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The Nonhistone Chromosomal Proteins of Vertebrate Liver and Kidney: A Comparative Study by Gel Electrophoresis

FLORA C. WU, SARAH C. R. ELGIN* and LEROY E. HOOD+
Division of Biology, California Institute of Technology, Pasadena, California 91109

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Summary. The nonhistone chromosomal proteins (NHC proteins) probably include enzymes of chromosomal metabolism, general structural proteins, and possibly control elements. In theory, these proteins may have been strongly conserved during evolution, as the histones have. We have used sodium dodecyl sulfate (SDS) disc gel electrophoresis to analyze and compare the NHC proteins of two tissues, liver and kidney, from rat, cat, cow, chicken, turtle, and frog. The gel patterns indicate that the NHC proteins have changed much more during evolution than have the histones; the total pattern of NHC proteins has not been conserved. However, there does appear to be a conservation of a subset of bands for each tissue investigated. Further chemical analysis will be required to establish the significance of the results.

Key words: Chromosomal Proteins - Nonhistone Chromosomal Proteins - Histones - Vertebrate Evolution - SDS Gel Electrophoresis

INTRODUCTION

Chromatin, the interphase form of the eukaryotic hereditary material, is a complex of DNA, RNA, histones, and nonhistone chromosomal proteins (Marushige & Bonner, 1966). Isolated chromatin possesses many of the biological properties of chromatin *in vivo,* including its capacity to act as a limited, tissue-specific template for RNA transcription (Marushige & Bonner, 1966; Paul & Gilmour, 1966, 1968; Smith et al., 1969;

* Fellow of the Jane Coffin Childs Memorial Fund for Medical Research.
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Axel et al., 1973). Accordingly, it is an appropriate starting material for the isolation and study of the macromolecules associated with DNA which are relevant to the control of gene transcription. The histones, 5-12 species of small basic proteins, are now well characterized. They are thought to be general elements in the mechanism of gene repression, as it has been established that the binding of histones to DNA will prevent transcription by RNA polymerase *in vitro* (Shih & Bonner, 1970; Smart & Bonner, 1971). The NHC proteins,[1] a more complex group, have only recently been studied. Most probably they include general structural proteins and enzymes of chromosomal metabolism, and possibly repressor/activator proteins relevant to tissue-specific gene expression. Analyses (by disc gel electrophoresis) of the NHC proteins of different tissues of a creature show a limited comparative heterogeneity. Most of the NHC protein bands are present in all tissues; nonetheless, each tissue exhibits a unique NHC protein band pattern as shown by both qualitative (presence or absence of band) and quantitative (density of band) considerations (Elgin & Bonner, 1970; Loeb & Creuzet, 1970; Shaw & Huang, 1970; MacGillivray et al., 1971; MacGillivray et al., 1972; Teng et al., 1971; Richter & Sekeris, 1972; Wu et al., 1973; Elgin et al., 1971; Spelsberg et al., 1972; DeLange & Smith, 1974; Johnson et al., 1974). See references for reviews on this topic.

The histones are among the most highly conserved proteins known. Data obtained by comparative gel electrophoresis and by amino acid sequence analysis have shown that histone IV (f2a1) is most conserved, with only two conservative amino acid substitutions in 102 positions having occurred since the divergence of cows and peas from a common ancestor one billion years ago. Histone III (f3) is also very conserved. Histones IIa and IIb (f2b and f2a2) show a more frequent change in primary structure. Histone I (f1), which is unlike the other histones in many characteristics, is almost species specific in number of subfractions and in their primary structure (DeLange et al., 1969; Panyim et al., 1970; Panyim & Chalkley, 1971; Panyim et al., 1971a; Panyim et al., 1971b; DeLange & Smith, 1974). The conservative evolution of histones II, III and IV implies that they are an integral component of the chromatin complex with few options for mutation. Presumably all portions of the molecule must interact critically with other components of chromatin during at least part of the cell cycle.

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[1]Abbreviations used are: NHC proteins, nonhistone chromosomal proteins; SDS, sodium dodecyl sulfate.

Accordingly, it is of interest to determine whether or not the nonhistone chromosomal proteins are in any instance similarly conserved. Studies to date indicate that structural proteins, including the histones and ribosomal proteins, are more conserved than enzymes. Frequently a very small percentage of the amino acid sequence of enzymes appears critical to activity and is conserved. Thus a NHC protein which is an important structural constituent of chromatin might well be present in all tissues and be conserved in protein structure as the histones have been. However, enzymes which have analogous functions in different chromatins may have diverged considerably in amino acid sequence and if so will not be easily recognizable. In addition to looking for conserved structural NHC proteins, one may consider the molecular weight patterns of NHC proteins. If these patterns of NHC proteins are important in terms of tissue-specific chromatin function, they might be conserved in comparisons of the same tissue from different organisms. To consider these questions we have used SDS-disc gel electrophoresis to analyze and compare the NHC proteins of two tissues, liver and kidney, from rat, cat, cow, chicken, turtle, and frog. These species include examples of all the major vertebrate classes except the fish.

METHODS

Preparation of Chromatin. Chromatin was prepared from the frozen liver and kidney tissues as described previously (Bonner et al., 1968; Elgin & Bonner, 1970). In brief, the frozen tissue was ground in a Waring blender and a crude nuclear pellet obtained by centrifugation. Following lysis of the nuclei, the chromatin was purified by centrifugation through 1.7 M sucrose, 0.01 M Tris, pH 8. The chromatin was then sheared in a Virtis homogenizer at 30 V for 90 sec and centrifuged at 12000 g for 30 min; the supernatant, referred to as purified chromatin, was used immediately as the starting material for the preparation of chromosomal proteins.

Preparations of Chromosomal Proteins. Histones and NHC proteins were prepared from the chromatin by the method of Elgin & Bonner (1970). The histones were extracted from chromatin with 0.4 N H_2SO_4, the pellet of DNA and NHC proteins was solubilized in SDS, and the DNA was removed by centrifugation. The supernatant containing the NHC proteins in SDS solution was concentrated by evaporation under N_2 at 37°C as necessary, and dialyzed to the sample buffer for gel electro-

phoresis.

Disc Gel Electrophoresis. The acid-extracted histones were dialyzed against 8 M urea, 0.01 M Tris, pH 8, and analyzed on 15% acrylamide gels, pH 4.3, in the presence of urea (Bonner et al., 1968). The NHC proteins were analyzed on SDS-phosphate gels (Shapiro et al., 1967) (data not shown) and on SDS-Tris glycine gels (Laemmli, 1970) as described in Elgin & Bonner (1970) and in Wu et al. (1973), respectively. Control samples of total chromosomal proteins were prepared by mixing an aliquot of freshly prepared chromatin with an equal volume of 2-fold concentrated SDS gel sample buffer. Such control samples were run on gels immediately. The gels were fixed with 50% TCA, stained with Coomassie Brilliant Blue, and scanned on a Gilford recording spectrophotometer at 600 μm as previously described (Wu et al., 1973). Protein loads were arbitrarily adjusted for maximum band resolution for each sample. Typically 100 μg total NHC protein was used per gel. In scanning, the optical density scale was set to approximately equalize major peak heights of the gels being compared. Scans were compared by superimposing them on a light box. In several instances samples were run on the same SDS gel with rat NHC protein standards to check homology assignments (data not shown). In discussing the band patterns, the nomenclature used is that given in Wu et al. (1973) with two minor additions; the high molecular weight shoulder of band θ_1, much more prominent in some tissues other than those of the rat, has been designated θ_0, and a similar low molecular weight shoulder of λ_2 has been designated λ_3. The SDS-Tris-glycine gels separate proteins primarily on the basis of molecular weight. The relative migration distance of a protein is linearly related to the log of its molecular weight in the range *ca.* 30,000 - *ca.* 100,000. All proteins of molecular weight 18,000 daltons or less move at the running front (U.G.Laemmli, personal communication; W.T.Garrard, personal communication).

Materials. Tissues from rat (white, Sprague-Dawley), domestic cat, turtle *(Chelydra serpentina),* and frog *(Rana pipiens)* were obtained from Pel-freez Biologicals, Inc., Rogers, Arkansas; tissues from cow were obtained at a local slaughterhouse; and tissues from chicken (white leghorn) were obtained at Caltech courtesy of Steve Beverly. In all cases tissues were removed quickly, washed in saline and individually frozen with dry ice or liquid nitrogen. Tissues were stored at 80°C until used. In all cases except the cow, where sex selection was not possible, only adult male animals were used. (Yolk

proteins can interfere with chromatin isolation from livers of egg-laying animals). Other materials used were as previously described (Wu et al., 1973).

RESULTS AND DISCUSSION

Reproducibility of Chromatin and Chromosomal Protein Preparations

Chromatin preparations were monitored in two ways - by examination of the UV spectrum, and by analysis of the histones by disc gel electrophoresis. The ratio of absorbance at 230 mμ to absorbance at 260 mμ of the chromatin was used as a routine measure of the protein-DNA ratio (Bonner et al., 1968). This number varied depending upon the tissue in question, but was reproducible for a given tissue with a standard deviation of 10%. In all cases this ratio had a value between 0.6 and 1.0. |It has been previously established by chemical determinations that the mass ratio of DNA: histone: NHC protein of chromatin preparations from a given tissue has a standard deviation of 10% (Elgin & Bonner, 1970)|. This indicates the anticipated level of quantitative variation among the NHC proteins. The high reproducibility of the NHC protein gel patterns has been previously demonstrated (Wu et al., 1973) and can also be assessed by comparing the three independent preparations of rat liver NHC proteins (Figs.1-3) as well as by comparing those of rat kidney NHC proteins (Figs.4-6).

The analyses of histones by disc gel electrophoresis, carried out routinely as a control, showed that the chromatin preparations were free of extraneous protein (no other basic proteins, such as basic ribosomal proteins, were observed) and that the fractionation of proteins into histones and NHC proteins was successful. The histone data (not shown) were in agreement with that of Panyim et al. (1971a,b).

To determine whether or not any degradation of the NHC proteins, detectable as a change in the gel patterns, occurs during the protein isolation procedure, the NHC protein gel patterns for rat liver and chicken liver were compared with those of the corresponding total chromosomal proteins on SDS-phosphate gels. The total chromosomal protein samples were run on gels immediately following the isolation of chromatin. The observed gel patterns are essentially identical to the sum of those of the NHC protein and of the histone (data not shown). A small increase in the amount of low molecular weight NHC protein would not be detected because

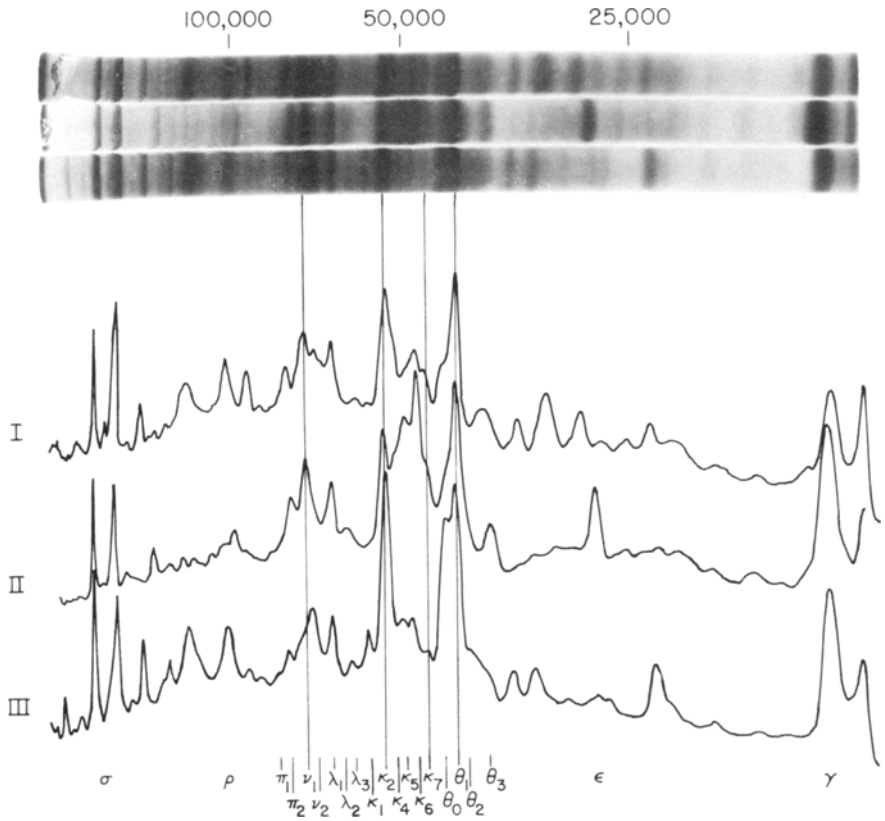


Fig.1. Comparison of the liver NHC proteins of cat, rat and cow. Gel origin at left

of the presence of comigrating histone bands in the total chromosomal protein sample. The only change observed was a relative increase in the amount of the protein band σ in the rat NHC protein sample, suggesting a small amount of aggregation during processing. There was no indication of any proteolytic degradation, which might introduce artifacts into the gel analyses, having occurred during the processing of the samples.

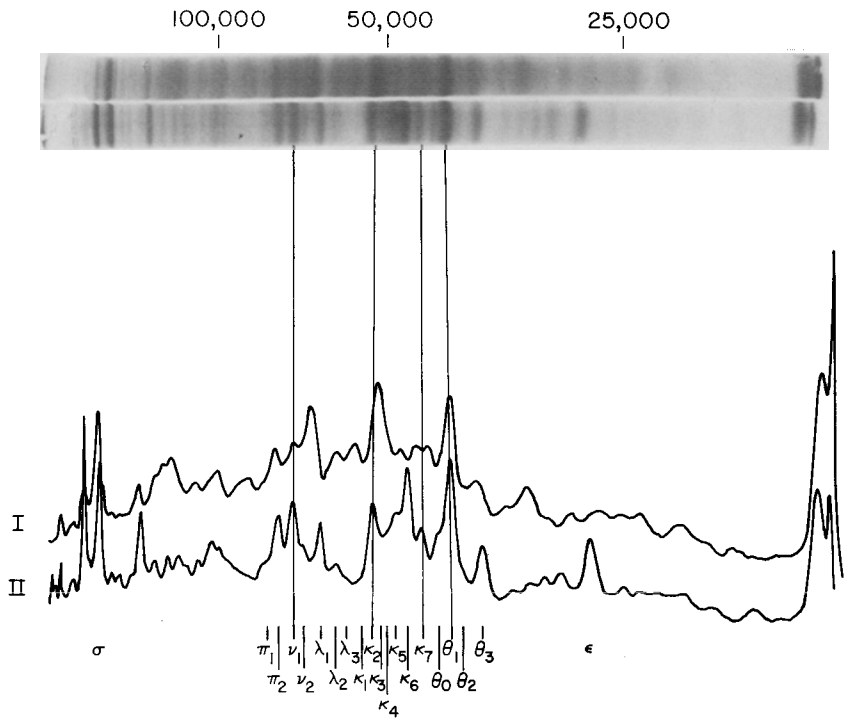


Fig.2. Comparison of the liver NHC proteins of chicken and rat

Comparative Gel Electrophoresis

The SDS-Tris-Glycine polyacrylamide gels of the liver NHC proteins are shown in Figs.1-3, and those of the kidney NHC proteins are shown in Figs.4-6. The gel scans are presented directly below. In all cases the patterns are compared to the rat NHC protein pattern as a standard. The band pattern in the prominent middle molecular weight region has been analyzed in detail, and the results tabulated (Tables 1 and 2).

The method of analysis used, comparative SDS gel electrophoresis, has obvious limitations for a study of this type. Each protein band may contain several different polypeptide chains of widely different function. However, preliminary studies of the rat liver NHC proteins indicate that several of the major bands consist of one or a few different polypeptide chains (Elgin & Bonner, 1972). In the simple case

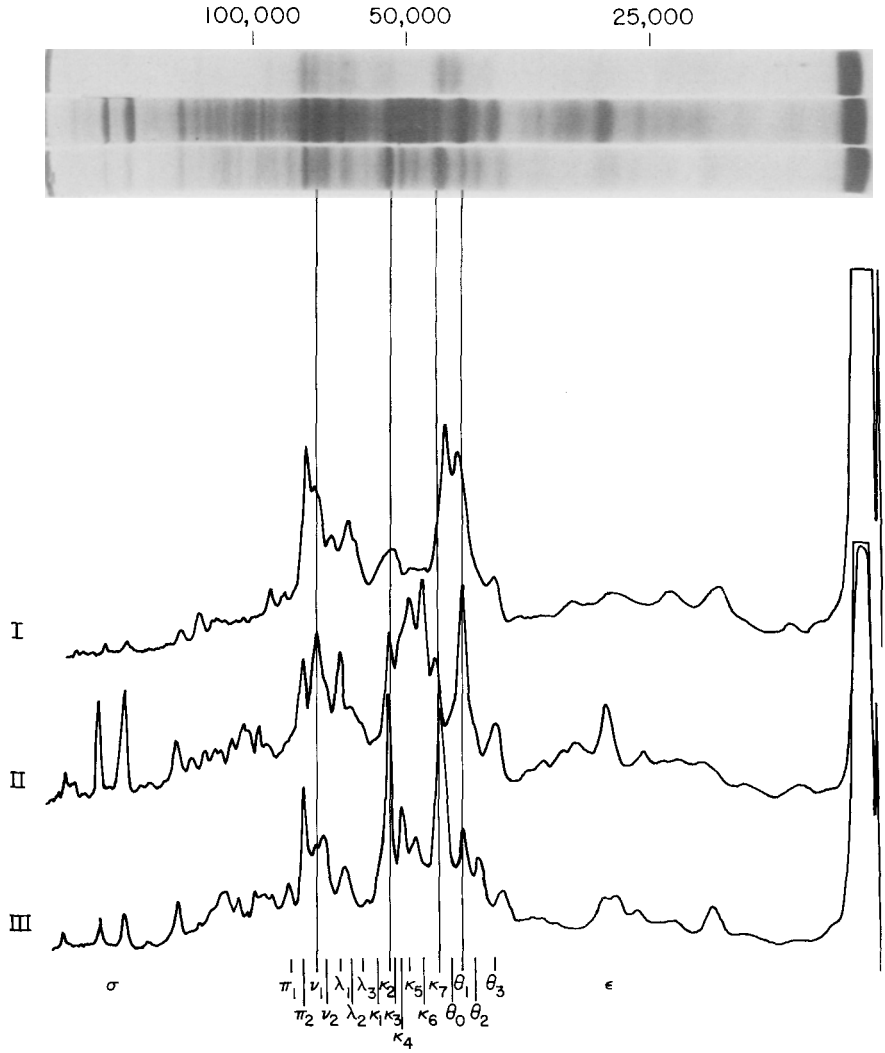


Fig.3. Comparison of the liver NHC proteins of turtle, rat and frog

of the histones, conclusions on their evolution based on studies by comparative disc gel electrophoresis have been substandiated by subsequent comparisons of their amino acid sequences (Panyim et al., 1971a,b; DeLange & Smith, 1974). Thus a conservation of bands or band patterns is a required (but not sufficient) condition for conservation of the NHC

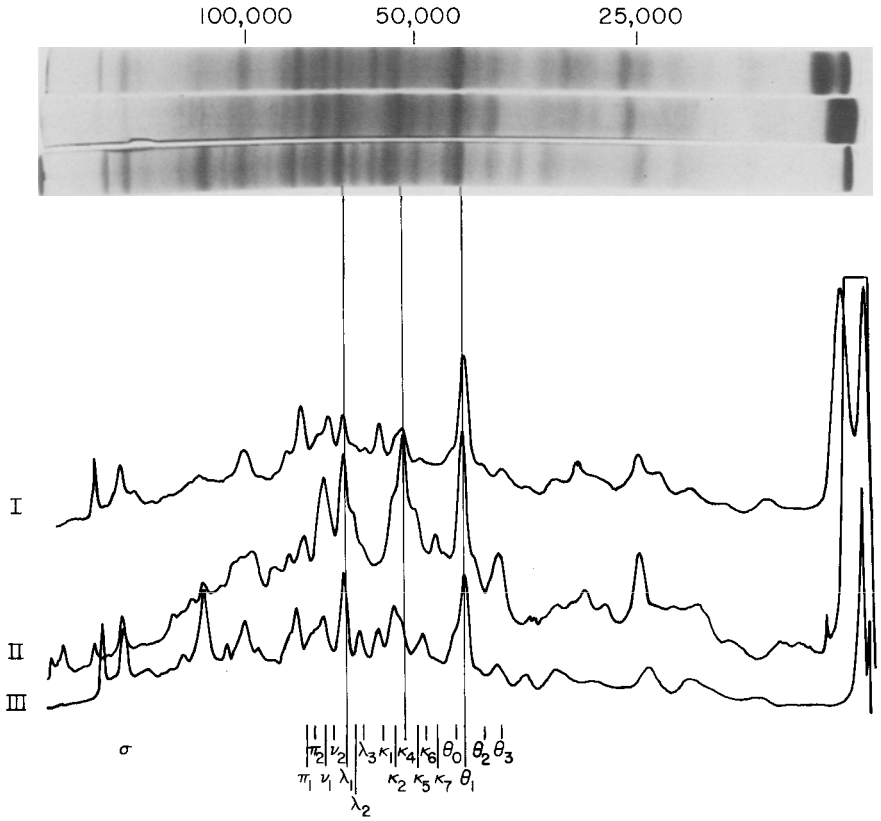
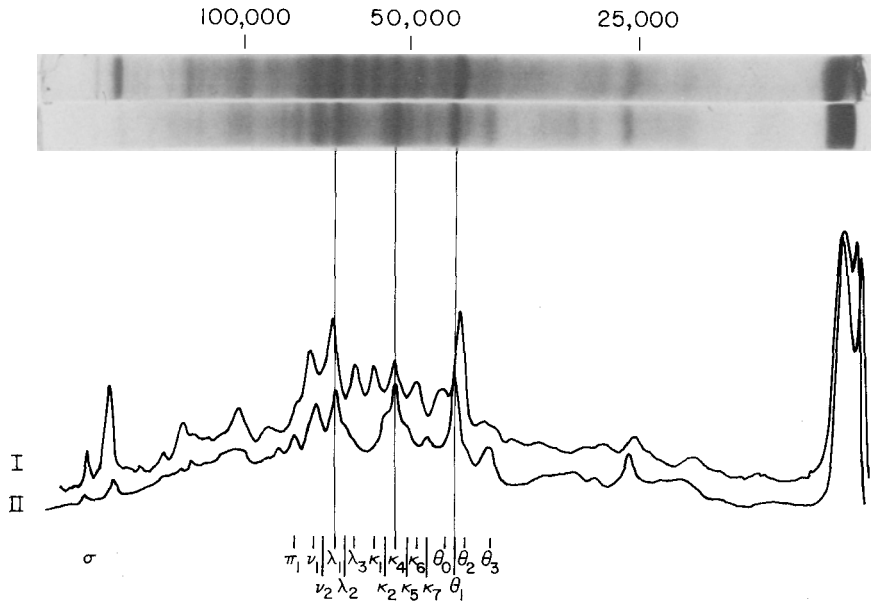


Fig.4. Comparison of the kidney NHC proteins of cat, rat and cow

proteins during evolution.

In considering the data, it is at once clear that there is an overall conservation of the distribution of protein mass along the molecular weight axis. In all cases the protein bands from *ca.* 40,000 to *ca.* 80,000 daltons molecular weight make up the bulk of the protein mass. There is also a considerable amount of protein at the gel front. This represents the major bands of *ca.* 15,000 and *ca.* 18,000 daltons, β and γ, which are resolved on SDS-phosphate gels (data not shown).

Examination of the qualitative aspects of the data (presence or absence of bands) demonstrates that the NHC proteins



I NHC PROTEINS OF CHICKEN KIDNEY
II NHC PROTEINS OF RAT KIDNEY

Fig.5. Comparison of the kidney NHC proteins of chicken and rat

as a class have had considerably fewer evolutionary constraints than have the histones; the patterns have changed considerably more during evolution. Further chemical studies of the histones have shown that the homologies indicated by band patterns do reflect real sequence homologies. Although the NHC proteins are a more complex class, and as yet chemical data are not available, some information may be obtained from a comparison of the band patterns. Seventeen major and eight minor bands of 40,000 to 80,000 daltons were identified in the analysis of liver and kidney NHC proteins (Tables 1 and 2), 24 bands in each. One way of quantifying the degree of homology is to count the number of times a common band occurs in the NHC protein pattern of a given tissue from two organisms. For example, bands π_1, ν_1, ν_2, λ_1, λ_3, κ_1, κ_2, κ_4, κ_5, κ_6, κ_7, θ_0, θ_1 and θ_3 occur in the liver NHC proteins of both rat and cat, for a pair homology score of 14/24 or 58%. On the average, each tissue possesses 16 protein bands in this molecular weight region of the gel. Thus if the bands are randomly distributed the pair homology will be (16/24)(16/24)=4/9 or 44%. A count of pair homology gives 49% for the liver NHC proteins and 43% for the kidney NHC

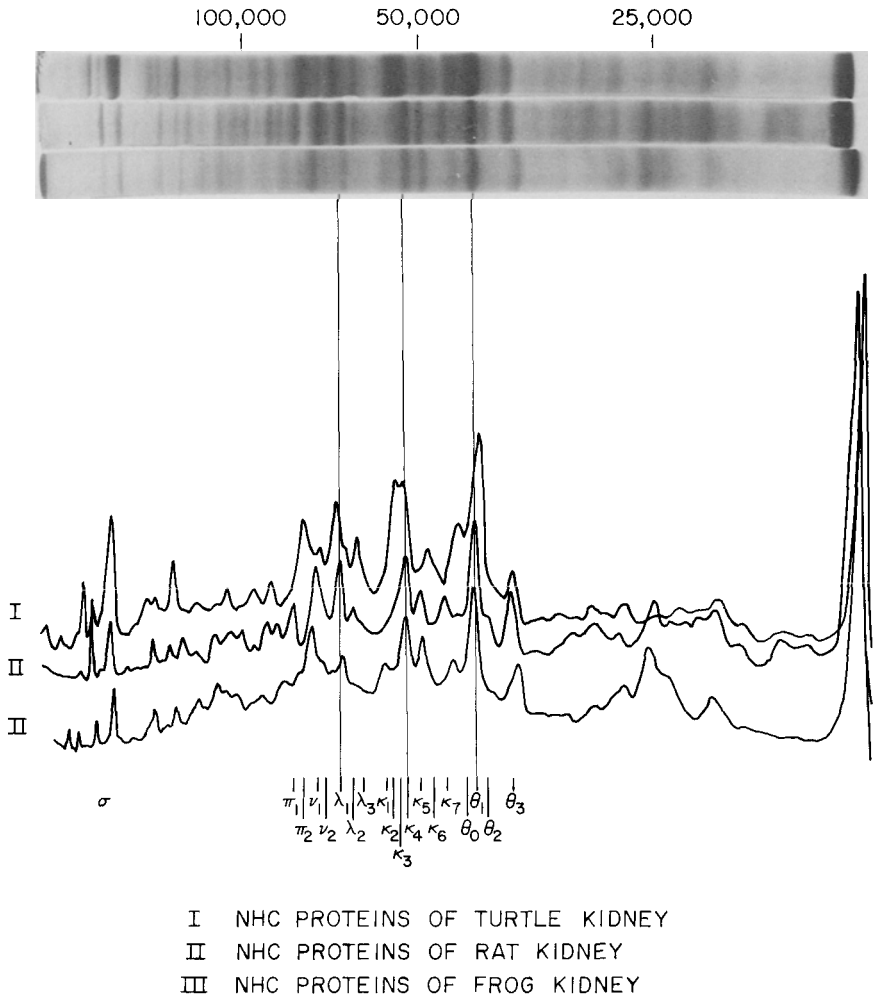


Fig.6. Comparison of the kidney NHC proteins of turtle, rat and frog

proteins, suggesting no conservation of the total pattern. However, there does appear to be a significant conservation of a subset of bands in each case (liver and kidney). Were distribution entirely random one would anticipate that only two of the 24 protein bands tabulated would occur in all six organisms. However, six protein bands occur in all of the liver NHC protein preparations (ν_1, κ_1, κ_2, κ_5, κ_7, θ_1) and six protein bands occur in all of the kidney NHC protein preparations (λ_1, λ_3, κ_4, θ_0, θ_1, θ_3). The occurrence of these band subsets and the lack of overlap between them (only θ_1 is common) suggests that the conserved elements may

Table 1. Presence (+) or absence (O) of liver NHC proteins. ++ indicates a dominant band; (+) indicates a minor band; ? indicates no information; * indicates a common band. See Figs.1-3

Band	Rat	Cat	Cow	Chicken	Turtle	Frog	Common
π_1	(+)	+	+	+	O	+	O
π_2	++	O	+	+	++	++	O
	O	O	O	O	+	O	O
ν_1	++	++	+	+	+	+	*
ν_2	(+)	+	+	O	+	+	O
	O	+	O	++	O	O	O
λ_1	++	++	+	O	O	+	O
	O	+	O	O	+	O	O
λ_2	+	O	+	+	+	(+)	O
λ_3	(+)	+	O	O	(+)	(+)	O
κ_1	+	+	+	+	+	+	*
	O	O	O	O	+	O	O
κ_2	++	++	++	++	+	++	*
κ_3	O	?	?	O	+	O	O
κ_4	+	+	O	(+)	O	+	O
κ_5	++	+	+	+	(+)	+	*
κ_6	++	+	O	O	+	O	O
	O	O	O	+	O	O	O
κ_7	+	+	+	+	+	++	*
	O	O	O	O	++	O	O
θ_0	+	+	++	O	++	O	O
θ_1	++	++	++	++	+	++	*
θ_2	+	O	+	O	+	+	O
θ_3	+	+	+	+	+	O	O

describe a tissue-specific pattern as discussed by Gierer (1973). Gierer has suggested that a state of differentiation may be defined by the presence of a certain combination of regulatory proteins. Such a combinatorial model has certain advantages of efficiency in terms of the number of regulatory proteins required and in terms of models of developmental processes. A similar result of conserved tissue-specific patterns has been obtained in a much more limited study (3 tissues) using a two-dimensional analysis (Barrett & Gould, 1973). However, in both cases the amount of data and the level of resolution are insufficient to make possible a final judgment on the significance of the results.

Only the band θ_1 is common to all tissues examined here. Further chemical characterization of the protein of this band from different tissues is required to verify if in fact θ_1 represents a conserved polypeptide. θ_1 is a prominent band

Table 2. Presence (+) or absence (O) of kidney NHC proteins. ++ indicates a dominant band; (+) indicates a minor band; ? indicates no information. See Figs.4-6

Band	Rat	Cat	Cow	Chicken	Turtle	Frog	Common
π_1	+	++	O	+	(+)	O	O
π_2	O	O	+	O	+	+	O
	O	O	O	+	O	O	O
ν_1	++	+	+	O	+	O	O
ν_2	+	+	O	+	O	+	O
λ_1	++	++	++	+	+	+	*
	O	O	O	O	(+)	O	O
λ_2	+	+	O	O	+	+	O
λ_3	+	+	+	+	+	+	*
	O	O	+	O	O	O	O
κ_1	O	+	O	+	+	+	O
κ_2	+	+	++	O	++	+	O
κ_3	O	O	O	?	?	O	O
κ_4	++	+	+	+	++	++	*
κ_5	+	O	+	O	(+)	+	O
κ_6	O	+	+	+	+	O	O
	O	O	O	O	+	O	O
κ_7	+	O	O	O	O	+	O
	O	O	O	+	O	O	O
θ_0	+	+	+	+	+	+	*
θ_1	++	++	++	++	++	++	*
θ_2	+	O	+	?	+	+	O
	O	+	O	+	O	O	O
θ_3	+	+	+	+	+	+	*

of 44,000 daltons molecular weight. It will be of interest to determine whether or not this band (or a component of it) shares other characteristics in common besides molecular weight with the protein isolated from nuclear ribonucleoprotein (transport) complexes (Samarina, Lukanidi, Molnar & Georgiev, 1968; Krichevskaya & Georgiev, 1969; Morel et al., 1973). Such a protein might well be a conserved component of chromatin, common to all tissues.

In conclusion, it is clear that the NHC proteins as a class have sustained considerably more change during evolution than have the histones; however, certain elements of the NHC protein band pattern appear to have been conserved in a tissue-specific manner. Further data are required to establish the biological significance of this observation.

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Dr.Sarah C.R.Elgin
The Biological Laboratories, Harvard University
16 Divinity Avenue, Cambridge, Mass.02138, USA