CHANGES IN THE DISTRIBUTION OF A HISTONE IN DORSAL ROOT GANGLION NEURONS DURING DEVELOPMENT

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ABSTRACT

The isolation and cellular localization of a basic protein (histone) from central nervous tissue have been previously reported. In the tissues previously studied (nervous tissue, testis, liver, spleen, kidney, ovary), the basic protein was restricted in distribution to the nuclei of neurons and spermatogonia. In the present study, the temporal appearance of the histone within neurons and the changes in its distribution during ontogenesis were examined. The reaction between a fluorescent immune γ-globulin prepared against this purified tissue-specific histone and the neurons from the dorsal root ganglia of the rat was investigated. The dorsal root ganglia examined were those from fetuses, 2-, 10-, and 40-day-old rats, and from adult rats. At the earliest stages, only the nucleoli reacted. Subsequently, threads of fluorescent material were seen to emerge from the nucleoli. The extent of this reaction between the immune globulin and the threads within the nuclei continued to increase with maturation. No changes in fluorescence localization during development could be seen in the nuclei of neurons in the cerebellum or brain stem. The role that this tissue-specific histone may play in cell function is discussed.

Studies on the chemical and biological properties of histones have been made by many groups since the Stedmans postulated that these polycationic proteins regulate genetic expression (1). As a result of the findings of Bonner and Huang (2, 3) and Allfrey and Mirsky (4), it has become evident that lysine-rich or arginine-rich histones can markedly inhibit DNA-dependent RNA synthesis. In addition, Hurlbert et al. (5) have shown that the base ratios of RNA synthesized in the nucleoli are dependent upon the type of histone added to the incubation mixture. The control of genetic expression by histones, therefore, may be manifest either by a generalized repression of DNA-dependent RNA synthesis or by a modification of the type of RNA synthesized. If the specificity required for correct repression is a function of the primary structure of the individual histones, different organs should have histones unique in primary structure, and any particular organ may be expected to have a different histone population during the various stages of its ontogenic development. Hnilica has recently reviewed the literature concerning the role of histones in differentiation (6).

In a previous report (7) the isolation and partial characterization of a basic protein from pig brain were described. The protein was shown to be homogeneous by the criteria of ultracentrifugation, electrophoresis on starch gel and polyacrylamide supports, and end-group analysis. The chemical properties of this molecule (7) are similar to those of histones (8). Subsequently, it was shown that this protein had a molecular weight of 27,000 (9).

With the aid of immunochemical and immunofluorescent techniques, it was determined that the protein is localized in the nuclei of neurons and spermatogonia but not in nuclei of the liver, kidney, spleen, or ovary from the pig. It was also demonstrated that nuclei of neurons and spermatogonia from a variety of animals, including frog, rat, guinea pig, cow, and monkey (macaque), react equally well with the antiserum to the pig brain basic protein, whereas nuclei of liver, kidney, spleen or ovary from these same animals do not. The chemical properties and nuclear localization of this protein indicate that it is a tissue-specific but not a species-specific histone.

It was then of interest to examine the temporal appearance of this basic protein and the changes in its distribution in neurons during ontogenic development. The white rat was chosen for this study, for the following reasons: a protein, antigenically similar to the pig brain protein, was shown to be present in the rat; dated pregnant female rats were readily available; and the fresh tissues could be rapidly processed. These studies are described in this report.

MATERIALS AND METHODS

Preparation of the Antisera to the Pig Brain Basic Protein

The basic protein was isolated from the pig brain as previously described (7). In brief, the purification procedure consisted of repeated extractions with chloroform-methanol (2:1 v/v), 10 per cent sodium chloride, and dilute hydrochloric acid. The pH 2.4–2.6 fraction obtained from the repeated acid extraction was further purified by pH and $(NH_4)_2SO_4$ fractionation, Sephadex G-100 gel filtration, and by chromatography on Amberlite IRC-50 with a gradient (5–20 %) of guanidinium chloride.

Three female albino rabbits, each weighing 4 lb., were bled, prior to immunization, to obtain the normal serum used as the control. Each rabbit was injected subcutaneously at seven sites (hind footpads, either side of the lumbar region, above the scapulae, and the cervical fat pads), each site receiving 0.2 ml of the complete Freund's adjuvant containing 2 mg of pig brain basic protein (14 mg per rabbit). 1 wk later, each of the rabbits was injected intravenously with 5 mg of the protein, once weekly for 6 wk, and then each was bled to collect the serum. When these sera were examined by the double diffusion method of Ouchterlony and by immunoelectrophoresis (10), only one precipitin line was seen. After immunoelectrophoresis the precipitin line was in the region of migration of the basic protein (9).

Preparation of Fluorescent γ-Globulin

Fluorescent γ-globulin was prepared (11) from the immune rabbit serum and from the normal serum obtained from the same rabbits prior to immunization. These sera were bought to 33 % saturation with solid ammonium sulfate at 0°C, and the percipitated γ-globulin fraction was collected by centrifugation. The precipitate was dissolved to a final concentration of 15 mg/ml in sodium bicarbonate (0.5 M, pH 9.0) and dialyzed against the same bicarbonate buffer for 16 hr.

The dialyzed γ-globulin fraction was diluted with an equal volume of saline, made 10% with respect to acetone, and cooled in a dry ice-acetone bath until ice crystals began to form. At this point the acetone concentration was brought to 14%, and fluorescein isothiocyanate was added to a final concentration of 2.5 mg/100 ml. The solution was stirred for 24 hr at 4°C and then applied to a column of Sephadex G-25 (1.5 × 30 cm) equilibrated with sodium phosphate (0.01 M, pH 7.2). The fluorescent γ-globulin fraction emerged with the front while the unreacted dye remained near the top of the column. The fluorescent γ-globulin was mixed with pig kidney acetone powder to remove nonspecifically reacting material. The fluorescent protein remaining in solution was passed through a 0.35 μ Millipore filter to give a clear solution.

Reaction of the Fluorescent γ-Globulin with Tissue Sections

Both the dated pregnant female albino rats and the young rats were anesthetized with Amytal. Fresh samples of nervous tissue from fetal, newborn, and adult rats were rapidly frozen in a cryostat, and 6-μ sections were cut from the frozen block. The samples of tissue were taken from dorsal root ganglia (cervical cord level), cerebellum, and brain stem at 20 days after conception, and at 2, 10, and 40 days after birth. The newborn rats were delivered on the 22nd day after conception. The sections were immediately placed in 95% ethanol at room temperature for 10 min, air dried, and placed in a moist chamber. A drop of fluorescent immune or normal γ-globulin was placed on each tissue section. The tissue sections were then incubated for 30 min at room temperature, extensively washed with 0.01 M sodium phosphate (pH 7.5), and mounted with glycerol containing 0.001 M sodium phosphate (pH 7.5). The sections were examined by dark-field microscopy with an ultraviolet light source. Sections 6 μ thick were taken from the same block, stained with Paragon multiple stain, and examined in the light microscope.

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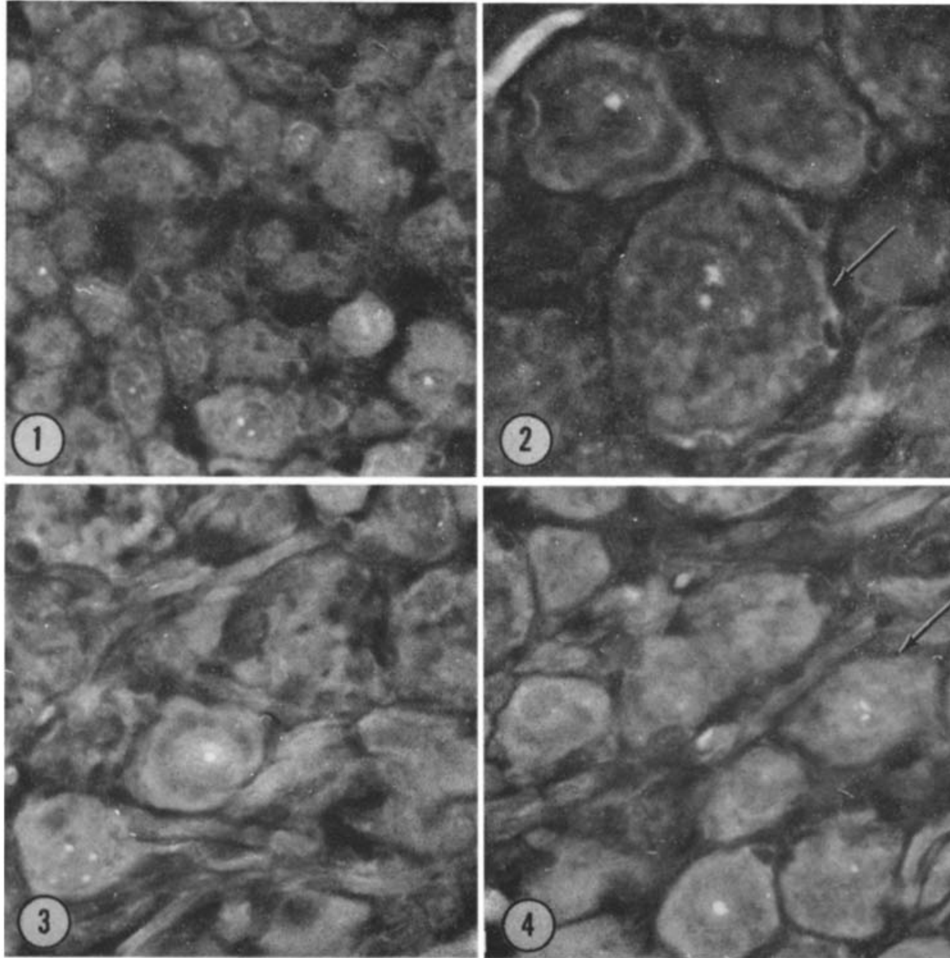
RESULTS

Of the three regions of nervous tissue examined (i.e. dorsal root ganglion, cerebellum, brain stem), only the dorsal root ganglion cells showed changes during the maturation period investigated. The nuclei of the dorsal root ganglion cells were observed to undergo changes in the extent and distribution of reactivity with the fluorescent immune γ-globulin. The nuclei of cerebellar neurons (i.e. Purkinje cells, granule cells) and of brain stem neurons from newborns reacted as extensively and with the same distribution as nuclei of comparable neurons from adults. No reaction was observed between a neuron of any



Figs. 1–4 are photographs of sections of dorsal root ganglia (cervical cord level) taken from 2- or 10-day-old rats. The sections were incubated with the fluorescent immune globulin.

FIGURE 1 Section from a 2-day-old rat. The primary reaction is with the nucleoli and the perinuclear region of the neurons. × 450.

FIGURE 2 Section from a 10-day-old rat. A thread of fluorescence may be seen between two nucleoli in one cell (arrow). × 600.

FIGURES 3–4 Sections from a 10-day-old rat. In Fig. 3, a nucleus containing four nucleolar-like regions of fluorescence can be seen at left (bottom). In Fig. 4, threads of fluorescence appear to emerge from some of the nucleoli in one neuron (arrow). × 400.

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age and either fluorescent normal γ-globulin or fluorescent immune γ-globulin that previously had been absorbed with the purified basic protein isolated from pig brain.

Reaction of Fluorescent Immune γ-Globulin with Dorsal Root Ganglion Cells

At the earliest time examined (20-day fetus), the nuclei of dorsal root ganglion cells were observed to react specifically with the fluorescent antiserum. At 2 days after birth, the extent and distribution of this reaction had not changed. At that stage of development, only the nucleoli (as determined by phase microscopic studies) of this cell population reacted intensely (Fig. 1); the rest of the nucleus did not react noticeably. Some cytoplasmic reaction, localized primarily in the perinuclear region, was seen, but its intensity was markedly lower than that of the nucleolar reaction. Satellite cells were not observed to react at any of the stages examined.

By the 10th day of extrauterine life, several marked changes occurred in the reaction of fluorescent antiserum with the dorsal root ganglion cells. The most striking of these changes was the appearance of a thread of fluorescence joining the several nucleoli (Fig. 2). In addition, fluorescent threads appeared to emerge from the nucleoli of the dorsal root ganglion cells and to travel into the nucleoplasm (Figs. 2, 4). The nucleolus was still the primary organelle involved in the reaction between the fluorescent immune globulin and the ganglion cell. From a comparison between Fig. 5 (taken under phase optics) and Fig. 3, it may be seen that all the nucleoli present in the section reacted with the fluorescent immune γ-globulin. The cytoplasmic reaction at this and all subsequent stages was weak, however all the binding with antiserum was distributed in the region of the Nissl substance (rough endoplasmic reticulum). The perinuclear reaction was minimal at this stage and all subsequent stages of development.

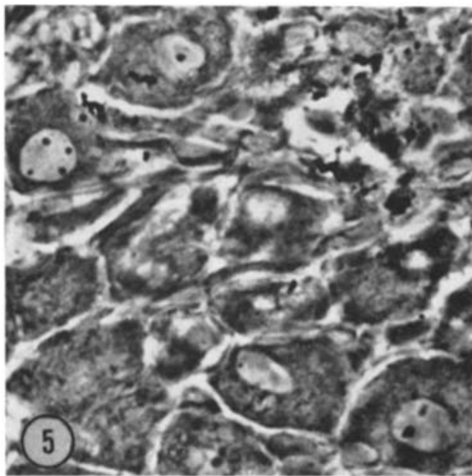

FIGURE 5 A micrograph (taken with phase optics) of the same region shown in Fig. 3. The four fluorescent spots in Fig. 3 appear as nucleoli in this photo. × 400.

At the 40th day of extrauterine life, more extensive changes were seen. The reaction between the dorsal root ganglion cells and the fluorescent immune globulin had extended to include a large area of the nucleus (Figs. 6, 7). The individual nucleoli were not so evident at this stage. The nuclear reaction was not homogeneous, however, since many fine threads of fluorescence were visible in this region. These threads were either overlapped or joined together. In at least one cell (Fig. 7) the perinucleolar region did not react with the immune γ-globulin; this was the only large nuclear region that did not interact with the immune γ-globulin.

In the dorsal root ganglion cells from the adult rat, the nucleus reacted very intensely. Even at this stage of development, the fluorescence was patterned rather then homogeneous throughout the nucleus (Fig. 8). In the nucleus of one of the neurons shown in Fig. 8, a loop of fluorescence may be seen extending from a region of intense fluorescence. This loop appears similar to the thread of fluorescence seen between two nucleoli in the dorsal root ganglion cell of the 10-day-old rat. The darkened center of the loop does not correspond to the nucleolus seen with phase optics.

In summary, the changes in reactivity of the dorsal root ganglion cells with the fluorescent immune γ-globulin observed during ontogenic development were as follows: at the earliest stages (20-day fetal and 2-day-old rats) the fluorescent immune globulin reacted primarily with nucleoli and to a small extent with the perinuclear region. At 10 days of postnatal life, threads of fluorescence were seen in the nuclei. These threads emerged from one nucleolus and ended either in the nucleoplasm or on a second nucleolus. The extent of this reaction between the immune globulin and the threads within the nuclei continued to increase with maturation.

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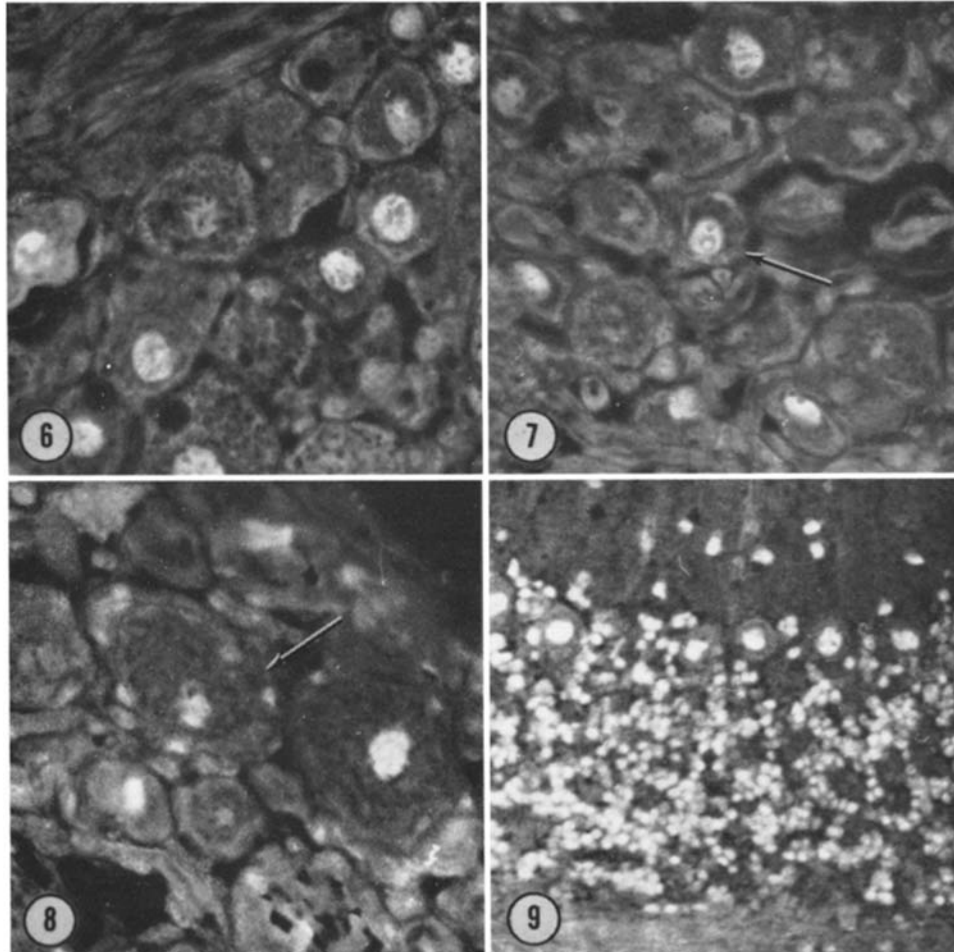
Reaction of Fluorescent Immune γ-Globulin with Cerebellar and Brain Stem Neurons

The extent of reaction between the fluorescent immune γ-globulin and the neurons of the cerebellar cortex of adult rats may be seen in Fig. 9. Fig. 10 illustrates the extent of reaction between the immune globulin and neurons in the brain stem of the adult rat. The nuclei of the Purkinje cells and granular cells of the cerebellar cortex and the nuclei of the neurons of the brain stem reacted with the same distribution and intensity at all



Figs. 6–8 are photographs of sections of dorsal root ganglia (cervical cord level) taken from 40-day-old or adult rats. The sections were incubated with the fluorescent immune globulin.

FIGURE 6 Section from a 40-day-old rat. Threads of fluorescence may be seen in several nuclei. The fluorescence appears to extend through a larger area of the nuclei than at earlier stages. × 400.

FIGURE 7 Section from a 40-day-old rat. In one cell (arrow) the perinucleolar region did not react with immune globulin. × 500.

FIGURE 8 Section from adult rat. A loop of fluorescence may be seen in the nucleus of one of the cells (arrow). × 400.

FIGURE 9 Photograph of a section from the cerebellar cortex of an adult rat. This section was incubated with the fluorescent immune globulin. The nuclei show fluorescence throughout the nucleoplasm. × 200.

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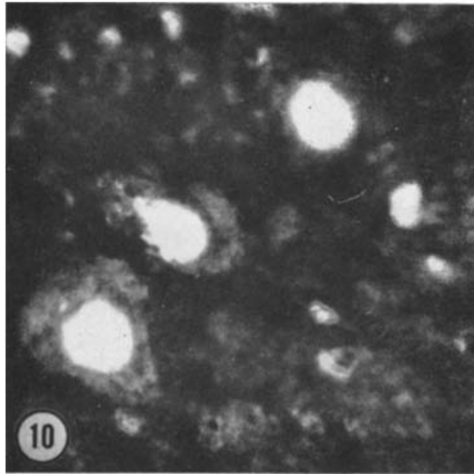

FIGURE 10 Photograph of a section from the brain stem of an adult rat. This section was incubated with the fluorescent immune globulin. The nuclei show fluorescence throughout the nucleoplasm. × 500.

stages of maturation. The nuclei of these neurons seemed to fluoresce throughout the nucleoplasm. A minor reaction appeared between the rough endoplasmic reticulum of the cytoplasm and the fluorescent immune γ-globulin.

DISCUSSION

In recent years, the relationship of histones to differentiation has been investigated in several laboratories. Two techniques have been applied to the study of tissues at various stages of differentiation: (*a*) reactivity of tissues with the dye, fast green; and (*b*) comparison of heterogeneous histone fractions with regard to absolute concentration, amino acid composition, and electrophoretic patterns. When the dye, fast green, is permitted to react with tissues under alkaline conditions (12), basic proteins, primarily histones, are stained selectively. With this fast green technique, Alfert (13) demonstrated that the male pronucleus of the newly fertilized mouse egg did not react with the dye; fast green staining was observed only during the late blastular stage. Horn (14) reported the absence of fast green staining in nuclei of oocytes and blastomeres. He observed nuclear staining with the dye at early gastrulation, however, and postulated that there was a shift in histone distribution during gastrulation. Some ambiguity arose as a result of Moore's (15) investigations which indicated that the absence of fast green staining in blastulae of Amphibia may be a consequence of dilution (the nuclear volume of the blastomeres was considerably larger than that of the cells of the gastrula). Bloch and Hew (16, 17) have used the fast green technique to study transition in histone populations during fertilization and early embryonic development of *Helix aspersa*. With this technique they have shown that during spermatogenesis there is a shift in the histone population from lysine-rich histones to arginine-rich histones, which can be viewed as a differentiation process. This technique, however, gives little information about changes within each of the histone subgroups.

Neidle and Waelsch (18) have compared the electrophoretic migration of histones isolated from young rat brain to that of histones isolated from adult rat brain. They observed differences in the pattern of migration and concluded that there were some differences between these histone populations. Dingman and Sporn (19) and Kischer and Hnilica (20) isolated total histones from embryonic and adult chickens. Dingman and Sporn found no change in the ratio of total histones to total DNA during development, and Kischer and Hnilica reported no change in the total amino acid composition of the mixtures of histones. However, since in neither of these studies were homogeneous histone preparations compared, changes within such histone populations could not be determined. Agrell and Christensson (21) isolated the lysine-rich (F_1), slightly lysine-rich (F_2), and arginine-rich (F_3) fractions from the chick embryo at various stages of development. They found no changes in the concentrations of these subgroups during the development of the brain. The major difficulty in the interpretation of these findings is that mixtures of proteins rather than single homogeneous histones were examined.

The present report has described changes in the distribution of a nuclear histone in neurons of the dorsal root ganglia of the rat during maturation. These findings demonstrate that this histone, in addition to showing a restricted organ localization in the adult (9), shows a change in its distribution in a given cell population during ontogenesis. Furthermore, the distribution of the histone during ontogenesis was found to be different in two different neuronal cell types, (i.e. the dorsal root ganglion cells and Purkinje cells). In the dorsal root ganglion cells the distribution of the histone was markedly different at the different stages of

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development, whereas in the Purkinje cells the localization of the histone did not change with the age of the animal (2 days to adult). At the earliest stage examined in the maturation of the dorsal root ganglion neurons (20-day fetal and 2-day-old rats), the fluorescent immune γ-globulin reacted only with the nucleoli. At a later stage, threads of fluorescence continuous with the nucleoli were observed. These threads could be seen either to traverse the space between the nucleoli or to emerge from one nucleolus and to have no obvious terminus. The striking similarity (in dimension and distribution) between these threads and the DNA-containing, Feulgen-positive filaments in nuclei from a variety of cells (22, 23) indicated that the fluorescent threads represented strands of chromatin containing DNA and the histone with which the fluorescent immune globulin reacted. Of particular interest is Olah's report (22) that, during early zygotene (in *Corypha umbraculifera*), an unusually great amount of Feulgen-positive substance appears at the nucleolar organizing region and then seems to be drained off by the chromosomal threads. This description could appropriately be applied to the changes observed in the developing dorsal root ganglion neurons of the rat when they are reacted with the specific fluorescent immune globulin.

The flow of histone from nucleolus to nucleoplasm could represent either the actual synthesis of basic protein within the nucleoli or a concentration of previously synthesized histone at these sites. There is evidence that nuclear histones are synthesized in the cytoplasm of spermatocytes (24) and HeLa cells (25). In the present studies, the reaction of fluorescent immune γ-globulin with the rough endoplasmic reticulum of dorsal root ganglion cells could be reflective either of the site of synthesis of the basic protein or of the presence of an antigenically similar protein in that organelle. In either case, the nucleolus or a region associated with it appears to be central to the initiation and perhaps to the complete elaboration of the basic protein within the nucleoplasm.

From the amino acid composition and electrophoretic mobility (7) of the basic protein isolated from pig brain, the authors have concluded that this protein could be classified as a histone of the F2al type (8). Both the pig brain protein and the F2al histone have the following properties: the ratio of arginine to lysine is 1.25/1; the total amount of basic amino acids (lysine, arginine, histidine) is 24 moles/100 moles of total amino acids, the ratio of basic to acidic amino acid residues is 1.9/1; no cystine is present; and the glycine content (15 moles/100 moles of total amino acids) is unusually high. Several properties of the F2a histones give some indication of the role that the pig brain protein may have in the function of the neuron and spermatogonia. Laurence (26) has shown that the F2a fraction binds the dye, 8-anilinonaphthalene-1-sulfonic acid (ANS), more strongly than do the other histone fractions, and that the F2a fraction in complex with DNA binds ANS as strongly as the uncomplexed F2a fraction. The ANS binds the F2a fraction in the nonpolar regions of the protein moelcule (26). In contrast to ANS, Biebrich scarlet and tetraphenyl porphine sulfonate dyes bind the cationic region of histones and do not interact with nucleohistones at pH 6–9 (27). Of interest in this regard are our observations that the specific immune globulin reacts strongly with free basic protein as shown by immunodiffusion and immunoelectrophoresis and with histone *in situ* as shown by immunofluorescence studies. The similarity between the immune reaction and the ANS reaction, in this respect, and the difference between the immune reaction and the Biebrich scarlet reaction suggest that the antigenic portion of the pig brain protein resides in the nonionic rather than in the ionic region of this histone. Bradbury et al. (28) have indicated that these nonpolar regions of the F2a histones are looped and interact hydrophobically with the deep groove of the DNA double helix.

Hurlbert et al. (5) examined the effect of each of the histone fractions on the base composition of RNA synthesized in nucleolar preparations from liver. They found that RNA synthesized in the presence of each of the histone fractions, except the F2a fraction, had increased cytosine and guanine contents (approaching the base ratios of ribosomal RNA). The RNA synthesized in the presence of the F2a histones had a base ratio more like that of DNA. In addition, the F2a histone inhibited the DNA-dependent RNA synthesis to a lesser extent than did the other histone fractions.

It does not appear likely that free DNA (i.e. uncomplexed with a polycation) can exist in the nucleoplasm because of the high charge on this polyanion. The expression of DNA, therefore, would be a function of the histone with which it is complexed. From the studies of Hurlbert et al. (5) and the observed high affinity of the F2a histone

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for DNA (25), the present authors suggest that the basic protein described in this report complexes the DNA in such a way as to effect a minimum of repression and to permit the synthesis of an RNA more similar in base ratio to DNA than other histones would permit.

This work was supported by Grant NB-05631 from NINDB.

This work was carried out during tenure of a Life Insurance Medical Research Fund Fellowship awarded to Mr. Lawrence G. Tomasi.

The authors would like to express their appreciation to Dr. John W. Anderson for the use of his equipment during this investigation and to Miss Judith Oldenburg and Mr. Grayson Scott for their valuable assistance.

Received for publication 5 March 1968, and in revised form 6 May 1968.

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