1, $H3_2$–$H4_2$ tetramer [according to (*5*)], and H2A–H2B oligomer were pure as shown by polyacrylamide gel electrophoretic patterns (Fig. 1). For immunization, they were dissolved in saline (2 mg/ml) and emulsified with equal volumes of Freund's complete adjuvant (Difco) before each immunization. New Zealand white rabbits were injected with 0.1 ml every 7 days in each of four toepads. The 7*S* globulins were routinely obtained from all serums by fractionation on DEAE-cellulose (*19*); neutral 7*S* globulins were tested by a quantitative micro complement fixation technique in a total volume of 1.4 ml (*9*).

Specific antibodies were produced to H1, to the $H3_2$–$H4_2$ tetramer, and to the H2A–H2B oligomer. Antibodies to H1 had the highest serum titer at 80 percent complement fixation (1 : 9870, based on

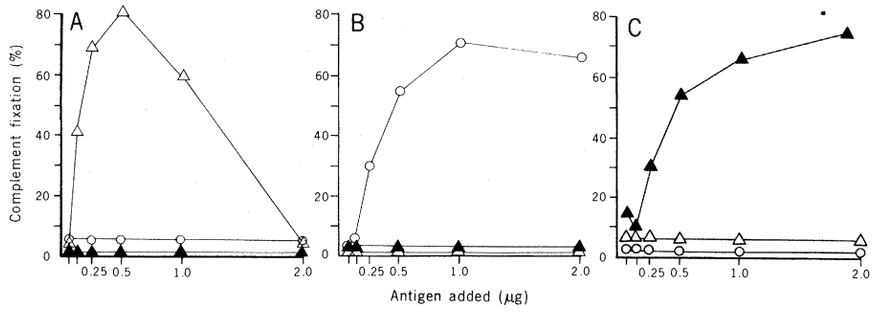

Fig. 2. Micro complement fixation reaction of histone fractions H1 (Δ), $H3_2$–$H4_2$ tetramer (▲), and H2A–H2B oligomer (○) with their homologous 7*S* antibodies. (A) Antibody to H1 (equivalent to serum dilution of 1 : 9870); (B) antibody to H2A-H2B (1 : 2820); (C) antibody to $H3_2$–$H4_2$ (1 : 1645). The titers given are the final dilutions in the reaction mixtures.

an estimated 7*S* globulin level in rabbit serum of 20 percent), while the antiserums to $H3_2$–$H4_2$ and H2A–H2B oligomer had titers of 1 : 1645 and 1 : 2820, respectively. No cross-reaction was observed between any of the antiserums (Fig. 2). No cross-reactions were observed at a 1 : 39 dilution of antiserums, a dilution within the range used in the chromosome studies (see below). Although the histone complexes, that is, $H3_2$–$H4_2$ and H2A–H2B, have considerably more secondary structure and higher molecular weights than H1, a stronger immune response was elicited by H1.

Histone 1, isolated by 0.6*M* NaCl extraction of chromatin and purified by Sephadex chromatography (as described above), fixed complement maximally at 0.5 μg of antigen added. Urea- and acid-treated H1 samples (legend to Fig. 3) also fixed complement maximally at 0.5 μg of antigen, but significantly less than the salt-extracted H1. In treating H1 samples by the commonly used acid procedures, we had followed the established method of washing the extracted histone with acetone (when the perchloric acid–trichloroacetic acid procedure was used) (*17*) or precipitating with ethanol during the sulfuric acid procedure (*14*). To test the possibility that acetone or ethanol treatment might contribute to a significant loss in complement-fixing activity, samples of salt-extracted H1 were treated with these solvents alone. No appreciable effect was found (Fig. 3).

We can conclude, therefore, that exposing H1 to dilute acid or to urea causes an irreversible change in its secondary structure, as evidenced by the ability of its antibody complex to fix only half as much complement as H1 treated with salts only. An antiserum to H1 (*20*), which was prepared to a fraction of acid-extracted H1, was unable to distinguish between salt-extracted and acid-treated preparations of H1, fixing complement equally with both. Presumably, it is specific for determinant groups common to both, but unaffected by conformational changes brought about by dilute acid.

The antibodies we describe should be useful probes of intact chromatin or chromosomes, particularly those preparations not exposed to acid fixation—a procedure known to remove a variable and often uncharacterized fraction of histones (*21*). Moreover, since we now report antiserums specific for the histone complexes, questions of histone-histone interactions in native chromatin can now be investigated by direct immunochemical methods. Antibodies to acid-extracted histones other than H1 have been shown to react with acid-fixed mammalian metaphase and insect polytene chromosomes (*22*). Recently, antibodies to H4, but not to other histone fractions, were reported to produce brilliant transverse banding of acid-fixed chromosomes from the Chinese hamster (*11*). Initial observations with our antiserums fractionated on DEAE-cellulose (*19*) indicate that antibodies to H1 and to the H2A–H2B complex fail to bind detectably to mammalian metaphase chromosomes fixed in methanol–acetic acid. However, when chromosome spreads were made by simple air drying, in a cytocentrifuge, antiserum to H1 bound strongly to the chromosomes; antiserum to H2A–H2B still showed no detectable binding, perhaps owing to the masking by H1 or by DNA. Selective removal of histone fractions or DNA without resorting to denaturing agents will provide more definitive answers. It is clear, however, that the antibodies we are using react differently with chromosomes than do antibodies to acid-extracted histones.

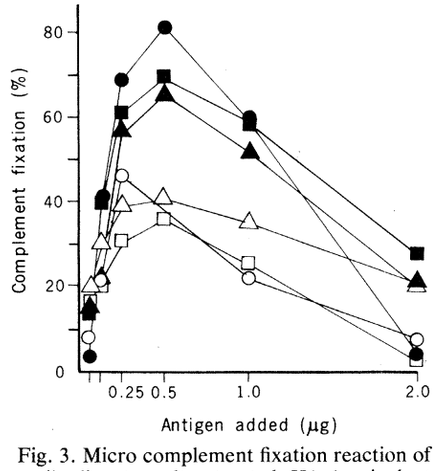

Fig. 3. Micro complement fixation reaction of antibodies to salt-extracted H1 (equivalent serum titer 1 : 9870) with H1 antigen exposed to various denaturing conditions. (□) Salt-purified H1 (10 mg) was dissolved in 5 ml of 0.74*N* perchloric acid for 1 hour at 4°C, precipitated with an equal volume of 36 percent (weight to volume) trichloroacetic acid, and washed with acetone (*17*). (△) H1 (10 mg) was treated with 2 ml of 0.25*N* sulfuric acid for 1 hour at 4°C, precipitated with four volumes of cold ethanol at –20°C for 24 hours, centrifuged, and washed with cold 95 percent ethanol (*14*). Other samples were treated (1 hour at 4°C) with 8*M* urea followed by dialysis (○), with cold acetone (▲), or with cold 95 percent ethanol (■). (●) Salt-extracted H1—that is, undenatured H1.

Our data show, therefore, that specific antibodies can be elicited to salt-extracted H1 and to histone complexes $H3_2$–$H4_2$ and H2A–H2B and that no cross-reactions occur among them. Moreover, with respect to H1, at least, these antibodies can detect conformational changes brought about by exposure to dilute acid or to urea. We also present evidence that some determinant groups, detectable by antibodies to salt-extracted histones, are not detected by an antibody preparation elicited by an acid-extracted histone antigen. Our data do not allow us to conclude that salt-extracted histones are "native," but they do raise serious questions about the validity of interpretations of reconstitution experiments carried out with acid-extracted histones (*23*).

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Neurotransmitter Regulation of Adenosine 3′,5′-Monophosphate in Clonal Nerve, Glia, and Muscle Cell Lines

Abstract. *Norepinephrine increases the intracellular level of adenosine 3′,5′-monophosphate (cyclic AMP) in clonal cell lines of nerve, glia, smooth muscle, and skeletal muscle. The largest response is in skeletal muscle, where the cyclic nucleotide concentration is elevated more than 500-fold. Glia and muscle cells, but not nerve cells, respond to dopamine with increased cyclic AMP accumulation. This response appears to be mediated through a beta-adrenoreceptor.*

The adenosine 3′,5′-monophosphate (cyclic AMP) content of many neural and muscle preparations is altered by exposure to putative neurotransmitters (*1–3*). However, because of the cellular heterogeneity in vivo or in tissue slices, it has proved difficult to characterize the responsive cells. To circumvent this difficulty, both primary cultures (*4, 5*) and clonal cell cultures (*5–9*) have been employed; only the latter can unambiguously define the responsive cell. Of the limited number of glia cell lines studied to date, some responded to catecholamines with the elevation of intracellular cyclic AMP, while the only neuronal cell line examined, the C1300 neuroblastoma, with one exception, did not (*5–10*). The recent availability of a larger collection of both nerve and glia cell lines from the rat central nervous system (*11*) and several skeletal (*12*) and smooth muscle (*13*) cell lines presents the opportunity to extend these studies to a larger number of cells. In this report we show that some nerve, glia, and muscle cells are capable of responding to catecholamines by elevating endogenous cyclic AMP; these cells appear to be much less sensitive to the other putative neurotransmitters.

Of the cell lines employed, B35 Cl3, B50 Cl5, B65 Cl27, B103 Cl4, and B104 Cl7 are neuronal, as defined by electrical excitability and neurotransmitter synthesis, while B11 Cl4, B12 Cl1, B19 Cl4, B23 Cl8, B27 Cl1, B28 Cl6, B49 Cl1, B90 Cl2, B92 Cl5, B111 Cl1, C6B, and RN2 are of glial origin (*6, 11, 14*). Line L6 is skeletal muscle (*12*) and BC_3H1 Cl9 is smooth muscle (*13*). In all cases early passages of the individual clones were used. All cells were grown in Vogt's modified Eagle's medium (*15*) containing 10 percent fetal calf serum, as described previously (*11*). The assay conditions were the same as those described for clonal glia cells (*6*). Cells were incubated for 60 minutes with $10^{-3}M$ theophylline, followed by the addition of the freshly prepared putative neurotransmitters, analogs, or drugs at the indicated concentrations. After 15 minutes, the cells were extracted with trichloroacetic acid and the cyclic nucleotides assayed (*16*). The data are expressed both as the actual amount of cyclic nucleotide and as the percentage change relative to control cultures, which were treated identically except for the addition of the test compound. Unless otherwise indicated, stationary phase cells were used since these cells usually express a more highly differentiated phenotype than exponentially growing cells (*11, 13*).

Table 1 shows that of the seven neurotransmitters or neurotransmitter analogs examined, only dopamine and norepinephrine had a stimulatory effect on cyclic AMP; dopa, γ-aminobutyric acid, histamine, carbamylcholine, and 5-hydroxytryptamine had no significant effects. Two of the five neuronal lines responded to norepinephrine with an increase in endogenous cyclic AMP, while nine of the eleven glial lines responded; the response was larger in the majority of the glial lines than in the nerve cells. The only qualitative difference between the nerve and glia cells was the dopamine response associated with glia cells. This may, however, be due to the generally higher catecholamine sensitivity of the glia cells. The basal cyclic AMP levels in nerve (16.6 ± 8.3 pmole per milligram of protein, $N = 5$) and glia (15.1 ± 8.1 pmole/mg, $N = 11$) were not significantly different.

The L6 skeletal muscle line responded to both norepinephrine and dopamine with increases in intracellular cyclic AMP, and the fused fibers were less responsive to norepinephrine than the exponentially dividing myoblasts. In contrast, the magnitude of the response to catecholamines was less in the BC_3Hl smooth muscle cells, and the dividing BC_3H1 myoblasts responded less to norepinephrine than did stationary phase cultures.

Both norepinephrine- and dopamine-responsive adenylate cyclase systems have been described in the mammalian central nervous system (*1, 2, 17*). These classes of receptors can be distinguished on the basis of their pharmacological properties. Thus the dopamine system is not activated by the potent β-adrenoreceptor stimulant isoproterenol, nor is the dopamine response blocked by β-receptor antagonists such as propanolol (*17*). The dopamine response is, however, blocked by high concentrations of α-adrenoreceptor antagonist drugs and by a defined sequence of antipsychotic drugs (*17*). When examined by these criteria, catecholamine-induced cyclic AMP responses in the cell lines appeared to function through a β-adrenoreceptor. In the cell lines where dopamine elicited a cyclic AMP increase greater than 1.5-fold, a similar increase was generated by at least a 10-fold lower concentration of isoproterenol or norepinephrine. In addition, the responses are inhibited by β-blocking agent propanolol but not as well by phentolamine, an α-blocking agent (Table 2). The tricyclic antidepressant imipramine and various antipsychotic drugs block the dopamine response in both the myoblast and glia cells to varying extents, but not in the order characteristic of the previously defined dopamine receptors (*17*).

On the basis of these data, it can be concluded that nerve, glia, and both smooth and skeletal muscle cells can respond to norepinephrine by accumulating intracellular cyclic AMP. The catecholamine response has been well studied in certain types of smooth muscle,