Role of Growth Hormone in the Multihormonal Regulation of Messenger RNA for α_{2u} Globulin in the Liver of Hypophysectomized Rats†

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ABSTRACT: Hypophysectomy in mature male rats results in complete abolition of the hepatic synthesis of α_{2u} globulin. Reversal of the effect of hypophysectomy on the synthesis of α_{2u} globulin requires simultaneous treatment with androgen, glucocorticoid, thyroxine, and growth hormone. The mode of action of growth hormone on the hepatic synthesis of α_{2u} globulin was investigated by a correlative study of the hepatic and urinary concentrations of α_{2u} globulin and hepatic concentration of the mRNA for this protein in hypophysectomized rats treated with dihydrotestosterone, corticosterone, and thyroxine and also dihydrotestosterone, corticosterone, thyroxine, and growth hormone. mRNA activity for α_{2u} globulin was quantitated by in vitro translation of the poly(A) containing hepatic RNA in wheat germ cell-free system and subsequent immunoprecipitation and sodium dodecyl sulfate–polyacrylamide gel electrophoresis of the α_{2u}–anti-α_{2u} immunoprecipitate. Results show that inclusion of growth hormone in the three-hormone combination (dihydrotestosterone, corticosterone, and thyroxine) caused more than a sevenfold increase in both hepatic and urinary concentrations of α_{2u} globulin and the hepatic concentration of the mRNA for this protein. The present observation, along with earlier results which have shown that androgen, glucocorticoid, and thyroxine also increase the hepatic concentration of the mRNA for α_{2u} globulin, indicates that the pituitary gland regulates the hepatic synthesis of α_{2u} through a multihormonal regulation of the mRNA for this protein.

In our laboratory, we have developed a model system for the study of hormone action involving hepatic synthesis of α_{2u} globulin. α_{2u} globulin, the principal urinary protein of the mature male rat, is synthesized and secreted by the parenchymal cells of the liver (Roy et al., 1966; Roy and Neuhaus, 1967; Roy and Raber, 1972). Hepatic synthesis of α_{2u} globulin in the normal male rat begins at the onset of puberty and ceases at senescence (Roy, 1973a; Roy et al., 1974). Although normal female rats do not synthesize α_{2u} globulin, treatment of castrated female rats with androgenic hormones results in the induction of α_{2u} globulin (Roy and Neuhaus, 1967; Roy et al., 1975; Roy, 1977). Androgen-dependent synthesis of α_{2u} globulin requires the presence of the pituitary gland (Kumar et al., 1969). Normal levels of α_{2u} globulin in the hypophysectomized animals can be induced with simultaneous administration of androgen, glucocorticoid, thyroxine, and growth hormone (Roy, 1973b). Androgenic induction and estrogenic suppression of α_{2u} globulin, as well as synergistic influence of glucocorticoids and thyroxine, have been found to be mediated through the regulation of the hepatic concentration of the mRNA for this protein (Sippel et al., 1975; Roy et al., 1976a, b, 1977; Kurtz et al., 1976a, b).

Although the role of growth hormone in the regulation of hepatic protein synthesis has been known for a long time (Geschwind et al., 1950), the exact mechanism of action of growth hormone on the liver has not been clearly established. Supplementation of growth hormone to hypophysectomized rats has been found to result in enhancement of chromatin template capacity (Spelsberg and Wilson, 1976), increased activity of nuclear RNA polymerase (Pegg and Korner, 1965; Widnell and Tata, 1966; Janne and Raina, 1969), increased ribosomal RNA synthesis (Talwar et al., 1964), increased synthesis of mRNA (Korner, 1964), increased efficiency of mRNA translation (Korner and Gumbley, 1966; Korner, 1969), and decrease in RNase activity (Brewer et al., 1969). In addition, Keller and Taylor (1976) have recently shown that hypophysectomy results in approximately 50% reduction in the level of albumin mRNA activity in rat liver. In spite of these indications of possible important regulatory role of growth hormone in the regulation of mRNA synthesis in rat liver, so far no study on the effect of growth hormone on regulation of a specific species of mRNA has been reported.

Recent improvements in the techniques for the isolation of mRNA through affinity chromatography and their efficient translation in heterologous cell-free system have added a new dimension to the identification and quantitation of specific mRNA (for various techniques, see Moldave and Grossman, 1974). By using some of these procedures, we have shown that the mRNA for α_{2u} globulin could be identified and quantitated in the heterologous cell-free systems derived from both ascites tumor cells and wheat embryo (Sippel et al., 1975; Roy et al., 1976a). In the present investigation, we have examined the effect of growth hormone in the regulation of the messenger RNA synthesis in rat liver in relation to the synthesis of α_{2u} globulin. The results show that growth hormone regulates the synthesis of α_{2u} globulin by specific modulation of the hepatic concentration of the mRNA for this protein.

Materials and Methods

1. Animals, Hormones, and Chemicals. Experiments were carried out on 300–350-g male albino rats of Sprague-Dawley strain. Hypophysectomy was performed by Zivic-Miller Laboratory, Allison Park, Pa. Hypophysectomized animals were maintained on 6% glucose solution instead of drinking water. The animals were given 2 weeks of postoperative rest before any treatment. L-Thyroxine, porcine growth hormone

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(0.4 IU/mg), corticosterone, and 5α-dihydrotestosterone were obtained from Sigma Chemical Co., St. Louis, Mo. Microgranular cellulose (Sigmacell-50) was also obtained from the same supplier. [^{14}C]Orotic acid (50 mCi/mM) and [^{3}H]leucine (59 Ci/mM) were products of Amersham/Searle.

Steroid hormones were administered into the animals through subcutaneous injections as emulsions in 0.1 M phosphate buffer (pH 7.2), propylene glycol, and Tween 80 (89.9:10:0.4, by volume). Growth hormone was administered subcutaneously as a solution in 0.01 M Tris-HCl (pH 8.0), containing 0.9% NaCl. Thyroxine was administered intraperitoneally as a solution in 0.005 M NaOH. The dosages used for the above hormones per 100 g of body weight were: dihydrotestosterone, 50 μg; corticosterone, 3 mg; growth hormone, 200 μg; thyroxine, 25 μg. Earlier studies have shown that the above dose levels produce maximum induction of α_{2u} globulin (Roy, 1973b).

2. Purification of α_{2u} Globulin and Preparation of Antiserum against the Protein. α_{2u} globulin was purified from male rat urine and antiserum against α_{2u} globulin was prepared in rabbits by repeated injections of the protein with Freund's complete adjuvant (Roy, 1973b, 1977). Purified α_{2u} globulin was found to give single protein band in sodium dodecyl sulfate–polyacrylamide gel electrophoresis (14% gel). In immunoelectrophoresis, rabbit antiserum against α_{2u} globulin was found to be monospecific for α_{2u} globulin.

3. Radioimmunoassay of α_{2u} Globulin in Urine and Cytoplasmic Proteins of the Liver. Conditions for the collection and preservation of 24-h urine samples, method for preparation of hepatic proteins (160 000*g* supernatant) and radioimmunoassay of urinary and hepatic concentrations of α_{2u} globulin have been described in earlier papers (Roy, 1973b, 1977). Total protein concentration was estimated by the method of Lowry et al. (1951), with bovine serum albumin as standard.

4. Extraction of Hepatic RNA and Its Translation in Wheat Germ Cell-Free System. Total hepatic RNA was extracted with phenol and sodium dodecyl sulfate according to the procedure of Rosenfeld et al. (1972). Poly(A) enriched RNA was obtained by chromatographic fractionation of the total RNA in cellulose column (Schutz et al., 1972), except that Sigmacell-50, instead of Sigmacell-38, was used for the preparation of the column. For in vivo labeling of hepatic RNA, 65 μCi of [^{14}C]orotic acid in 0.2 mL of Tris[1]-HCl (10 mM, pH 8.0) was administered intraperitoneally and the animals were sacrificed 60 min later. Wheat germ S-30 was prepared according to the standard procedure (Roberts and Patterson, 1973; Rosen et al., 1975), with only minor modification. Wheat germ, 11.0 g, was ground at 0 °C with 11.0 g of acid-washed glass beads and 30 mL of buffer. The remaining procedure was as described by Rosen et al. (1975). The reaction mixture for mRNA translation was as follows: 20 mM 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (pH 7.6), 2 mM-dithiothreitol, 1 mM ATP, 100 μM GTP, 60 μM CTP, 5 mM phosphocreatine, 12.8 μg of creatine kinase (3.2 mg/mL), 60 mM KCl, 1.6 mM magnesium acetate, 40 μM each of 19 unlabeled amino acids. A 200 μL reaction mixture contained 100 μL of wheat-germ S-30 fraction, 60 μg of poly(A)-containing RNA, and 60 μCi of [^{3}H]leucine (59 Ci/mmol). The mixture was incubated for 60 min at 24 °C.

At the end of the incubation period, 5 μL of the reaction mixture was assayed on filter paper discs for total protein synthesis according to Bollum (1968). The rest of the reaction mixture was centrifuged at 160 000*g* for 90 min at 4 °C to obtain the supernatant containing released polypeptide chains. An aliquot of the supernatant (5 μL) was assayed for leucine incorporation into released polypeptide chains by the filter disc procedure. Aliquots of the 160 000*g* supernatant containing 10^6 cpm of incorporated leucine were used for immunoprecipitation of α_{2u} globulin and subsequent sodium dodecyl sulfate–polyacrylamide gel electrophoresis. The antigen–antibody precipitate was washed through sucrose (Rhoads et al., 1973) and was dissolved by incubating for 2 min at 100 °C in 100 μL of 0.625 M Tris-HCl, pH 6.8, 2% sodium dodecyl sulfate, 10% glycerol, 5% β-mercaptoethanol, and 0.001% bromophenol blue. The dissolved immunoprecipitate was then subjected to sodium dodecyl sulfate–polyacrylamide gel electrophoresis according to Laemmli (1970). After electrophoresis, gels were fractionated through a Gilson gel fractionator and minced gel fractions obtained from 2-mm portions of the gel were collected in scintillation vials with 5 mL of a counting medium containing 0.5 g of Omnifluor, in 100 mL of liquid base consisting of toluene (67%), protosol (3%), and Triton X-100 (30%). The mixture was incubated 16 h at 37 °C and counted in a Packard liquid scintillation spectrometer. Further details of the above procedures have appeared elsewhere (Roy et al., 1976a, 1977).

5. Agarose Gel Electrophoresis of RNA. Analytical disc gel electrophoresis of RNA was performed in 2% agarose gel at 4 °C according to the procedure described by Rosen et al. (1975). RNA samples (0.7 OD_{260} unit) were dissolved in 0.025 M citric acid, pH 3.5, containing 6 M urea, 20% sucrose, and 0.005% bromophenol blue. Electrophoresis was carried out in 0.025 M citric acid at 2 mA/gel at 4 °C. Gels were stained for 30 min in 1% methylene blue in 15% acetic acid solution and destained in water. Gels were then photographed in 50% glycerol. In the case of labeled RNA, the gels were fractionated at 2-mm thickness through Gilson gel fractionator. The minced gels were counted for radioactivity in a manner similar to polyacrylamide as described under section 4.

Results

1. Effect of Hormone Supplementation in the Incorporation of Labeled Orotic Acid in the Heterodisperse Poly(A) Enriched Hepatic RNA in Hypophysectomized Rats. Because of the deprivation of various trophic hormones, hypophysectomy is known to induce multiple hormone deficiency. Definite reversal of the effect of hypophysectomy on the synthesis of α_{2u} globulin requires simultaneous administration of androgen, glucocorticoid, thyroxine, and growth hormone. Growth hormone by itself was found to be ineffective in promoting the androgen-dependent synthesis of the α_{2u} globulin (Roy, 1973b). In order to examine the effects of various hormone treatments on the hepatic RNA synthesis in the hypophysectomized rats and to establish the specificity of growth hormone action on the synthesis of α_{2u} globulin and its mRNA, we have studied the rate of incorporation of labeled orotic acid into the heterodisperse poly(A) enriched hepatic RNA from hypophysectomized male rats treated daily for 8 days with dihydrotestosterone, corticosterone, thyroxine plus growth hormone. Results are shown in Figure 1. Both in agarose gel (Figure 1) and in sucrose density gradient centrifugation (data not shown), the nonribosomal fraction of the labeled poly(A) enriched RNA was found to be heterodisperse in nature. Approximately 60% increase in the incorporation of labeled orotic acid into various fractions of the heterodisperse poly(A) enriched hepatic RNA was observed after treatment of the hypophysectomized animals with dihydrotestosterone, corticosterone, and thyroxine. Addition of growth hormone into the combination was found to result in a significant decrease in the

[1] Abbreviation used: Tris, tris(hydroxymethyl)aminomethane.

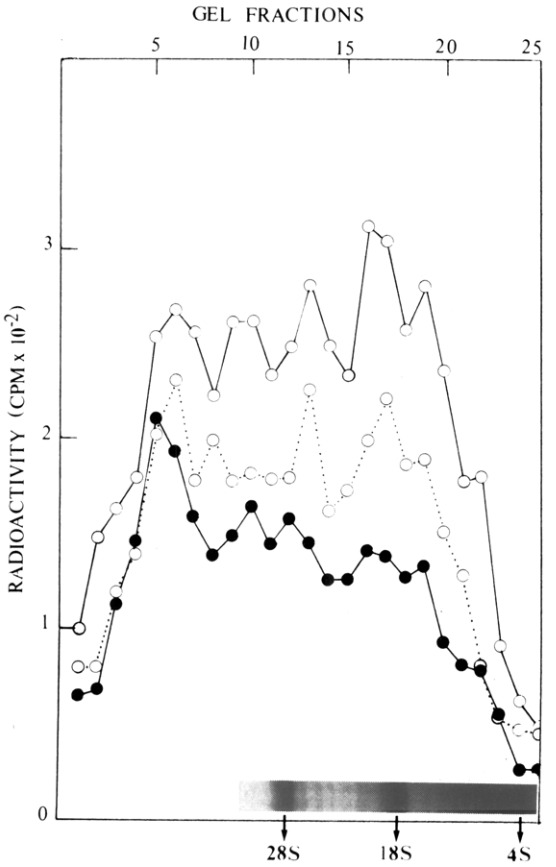

FIGURE 1: Representative electrophoretic patterns of pulse-labeled poly(A) enriched hepatic RNA from hypophysectomized animals with and without hormone supplementations. RNA samples were obtained from animals 60 min after administration of [^{14}C]orotic acid and 0.7 A_{260} unit of the cellulose chromatographed RNA was analyzed in agarose gel electrophoresis. RNAs from various experimental animals are expressed with the following symbols: (●—●) hypophysectomized male without any hormone supplementation; (○—○) hypophysectomized male treated daily for 8 days with dihydrotestosterone, thyroxine, and corticosterone; (○····○) hypophysectomized male treated for 8 days with dihydrotestosterone, thyroxine, corticosterone, and growth hormone. The bottom inset shows the photograph of the distribution patterns of cellulose chromatographed RNA from hypophysectomized rat in the agarose gel stained with methylene blue. No difference in the pattern of stained gels run with RNA samples from hypophysectomized animals with and without hormone treatment was detected. Purified ribosomal RNAs and tRNAs were used as markers.

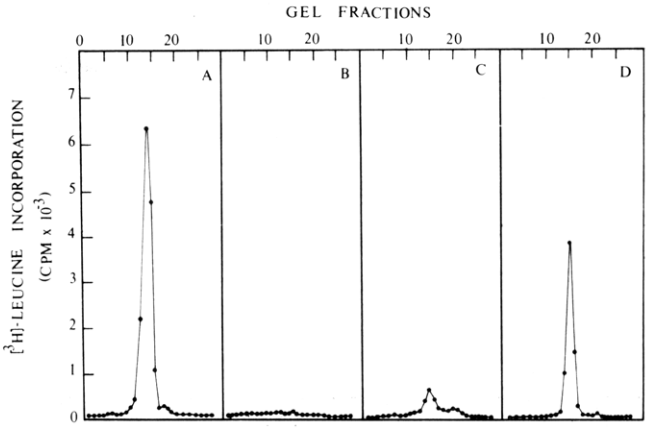

FIGURE 2: Effect of hypophysectomy and multiple hormone supplementation on the hepatic concentration of mRNA for α_{2u} globulin. Each frame shows a representative pattern of radioactivity in the sodium dodecyl sulfate–polyacrylamide gel electrophorogram of immunoprecipitated α_{2u} globulin from labeled proteins (10^6 cpm) synthesized in vitro under the direction of poly(A) enriched hepatic RNA obtained from different experimental animals. RNA samples obtained from various experimental animals are represented as follows: (A) unoperated control; (B) hypophysectomized rat without any hormone treatment; (C) hypophysectomized rat treated daily for 8 days with dihydrotestosterone, corticosterone, and thyroxine; (D) hypophysectomized rat treated daily for 8 days with dihydrotestosterone, corticosterone, thyroxine, and growth hormone.

incorporation of labeled orotic acid into the heterodisperse poly(A) enriched RNA over the three-hormone-treated animals.

2. Effect of Hormone Supplementation on the Hepatic Concentration of the mRNA of α_{2u} Globulin in Hypophysectomized Male Rats. Hepatic concentrations of the translatable mRNA activity for α_{2u} globulin in the normal male, hypophysectomized male, hypophysectomized male treated with dihydrotestosterone, corticosterone, and thyroxine and hypophysectomized male treated with the above three hormones plus growth hormone are shown in Figure 2. Hypophysectomy completely abolished the mRNA activity for α_{2u} globulin within the poly(A) enriched RNA fraction of male rat liver. Treatment of hypophysectomized animals with a combination of three hormones caused only slight enrichment of the α_{2u} mRNA within the total population of the translationally active hepatic mRNA. However, addition of growth hormone to the hormone combination caused more than a sevenfold increase in the hepatic concentration of the mRNA for α_{2u} globulin over the level found in the three hormone treated animals.

Table I summarizes relative concentrations of hepatic and urinary α_{2u} globulin and hepatic mRNA for α_{2u} globulin in the hypophysectomized rats with and without multiple hormone supplementation. The results show a strong correlation between both the urinary and hepatic concentration of α_{2u} globulin and the hepatic concentration of the mRNA for α_{2u} globulin. Inclusion of growth hormone in the three-hormone combination (dihydrotestosterone, corticosterone, and thyroxine) caused more than a sevenfold increase in the concentrations of α_{2u} globulin and its corresponding mRNA. These results show that the effect of growth hormone on the hepatic synthesis of α_{2u} globulin is mediated by corresponding changes in the concentration of α_{2u} mRNA.

Discussion

The observed correlation between the hepatic and urinary concentrations of α_{2u} globulin and the hepatic concentration of the mRNA for this protein in the hypophysectomized animals treated with and without growth hormone shows that the permissive effect of growth hormone on the synthesis of this protein is mediated through the modulation of its mRNA concentration in the liver. The above finding, for the first time, provides evidence for the role of growth hormone in regulation of a specific mRNA. In addition to its effect on the induction of α_{2u} globulin, growth hormone is also known to be involved in specific induction of ornithinine decarboxylase in rat liver (Janne and Raina, 1969; Russel et al., 1970), and Spelsberg and Wilson (1976) have recently shown that one of the early effects of growth hormone is to cause derepression of liver chromatin. These observations support the concept that growth hormone is involved in the regulation of specific genetic activity in rat liver. Unlike steroid hormones (Chan and O'Malley, 1976), very little is known about the role of peptide hormones in the regulation of specific mRNA levels in their target tissues. Recently, the specific role of another peptide hormone, i.e., prolactin in the regulation of casein mRNA, has been investigated. Houdebine (1976) has shown that treatment of pseudo-pregnant rabbits with prolactin causes an increase in

TABLE I: Quantitative Relationship between Hepatic and Urinary Concentrations of α_{2u} Globulin and mRNA for α_{2u} Globulin in Hypophysectomized Animals with and without Multiple Hormone Supplementation.

Animals and treatment[a]	24-h urinary output of α_{2u} globulin (mg)	Hepatic concn of α_{2u} globulin ng/mg of protein)	α_{2u} mRNA[b] act. (cpm α_{2u} globulin per 10^6 cpm of released chains)
Hypophysectomized male, no hormone supplementation	0	0	0
Hypophysectomized male, treated daily for 8 days with dihydrotestosterone, corticosterone, and thyroxine	0.7	190	920
Hypophysectomized male, treated daily for 8 days with dihydrotestosterone, corticosterone, thyroxine, and growth hormone	9.3	1320	6530
Normal male (unoperated control)	17.6	3260	15100

[a] Each value represents an average of three experimental animals. [b] mRNA activity for α_{2u} globulin is expressed as cpm of immunoprecipitated α_{2u} globulin in the sodium dodecyl sulfate-polyacrylamide gel electrophoresis per 10^6 cpm of released chains synthesized in vitro in the wheat germ cell-free system primed with poly(A) enriched hepatic RNA.

the concentration of casein mRNA in the mammary gland, and Rosen (personal communication) has also made a similar observation in rat mammary explants in vitro.

The contention of growth hormone exerting a specific effect on the hepatic synthesis of the mRNA for α_{2u} globulin and not via its general stimulatory action on hepatic RNA synthesis is greatly strengthened by the observation that inclusion of growth hormone in the hormone combination containing dihydrotestosterone, corticosterone, and thyroxine does not cause any further increase but a significant decrease in the incorporation of labeled orotic acid into poly(A) enriched fraction of the total hepatic RNA in hypophysectomized animals over those treated with only three hormones (dihydrotestosterone, corticosterone, and thyroxine). However, unlike its effect on total poly(A) enriched RNA synthesis, inclusion of growth hormone into the three-hormone combination caused more than a sevenfold increase in both the hepatic concentrations of α_{2u} globulin and its mRNA. The negative effect of growth hormone on the hepatic incorporation of labeled orotic acid into poly(A) enriched RNA fraction in the hypophysectomized rat when supplemented with dihydrotestosterone, thyroxine, and corticosterone may be the result of a complex interaction between these hormones on the rate of RNA synthesis and cellular uptake of orotic acid. Although enhancement of hepatic RNA synthesis by all of the above hormones, when administered separately or in combinations of two has been reported (Talwar et al., 1964; Greenman et al., 1965; Tata and Widnell, 1966; Widnell and Tata, 1966), analyses of the interacting role of androgens, glucocorticoids, thyroxine, and growth hormone at various combinations and dose levels on hepatic RNA synthesis and intracellular orotic acid pool have not been performed. Our earlier studies have established that androgen, glucocorticoids, and iodothyronines promote the synthesis of α_{2u} globulin by increasing the hepatic concentration of mRNA for this protein (Sippel et al., 1975; Roy et al., 1976a, b, 1977). The present observation that growth hormone also operates through modulation of the mRNA for α_{2u} globulin indicates that the overall effect of the pituitary gland is on the maintenance of the hepatic concentration of the mRNA for this protein. These findings raise an interesting question concerning the possible mechanism of the regulation of a single gene product by four different hormones. Recent studies of Weintraub and Groudine (1976) have indicated that regulation of a single gene at the level of chromatin may involve at least two discrete steps. Moreover, on the basis of presently available information, the possibility of specific regulation at extrachromosomal steps cannot be ruled out. Therefore, it is tempting to speculate that the pituitary gland coordinates the multihormonal regulation of α_{2u} globulin, operating through sequential effects of androgens, glucocorticoids, thyroxine, and growth hormone at distinct regulatory steps antecedent to the transcription of α_{2u} gene.

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Studies on the Reactive Properties of Histone Amino Groups: Reactivities of Free Histones and Histones in Chromatin as a Function of Ionic Strength†

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ABSTRACT: The reactivity of the amino groups of the five histones towards acetic anhydride has been measured and with the exception of histone IIb2 the reactivities are very similar to those of exposed lysines with an average pK of 9.5. In addition the reactivities of these groups from 0.20 to 1.0 M NaCl and the reactivity of a peptide containing lysines 5, 8, 12 and 16 of histone IV have been measured in chromatin. It is concluded that at the lower ionic strengths the large proportion of the amino groups are buried for both the histones and the region of histone IV studied. Data obtained from the measurement of the reactivity of standard proline compounds and from a pH and ionic strength study indicate that the N-terminal proline of histone IIb2 is exposed.

The structure of the chromatin of a cell nucleus is a primary factor in determining the nature of the processes of transcription, replication and differentiation. These processes involve the interactions of DNA, RNA, histones, nonhistone proteins, and a number of enzymes. Recent developments have resulted in a widely accepted model for the structure of nucleohistone in which 4 of the 5 histones interact in pairs to form an octomeric complex with 200 base pairs of DNA (Kornberg, 1974). This structure is consistent with evidence from studies on histone–histone interactions, chemical cross-linking studies, nuclease digestion studies, observations by electron microscopy, and semiquantitative studies on the quantities of each of the histones (Kornberg, 1974; Noll, 1974; Olins and Olins, 1973). Although this model is consistent with many experimental observations, it has not explained many other aspects of nucleohistone structure. In particular it is not possible at present to account for the significance and timing of the variety of chemical modifications of histones that occur in chromatin or to account for the slow rate of evolution of histone primary sequence. It appears that further studies relevant to histone function are required to explain these phenomena.

One approach that has been used in the analysis of chromatin structure is that of measuring the chemical reactivities of potential histone reactive groups by the method of competitive labeling (Malchy and Kaplan, 1974). In this procedure an internal standard is included directly in the reaction mixture and chemical reactivities relative to this standard are determined by measuring the extent of reaction of the groups under study (Kaplan et al., 1971). This procedure also allows estimation of the degree of exposure of any group by the determination of its "reactivity index" (Visentin et al., 1973). Even with a system as complex as chromatin, it is possible to obtain precise information on the properties of individual reactive amino acids. Thus the object of this approach is to provide information about the chemical properties of specific areas within the nucleohistone structure and to assess these properties in terms of nucleohistone function.

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