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THE ANTIGENICITY OF THE
LYSINE-RICH HISTONES

by

CATHERINE ELIZABETH SHAY, B.Sc.(Hons.)

A thesis submitted to the
Faculty of Graduate Studies and Research
in partial fulfilment of
the requirements for the degree of
Master of Science

Department of Biology
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July 6, 1984.

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ABSTRACT

Sequence homology of chicken and goose histone 5 has been compared using the immunochemical techniques of microcomplement fixation and enzyme-linked immunosorbent assay. The published relationship between immunological distance and percent amino acid sequence difference did not apply to these two sequenced histones. Indeed no mathematical model adequately described the relationship among histone proteins of known sequence. The species of immunization or the inclusion of ribonucleic acid with the immunogen had no effect on the production of antisera capable of recognizing degrees of antigenicity among specific domains of chicken histone 5. A variety of antisera showed no localization of antigenicity within the central globular domain of chicken histone 5, the most highly conserved domain within the lysine-rich histones. Therefore, even tentative structural and functional homologies among lysine-rich histones should not be based solely on immunochemical cross-reactivities.

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LIST OF ABBREVIATIONS

Ab.....	antibody
ABTS.....	2,2'-azino-di(3-ethyl-benzthiazoline sulfonic acid-6)
Ag.....	antigen
Anti-H1 or H5.....	antiserum against histone H1 or histone H5
BSA.....	bovine serum albumin
BSB.....	borate buffered saline
C'F.....	complement fixation
ChH1.....	chicken histone H1
ChH5.....	chicken histone H5
CP.....	chymotryptic degradation product
CTAH.....	1-chloro-3-tosylamido-7-amino-heptanone
DNS-Cl.....	5-dimethylamino-naphthalene-1-sulphonyl chloride
DPCC.....	diphenyl carbamyl chloride
GH5.....	goose histone H5
GP.....	guinea pig
GuCl.....	guanidinium-hydrochloride
H1, H5, H1 , H1	lysine-rich histones
H2A, H2B, H3, H4.....	four core histones
IgG.....	immunoglobulin G
IgM.....	immunoglobulin M
MS222.....	tricaine methane sulfonate
NBS.....	N-bromosuccinimide

OD.....optical density
 o/n.....overnight
 PBS.....phosphate buffered saline
 PCA.....perchloric acid
 PMSF.....phenyl methyl sulfonylfluoride
 Rab.....rabbit
 SDS.....sodium dodecyl sulfate
 TCA.....trichloroacetic acid
 TEMED.....tetramethylethylenediamine
 TP.....limit tryptic digest peptide
 TPCK.....L-1-toslamide-2-phenyl-
 ethylchloromethyl ketone
 TrH1, TrH5.....trout lysine-rich histones

INTRODUCTION

I. Histones and Chromatin Structure

A fundamental area of research in molecular biology today is the study of the processes involved in cell proliferation and differentiation that enables single cells to evolve and develop into multicellular organisms. This "blueprint for development" is all stored in the chromatin of the eukaryotic nucleus. As a minimum, chromatin consists of deoxyribonucleic acid(DNA) associated with an equal weight of small basic proteins called histones. Histones are classified according to their basic amino acid content into five major classes: very lysine-rich histone H1, moderately lysine-rich histones H2A and H2B, and arginine-rich histones H3 and H4 (for reviews see Hnilica, 1967; Elgin & Weintraub, 1975; Isenberg, 1979).

The fundamental subunit of eukaryotic chromatin is the nucleosome; a 10nm diameter structure composed of about 140 base pairs of DNA coiled twice around an octameric core particle consisting of each of the four "core" histone classes- H2A, H2B, H3, H4(for reviews see Worcel, 1977; Kornberg, 1977; Felsenfeld, 1978 and McGhee et al, 1980). The internucleosome linker DNA varies from

20-70 base pairs in length and interacts with the lysine-rich histone class(Worcel, 1977; Belyavsky et al, 1980).

A consideration of the amino acid sequence of the four core histones of the nucleosome shows the N-terminus of each to be highly basic, whereas the carboxyl region has an amino acid constitution characteristic of more hydrophobic proteins. The apolar globular regions of the core histones are involved in the protein-protein interactions of the nucleosomal core(Bradbury & Rattle, 1972) whereas the very basic regions are thought to be the major sites of interaction with DNA(Isenberg, 1979).

Comparisons of the sequences of lysine-rich histones show this class of proteins to be comprised of three distinct structural domains: the basic N-terminal domain, rich in alanine or serine, and proline, a more hydrophobic region that forms a globular domain at high pH and ionic strength, and a very basic C-terminal domain where alanine or serine, lysine or arginine, and proline comprise 80% of the amino acid residues(Hartman et al, 1977).

At very low ionic strength chromatin assumes the dispersed 10nm conformation often described as "beads on a string" (Worcel & Benyanati, 1977). The globular domain of the lysine-rich histone binds to the nucleosomal DNA at the site of the DNA entrance and exit from the nucleosome core particle(Allan et al, 1980; Belyavsky et al, 1980) at a ratio of approximately one molecule per nucleosome(Bates

& Thomas, 1981). Tryptic digestion of nuclei has shown the trypsin-resistant globular domain of chicken H1 and H5 to exist in native chromatin as it does in isolated H1 and H5 in moderately concentrated salt solution(Allan et al, 1981; Puigdomenech et al, 1983).

Increased ionic strength induces the relaxed nucleosomal filament to supercoil and condense into the 25-30nm superhelical structure with 5-7 nucleosomes per turn(for reviews see Worcel, 1977; McGhee et al, 1980 ; Thoma & Koller, 1981). The presence of the lysine-rich histones is an essential requirement for this organized condensation regardless of ionic environment(Brasch, 1976; Thoma & Koller, 1981; Allan et al, 1981).

The binding of lysine-rich histone to DNA is largely the result of electrostatic interaction between the negatively charged phosphate groups of the DNA and the positively charged lysine and arginine residues (Belyavsky et al, 1980), concentrated in the N- and C-terminal regions of the H1 molecule. These more variable, less organized N- and C-terminal arms of the lysine-rich histone are essential to effect the condensation of the chromatin fiber in the physiological range of ionic strength(Allan et al, 1980).

It has been proposed that the N-terminal arm interacts loosely with the outside of the nucleosomal DNA and protects the DNA from nucleolytic attack at regularly spaced intervals;i.e. it protects 10-20 base pairs near

the DNA entry to the nucleosomal core(Boulikas et al, 1980; Ishimi et al, 1981). The C-terminal arm binds more strongly to the DNA, presumably because of its greater lysine or arginine content. It generates salt-induced chromatin condensation equivalent to the condensation generated by the whole molecule(Rattle et al, 1977) and interacts with the 20 base pairs of linker DNA on the exit side of the nucleosomal core(Boulikas et al, 1980). The C-terminal regions may also interact with the linker or nucleosomal DNA of other nucleosomes thereby cross-linking nucleosomes and participating in chromatin condensation (Allan et al, 1980; Belyavsky et al, 1980). As well, H1 interactions between the globular regions, known to occur for H1 residues 47-106(Bradbury & Rattle, 1972), may be important for maintaining higher order structure(Thoma et al, 1979). In the cell, the observations of salt induction of chromatin condensation are proposed to be controlled by histone modification; i.e. phosphorylation/dephosphorylation(Rattle et al, 1977).

II. Lysine-Rich Histones and Gene Expression

Transitions between loose and disordered polynucleosome chains and compact and regular higher order structure can be expected to exert a major effect on the

template activity of the chromatin, both for replication and transcription. Transcriptionally active or potentially active genes possess a structural organization different from that of repressed genes. Electron microscopy and nuclease digestion of transcriptionally active or potentially active genes show the chromatin to be in the "disperse" conformation in contrast to the highly condensed tertiary conformation of repressed genes (for review see Elgin, 1981). Cross-linking studies on heat shock protein genes have indicated that actively transcribing genes are linearized and stripped of histone. Moderately active genes assume the 10nm conformation of DNA and inactive DNA assumes the highly condensed 25nm conformation. The coding region is depleted of H1 first, followed by depletion of the cores (Karpov et al, 1984). The association of lysine-rich histone with the regulation of chromatin structure leads one to speculate on its involvement in control of gene expression.

The specialized cell differentiation needed for the development of multicellular eukaryotic organisms could be achieved by the regulation of chromatin structure and thus control of gene expression mediated by the lysine-rich histones, if different lysine-rich histone subtypes existed with different functions. The amino acid sequences of the lysine-rich histones have been the least conserved of the histone class of proteins throughout evolution. The rate of protein evolution to establish a

1% difference in amino acid sequence between two lineages of lysine-rich histones has been estimated to be 8 million years, whereas rates of protein evolution have been estimated to be 400 million years for H4, 330 million for H3, and 60 million for H2A and H2B(Wilson et al, 1977).

Lysine-rich histones are not only the most divergent class in terms of evolution but also in terms of the number of subfractions within any given tissue or species. As many as six subtypes of H1(reviewed in Cole, 1984), all having molecular weights in the 20,000-22,000 dalton range(Elgin & Weintraub, 1975) are known to occur in one tissue. Although all H1 subtypes show closely-related primary structures with three distinct structural domains(Cole, 1977; Isenberg, 1979; Yaguchi et al, 1977, 1979), they exhibit characteristically different abilities to protect DNA against nucleolytic attack (Gorka & Lawrence, 1979). The proportions of H1 subtypes vary with progression of differentiation in embryonic development (Newrock et al, 1978; Hentschel & Birnstiel, 1981; Urban et al, 1979) and continue to change at later stages of differentiation associated with hormone induction (Pehrson & Cole, 1982). H1 subtypes are known to be selectively phosphorylated(Pehrson & Cole, 1982) and involved in spermatogenesis and terminal differentiation(Seyedin & Kistler, 1980). Differential synthesis of H1 subtypes during S-phase in cultured HeLa cells has also been demonstrated(Sizemore & Cole, 1981). These findings

suggest an important role of H1 subtypes in maintaining the schedule of chromatin dynamics through the cell cycle and perhaps through patterns of differentiation(Cole, 1984), and thus implicate H1 subtypes in the control of differential gene expression(Cole, 1977).

III. Lysine-Rich Histone Satellites

The occurrence of a distinct subclass of H1 satellites in cells of highly differentiated tissues exhibiting little proliferative activity may be a form of functional specificity that regulates tissue-specific gene expression. Because of their similarity in primary structure, histone H5, the erythrocyte specific lysine-rich histone found in birds and fish(Neelin et al, 1964; Miki & Neelin, 1975), histone H^P, found in non-proliferative mammalian tissue(Panyim & Chalkley, 1969), and histone H1^S found in terminally differentiated tissues of reptiles and amphibians (Rutledge et al, 1981; Shimada et al, 1981; Risley & Eckhart, 1981; Brown et al, 1981; Svinarchuk et al, 1982) are believed to be highly divergent variants of H1(Yaguchi et al, 1977, 1979). The lysine-rich histones of this subclass are shorter in length than H1 and differ somewhat in amino acid composition. H5 has more serine replacing alanine and

arginine replacing lysine than H1, whereas H1^O and H1^S, which share electrophoretic and chromatographic properties as well as similar amino acid compositions, have an intermediate lysine and arginine content between H1 and H5. All known H1 satellites contain one methionine residue at approximately residue 30 which is absent in H1.

H5 and H1^O possess similarities in secondary and tertiary structure with H1 (Chapman et al, 1976; Hartman et al, 1977; Aviles et al, 1978; Cary et al, 1981) with the apolar globular domain of H1^O being 70% homologous to the globular domain of H5 whereas it is only 42% homologous to H1 (Pehrson & Cole, 1981). Preliminary amino acid sequencing of this apolar region from H1^S also indicates the closer homology to H1^O and H5 than H1 (Brown, 1982).

The globular domain of H5 and H1^O interacts with nucleosomal DNA in the same manner as H1 (Allan et al, 1981; Cary et al, 1981; Puigdomenech et al, 1983). On the surface of the globular domain of H5 are situated 6 arginine and 8 lysine residues that have the potential to bind more tightly than H1 to nucleosomal DNA. In fact, the increased arginine content of intact H5 and H1^O allows tighter binding of all three domains to DNA which could modulate chromatin higher order structure; thus H5-containing chromatin has a greater stability in higher order structures than H1-containing chromatin (Thomas & Rees, 1983).

Tissue-specific subtype variants, seen in H1, have

also been shown to exist for H1^O (Smith & Johns, 1980; Pehrson & Cole, 1980; Cary et al, 1981; Harris & Smith, 1983) and for H1^S (Rutledge et al, 1981; Brown et al, 1981; Risley & Eckhart, 1981).

Other examples of tissue-specific H1 satellites include histone $\emptyset$ I, which completely replaces H1 in the transcriptionally inert sperm of marine invertebrates (Palau et al, 1969) and shares H5's increased levels of arginine. Histone 1 from the fruit flies, Drosophila melanogaster and Ceratitis capitata, contain one methionine at approximately residue 30 and have 55% identity with chicken H5 within the central domain compared to 30% identity with mammalian H1 (Alfageme et al, 1974; Barbero et al, 1982). TH1^{met} (Brown & Goodwin, 1983; Srebrenja & Zlatanova, 1983), an H1^O-like protein found in trout liver exhibits characteristics more like H5 than H1. Trout testes H1^t from terminally differentiated spermatocytes shares certain compositional similarities with both sea urchin sperm H1($\emptyset$ I) and avian H5 but it has a lower lysine and higher arginine content than H1^O (Seyedin et al, 1981).

With the exception of the insect H1, the H1 satellite subclass of proteins occurs in tissues that are terminally differentiated and transcriptionally inert. H5 is synthesized during decreased DNA synthesis (Tobin & Seligy, 1975; Ruiz-Carillo et al, 1976) or after the cessation of DNA synthesis (Appels & Wells, 1972; Urban et al, 1980),

and it is thought to be involved in the progressive nuclear condensation occurring during erythropoiesis which is related to the transcriptional inactivation of erythrocyte chromatin (Seligy et al, 1973).

One possible mechanism of regulation of chromatin condensation involves phosphorylation/dephosphorylation of the highly basic terminal regions of H5. In immature erythroid cells newly synthesized H5 is phosphorylated at serine 3 or 7 introducing a negative charge into the N-terminus which would weaken electrostatic binding to the DNA and thus prevent premature or inappropriate chromatin condensation (Seligy et al, 1973; Tobin & Seligy, 1975; Sung et al, 1977). H5 is dephosphorylated in mature erythrocytes, allowing condensation to occur (Brasch et al, 1974; Seligy et al, 1973). In the regenerating blood cells from Xenopus laevis, synthesis of H1^S also continues into later stages of erythropoiesis after the synthesis of H1, other histones and DNA has ceased (Rutledge et al, 1984), but it is not known if phosphorylated forms of H1^S exist.

H1^O was first observed in greatest relative amounts in mammalian tissues possessing low levels of cell replication (Panyim & Chalkley, 1969). Studies on pancreatic (Marsh & Fitzgerald, 1973) and liver cell regeneration (Benjamin, 1971; Balhorn et al, 1972; Garrard & Bonner, 1974) showed that the levels of H1^O were reduced as long as DNA synthesis continued and that normal levels

of $H1^O$ were regained upon full tissue regeneration. This inverse relationship between abundance of $H1^O$ and mitotic activity of tissue was also demonstrated in liver, pancreas (Varrichio, 1977) and spleen development (Medvedev & Medvedeva, 1980). Both the chemical induction of Friend erythroleukemia cells (Keppel et al, 1977, 1979; Zlatanova & Swetly, 1978) and neuroblastoma cells (Pieler et al, 1981) and the induction of neuroblastoma cells by density inhibition and serum deprivation (Pehrson & Cole, 1980, 1982) resulted in declining proliferation and differentiation as well as a 2- to 7-fold increase in $H1^O$ concentration. The attribution to $H1^O$ of the function of arresting cell division only may not necessarily be valid. $H1^O$ is present in some cells that are actively proliferating, and it is not present in others which do not divide (Gjerset et al, 1982; Lennox & Cohen, 1983).

The involvement of $H1^O$ in cellular differentiation has been convincingly proven in a variety of mammalian models which permit a clear distinction between proliferation and differentiation. Analysis of the level of $H1^O$ during rat liver development (Larue et al, 1983) showed that $H1^O$ emerges as rat liver cell proliferation has largely terminated but accumulates mostly during the cyto-differentiation stages; this corresponds to the levels of H5 found in chick embryonic blood cells which are undergoing the distinct proliferative and maturation stages of erythropoiesis (Urban et al, 1980).

While H1⁰ accumulation has been correlated with limited cell proliferation and commitment to tissue differentiation, it is not known if it acts by mediating replicational or transcriptional control. H1⁰ does cause a greater condensation of chromatin (like H5) as it replaces H1 in the linker (Smith & Johns, 1980b), but it does not bind as strongly to DNA as H5 (Smith & Johns, 1980b; Cary et al, 1981). H1⁰ also becomes highly phosphorylated during mitosis (D'Anna et al, 1981). However, because its induction follows rather than precedes a decline in DNA synthesis, H1⁰ would seem to have a maintenance function rather than a role in the induction of the repressed state (Biard-Roche et al, 1982).

These multiple examples of lysine-rich histones, variable in sequence yet similar in structure, not only among the H1 subtypes but also within the subclass of H1 satellites, and the correlation of H1 subfraction specificity with functions that can be regulated through control of DNA replication or transcription, leads one to speculate that lysine-rich histones have a direct effect on the control of gene expression. From the studies reported to date however, one can only conclude that H1 satellites are involved in an event that occurs during the final stages of tissue maturation and is associated with chromatin condensation.

IV. Immunochemistry of the Lysine-Rich Histones

A. Antibodies as Probes of the Relatedness among the Lysine-Rich Histones

In the absence of a functional assay for lysine-rich histone action it is hoped that a detailed analysis of the structure of a wide range of lysine-rich histones will provide insight into their biological function or mode of action. Because histones have no assayable function, they have been characterized by their solubility, chromatographic and electrophoretic behaviour and amino acid composition. The immunological comparison of structure of homologous proteins from different species has contributed towards an understanding of the relationship between protein structure and function (Arnheim & Wilson, 1967). As early as 1900 antisera were used to show differences between homologous proteins in different species (Sibley et al, 1974). In 1904 Nuttall published the results of a report of precipitin tests with antisera from four species of bird cross-reacted with blood serum proteins from over 500 species of animals. He concludes "with care, we shall perhaps be able to 'measure species' by this method." Since then, innumerable studies have compared suspected homologies immunologically among antigens from countless species. Consequently this study

was undertaken with the purpose of using immunochemistry to compare the lysine-rich histone structure from different species in order to gain insight into their function.

The technique of complement fixation is sensitive to small differences in antigen structure, including amino acid sequence in proteins, thus providing an approximate measure of the degree of structural relatedness between homologous proteins(Arnheim & Wilson, 1967). The immunological distance, a measure derived from complement fixation results, appears to be proportional to the actual amount of change which has occurred since two proteins diverged from a common ancestor(Sarich & Wilson, 1966). Comparisons using complement fixation on avian lysozymes(Prager & Wilson, 1971), pancreatic ribonucleases(Prager et al, 1978), bacterial azurins(Champion et al, 1975) and serum albumins(Sarich & Wilson, 1966; Arnheim & Wilson, 1967) have indicated that the immunological distance between these proteins is directly related to the percent difference in amino acid sequence. Comparisons using complement fixation are much less time-consuming and require much less protein than amino acid sequencing. This relationship is supposedly valid for naturally occurring, globular, monomeric isofunctional proteins(Champion et al, 1975).

Antisera elicited against histones have been used to establish the phylogenetic and ontogenetic relationships

within this class of proteins(Bustin & Stollar, 1972). Using microcomplement fixation(Wasserman & Levine, 1960), immunological comparisons have been made of the lysine-rich histones of species, tissues and subfractions within tissues. The chromatographically purified subfractions of calf thymus H1 histones showed various reactivities with whole H1 preparations from calf thymus, lamb thymus, calf liver, rat thymus and mouse spleen, as well as three chromatographically separated subfractions of calf thymus(Bustin & Stollar, 1972). Antibodies to purified rat thymus H1 subfractions were cross-reacted with each other as well as with purified subfractions from calf thymus and rat liver H1(Bustin & Stollar, 1973a). Antisera elicited by subfractions from rat thymus H1 and unfractionated rat hepatoma H1 histones have been studied(Sluyser & Bustin, 1974). An immunochemical investigation into the cross-reactivity of the lysine-rich histones from chicken and pigeon has been undertaken using antibodies to pigeon red blood cell H5(Bers et al, 1979). Species comparisons have been made among the lysine-rich histones of a mammal(calf thymus) , two sea urchins(Arbacia lixula and Paracentrotus lividus) and a sea cucumber (Holothuria tubulosa)(Martinez & Palau, 1983), as well as between calf thymus and rye(Roland & Pallotta, 1978).

Microcomplement fixation has also been used to compare species-relatedness between the core histones.

Stollar & Ward(1970) compared the cross-reactivity of antisera elicited against calf thymus H4, H2A and H2B with the corresponding fractions from human, calf, chicken, frog and lobster tissues. Other immunological comparisons have been performed between calf thymus core histones and rye and yeast histones(Roland & Pallotta, 1978; Tessier et al, 1980) and insect histones(Bustin et al, 1977).

Antisera to intact calf thymus H2B and two peptides derived from it have been used to make immunological comparisons with the H2Bs from mouse, chicken, frog, trout, and sea urchin(di Padua Mathieu & Stollar, 1980). Microcomplement fixation has also been used to detect variants within the H2A core histone class(Blankstein et al, 1977).

Immunological techniques other than microcomplement fixation have also been used to survey homologies between histones. Antibodies to chicken H5 were used to detect inter-species variations within the lysine-rich histones of fish erythrocytes using radioimmunoprecipitation(Goetz et al, 1978). The indirect immunofluorescence method was used to study the immunological distances between the five classes of calf thymus histones and the corresponding fractions from four species of amoeba(Sopina et al, 1979).

Thus much work has been done relating the immunological cross-reactivity quantitatively and qualitatively to sequence differences between all classes of histones. Although histone proteins meet the criteria

set by Champion et al (1975), despite their relatively small size and great basicity, the relationship between the derived quantity of immunological distance and percentage amino acid sequence difference, as defined by Prager & Wilson (1971), has never been demonstrated for fully sequenced histones. Since the complete amino acid sequences of both chicken H5(Briand et al, 1980) and goose H5(Yaguchi et al, 1979) are known, it is the intention of this study to test the relationship between the immunological cross-reactivity and percent amino acid sequence difference using a variety of antisera to these lysine-rich histones of known sequence.

B. Antibodies as Probes of Lysine-Rich Histone Structure

Antibodies to histones have served as probes of chemical modifications(Bers et al, 1979), conformational changes in isolated histones(Mihalikis et al, 1976), or of accessibility of H1 in chromatin under various experimental conditions (Zick et al, 1975; Takahashi & Tashiro, 1979; Goldblatt & Bustin, 1980). They have been used to localize lysine-rich histones in various cell types under different metabolic conditions using immunofluorescence(Hekman & Sluyser, 1973; Mura et al, 1974; Mura et al, 1978a,b; Bustin, 1977; Mura & Stollar, 1981; Mura et al, 1982; Destree et al, 1979). More

recently chromatin structure has been probed using lysine-rich histones and immunoelectron microscopy (de Murcia et al, 1980; Mazen et al, 1982; Frado et al, 1983). When using antibodies for comparative studies or as probes of histone conformation in chromatin organization, it is imperative to know whether the recognition of the different parts of the histone molecule is random or whether certain parts are preferentially reactive with the antibody(di Padua Mathieu & Stollar, 1980).

Early work using mammalian H1 indicated that the C-terminal two-thirds of the molecule was preferentially detected by the antisera(Bustin & Stollar, 1973b; Hekman & Sluyser, 1973; Sluyser & Bustin, 1974). Other workers using inhibition of cross-reaction with a variety of immunological techniques, have localized three determinants in chicken H3(Sapieka et al, 1976; Absolom & Van Regenmortel, 1977), at least one determinant in calf thymus H2B(di Padua Mathieu & Stollar, 1980) and two determinants in chicken H4(Muller et al, 1983).

Using two antisera raised with chicken H5 in guinea pigs in assays for inhibition of complement fixation by eight peptides derived enzymatically and chemically from this protein, two precisely delineated regions of antigenicity were located, specifically amino acid residues 59-65 and 94-99(Mura et al, 1980). Two less-defined antigenic regions were localized in amino acid residues 66-93 and 100-189.

Thus, for these antibody populations, the serological specificity resides within the globular domain, the region of the lysine-rich histones which is known to have the greatest conservation of sequence homology. This conserved region is also critical to the structural role played by the lysine-rich histones in chromatin. An immunological comparison of unsequenced lysine-rich histones; i.e. H1^S satellites found in reptiles and amphibians (Rutledge et al, 1981; Brown et al, 1981), using antisera with serological specificity to the conserved globular domain, might illuminate their biological function.

Antisera to histones complexed with nucleic acid and free histones have been raised in both guinea pigs and rabbits (Mura et al, 1980). It is not known whether the serological specificity of these antisera are a consequence of their mode of immunization. Thus an immunological comparison was undertaken using antisera elicited in two different species against both free and complexed chicken and goose H5 to determine the localization of serological reactivity. These characterized antisera were then cross-reacted with lysine-rich histones of known and unknown sequence to establish homologies of structure and therefore function among them.

MATERIALS AND METHODS

All chemicals used, unless otherwise specified, were reagent grade and obtained from either BDH(Toronto), Fisher Scientific(Toronto) or J.T. Baker & Co.(Toronto). All centrifugations were done in a Sorval RC-2 high speed centrifuge equipped with an SS-34 rotor. Optical density measurements were performed on a Beckman Acta C-III spectrophotometer.

Enzyme-linked immunosorbent assay measurements in microtiter plates were read on a Biotek EIA Reader(Mandel Scientific Ltd., Toronto).

I. Preparation of Histones

A. Preparation of Chicken Erythrocyte Nuclei

Blood was obtained from adult White Leghorn chickens (McEwen's Poultry Farm, North Gower, Ont.) by either heart puncture or severance of the jugular vein under local anaesthetic. To prevent coagulation during the collection of blood, 3.8%(w/v) sodium citrate was added to give a final concentration of 0.38%. The blood was immediately

chilled and all further operations were carried out at 4°C. The red blood cells were pelleted by centrifugation at 500g for 10 min. Serum and white cells were removed by aspiration, and after two successive washes in 0.15M NaCl, the pelleted cells were resuspended to half the original blood volume in 0.15M NaCl. The cells were lysed by the addition of one-twentieth volume of fresh 5% saponin in 0.01M sodium phosphate, pH 6.8, and subsequent stirring on ice for 15 min. Nuclei were then pelleted at 12,000g for 15 min and major cytoplasmic contaminants (mostly heme and unlysed red blood cells) were removed by approximately three successive washes in 0.15M NaCl at 12,000g for 10 min until a clean white nuclear pellet remained.

B. Preparation of Xenopus Erythrocyte Nuclei

Adult Xenopus laevis (Boreal Laboratories, Mississauga, Ont.) were anaesthetized for one-half hour in 0.2% MS222 (Sigma Chemical Co., St. Louis, Mo.), rinsed in distilled water to remove excess MS222 and blotted dry.

An I-shaped ventral midline incision was made to expose the body cavity, which was rinsed with citrate-saline (0.15M NaCl containing 1.14% sodium citrate and the protease inhibitor, 1mM PMSF (Sigma Chemical Co.; from a stock 50mM solution in isopropanol)). The blood was collected with a pasteur pipette from the

severed hepatic portal vein and abdominal cavity, mixed with one-tenth volume citrate-saline-PMSF solution and centrifuged at 500g for 10 min. The serum and white cells were removed by aspiration and after two or three washes (until the supernatant was clear) in saline-PMSF, the pelleted red blood cells were resuspended to one-half the original blood volume in saline-PMSF.

The cells were lysed by the addition of one-one hundredth volume of fresh 5% saponin in 0.01M sodium phosphate, pH 6.8, and subsequent stirring on ice for 7-10 min. Nuclei were then pelleted at 12,000g for 10 min. Major cytoplasmic contaminants were removed by approximately 3 centrifugal washes in saline-PMSF at 12,000g for 10 min as before.

C. Histone Extraction

Clean nuclear pellets were resuspended in 0.01N HCl to one-half the original blood volume. The lysine-rich histones were selectively extracted by homogenization in an equal volume of 10% PCA(w/v), followed by incubation on ice with stirring for one-half hour.

After centrifugation at 27,000g for 10 min the pellets were resuspended in 5% PCA, 0.005N HCl, re-extracted as above for one-half hour and pelleted. The supernatant extracts, containing the lysine-rich histones, were

pooled. The core histones were solubilized from the residual PCA-extracted nuclear pellet by three successive homogenizations and centrifugations(27,000g for 10 min) in one-quarter the original blood volume of 0.25N HCl. The supernatant extracts containing the core histones were pooled. All homogenizations were done using a glass dounce homogenizer(Kontes Glass Co., N.J.) with a loose-fitting A-type pestle.

The solubilized lysine-rich histones were recovered from the pooled PCA supernatants by precipitation with 20% TCA, added as one-third volume of 80% TCA, followed by a room temperature incubation of 30-60 min and centrifugation at 27,000g for 10 min. Protein pellets were washed once in acidified acetone(1 ml conc.HCl in 500 ml acetone), repelleted(27,000g for 10 min), washed once more in acetone, repelleted, and air dried.

The solubilized core histones were recovered from the pooled HCl-supernatants by precipitation in 9 volumes of acetone o/n at -20°C. Protein was pelleted(27,000g for 10 min) and air dried. All histones were lyophilized from distilled water and stored in air-tight vials at -20°C.

D. Fractionation of Histone Extracts by Exclusion Chromatography

PCA-extracts(1-50 mg), dissolved in 0.5 ml of 8M urea

containing 5% (v/v) β -mercaptoethanol were applied to a 2.5 x 100 cm glass column containing either Bio-Gel P-60, or P-150 (Bio-Rad Laboratories Ltd., Mississauga, Ont). Proteins were eluted from the column with 0.01N HCl at a flow rate of 20 ml/h. Two-ml or 5-ml fractions were collected and monitored for protein content by measurement of the absorbance at 230 nm. Pooled fractions were either dialyzed against 3 changes of distilled water, frozen and lyophilized, or protein was precipitated with 20% TCA (added as 80% TCA). The precipitate was collected by centrifugation (27,000g for 10 min), washed once in acidified acetone, pelleted, washed again in acetone, air-dried and lyophilized from solution in distilled water.

Goose erythrocyte H5, prepared by differential pH extraction of fractionated nuclei (Seligy et al, 1976), was a generous gift of Dr. V.L. Seligy, National Research Council, Ottawa, Ont.

E. Preparation of Globular Domain Peptides

The tryptic digest limit peptides equivalent to the mid-globular domain from chicken H5 and H1 and from goose H5 were prepared following the method of Aviles et al, 1978. Histones (20 mg/ml) were dissolved in 0.2M K_2SO_4 , 50mM Tris-HCl buffer, pH 8.0. Trypsin (Sigma Chemical Co.)

was added at an enzyme to substrate ratio of either 1:100 or 1:1000 and the digestion was carried out at room temperature. Aliquots were taken at intervals up to 180 min, the reaction was quenched with 0.02% CTAH (Sigma Chemical Co.) and the pH of the reaction mixture was adjusted to 3.0 with HCl. The limit peptides were recovered from the reaction mixture by precipitation in 20% TCA (added as 80% TCA) and subsequent acid-acetone, acetone washes as described for histone extraction. The limit peptides were lyophilized from distilled water and stored in air-tight vials at -20°C .

F. Polyacrylamide Gel Electrophoresis

The heterogeneity of protein extracts was characterized using SDS polyacrylamide gel electrophoresis according to the method of Laemmli (1970). Slab gels (0.07 x 20 x 12 cm) were cast in a plexi-glass gel apparatus built by the Science Technology Centre, Carleton University, Ottawa, Ont. Separating gels contained 0.1% SDS, 1mg/ml fresh ammonium persulfate, 0.4M Tris, 0.5µl/ml TEMED, pH 8.8, and either 12.5% or 15% acrylamide, prepared from a stock solution of 30% w/v acrylamide (Bio-Rad Laboratories Ltd.) and 0.8% w/v n,n'-bis-methylene acrylamide (Eastman Kodak, Rochester, N.Y.).

The stacking gel consisted of 7.5% acrylamide, 0.1% SDS, 1 mg/ml ammonium persulfate, 1 μ l/ml TEMED and 0.12M Tris, pH 6.8. The electrode buffer (pH 8.3) contained 0.025M Tris, 0.2M glycine and 0.1% SDS and the samples were dissolved in 0.062M Tris (pH 6.8), 10% glycerol, 2.5% SDS, and 2.5% β -mercaptoethanol at a concentration of 2 mg/ml. Samples were heated to 100°C for one minute before loading approximately 2 μ g/well of the slab from a 10 μ l Hamilton syringe (Chromatographic Specialities Ltd, Brockville, Ont.) Electrophoresis was conducted at 0-5°C at a constant voltage of 350V until the leading edge of the tracking dye (0.1% bromophenol blue) reached the bottom of the gel. Gels were stained o/n in 0.25% Coomassie Blue R250 (Sigma Chemical Co.), 40% ethanol and 5% acetic acid. The gels were destained in successive washes of 20% ethanol and 5% acetic acid. Gels were photographed using Technical Pan film 2415 (Eastman Kodak Co.) and a red, No. 25(A) filter (Vivitar Can. Ltd.)

G. Amino Acid Analysis

Approximately 0.5-1.0 mg of purified histone or peptide was hydrolyzed in 0.5 ml of 6N HCl in vacuo for 22 h at 110°C. After drying the hydrolysates under vacuum, the amino acid mixtures were separated and quantitated using a Beckman 119BL Amino Acid Analyser (Beckman

Instruments, Palo Alto, Ca.) equipped with a peak integrator(Beckman model 126).

H. N-Terminal Determination

The N-terminal amino acid of the globular domain peptides as well as other control proteins such as intact chicken H5, a chicken core histone mixture, and lysozyme were determined by a modification of Hartley's dansylation procedure(Hartley, 1970). Approximately 1 nmol of peptide or protein in 100 μ l of 0.2M NaHCO_3 and 0.1% SDS was briefly heated(1 min) before addition into a 10 x 100mm glass tube. After evaporation, 100 μ l of a 1:1 mixture of DNS-Cl(Sigma Chemical Co.) in acetone(2.5 mg/ml) and water was added to the tube which was then sealed and incubated at 45°C for 30 min. The reaction mixture, was evaporated to dryness under N_2 before the addition of 20 μ l of 6N HCl for hydrolysis at 110°C for 4 h. The tube was opened and the HCl evaporated. The sample was taken up in either 5 μ l of acidified acetone or 95% ethanol and 2 μ l were spotted on each side of a double-sided 5cm x 5cm polyamide layer plate(BDH Chemicals, Toronto, Ont.) about 1 cm from the corner. One μ l of marker DNS-amino acids 1mg/ml each(Sigma Chemical Co.) was applied to one side of the plate. Chromatography proceeded in the first dimension for 10 min in solvent 1 (1.5% formic acid(v/v))

in H_2O), and for 15 min in the second dimension in solvent 2 (toluene:acetic acid; 10:1(v/v)), than for 15 min in the second dimension in solvent 3 (ethyl acetate:methanol:acetic acid; 20:1:1(v/v)). The plate was dried thoroughly and examined under long uv light between each solvent run. If a fourth solvent was needed to resolve arginine from ϵ -lysine and serine, the plate was run in 1% NH_4OH in H_2O for 10 min.

I. Protein Concentration Determination

Histone protein concentrations were measured by TCA turbidity (Rasmussen et al, 1962). One hundred μl of histone sample were added to 0.5ml of 21.8% TCA (to make a 1.1M TCA solution). Approximately 15 min after the addition of TCA, the optical density was measured at 400nm and compared to a standard curve prepared from a mixture of the core histones over the range of 25-200 $\mu g/ml$.

II. Preparation of Antibodies

A. Preparation of Guinea Pig Antisera

Purified lyophilized histone (either chicken H5 or goose H5) was dissolved in sterile isotonic saline (0.15M NaCl) to make a 100 µg/ml solution; when the protocol dictated RNA in the immunogen, an equal weight of yeast RNA (Sigma Chem. Co.) was added to this solution to form an insoluble complex. Immunogen solution or suspension was emulsified with one part complete Freund's adjuvant containing 10 mg of Mycobacterium tuberculosis H37 RA/ml (Bacto, Difco Laboratories, Detroit, Mich.).

Groups of six randomly-bred Hartley strain female guinea pigs (Canadian Breeding Farms, P.Q.) were immunized with each antigen regimen; i.e. chicken H5 + RNA, goose H5 and goose H5 + RNA. Antisera elicited against CH5 alone in both guinea pig and rabbit were a gift of C.V. Mura and were prepared as described here. Each guinea pig received 0.1 ml intradermally, into two sites on the back for a total dose of 20 µg of histone. Booster injections consisting of 10 µg histone in sterile isotonic saline, emulsified in incomplete Freund's adjuvant, were administered subcutaneously in five sites on days 8, 15 and 22. The ether-anaesthetized animals were bled per

cardium on the 30th and 39th day after primary immunization. Early bleeds used for the detection of antibody production were pooled from the six guinea pigs (total ~2ml). Serum was prepared by allowing the blood to thoroughly clot at room temperature for 1-2 h and then allowing the clot to contract o/n at 4°C. The serum was pipetted off and the residual red cells removed by centrifugation (480g for 10 min). Sodium azide was added as a preservative at a final concentration of 0.1% and the serum was stored at -20°C.

The same guinea pigs were re-immunized nine months later, starting with an initial injection of 20 µg histone per guinea pig in complete adjuvant followed by a booster of 10 µg histone on day 8, a test bleed for quantitation of antibody titer on day 15 and exsanguination on day 22. The serum (approximately 10 ml/guinea pig, prepared as above) from this final bleed was not pooled from individual guinea pigs.

B. Preparation of Rabbit Antisera

Purified lyophilized histone (chicken H5 or goose H5) was dissolved in sterile isotonic saline at a concentration of 100 µg/ml; when RNA was included, 0.5 mg was added to the histone solution to form an insoluble complex. Both immunogens were emulsified with one part

complete Freund's adjuvant. Groups of two white New Zealand female rabbits, six to eight weeks old (Double J Rabbit Ranch, Smiths Falls, Ont.) were immunized with each antigen regimen; i.e. chicken H5 + RNA, goose H5 and goose H5 + RNA. Each rabbit received a total dose of 2 ml containing 100 μ g histone intradermally, spread over numerous sites on the rabbit's back. Booster injections consisting of 100 μ g histone in sterile isotonic saline emulsified in incomplete Freund's adjuvant were administered subcutaneously or intramuscularly on days 8, 15 and 22. A blood sample was taken from the ear vein on day 30 to detect antibody production.

The six rabbits were re-immunized in 7 months' time starting with an initial subcutaneous injection of 100 μ g histone in incomplete adjuvant per rabbit, followed by a test bleed on day 8. This test bleed indicated that the antibody titer was low so the rabbits were again re-immunized 2 months later starting with an initial subcutaneous injection of 100 μ g histone in complete adjuvant followed by one subcutaneous booster of 100 μ g histone in incomplete adjuvant on day 8 and a test bleed on day 18.

The ether-anaesthetized rabbits were completely exsanguinated per cardium on day 31. The rabbit serum was prepared and stored identically to the guinea pig serum but in no case were the serums pooled among rabbits. Test bleeds resulted in approximately 2 ml of serum whereas the

final bleed produced approximately 25 ml of serum/rabbit.

C. Purification of Antibodies by Affinity Chromatography

Two immunoadsorbent affinity columns (prepared by Peter Foster in this laboratory) linked purified chicken H5 and H1 to CNBr-activated Sepharose 4B (Pharmacia Chemicals, Uppsala, Sweden), following the method outlined by Cuatrecasas and Anfinsen (1971). A protein A-agarose affinity column (Bethesda Research Laboratories, Gaithersburg, M.D.) was also used to purify IgG. Approximately 5 ml of serum were added to the Sepharose 4B-antigen complexed resin (25 ml in 0.1M BSB buffer, pH 8.3), stirred at 37°C for 1 h and 4°C o/n. The antibody-coupled affinity resin was then poured into a glass column (1 X 30 cm) and washed extensively with BSB until $A_{280} < 0.02$. This eluant was saved when the antiserum was heterologous to the bound antigen. Bound antibody was eluted with 4N Guanidinium-HCl, pH 8.0, and the protein concentration in the eluant was monitored at 280nm. Pooled antibody fractions were dialyzed against 0.01M PBS (0.15M), pH 7.5 and concentrated by ultrafiltration using a Millipore Immersible-CX Molecular Separator Kit (Millipore Corp., Bedford, MA.).

III. Assay of Antibodies

A. Complement Fixation

The immunochemical technique of microcomplement fixation(Stollar, 1978) was used to detect, characterize and quantitate the antibodies produced in this study. Complement, a set of serum proteins, recognizes and tightly binds to antibody-antigen complexes. If the complex is sheep red blood cells covered with antibodies(hemolysin) to cell-surface antigens, the complement will bind and lyse the red blood cell, thus releasing hemoglobin which is detectable spectrophotometrically.

If, however, the antigen-antibody complex is unknown histone-antihistone, some complement becomes "fixed" and is unable to lyse a standardized amount of hemolysin-sensitized red blood cells. Thus the amount of hemoglobin detected is inversely related to the amount of complement fixation occurring in the reaction.

i) Preparation of Standardized Components

The diluent for microcomplement fixation was freshly

prepared from 10X concentrated stock solution containing 1.4M NaCl, 0.1M Tris-HCl, 1.5mM CaCl₂ and 5mM MgCl₂, pH 7.4. This diluent or "Isotris-M" buffer was used to wash the erythrocytes, to dilute antisera and antigens, and in the complement fixation test itself. "Isotris-BSA" buffer(Isotris-M + 0.1% BSA(Sigma Chemical Co.)) or "Isotris-gel" buffer(Isotris-M + 0.1% gelatin(Bacto-Difco)) was used for the final suspension and dilution of the erythrocytes in the "sensitized" erythrocyte preparation and for the dilution of hemolysin and complement.

ii) Standardization of Sheep Erythrocyte Suspension

Cells(1.5-2.0 ml) were aseptically withdrawn from a commercially available sheep blood preparation (50% in Alsever's solution; Qualicum Scientific Ltd, Ottawa, Ont.) and centrifuged(600g for 10 min). The plasma supernatant was discarded and the pellet of red blood cells was washed several times in 10 volumes of Isotris-M until the supernatant was clear. An approximate 5% suspension of cells was prepared by the addition of 6-7 ml of Isotris-BSA. Exactly 0.5 ml of this cell suspension was added to exactly 7.0 ml of 0.1% Na₂CO₃, mixed well and the absorbance of the lysate was read at 541nm. The required cell concentration of 10⁹ cells/ml had an absorbance of

0.680 O.D. The cells were diluted to this value using the relation:

$$v_f = \frac{v_i \times A}{0.680}$$

where v_f = final volume; v_i = initial volume; A = absorbance of 5% suspension. This standard cell suspension was prepared fresh daily.

iii) Sensitization of Erythrocytes

Hemolysin(Bacto Antisheep Hemolysin, glycerinated; Difco, Detroit, Mich.) at its titered dilution(1:400) was slowly added to 0.5 ml of standardized cell suspension with constant swirling. After incubation at 37°C for 15 min the "sensitized" erythrocyte suspension was placed on ice and diluted with 90 ml of cold Isotris-BSA to give a final concentration of 5×10^7 cells/ml.

iv) Complement Titration

Initial experiments using "Bacto" lyophilized complement from guinea pig serum purchased from Difco gave erratic results; thus we began using normal fasted guinea

pig serum as a source of complement. This complement was titrated by adding to tubes in an ice bath 1 ml Isotris-BSA and 0.2 ml complement diluted successively from 1:10 to 1:300 from a 1:10 diluted stock of normal guinea pig serum that had been stored frozen at -20°C in 1-2ml aliquots. The tubes were incubated 16-18 h at 4°C before the addition of 0.2 ml sensitized erythrocyte suspension. After incubation at 37°C with occasional swirling for 60 min, the tubes were centrifuged (600g for 10 min) and the absorbance of the supernatants was read at 413nm. The dilution of complement that gave 80-90% hemolysis was used in the complement fixation test.

v) Titration of Hemolysin

Commercial hemolysin (Difco) was diluted with Isotris-BSA over the range 1:200 to 1:3200 before addition (1:1) to the standardized erythrocyte suspension. After incubation at 37°C for 30 min with occasional swirling, the "sensitized" erythrocytes were placed on ice and diluted as described in sensitization of erythrocytes (Methods III, A (iii)). A series of tubes were prepared in duplicate containing 1 ml Isotris-M and 0.2 ml complement diluted to give 90% hemolysis. After incubation for 16-18 h, 0.2 ml of the sensitized erythrocyte suspension were added and the tubes were

further incubated at 37°C for 60 min with occasional swirling. The unlysed red blood cells were pelleted by centrifugation (600g for 10 min) and the absorbance of the supernatants was recorded at 413 nm. The optimum dilution of hemolysin was taken as the point where the curve of percentage hemolysis versus dilution hemolysin just reaches a plateau.

vi) Complement Fixation Test

All sera to be assayed were first incubated at 60°C for 15 min in order to destroy their own complement systems. The concentrations of serum and antigen were found empirically. The antigen concentrations were chosen to give a complete complement fixation curve with zones of antibody excess, equivalence and antigen excess. Each complement fixation experiment was set up in duplicate in a series of disposable glass culture tubes (10 x 75 mm) containing antibody, antigen (serial dilutions), complement and buffer as outlined in Table I.

TABLE I: Experimental Protocol for Complement Fixation Test

	Tube Number										
	1	2	3	4	5	6	7	8	9	10	11
Diluted Serum (ml)	0.2	0.2	0.2	0.2	0.2	0.2	0.2	-	-	-	-
Diluent(ml) Isotris-M	0.6	0.6	0.6	0.6	0.6	0.6	0.8	0.8	1.0	0.8	1.2
Complement(ml)	0.2	0.2	0.2	0.2	0.2	0.2	0.2	0.2	0.2	0.4	-
Antigen(ml)	0.2	0.2	0.2	0.2	0.2	0.2	-	0.2	-	-	-
Antigen(μ g/ml)	0.31	0.63	1.25	2.5	5.0	10.0	-	10.0	-	-	-

Figure 3: SDS-polyacrylamide gel electrophoresis of a time course digestion of chicken H5 by trypsin(1:1000).

Samples were taken at 0, 2, 10, 40, 60, 90, 120, 150, 180 min(Lanes 2, 4, 5, 6, 7, 8, 9, 10, 11 respectively). After inhibition of tryptic activity with CTAH, the pH was lowered to 3.0 with HCl and samples were loaded onto the gel. Intact ChH5(lane 1) was rapidly digested to two limit peptides, TP and CP, by 90 min digestion time(Lane 8). The larger peptide (LGH5) present from 10-60 min(Lanes 5, 6, 7) is supposedly peptide 22-102(Aviles et al, 1978).

B. Inhibition of Complement Fixation

For complement to be fixed by antigen-antibody complexes, the bivalent antibody must react with multivalent antigens to form a "lattice-work" structure. Monovalent fragments(haptens) react with the antibody without fixing complement. If the conditions of antigen and antibody concentration are chosen so that all antibody is saturated with antigen and all the complement is fixed, and monovalent haptens are added to the experimental tubes, a reduction in complement fixation indicates that hapten is competing with the intact antigen for antibody binding sites and therefore shares a common determinant with the intact antigen. This technique of inhibition of complement fixation was used to characterize the antigenicity of the globular domain peptides. Various concentrations of whole antigen or fragments were incubated for 15 min at 37°C with anti-histone serum which had been diluted to a titer that gave 80-90% maximum complement fixation in inhibitor-free controls. A series of H5 concentrations were then added and the complement fixation test performed at 4°C as described above.

C. Enzyme-Linked Immunosorbent Assay

i) Direct ELISA

The technique of enzyme-linked immunosorbent assay (ELISA) as first described by Engvall and Perlmann in 1971 was adapted in conjunction with Peter Foster for use with our histone-anti-histone system. This four step assay involves the adsorption of antigen to a solid phase; the addition of antibody in a liquid phase (diluted serum) which combines with bound antigen; the addition of a secondary antibody-enzyme conjugate specific for the immunoglobulins of the first antibody and the detection of the antigen-antibody-conjugate complex by the addition of the conjugated enzyme's substrate. The amount of coloured product measured spectrophotometrically is directly proportional to the number of primary antigen-antibody complexes in the reaction.

For the direct measurement of antigen-antibody interaction, wells of flat-bottom, polystyrene EIA microtiter plates (Flow Labs, Mississauga, Ont.) were coated at 37°C for 1-2 h with 100 μ l (1.0 μ g/ml) of purified histone in 0.1M Na_2CO_3 , pH 9.6. Plates were then rinsed three times with PBS (0.15M NaCl, 0.01M phosphate buffer, 0.01% sodium azide, pH 7.2) containing 0.05% Triton-X 100. Any remaining protein binding sites on the plastic were

blocked with an incubation of PBS + 1% BSA(100 μ l) for 15 min at room temperature. Five-fold dilutions of anti-histone sera in PBS + 1% BSA(100 μ l) were incubated in the coated wells at room temperature, initially o/n, then later from 1-2 h. After rinsing the plates three times with PBS + Triton, one of the enzyme conjugates(100 μ l) protein-A-alkaline phosphatase or anti-IgG, IgM-peroxidase (Flow Labs Ltd.) was added for a room temp. incubation of 1-2 h. After rinsing 3X with PBS + BSA, the bound enzyme conjugate was detected by adding 250 μ l of p-nitrophenyl phosphate(Sigma Chemical Co.) at a concentration of 1mg/ml in 0.1M diethanolamine buffer (0.1M diethanolamine, 0.1M $\text{MgCl}_2 \cdot 6\text{H}_2\text{O}$, pH 9.8). Hydrolysis usually took place o/n at 37°C and the absorbance at 405 nm was recorded. The bound peroxidase was detected by the addition of 100 μ l of 1mg/ml ABTS(in 0.1M citrate phosphate buffer, pH 4.0 + 0.003% H_2O_2).

ii) Competitive or Indirect ELISA

If heterologous antigens or haptens are first allowed to react with the primary antibody before its addition to the homologous antigen bound in the microtiter plate well, then the inhibition of antibody binding by the competing heterologous antigen will be reflected in a decreased response by the substrate, and thus a reduction in the

optical density measurement.

The competitive ELISA involved coating the wells of the microtiter plates with 100 μ l of 0.5 μ g/ml purified histone, incubation of the five-fold dilutions of anti-histone serum at room temperature for 1-2 h with 10 μ g/ml intact heterologous or homologous histone or 5 μ g/ml globular domain peptide as inhibitors, before addition to the coated plates. All other procedures were identical to the direct ELISA method.

RESULTS AND DISCUSSION

I. Preparation and Separation of Histone Fractions

A. Intact Histones

PCA-extracts of lysine-rich histones and HCl-extracts of core histones were prepared from fractionated nuclei as described in Methods(I) and analyzed by polyacrylamide gel electrophoresis to check for cross-contamination or protein degradation. To obtain purified, fractionated lysine-rich histones clean PCA-extracts were applied to gel filtration columns. Chicken erythrocyte lysine-rich histones were fractionated on Bio-gel P-60(Fig. 1A), whereas Xenopus erythrocyte lysine-rich histones were fractionated on Bio-gel P-150(Fig. 1B). The purity of pooled chromatographic fractions was ascertained by polyacrylamide gel electrophoresis(Fig. 2A & 2C) and amino acid analysis(Table II). The purity of goose H5 was also determined by polyacrylamide gel electrophoresis(Fig. 2B, lane 4). Core histones were not fractionated by column chromatography and were used as a mixture in subsequent control experiments.

TABLE II: Summary of Amino Acid Compositions of
Chicken H1 and H5, Xenopus H1 and H1^S

Amino Acids	Moles per 100 mol of recovered amino acids ^(a)			
	Chicken H1	Chicken H5	Xenopus ^(b) H1	Xenopus H1 ^S
Aspartate	1.7	1.8	2.9	3.8
Threonine	3.8	2.7	3.4	3.5
Serine	6.2	11.1	8.0	9.6
Glutamate	3.3	4.0	5.2	6.5
Proline	11.1	7.8	9.5	8.5
Glycine	6.2	4.9	4.3	5.1
Alanine	27.3	16.3	25.1	17.3
Valine	5.0	4.3	5.0	5.9
Methionine	0.0	0.4	0.0	0.3
Isoleucine	0.9	2.9	0.3	2.3
Leucine	4.6	4.3	5.5	4.6
Tyrosine	0.3	1.5	0.6	1.3
Phenylalanine	0.5	0.5	0.5	0.7
Histidine	0.2	1.5	0.1	0.7
Lysine	27.1	23.5	28.7	26.9
Arginine	1.9	11.1	1.0	2.8
Lysine/Arginine	14.3	2.1	28.7	9.6

(a) Corrected for 10% Serine and 5% Threonine hydrolytic losses.

(b) Reported in Brown et al, 1981.

Figure 1: Lysine-rich histone fractionation by exclusion chromatography of PCA-extracts of chicken and Xenopus erythrocyte nuclei.

Fig. 1A: Gel filtration of chicken PCA-extract (65 mg) on Bio-Gel P-60. Proteins were eluted with 0.01 N HCl and collected in 5 ml fractions. Fractions in peaks A & B were pooled, recovered by TCA precipitation and lyophilized.

Fig. 1B: Gel filtration of Xenopus PCA-extract (4 mg) on Bio-Gel P-150. Proteins were eluted in 0.01N HCl and collected in 2.5 ml fractions. Fractions in peaks A & B were pooled, recovered by TCA precipitation and lyophilized.

Figure 2: SDS-polyacrylamide gel electrophoresis of the lysine-rich histones of chicken, goose and Xenopus erythrocytes.

Fig. 2A: Chicken H1, peak A(lane 1) and chicken H5, peak B(lane 2) from exclusion chromatography on P-60(Fig. 1A).

Fig. 2B: The purity of goose H5(lane 3) was checked with chicken H5(lanes 1, 2) for comparison.

Fig. 2C: Xenopus H1, peak A(lanes 2, 3) and Xenopus H1, peak B(lanes 4, 5) from exclusion chromatography of Xenopus PCA-extract(lane 1) on Bio-Gel P-150(Fig. 1B).

A
1 2

B
1 2 3

C
1 2 3 4 5

B. Globular Domain Peptides

It is known that at high ionic strength lysine-rich histones form a maximum amount of secondary and tertiary structure; i.e. a compacted middle domain with disordered N- and C-terminal arms (Crane-Robinson et al, 1976). Digestion of such material with trypsin at high pH produces a limit peptide equivalent to this middle domain, which retains all the helicity and features of tertiary folding that are observed in the intact H5 molecule (Aviles et al, 1978). This is the most highly conserved portion of these proteins (Yaguchi et al, 1979). The preparation of this peptide was considered essential to this project in order to assess the serologically reactive components of my antisera, since previous studies in this laboratory (Mura et al, 1980) had revealed that the strongest determinant activity of chicken H5 resided in the globular or middle domain, particularly at residues 59-65 and 94-99.

Results of polyacrylamide gel electrophoretic analysis of a time-course digestion experiment in the preparation of the limit tryptic peptide (TP) from chicken H5 (Fig. 3) were similar to published results (Aviles et al, 1978). Many large histone fragments appeared very early during digestion as the disordered N- and C-terminal arms were rapidly cleaved. By 90 min digestion time only the

Figure 3: SDS-polyacrylamide gel electrophoresis of a time course digestion of chicken H5 by trypsin(1:1000).

Samples were taken at 0, 2, 10, 40, 60, 90, 120, 150, 180 min(Lanes 2, 4, 5, 6, 7, 8, 9, 10, 11 respectively). After inhibition of tryptic activity with CTAH, the pH was lowered to 3.0 with HCl and samples were loaded onto the gel. Intact ChH5(lane 1) was rapidly digested to two limit peptides, TP and CP, by 90 min digestion time(Lane 8). The larger peptide (LGH5) present from 10-60 min(Lanes 5, 6, 7) is supposedly peptide 22-102(Aviles et al, 1978).

1 2 3 4 5 6 7 8 9 10 11

H5

LGH5-
TP-
CP-

0 2 10 40 60 90 120 150 180

S

limit peptide and what has been called a "chymotryptic degradation product"(CP; Aviles et al, 1978) were visible in the gel(Fig. 3, lane 8). The large amount of degradation of H5 at the initial sampling time(0) was due to the slow action of the inhibitor CTAH on the trypsin(Fig. 3, Lane 2). There is no evidence of a trypsin band because the level present was below the detection limit of Coomassie Blue dye.

Attempts to separate TP from CP by exclusion chromatography on Bio-gel P-30 were unsuccessful, because all protein eluted at the void volume(Results not shown). Other workers have purified the globular domain peptide by exclusion chromatography using Sephadex(Aviles et al, 1978; Frado et al, 1984).

TP was successfully recovered from peptide mixtures by precipitation in 20% TCA, but CP also precipitated(Fig. 4A, Lane 11). Amino acid analysis, performed on this TCA-precipitated digest(Table III), agreed well with published results(Aviles et al, 1978) despite contamination with CP.

Because pure globular domain peptide was desirable for valid antibody work, attempts were made to avoid production of CP. Two time-course analyses of trypsin digestion of chicken H5 in the presence of the chymotryptic inhibitors DPCC and TPCK(Schoellman & Shaw, 1963) are shown in Fig. 4A & B, respectively. CP is evidently not a chymotryptic degradation product because

TABLE III: Summary of Amino Acid Compositions of Globular Domain Peptides of Chicken H5

Amino Acids	Mole Percent (a)			Number of Residues		
	Published Experimental TP(b)	TP	CP(c)	Intact Chicken H5	TP(d)	TP(e)
Aspartate	3.8	2.9	5.5	3	3	2
Threonine	2.4	2.1	2.9	6	2	2
Serine	14.9	13.7	10.6	26	12	11
Glutamate	7.0	8.4	9.4	8	6	2
Proline	1.6	2.6	2.6	13	1	2
Glycine	9.1	7.2	7.6	9	7	6
Alanine	13.8	12.7	12.3	29	10	12/13
Valine	4.0	3.8	4.4	8	3	3
Methionine	1.2	1.2	0.5	1	1	1
Isoleucine	6.8	7.7	8.9	6	6	6
Leucine	7.5	7.7	8.1	8	6	6
Tyrosine	3.6	3.7	3.9	3	3	3
Phenylalanine	1.6	1.0	1.0	1	1	1
Histidine	3.8	3.1	4.3	3	3	3
Lysine	11.8	12.0	11.0	44	9	10
Arginine	7.1	7.9	7.0	21	6	7
Molecular Weight	8,750	10,600(f)	9,770(f)	20,580	8,592	9,043
Total Number of Residues				189	79	82

(a) Corrected for 10% serine and 5% threonine hydrolytic losses.

(b) TP-tryptic peptide; composition from Aviles et al, 1978.

(c) CP-chymotryptic peptide

(d) From published sequence of Briand et al, 1980; residues 22-100.

(e) Experimental results normalized to 3 residues for tyrosine.

(f) Calculated from Figure 5.

Figure 4: SDS-polyacrylamide gel electrophoresis of time course digestions of chicken H5 (Fig. 4A, lanes 12, 14) in the presence of trypsin(1:100) and DPCC (Fig. 4A) or trypsin(1:1000) and TPCK (Fig. 4B).

The chymotryptic degradation product(CP) as well as the globular domain peptide(TP) persisted. The larger peptide(LGH5) persisted for 10 min in Fig. 4A(Lane 4) and for 60 min in Fig. 4B(Lanes 3, 4). Previous preparations of TP and CP were used as standards(Fig. 4A, Lanes 11, 13).

A
 1 2 3 4 5 6 7 8 9 10 11 12 13 14 - H5
 B
 1 2 3 4 5

it persists in all experiments. When chicken H5 was digested with DPCC-treated trypsin at 1:100 (Fig. 4A), the globular domain peptide diminishes as the CP band increases. This was not due to the higher concentration of trypsin used, because when DPCC-treated trypsin was used at 1:1000 for 90 min little degradation took place (Results not shown).

The fractions enriched in CP from Fig. 4A, Lanes 8, 9 & 10, were pooled and after recovery by TCA precipitation, the protein was subjected to amino acid analysis (Table III, Experimental CP). Amino acid compositions for TP and CP did not differ substantially. If the value for the tyrosine residues is normalized to three and the other amino acid values adjusted accordingly, the results indicate that the amino acid composition for the globular domain peptide makes 8 perfect matches to the number of residues present in the globular domain (22-100) from the known sequence of chicken H5 (Briand et al, 1980) and 7 matches that differ by only ± 1 residue. CP, again normalizing the amino acid composition to 3 tyrosines, results in 9 perfect matches to the globular domain sequence and 6 that differ by ± 1 residue. More importantly, the major amino acids that distinguish the globular domain; i.e. 1 methionine, 1 phenylalanine, 3 histidines and 3 tyrosines, are common to the two bands. Therefore it was concluded that TP and CP are peptides of different lengths, both containing the conserved globular

domain of chicken H5.

To allow further characterization of these two peptides, N-terminal determinations were performed. As controls, a mixture of the four core histones, reacted in molar equivalents to the lysine-rich peptides, produced DNS-alanine and DNS-proline for N-termini, lysozyme gave di-DNS-lysine and chicken H5, which has an acetylated N-terminus produced a negative result. The limit tryptic peptide, equivalent to the globular domain of H5, and CP both gave DNS-serine for an N-terminus. Therefore, it appears that the tryptic cleavage point is well-defined on the N-terminal side of the globular domain at arg[↓]-ser²² but is not so specific on the C-terminal side, so that a mixture of peptide chains migrates as two bands in an SDS gel. The second band has nothing to do with chymotryptic contamination of the trypsin preparation but is simply a shorter version of the globular domain peptide.

Aviles et al (1978) also obtained the same result when they characterized GH5 and LGH5, a peptide longer at the C-terminal end, allegedly stretching from residues 22-102. The band equivalent to their LGH5 is visible in Fig. 3, Lanes 4, 5 & 6; Fig. 4A, Lane 4; Fig. 4B, Lanes 3, 4. Puigdomenech et al (1980) suggest that the relative proportions of LG01 and G01, equivalent globular peptides derived from sea urchin sperm H1, can be altered by changing the pH of the trypsin digestion. They found that equal amounts of both peptides result at pH 7.4 whereas

pure G ϕ 1 production is favoured from tryptic activity at pH 8.0. Giancotti et al(1981) added 1.5M NaCl to the digestion mixture to ensure complete folding of their sperm testis H1 molecules. Thus it seems possible that in a mixture of H5 molecules, the tryptic cleavage site at the globular domain boundary of the N-terminal arm is always accessible and the same, whereas the tryptic cleavage site at the C-terminal side may vary depending on the folded state of the whole molecule, which is a function of both pH and ionic strength. Aggregation of chicken H5 molecules is probably not a factor because it is known that lysine-rich histones and their fragments do not show a tendency to aggregate in solution(Bradbury et al, 1975).

The tryptic digestion-resistant globular domains were also prepared from purified chicken H1(Fig. 5, Lane 7, 8) and goose H5(Fig. 5, Lane 9, 10) using a 1:1000 dilution of trypsin for a digestion time of 90 min followed by TCA precipitation. The molecular weights for all the globular domain peptides were calculated from their mobilities in an SDS gel by using as molecular weight markers(Panyim & Chalkley, 1971) intact goose H5, chicken H5, H1, H3, H2A, H4 and chicken H5 peptide 59-189 (courtesy of Alice Mazen, Institut de Biologie Moléculaire et Cellulaire du Centre National de la Recherche Scientifique, Strasbourg, France), characterized by Garel et al(1975).

A plot of $\log M_r$ against mobility(Fig. 6) yields

Figure 5: SDS-polyacrylamide gel electrophoresis of 90 min digests of chicken H5(lanes 5, 6), chicken H1(lanes 7, 8) and goose H5(lanes 9, 10) using trypsin at 1:1000.

Marker proteins included intact ChH1(lane 1), intact ChH5(lane 2), intact GH5(lane 3), a previous preparation of tryptic digest peptides from ChH5(lane 4) and peptides 32-189(lane 11) and 59-189(lane 12) from chicken H5. The 90 min tryptic digest from all three lysine-rich histones resulted in two peptides; tryptic peptide(TP) and chymotryptic peptide(CP), lanes 4-10 inclusively.

1 2 3 4 5

HL

H5

22-1000

55-1586

TP-

CP-

Figure 6: Estimation of molecular weight by mobility in SDS-polyacrylamide gels.

The log molecular weight of histones of known molecular weights, goose H5 (Δ), chicken H1 ($\blacksquare$), H5 ($\bullet$) and its peptide 59-189 ($\bullet$), H3 ($\circ$), H2B ($\diamond$), H2A ($\blacklozenge$) and H4 ($\square$) was plotted versus mobilities(RF to dye band from Fig. 5). The line of best fit was calculated to be $y = -0.610x + 3.969$ with a correlation coefficient of $r = 0.956$.

molecular weights for the two chicken H5 peptides (TP & CP) of 10,630 and 9770(± 2000). The apparent molecular weights from the amino acid compositions are approximately 9040 and 8230 and the molecular weight for the globular domain(22-100) from the known sequence is 8590. The molecular weights for the peptides derived from goose H5 and chicken H1 did not differ significantly from those for chicken H5, indicating that although goose H5 differs from chicken H5 by 4 amino acids and chicken H1 differs by 28 amino acids the size of the globular domain is conserved and the variability in length occurs in the N- and/or C-terminal arms(Cole, 1977).

Amino acid analysis was performed on the hydrolysates of the globular domain peptides derived from chicken H1 and goose H5 and recovered from the reaction mixture by TCA precipitation. The results are summarized in Table IV for chicken H1 and Table V for goose H5. Again, if the amino acid compositions are normalized to 1 tyrosine residue the results for chicken H1 make 9 matches to the published sequence(Sugarman et al, 1983) and 3 matches that differ by ± 1 residue. Goose H5 gives 4 matches to the published sequence(Yaguchi et al, 1979) with 8 matches that differ by ± 1 residue, when normalized to 3 tyrosine residues.

N-terminal analysis of the globular domain peptide from chicken H1 revealed DNS-alanine, thus pinpointing the cleavage site to be lys -pro[↓] -ala³⁶, because it is known

Table IV: Summary of Amino Acid Compositions of
Globular Domain Peptides of Chicken H1

Amino Acids	Mole Percent ^(a)	Number of Residues		
	Experimental ^(b) TP	Intact Chicken H1	TP ^(c)	TP ^(d)
Aspartate	3.6	4	4	3
Threonine	4.9	12	7	5
Serine	10.5	15	9	9/10
Glutamate	6.6	7	6	6
Proline	4.4	20	2	4
Glycine	9.9	14	10	9
Alanine	15.0	55	10	14
Valine	6.2	9	5	6
Methionine	0.2	0	0	0
Isoleucine	2.8	2	2	2/3
Leucine	11.6	10	10	10/11
Tyrosine	1.2	1	1	1
Phenylalanine	1.1	1	1	1
Histidine	0.2	0	0	0
Lysine	17.8	63	16	16
Arginine	4.3	4	2	4
Molecular Weight	10,630 ^(e)	21,800	9,026	9,223
Total Number of Residues		217	85	90

(a) Corrected for 10% serine and 5% threonine hydrolytic losses.

(b) TP-tryptic peptide of Chicken H1.

(c) From published sequence of Sugarman et al, 1983; residues 36-120.

(d) Experimental results normalized to 1 tyrosine residue.

(e) Calculated from Figure 5.

TABLE V: Summary of Amino Acid Compositions of
Globular Domain Peptides of Goose H5

Amino Acids	Mole Percent ^(a)	Number of Residues		
	Experimental ^(b) TP	Intact Goose H5	TP ^(c)	TP ^(d)
Aspartate	4.2	4	3	4
Threonine	4.7	8	3	5
Serine	9.7	21	8	9
Glutamate	7.7	6	6	7/8
Proline	4.1	18	0	4
Glycine	6.9	10	8	7
Alanine	15.2	33	8	15
Valine	5.0	5	4	5
Methionine	1.4	1	1	1
Isoleucine	5.8	6	5	6
Leucine	7.0	7	6	7
Tyrosine	3.1	3	3	3
Phenylalanine	1.6	1	1	1
Histidine	2.1	3	2	2
Lysine	13.0	45	8	13
Arginine	8.3	22	6	8
Molecular Weight	10,630 ^(e)	20,903	7,835	10,491
Total Number of Residues		193	72	97

(a) Corrected for 10% serine and 5% threonine hydrolytic losses.

(b) TP-tryptic peptide of Goose H5

(c) From published sequence of Yaguchi et al, 1979; residues 29-100.

(d) Experimental results normalized to 3 residues for tyrosine.

(e) Calculated from Figure 5.

that trypsin will cleave C-terminal to pro when lys-pro occur consecutively (Sautiere et al, 1970). Since N-terminal analysis of the globular peptide from goose H5 revealed DNS-alanine and DNS-serine it was deduced that the trypsin was cleaving at lys²²-pro[↓]-ala[↓]-ser²⁵.

Again, although the amino acid composition values for the globular domain peptides did not correspond exactly to published sequence values for the mid-globular domain regions, it was reasoned that, as in the case for chicken H5, the peptides obtained experimentally were derived from the globular region, differing in their C-termini. Although the peptides used in the antibody work to be described were not a homogeneous band on a gel electropherogram, they were composed primarily of peptides from the same area of the protein; i.e. the globular domain.

II. Preparation and Detection of Antibodies

A. Micro-complement Fixation

The central localization of antigenicity in chicken H5 (Mura et al, 1980) may be peculiar to guinea pig antisera or to the absence from the immunogen of nucleic

acid, which may impose structure on the loose N- and C-terminal ends of the histones. Therefore purified chicken H5 and goose H5 were used as antigens, alone or in combination with RNA, to produce antibodies in both guinea pigs and rabbits. Antibody complexes with H5 were detected by microcomplement fixation (Wasserman & Levine, 1961).

If varying amounts of antigen are added to several tubes containing a constant amount of antibody and complement, and the extent of complement fixation is plotted as a function of the amount of antigen added, then the resulting curve is analogous to a quantitative precipitin curve, with zones of antibody excess, equivalence and antigen excess. The curve can be used to quantitate and compare antigens as well as give a relative serum titer if the curves are measured with varying serum dilutions. The titer of an antiserum is defined as the reciprocal of the factor by which the antiserum is diluted in order to give 80% complement fixation with the homologous antigen at the peak of the microcomplement fixation curve. The term homologous is used here in the immunological sense; i.e. the antigen used for immunization rather than in the genetic sense; i.e. referring to proteins that are similar enough in sequence that one can say that the genes coding for them stemmed from the same ancestral gene.

After an initial titration showed that the optimal

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reaction required a concentration of 0.31-10 μ g/ml purified H5, the serum titers at 80% complement fixation were determined for most bleedings and are summarized in Table VI. Figure 7 depicts a sample complement fixation curve for determining serum titer. The height of the complement fixation curve was found to be approximately linearly related to the logarithm of the antiserum concentration. Preimmune serum from both guinea pigs and rabbits showed no complement fixation with either chicken or goose H5, and immune sera showed no cross-reactivity with either core histones or RNA (Results not shown).

It can be seen (Table VI) that potent antibodies (titer > 2000) are raised in guinea pigs in one month's time whereas rabbits produced weak antibodies (titer < 1000) during this time. In fact, it took two booster series after the initial injection schedule to produce antibodies in rabbits as potent as that produced in guinea pigs. The inclusion of RNA in the immunization regimen had no stimulatory effect on antibody production (Stollar, 1978); in fact, the antibody elicited against goose H5 alone in both guinea pigs and rabbits was more potent than that elicited against goose H5 + RNA. A broad range of responses to the immunogen was also noted among individual animals (Prager & Wilson, 1971).

Reciprocal cross-reactions were performed using microcomplement fixation to determine immunological distances in order to easily assess the differences in the

TABLE VI: Summary of Serum Titers from Most Bleeds

Bleed Day	Immunogen		
	CH5 + RNA	GH5	GH5 + RNA
Guinea Pigs:			
Injection 1:			
1 Day 30 ^(a)	nt ^(c)	nt	nt
2 Day 39 ^(a)	2000 ^(d)	3000	2000
Injection 2:			
3 Day 15 ^(a)	2400	3200	800
4 Day 22 ^(b)	nt ^(c)	2000	nt
Rabbits:			
Injection 1:			
5 Day 30 ^(b)	800	200	800
6	2000	50	200
Injection 2:			
7 Day 8 ^(b)	50	400	200
8	100	nt	nt
Injection 3:			
9 Day 18 ^(b)	2000	3600	3200
10	nt ^(c)	1300	3200
11 Day 31 ^(b)	nt ^(c)	nt	nt
12	nt ^(c)	nt	nt

(a) Pooled antisera.

(b) Antisera from individuals.

(c) Some sera were tested for antibody content using ELISA rather than microcomplement fixation.

(d) Titers derived from microcomplement fixation results are averages of replicates performed on the same day.

Figure 7: Determination of serum titer by complement fixation.

Fig. 7A: Percent complement fixation was plotted over serial dilutions of homologous Ag concentrations (0.31-10 μ g/ml) for various Ab concentrations (1:400- $\diamond$ — $\diamond$, 1:800- $\blacktriangle$ — $\blacktriangle$, 1:1600- $\circ$ — $\circ$, 1:3200- $\blacksquare$ — $\blacksquare$, 1:6400- $\square$ — $\square$).

Fig. 7B: Percent complement fixation was plotted versus the log Ab dilution for the Ag concentration at the inflection point (1.25 μ g/ml) of the curves in Fig. 7A. The titer at 80% C'F obtained from this plot was 1300.

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serological reactivity among the antibody populations present in each of the following 8 groups of antisera:

1. anti-chicken H5 raised in guinea pigs-courtesy of Casilda Mura; serum titer at 80% C'F=6500
2. anti-chicken H5 + RNA in guinea pigs
3. anti-chicken H5 raised in rabbits-courtesy of Casilda Mura; serum titer at 80% C'F=3000
4. anti-chicken H5 + RNA in rabbits
5. anti-goose H5 in guinea pigs
6. anti-goose H5 + RNA in guinea pigs
7. anti-goose H5 in rabbits
8. anti-goose H5 + RNA in rabbits

Since the microcomplement fixation assay employs antiserum at very low concentrations it is likely that only the antibodies of highest affinity are being measured. A change which alters the complementarity between one of the antigenic determinants on the protein and its specific antibody may also decrease the affinity between them. Thus even a local change in only one of several antigenetic determinants may be magnified during the process of antibody-antigen aggregation and be sufficient to alter the complement fixation curve (Levine, 1973). Therefore microcomplement fixation is sensitive to small differences in amino acid sequence.

Sarich & Wilson (1966) showed direct evidence for a correlation between immunological cross-reactivity as measured by microcomplement fixation and structural

relatedness of primate albumins. For any one species of protein, the peak height of the complement fixation curve bears a linear relationship to the logarithm of the antiserum concentration over a range of 20%-90% fixation; i.e. the slope of the lines are identical. The fact that the slopes are alike provides a basis for measuring the immunological distance between related proteins using the same antiserum. A measure of cross-reactivity (or index of dissimilarity; Prager & Wilson, 1971) is defined as the factor by which the antiserum concentration must be raised in order that the heterologous antigen gives a peak height equal to that of the homologous one at 50% C'F. The immunological distance (y) is defined as $100 \times$ the log of the index of dissimilarity (Sarich, 1969) and was shown by Prager & Wilson (1971) using the avian lysozyme protein family to be related to the percent difference in amino acid sequence (x) by the equation $y=5x$. This relationship has been used to establish the relatedness among all classes of histones.

Because the sequence of both chicken H5 (Briand et al, 1980) and goose H5 (Yaguchi et al, 1979) were published and therefore the percent amino acid sequence difference could be ascertained indisputably, it was reasoned that I would be assessing the serologically reactive components of the different antisera rather than assessing the homology between protein sequences by calculating the immunological

distances. A summary of cross-reactivity; i.e. index of dissimilarity, of the 3 antisera, measured by microcomplement fixation with homologous and heterologous antigens is presented in Table VII. Figure 8 shows a sample graph for the calculation of index of dissimilarity.

In general, the anti-goose H5 antisera from both species with or without RNA detected different determinants in chicken H5 much better (immunological distance average = 23) than the anti-chicken H5 antisera detected differences in goose H5 (immunological distance average = 0). This finding may however be peculiar to this study, as other investigators in this laboratory (Mura, 1979) have elicited antibodies in guinea pigs against chicken H5 without RNA that gave an immunological distance of 44 when cross-reacted with goose H5.

Table VII shows that the immunological distance for reciprocal crosses; i.e. GP-anti-ChH5 x GH5 versus GP-anti-GH5 x ChH5, vary considerably. This variation in reciprocity was noted by Prager & Wilson (1971) and they suggested averaging the two immunological distance values obtained for like-crosses. Average immunological distance values are presented in Table VIII.

From the published sequences of chicken H5 and goose H5 the percent amino acid sequence difference for the intact molecules is 14%. The mid-globular domain, which shows the most conserved amino acid sequence among the

TABLE VII: Summary of Cross-Reactivity of Antisera with Homologous and Heterologous Antigens using Microcomplement Fixation

Serum	Antigen	Dilution for 50% C.F.(a)		Index of Dissimilarity (b)		Immunological Distance (c)	
G.P. Chick H5 (d)	CH5 GH5	8710 8500	8320 7940	1.00 1.02	1.00 1.04	0 1	0 2
G.P. Chick H5 + 2 RNA (e)	CH5 GH5	3200 3200	6400 4200	1.00 1.00	1.00 1.52	0 0	0 18
Rab. Chick H5 (d)	CH5 GH5	4900 4900		1.00 1.00		0 0	
Rab. Chick H5 + 5 RNA	CH5 GH5	2510 2820	3200 4570	1.00 0.89	1.00 0.70	0 -5	0 -15
G.P. Goose H5 2	GH5 CH5	2400 1230		1.00 1.95		0 29	
G.P. Goose H5 + 2 RNA	GH5 CH5	2950 2140	3200 2340	1.00 1.37	1.00 1.36	0 14	0 14
Rab. Goose H5 7	GH5 CH5	480 320	580 300	1.00 1.50	1.00 1.93	0 18	0 29
Rab. Goose H5 + 5 RNA	GH5 CH5	1260 1120	3200 1150	1.00 1.13	1.00 2.78	0 5	0 44

(a) Values reported are averaged replicates for experiments performed on different days.

(b) Index of Dissimilarity = $\frac{\text{Homologous Titer}}{\text{Heterologous Titer}}$, at 50% C.F.

(c) Immunological Distance = $100 \times \text{Logarithm of Index of Dissimilarity}$.

(d) Antisera prepared by C. Mura.

(e) Antiserum number corresponds to numbers in Table VI.

Figure 8: Calculation of index of dissimilarity.

Fig. 8A: Percent complement fixation was plotted over serial dilutions of heterologous Ag concentrations ($0.31-10 \mu\text{g/ml}$) for varying Ab concentrations (1:50-◆—◆, 1:100-○—○, 1:200-●—●, 1:400-△—△, 1:800-□—□, 1:1600-▲—▲, 1:3200-■—■).

Fig. 8B: Percent complement fixation was plotted versus the log Ab dilution for both homologous (○—○) and heterologous (▲—▲) Ag concentrations at the inflection point of the curves. The titer for both homologous and heterologous antigen obtained at 50% C'F was 570 and 360 respectively and the resulting index of dissimilarity was 1.60.

TABLE VIII: A. Summary of Immunological Distances
using various antisera versus Chicken H5
and Goose H5

Antiserum	Immunological Distance	Average
G.P. ^(c) Chick H5	2	15
G.P. Goose H5	29	
G.P. Chick H5 + RNA	9	12
G.P. Goose H5 + RNA	14	
Rab. ^(d) Chick H5	0	12
Rab. Goose H5	24	
Rab. Chick H5 + RNA	-10	8
Rab. Goose H5 + RNA	25	

B. Theoretical values of Immunological Distance using
the formula $y = 5x$ ^(a)

Peptide	Immunological Distance	% Amino Acid (b) Sequence Difference
intact molecule	70	14
N-terminus	250	50
C-terminus	65	13
Mid-domain	50	10

(a) Prager & Wilson, 1971.

(b) from published sequences.

(c) Antisera elicited in guinea pigs (G.P.).

(d) Antisera elicited in rabbits (Rab.).

lysine-rich histones(Yaguchi et al, 1979) differs by 10%, whereas the highly variable N-terminal arm varies by 50% and the less complex C-terminal arm varies by 13%. Using the criterion of Prager & Wilson(1971) for lysozyme to determine the percent amino acid sequence difference by the equation $y=5x$, these known sequence values for chicken and goose H5 predict an immunological distance value of 70 for the intact molecules. If the antigenicity resided within the middle globular domain the immunological distance value would be 50. If the antigenicity was within the N- or C-terminal arms the immunological distance values would have to be 250 or 65 respectively.

No antiserum raised for this study gave these values for immunological distance(Table VIII). Therefore, the antibodies raised in guinea pigs and rabbits do not recognize the mid-globular domain as a specific determinant on the immunogen and the inclusion of RNA with the immunogen does not impart structure to the loose ends of the H5 molecule, thus enabling antibodies to be elicited against specific N- and C-terminal determinants. These results also clearly show that the formula for the derivation of the percent amino acid sequence difference from immunological distance used for avian lysozymes(Prager & Wilson, 1971) does not apply in this study of the lysine-rich histones. Figure 9 presents the graph of percent amino acid sequence difference versus immunological distance for the lysine-rich histones of

Figure 9: Percent amino acid sequence difference versus immunological distance for selected lysine-rich histones of known sequence; i.e. chicken H1 and H5, goose H5 and trout H1.

The line of best fit was calculated to be $y=1.57x + 12.5$ with a correlation coefficient of $r=0.726$.

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known sequence, chicken H5 and H1, goose H5 and trout H1 (results taken from this study and from work performed in this laboratory by Peter Foster and summarized in Appendix II). The calculation of the line of best fit from this graph indicates that if any relationship exists between percent amino acid sequence difference and immunological distance ($r=0.726$) for these lysine-rich histones it would be $y=1.6x$.

Prager & Wilson(1971) state that the determination of immunological distance from cross reactions is affected by the length of the immunization program. They also state that because of the variability between animals in antibody production response, indices of dissimilarity gradually decline to a constant value during the course of immunization. The serum protein concentration can vary considerably due to the animals' health and recent fluid consumption, the amount of anticoagulant used(if any) and the amount of hemolysis which occurs. Therefore, they suggest that antisera used for cross reactions should be pooled in the inverse proportion to the serum titers of individuals. The guinea pig antisera in this study are equal pools of sera taken late in the immunization program from six animals. The rabbit antisera are from individual animals bled early in the immunization program. The values for the indices of dissimilarity range from 1.0 to 2.8 for all comparisons and Prager & Wilson also report a three-fold range of 4.6 to 13.3 from individual animals.

Perhaps my values would not have fluctuated as much if the sera had been pooled in inverse proportion to the titers for both guinea pigs and rabbits from later bleeds in the immunization program, but these inconsistencies to Prager & Wilson's method do not account for the low factor relating immunological distance to percent amino acid sequence difference. In fact, the broadening of antiserum specificity with time would only bring the indices of dissimilarity closer, and therefore lower the calculated immunological distances and the factor even more.

In a study on cross reactions between antibodies to hemoglobins-S, C and A₁ using microcomplement fixation (Reichlin et al, 1964), the index of dissimilarity was calculated in each case to be 1.3 and these proteins differ by only one amino acid substitution. Another study investigating the relatedness of pancreatic ribonucleases (Prager et al, 1978) reported an index of dissimilarity of 1.5 between dromedary and camel ribonucleases which once again only differ by one amino acid substitution. My indices of dissimilarity are in this range, but chicken H5 differs from goose H5 by 25 amino acid substitutions.

B. Peptide Inhibitions

Because it was not apparent from immunological distance measurements which portion of the histone

molecule the various antibodies were recognizing, inhibition of complement fixation studies were undertaken (Methods III, B). The middle globular domains of both chicken and goose H5, prepared as limit tryptic digests (Fig. 5, Lanes 6, 10) were used to inhibit the formation of H5/anti-H5 complexes in the microcomplement fixation reaction. The initial experiments for inhibition of complement fixation were tried using GP-anti-ChH5 + RNA with intact chicken H5 and the globular domain peptide from chicken H5 (Fig. 10A & B) and GP-anti-GH5 with intact goose H5 and its globular domain peptide (Fig. 11A & B). A 1:5000 dilution of anti-ChH5 serum and a 1:4000 dilution of anti-GH5 serum were divided into several aliquots and serial dilutions (from 5 $\mu\text{g}/\text{ml}$ to 0.31 $\mu\text{g}/\text{ml}$) of intact ChH5 and globular domain peptide (TP-ChH5) or intact GH5 and globular domain peptide (TP-GH5) were added in replicate and incubated with the serum for 15 min at 37°C.

This serum was then used with a series of antigen concentrations (either ChH5 or GH5) to measure the percentage complement fixation at 4°C in the usual manner as described in Methods III, A. Intact ChH5 at concentrations of 0.31-5 $\mu\text{g}/\text{ml}$ reacted with a 1:5000 dilution of homologous guinea pig antiserum to fix 99% of the complement (Fig. 10A). The no-inhibitor control line rises quickly to 100% C'F indicating that a higher dilution of antiserum (e.g. to give 90% C'F maximum) might have given more sensitive results in this experiment.

Figure 10: Inhibition of complement fixation by ChH5/anti-ChH5 complexes with ChH5 peptides and a 1:5000 antiserum dilution.

Prior to the addition of Ag, Ab was incubated for 15 min at 37°C with inhibitor. Ab was then reacted with homologous Ag in the normal complement fixation test at 4°C . Percent complement fixation was plotted over serial dilutions of homologous Ag concentrations (0.31-10 $\mu\text{g/ml}$).

Fig. 10A: The inhibitor was intact ChH5 at 0.09 $\mu\text{g/ml}$ (Δ), 0.31 $\mu\text{g/ml}$ ($\square$), 0.63 $\mu\text{g/ml}$ ($\blacksquare$), 1.25 $\mu\text{g/ml}$ ($\blacklozenge$), 2.5 $\mu\text{g/ml}$ ($\bullet$), 5.0 $\mu\text{g/ml}$ ($\circ$).

Fig 10B: The inhibitor was the globular domain peptide (TP) of chicken H5 at concentrations corresponding to intact ChH5 (Fig. 10A).

Figure 11: Inhibition of complement fixation by GH5/anti-GH5 complexes with GH5 peptides and a 1:4000 antiserum dilution.

Prior to the addition of Ag, Ab was incubated for 15 min at 37°C with inhibitor. Ab was then reacted with homologous Ag in the normal complement fixation test at 4°C. Percent complement fixation was plotted over homologous Ag concentrations (0.31-10 µg/ml).

Fig. 11A: The inhibitor was intact GH5 at 0 µg/ml (◆), 0.31 µg/ml (□), 0.63 µg/ml (▲), 1.25 µg/ml (△), 2.5 µg/ml (●), 5.0 µg/ml (○).

Fig. 11B: The inhibitor was the globular domain peptide (TP) of goose H5 at concentrations corresponding to intact GH5 (Fig. 11A).

Using large excesses of inhibitor concentration might also have helped.

Pre-incubation at 37°C with intact ChH5 inhibited this complement fixation reaction at 4°C in proportion to the amount of pre-incubated H5 at concentrations of antigen at or above optimum (1.25 µg/ml). This self-inhibiting feature of guinea pig antiserum was also observed by Mura et al, 1980.

The interesting result is however, that TP-ChH5 behaves in almost an identical manner to intact ChH5. TP-ChH5 inhibits the formation of H5/anti-H5 complexes with intact ChH5 in proportion to the amount of pre-incubated peptide (Fig. 10B). There was no shift in the optimum antigen concentration which might have indicated inactivation of antigen or the typical effect of antigen excess (Bustin & Stollar, 1973a).

To compare the ability of the TP-ChH5 fragment to inhibit complement fixation by H5/anti-H5 with that of the intact TP-ChH5 molecule the fractional inhibition of maximal C'F was plotted against the logarithm of the molar ratios of peptide to antigen (Fig. 12). The globular domain peptide of ChH5 inhibits C'F almost as well as the intact chicken H5 molecule.

It may not be surprising that intact chicken H5 can inhibit the complement-fixing abilities of its own anti-ChH5/ChH5 complexes. In reactions with soluble antigens (histones) and immunoglobulin G's (anti-histones)

Figure 12: Relative inhibition of maximal complement fixation by TP-ChH5 compared with intact ChH5 calculated from results presented in Figure 10.

The ordinate is inhibition plotted as a fraction of optimal complement fixation in the absence of inhibitor and the abscissa is the molar ratio of inhibitor peptide to chicken H5. Inhibitors used were intact ChH5(●—●) and the globular domain peptide (TP) from ChH5(O—O).

complement fixation is known to be more efficient at 4°C rather than at 37°C (Cunniff & Stollar, 1968), the temperature used for pre-incubation with inhibitors. Any alteration of conformation of the antigenic or inhibitory H5 with temperature (Crane-Robinson et al, 1976) may affect the H5/anti-H5 complexes in some allosteric way so that they are less able to bind complement.

However, that the ability of intact whole antigen to inhibit subsequent complement fixation is not necessarily a peculiar feature of guinea pig antiserum (Mura et al, 1980) is shown in Fig. 11A. Intact GH5 at a concentration of 0.31-10 µg/ml reacted with a 1:4000 dilution of homologous guinea pig antiserum to fix 98% complement. Unlike intact chicken H5, goose H5 added as inhibitor for 15 min at 37°C produced no change in the complement fixation reaction except perhaps at the equivalence value of 1.25 µg/ml inhibitor. The addition of TP-GH5 as inhibitor (Fig. 11B) also produced no change in the C'F reaction at optimal H5 concentrations, indicating that for this particular antiserum no determinants were recognized in the region of 29-100 on the goose H5 molecule.

An experiment was performed to see if TP-ChH5, when reacted with anti-ChH5 in the presence of complement at 4°C, was capable of fixing complement in the regular manner (Fig. 13). TP-ChH5-anti-ChH5 complexes were found capable of fixing complement and gave a titer at 80% C'F for TP-ChH5 at 2.5 µg/ml of 930. The titer of anti-ChH5

Figure 13: Complement fixation by the globular domain peptide(TP) of chicken H5.

Fig. 13A: Percent complement fixation was plotted over serial dilutions of TP concentrations (0.31-10 μ g/ml) for varying Ab dilutions (1:400- $\blacklozenge$ — $\blacklozenge$, 1:800-O—O, 1:1600- $\bullet$ — $\bullet$, 1:3200- Δ — Δ , 1:6400- $\blacksquare$ — $\blacksquare$, 1:12800- $\square$ — $\square$).

Fig. 13B: Percent complement fixation was plotted versus the log Ab dilution for both intact ChH5(O—O) and TP($\blacktriangle$ — $\blacktriangle$) at the antigen concentration of the inflection point of the curves.

with intact ChH5 at 5.0 ug/ml(i.e. molar equivalents) in the same experiment was 2750. The antigen control tube(see methods) showed no anticomplementarity indicating that TP-ChH5 aggregates were not fixing complement themselves.

An essential requirement for immunoglobulins to bind complement is the presence of multivalent antigens possessing 3 or more determinants(Reichlin & Noble, 1977), so that an aggregate of at least 4 immunoglobulin molecules(Hyslop et al, 1970) forms with their associated antigen molecules to interact with complement. However, the presence of only one determinant will serve to inhibit complement fixation. Thus the globular domain peptide from chicken H5 possesses at least three determinants and is capable of fixing a reduced amount of complement compared to intact chicken H5.

C. Enzyme-Linked Immunosorbent Assay

During the previous set of experiments a variety of problems was encountered with the complement fixation technique. Titration results varied from day to day and experiment to experiment. The results from the inhibition studies were very hard to interpret; it was not obvious whether the addition of intact H5 or TP-ChH5 as inhibitor was acting solely as an inhibitor or if it was also fixing

complement, because one could not observe the diagnostic shift to the left with these curves.

Enzyme-linked immunosorbent assay(ELISA) afforded another method of examining the serologically reactive components of the antibody populations that were raised for this study. With ELISA, antigen adsorbed to the plastic surface of a microtiter plate binds primary antibodies. Non-specific antibodies are washed free and the remaining antibody-antigen complexes are detected with a secondary antibody(in this case, Protein A) conjugated with an enzyme such as alkaline phosphatase or horse radish peroxidase. Protein A is a cell-wall component of Staphylococcus aureus which binds specifically to the F_c fragment of immunoglobulin G at sites distal to the antigen combining site, thereby not inhibiting the immune reaction. It serves as a general tracer and its use eliminates the need to purify and label individual anti-immunoglobulins for each species (i.e. guinea pig and rabbit). The labelling with enzyme tracers does not render it unstable or unreactive(Gee & Langone, 1981) as may happen with antibodies. When chromogen substrate is added to the bound antigen-antibody in the wells, the degree of colour production is directly proportional to the number of antigen-antibody complexes present.

The use of the secondary antibody reaction provides an amplification of the primary antigen-antibody response. In the example of an antigen containing 3 determinants it

is possible for 3 antibody molecules to bind. This would in turn bind 3 secondary antibodies producing an increase in the colour development of substrate. The sensitivity limit is not set by the affinity between antigen and primary antibody as in complement fixation(Engvall, 1980).

The ability of heterologous antigens, either monovalent or multivalent, to inhibit the primary antibody binding to homologous antigen in the well is measured as a reduction in optical density as a result of the reduced binding of the secondary antibody, presenting less enzyme to the reaction mixture. This "competitive ELISA" avoids the discrepancies of variation in protein binding to the plastic wells. The direct passive adsorption of histones onto the plastic was found to be time, temperature and concentration dependent (Foster, unpublished observations). Since the adsorption process is non-specific, a non-ionic detergent is added which does not interfere with the antigen-antibody reaction but does prevent the formation of new hydrophobic interactions between the added proteins(antibodies) and the solid phase without disrupting the hydrophobic bonds already formed between the previously adsorbed protein and the plastic surface. Control experiments were performed to examine the optimum type of plate and the type and concentration of detergent(Results not shown). To control for the different adsorbing characteristics of various proteins and peptides only chicken H5 was adsorbed to the well of

the plate and all other peptides or proteins were tested for their ability to inhibit chicken H5-anti-ChH5 formation.

All antisera raised against chicken H5 were screened to determine their serological reactivity. The calculation of percent inhibition from a sample plot of optical density versus the logarithm of antibody concentration is shown in Fig. 14. Table IX presents a summary of percent inhibition by the globular domain peptide of chicken H5 with all antiserums elicited against chicken H5 + RNA in a time course progression of successive bleedings. Antiserum from earlier bleeds (Rabbits 5, 7) show more specificity for the middle globular domain than for the intact molecule. This specificity broadens with time to include the whole molecule so that later antibodies (Rabbits 9, 11) show the intact chicken H5 molecule to be a better inhibitor than the globular domain peptide, contrary to reports that the major determinant resides in the middle globular domain (Mura et al, 1980). Again, the variability in immune response among animals was noted. Species of immunization as well as inclusion of RNA in the immunogen had no effect on serological reactivity of the antibody populations. These experiments confirm the complement fixation results.

All of these antisera were also tested using competitive ELISA for their ability to recognize

Figure 14: Calculation of percent inhibition of antibody binding by competitive ELISA.

A range of antiserum dilutions are first reacted with inhibitors-no inhibitor ($\bullet$ — $\bullet$), ChH5 ($20\mu\text{g/ml}$, $\circ$ — $\circ$), TP-ChH5 ($10\mu\text{g/ml}$, Δ — Δ) and ChH1 ($20\mu\text{g/ml}$, $\square$ — $\square$) before addition to the microtiter plate wells containing ChH5 as antigen. Optical density was measured at 405nm. Percent inhibition was calculated in relation to no inhibitor = 0% inhibition and ChH5 = 100% inhibition at one-half maximum OD. For this graph TP-ChH5 gave 20% inhibition and ChH1 gave 60%.

TABLE IX: Competitive ELISA - Summary of Serological Specificity with Homologous Antigen and with Other Lysine-Rich Histones

Antiserum ^(a)	Inhibitors								
	No I	CH5	TPCH5	CH1	GH5	TPGH5	XH1	XH1 ^s	
Guinea Pig									
1(b)	0 ^(c)	100	61	42	68	58 [*]			
2	0	100	58	40	36	0			
3	0	100	33	79					
4i	0	100	47	65	63	0			
4ii	0	100	45	61	75		17	39	
4iii	0	100	64	74	69	43			
4iv	0	100	74	76	79	36			
Rabbit									
5	0	100	151	61	78	52			
7	0	100	131	42			6	23	
9	0	100	42	32	62	38			
11	0	100	53	61	42	29			
6	not tested								
8	0	100	55	52					
10	0	100	58	33	38	0			
12	0	100	14	13	35	15			

(a) All antisera raised against CH5 + RNA.

(b) Antisera numbers correspond to numbers in Table VI.

(c) Reported as percent inhibition.

similarities and differences with other lysine-rich histones (Table IX). All antisera elicited against chicken H5 cross-reacted with other lysine-rich histones from the same and different species over a range of antiserum dilutions. Of the 14 antisera tested, 6 showed subtype-specificity, 4 showed species-specificity and 4 did not discriminate between the two. Thus, although the percent amino acid sequence differences between chicken H1 and H5 and between goose H5 and chicken H5 are 71% and 14% respectively, some antisera recognized ChH1 as a better inhibitor than GH5 of ChH5/anti-ChH5 complexes.

Since the percent amino acid sequence difference between the globular domain peptides of goose H5 and chicken H5 is only 10%, they should be approximately equivalent in inhibitory activity. However, this is not evident in antisera GP 2,4i,4iv; Rab 5,10,11 (Table IX), where the globular peptide from goose H5 is a poorer inhibitor than both the globular peptide from chicken H5 and intact chicken H1. Species-specificity of antiserum was also evident in another comparison between lysine-rich histones done in this laboratory by Peter Foster (Appendix III). Of 17 comparisons made, 11 were found to be species-specific, 4 subtype-specific and 2 did not distinguish between the two. The origin of the antibody (i.e. guinea pig or rabbit) or the inclusion of RNA with the immunogen were not a factor. This species-specific nature of antibodies elicited against

lysine-rich histones has been reported elsewhere (Bustin & Stollar, 1973b) but it has not been reported that species similarities over-ride differences between sub-classes of histones. Consequently, using the cross-reactive abilities of these antisera to predict or pinpoint structure or sequence homologies among the lysine-rich histone satellites would be unilluminating, because results would indicate that chicken H1 and H5 are more closely related than chicken H5 and goose H5.

D. Affinity Chromatography

Attempts were made to eliminate the cross-reactivity of anti-ChH5 antisera with chicken H1 using affinity chromatography. Initial experiments using 0.1M glycine-HCl, pH 3.0 to elute bound antibodies from the column resulted in very low yields comprised of very weak antibodies (200-fold reduction in titer; results not shown). Other researchers have experienced only a 2-fold reduction in antibody titer when using this technique (Absolom & Van Regenmortel, 1978; Mazen et al, 1982). Since there is evidence that exposure to low pH can inactivate antibodies, while high salt is found to be significantly less harmful (Cuatrecasas et al, 1968), further attempts at antibody purification were tried using 4M Guanidinium-HCl, pH 8.0 for antibody elution.

One such experiment is summarized in Figure 15.

Antisera raised against chicken H5⁺ RNA in rabbit was allowed to react with Sepharose-4B-ChH5 overnight before elution of bound antibody with 4M Guanidinium-HCl, pH 8.0 the next day. Competitive ELISA results for the unbound and ChH5-bound antibodies, dialyzed and concentrated, are shown in Figure 15A and 15B respectively. The bound antiserum was more specific for chicken H5 than H1 at 1:50 dilution but the titer of the antiserum was reduced over 100-fold. This ChH5-bound antiserum was then re-adsorbed on a Sepharose-4B-ChH1 column. Competitive ELISA results for the unbound, chicken H5-specific antibodies and the bound, chicken H1-specific antibodies are presented in Figure 15C & D. The titer of the antiserum was again reduced 5-fold but the cross-reactivity with chicken H1, although reduced, was not totally removed. It was also evident from Figure 15D that the chicken H1 adsorption column was binding chicken H5 antibodies. Thus it was reasoned that both columns were non-specifically binding antibodies, and this fact, coupled with the extreme loss in antibody activity, led me to abandon the use of affinity chromatography to purify specific antibody populations. Others have recommended a wash with borate-buffered saline containing 0.1% Tween 20 after addition of the antiserum to elute any protein non-specifically bound to the column (Blankstein et al, 1980). Addition of isolated IgG rather than whole

Figure 15: Summary of assays of affinity chromatographic experiments using Sepharose-4B-conjugated antigens to remove chicken H1 cross-reactivity from antisera.

The ability of ChH1 (Δ — Δ) and ChH5 (O—O; both at 20 ug/ml) to inhibit the formation of ChH5/anti-ChH5 complexes ($\bullet$ — $\bullet$) over a range of Ab dilutions using competitive ELISA were plotted as OD_{405} versus Ab dilution⁻¹.

Fig. 15A: The original antiserum: GP Anti-ChH5 + RNA (#3, Table VI).

Fig. 15B: ChH5-bound antibodies after passing through ChH5-Sepharose-4B column. Effluent from this column gave no reaction.

Fig. 15C: ChH5-bound antibodies from Fig. 15B passed over a ChH1-Sepharose-4B column (i.e. effluent).

Fig. 15D: ChH1-bound antibodies from column in Fig. 15C.

antiserum to the affinity column might have minimized non-specific interaction of serum proteins with the immobilized histones(Stollar & Rezuze, 1978). Use of a large diameter, short column rather than a small diameter, long column might have reduced the dilution of the antibody upon elution.

GENERAL DISCUSSION

I. Microcomplement Fixation and Histone Sequence Homology

Initially it was the intention of this study to use the technique of microcomplement fixation to calculate immunological distances in order to distinguish between the serologically reactive components in the antibody populations present in sera raised in two species against protein antigens of known sequence in the presence and absence of RNA. When the most appropriate antibody population was found; i.e. the one specific for the mid-globular domain of chicken H5 and goose H5, it would be used to survey homologies among the lysine-rich histones of unknown sequence. However, the immunological distance data on the antibody populations indicated no serological specificity for the globular domain. In fact, the immunological distance data did not give a good estimation of the percent sequence difference between the two known histones when the usual relationship of immunological distance = $5 \times (\% \text{ sequence difference})$ was applied to the results (Prager & Wilson, 1971). The data suggests that if a relationship exists at all between immunological distance and percent sequence difference ($r=0.726$) it would be $y=1.6x$.

In the light of these results it was interesting to analyze previous published work that used this immunological relationship to establish similarities between other histone classes. Using published microcomplement fixation data for histones of known sequence, a comparison between percent immunological distance and amino acid sequence difference is presented in Table X. Figure 16 graphs the immunological distance versus percent sequence difference for known histone sequences. The line of best fit calculated from these results is $y=1.7x$ with a correlation coefficient of $r=0.819$. Thus even with the inclusion of many more data points the line of best fit does not differ significantly from results in this study. Only the correlation coefficient(r) is improved because there is less scatter with the addition of more points. Microcomplement fixation results using chicken and trout H2A comparisons have established a relationship of $y=2x$ (Neelin, unpublished results). The relationship established by Prager & Wilson(1971) and Champion et al(1975) between immunological distance and percent amino acid sequence difference is clearly not applicable to histone proteins. Recently the applicability to histones of extensive specificity studies, using whole serum to determine immunological distance and hence primary structure differences from the extent of cross-reactivity in complement fixation assays, has been questioned(Srebrev &

TABLE X: Immunological Distance Values versus Percent
Amino Acid Sequence Differences derived from
Published Histone Sequences

Antigens	Percent Sequence Difference	Immunological Distance
1 Chicken H5	0	0
2 Goose H5	14	12
3 Trout H1	67	140
4 Chicken H1	71	71
5 Chicken H1/Trout H1	38	140
6 Calf H2A	0	0
7 Chicken H2A	3	0
8 Human H2A	2	0
9 Calf H2B	0	0
10 Chicken H2B	5	4
11 Human H2B	5	4
12 Chicken H2B(1-58)	9	1
13 Chicken H2B(62-125)	2	2
14 Trout H2B(1-58)	14	50
15 Trout H2B(62-125)	2	0
16 Drosophila H2B	14	50
17 Yeast H2B	33	25
18 Calf H3	0	0
19 Yeast H3	11	44
20 Calf H4	0	0
21 Chicken H4	0	15
22 Human H4	1	0

Sequence References:

- 1- Briand et al, 1980.
- 2,3,6,9,14,15,18,20-Von Holt et al, 1979.
- 4,21-Sugarman et al, 1983.
- 7-D'Andrea et al, 1981.
- 8,11,22-Zhong et al, 1983.
- 10,12,13-Van Helden et al, 1982.
- 16-Elgin, 1979.
- 17-Wallis et al, 1980.
- 19-Brandt & Von Holt, 1982.

Immunological References:

- 1-5 This study
- 6,7,8,9,10,11,20,21,22-Stollar & Ward, 1970.
- 12,13,14,15,16-di Padua Mathieu & Stollar, 1980.
- 17,18,19-Tessier et al, 1980.

Figure 16: Percent amino acid sequence difference versus immunological distance for histones of known sequence as listed in Table X.

The values for chicken H1 and H5, goose H5 and trout H1 (Fig. 9) are included (⊙). The line of best fit was calculated to be $y=1.680x + 2.93$ with a correlation coefficient of $r=0.819$.

Zlatanova, 1983), and my results strengthen this skepticism.

The relationship between immunological distance, as measured by microcomplement fixation, and percent sequence difference has been shown to be unreliable in comparisons with high mobility group proteins (HMGs) of known sequence (Mayes, 1982). Sequence differences between calf thymus HMGs' 1, 2, 3, 8, 14 and 17, when determined immunologically by microcomplement fixation (Bustin et al, 1978) using the relationship of $y=5x$, were considerably lower than those obtained by primary structural comparisons.

Although the molecular basis for the correlation of sequence with immunological data is uncertain (Wilson et al, 1977), the use of immunological studies to detect sequence relatedness between proteins is based on the premise that complement fixation is very sensitive to structural differences in related proteins. The original observation that microcomplement fixation detected serological differences between hemoglobins (Reichlin et al, 1964) and ribonucleases (Prager et al, 1978) which differed by one amino acid supported this theory. The theory is based on a comparison of antiserum titers; i.e. the ability of homologous antibody-antigen complexes to fix complement, compared to that ability by heterologous antibody-antigen complexes. When the peak heights of the complement fixation curves are plotted over a range of

antibody dilutions, the slopes are equivalent. The factor by which the heterologous titer at 50% complement fixation has to be raised to give the homologous titer at 50% complement fixation is the index of dissimilarity. The immunological distance and percent amino acid sequence difference are then calculated from this index of dissimilarity. But the assumption that the complement fixation titer(peak height) of an antiserum is a measure of its antibody content is not valid(Kabat & Mayer, 1961). Equivalent weights of antibody in different serum reacting with equivalent concentrations of antigen may vary in their capacity to fix complement. It was also found that specific complement fixation potency per weight of cross-reacting antibody was far lower than that for homologous reactions and that different cross-reacting antisera varied widely in this respect. It is evident from these observations that the titer at 50% complement fixation of an antiserum in a cross-reaction does not furnish a reliable index of its antibody content.

One measure of reliability of immunological distance values related to sequence differences is the reciprocity of cross-reactions. Champion et al(1974) have stated that an acceptable deviation from perfect reciprocity is 20%. Sarich & Wilson(1966) reported a discrepancy of 6.6% for mammalian serum albumins, and Prager & Wilson(1971) reported 17% for bird lysozymes, but Prager et al(1978) reported 43.8% for mammalian ribonucleases (if no values

are excluded from their mean). Bustin & Stollar(1973a) found reciprocity tests to be unsatisfactory in an immunological comparison of mammalian lysine-rich histones where they report a mean percentage deviation of 32%. Calculations from the results of an immunological comparison of rye and calf core histones(Roland & Pallotta, 1978) gives a measure of deviation from perfect reciprocity of 61%. Calculation of the measure of deviation from perfect reciprocity(Champion et al, 1975) by antisera against histone proteins in my assays is 134%, and combined data for lysine-rich histones gives a value of 47%(Table XI). Therefore histone proteins give unacceptable deviation measurements from perfect reciprocity.

It has been stated(Atassi, 1977) that in the cross-reaction of protein 1 with antisera to protein 2, a given antigenically reactive region on protein 1 may take an active part whereas its equivalent structural counterpart on protein 2 may not even participate in the reverse cross-reaction. In other words, the cross-reaction is not necessarily effected through all equivalent antigenic regions on the surface of the two proteins. When equivalent regions on the two proteins do participate in the reciprocal cross-reactions, they may well not do so with equal affinity. The affinity of the reactive region or its share of the total reactivity of the antibody may vary with the antiserum. Immunological

TABLE XI : Calculation of the Measure of Deviation
from Perfect Reciprocity^(a) for:
A. Antisera raised in this study
B. Lysine-Rich Histone Antisera

A.	Protein A	Protein B	Immunological Distance		Percent Deviation from Average
			Anti-A vs B	Anti-B vs A	
	CH5	GH5	2	29 (b)	87
	CH5	GH5	9	14 (c)	22
	CH5	GH5	0	24 (d)	100
	CH5	GH5	-10	25 (e)	233
Measure of deviation from perfect reciprocity = 134%					

B.	CH1	CH5	116	26 (f)	63
	CH1	TrH1	138	141	1
	CH1	TrH5	186	-	-
	CH5	TrH1	139	141	1
	CH5	TrH5	104	133	12
	CH5	GH5	2	23	84
	TrH1	TrH5	67	-	-
Measure of deviation from perfect reciprocity = 47%					

(a) Champion et al, 1975.

(b) Antiserum from G.P. - RNA.

(c) Antiserum from G.P. + RNA.

(d) Antiserum from Rab. - RNA.

(e) Antiserum from Rab. + RNA.

(f) Antisera reported in Appendix II from G.P. - RNA.

comparisons using whale myoglobin from different species gave cross-reactivity results of 34-41% for protein 1 X anti-2 and 58% for protein 2 X anti-1 (Atassi & Saplin, 1971). However, with all antisera studied a reactive region was invariably a reactive region but its potency or efficiency varied with the individual serum. Thus prolonging immunization schedules and pooling sera in inverse proportion to titer (Prager & Wilson, 1971) may only enhance reciprocity at the expense of index of dissimilarity.

The linear correlation developed by Prager & Wilson (1971) between immunological distance and the percent amino acid sequence difference would be optimal only if all parts of the protein sequence contributed to the antigenic reactivity and if all antibodies in the analytical test serum were distributed equally to all antigenic determinants. Prager & Wilson (1971) felt that in many systems the variable contributions of amino acids to antigenic sites and the variable distribution of antibodies to different antigenic sites may be statistically averaged, especially in large molecules with many determinants. The antigenic effects of amino acid substitutions were thus approximately equal and additive.

There is evidence from fossil records (Champion et al, 1975) for evolutionary biases (i.e. natural selection) against substitutions in globular, isofunctional proteins that are internal or cause large conformational changes.

Therefore amino acid substitutions occur on the surface of globular proteins and should be easily detectable using antibodies. It is likely however, that in most proteins conformation-dependent clusters of particular surface amino acids contribute disproportionately to antigenic determinants and that the distribution of antibodies to different determinants is biased (di Padua Mathieu & Stollar, 1980). Amino acid substitutions in immunosilent areas of the protein would therefore go undetected.

The precise determinant locations in a few globular proteins of known amino acid sequence and three-dimensional structure have been elucidated. These include sperm whale myoglobin (Atassi, 1977), hemoglobin (Reichlin & Noble, 1977) and cytochrome c (Margoliash et al, 1970). The number of determinants is small (approximately 3/100 amino acids) and comprise only 20% of the amino acids of the whole protein. Champion et al (1975) felt that these were not typical of protein antigens because the amino acid sequences of these hemo-proteins were very similar to the corresponding proteins in rabbit (species of immunization) and were therefore not totally recognized as foreign by the rabbit's immune system resulting in antisera that reacted with only a few determinants. Despite their previous comment (Wilson et al, 1977) that rabbit antisera to mammalian proteins were probably more discriminatory by a factor of 1.2-1.8 than rabbit antisera to bird proteins in

detecting structural differences by cross-reactivity, they insist that proteins whose sequence differs greatly from that of any rabbit protein(eg. lysozyme) elicits production of antibodies to a larger number of sites and a larger fraction of the protein's surface. Therefore the sequence-immunology relationship derived for these "foreign proteins" would be reliable. This argument should apply to the lysine-rich histones chicken H5 and goose H5 which differ in sequence from rabbit thymus H1 by 750%.

The antigenic determinant regions of lysozyme have since been delineated(Atassi, 1977). There are three determinant regions comprising 12% of the total amino acids of the protein. Therefore the antibodies are not seeing many surface amino acid substitutions. Similarly, studies on determinant localization of histone proteins have indicated the presence of only two or three reactive regions per 100-200 amino acids (Sapieka et al, 1976; Absolom & Van Regenmortel, 1977; di Padua Mathieu & Stollar, 1980; Mura et al, 1980; Muller et al, 1983).

It is interesting to note that all the protein families that have been studied for sequence-immunology relationships; i.e. albumins, azurins, lysozymes and ribonucleases, contain disulphide linkages. These globular proteins are therefore very constricted conformationally and any amino acid substitution that disrupted this conformation would be strongly selected

against. The three determinants of lysozyme are three disulphide-containing peptides and are thus conformational rather than sequential.

Histone proteins, in contrast, are known to have secondary and tertiary structure that is not dependent on disulphide bridges but is more probably dependent on ionic environment (Crane-Robinson et al, 1976). It is likely that without the rigidity of conformation imposed by disulphide bridges the determinants on histone proteins are much more susceptible to conformational perturbation. Ionic environment is known to affect the immunochemical behaviour of histone protein presumably through alteration in conformation (Mihalikis et al, 1976; Feldman & Stollar, 1977).

Later studies in this field (Champion et al, 1975; Nei, 1977) relate the immunological distance to the number of amino acid substitutions that have occurred since the proteins diverged from a common ancestral gene rather than to the percent amino acid sequence difference. They rationalize that one amino acid substitution will have little effect on the antibody-antigen reaction in polyclonal antiserum along the whole molecule, if there are a large number of antibodies to a large number of antigenic sites. But one amino acid substitution changing size or charge in an antibody-combining site could drastically alter the local environment or conformation at that site. This small conformational change, not enough

to affect the function or conformation of the whole protein and therefore not selected against by natural selection, would be detected in the microcomplement fixation test, especially when there are only two or three antibody combining sites along the protein molecule. Thus maleylation of H1, which modifies the charge of lysine, causes immunochemical changes in H1, whereas amidination of the lysyl residues of H1, which has no effect on charge, does not alter the immunochemical properties of H1 (Bustin, 1978).

Other workers have applied the immunological relationship derived from complement fixation results to the percent difference in amino acid composition rather than sequence of histone proteins (Bustin et al, 1977; Bustin, 1977; Martinez & Palau, 1983). It is true that amino acid compositions are related to the maximum number of amino acid sequence substitutions allowed between proteins. For example, if the minimum number of sequence differences compatible with the compositional differences between two proteins is taken as either the sum of the larger of either the excesses or deficits in the number of residues of all the amino acids, an amino acid composition difference of 12% could reflect from 7 to 25% sequence differences (Prager & Wilson, 1971). The actual number of sequence differences are more likely near the maximum value since there are usually sequence differences between homologous proteins that do not affect

composition(Alfageme et al, 1974). Table XII presents the percent amino acid composition difference compared with the percent sequence difference for histones of known sequence. These values are plotted in Figure 17. The line of best fit($r=0.984$) is calculated to be $y=0.7x$ demonstrating direct correlation between composition difference and sequence difference.

Other mathematical methods for comparing sequence relatedness of proteins from their respective amino acid composition have appeared in the literature(Metzger et al, 1968; Marchalanis & Weltman, 1971; Cornish-Bowden, 1979, 1980). It has been shown that with proteins of known sequences the test of composition data(Cornish-Bowden, 1979, 1980) is nearly certain to give a negative result when applied to proteins with unrelated sequences and is nearly certain to give a positive result when applied to proteins with a substantial degree of sequence similarity. Table XIII summarizes the statistical comparison of amino acid compositions for all the histones for which there is published immunological distance data and complete sequence data available. Cornish-Bowden's(1980) mathematical application to amino acid composition data does give a genuine relatedness when applied to histones of known sequence but within each histone class the ordering of critical values does not follow the relationship of percent sequence difference from known histone sequences.

TABLE XII: Percent Composition Difference versus Percent
Sequence Difference of Histones of Known
Sequence

Histones	Percent Sequence Difference	Percent Composition Difference ^(a)
Chicken H5	0	0
Goose H5	14	14
Trout H1	67	50
Chicken H1	71	56
Calf H2A	0	0
Human H2A	2	3
Chicken H2A	3	5
Calf H2B	0	0
Human H2B	5	9
Chicken H2B	5	5
Trout H2B	14	7
Drosophila H2B	14	15
Yeast H2B	33	22
Calf H3	0	0
Yeast H3	11	15
Calf H4	0	0
Chicken H4	0	0
Human H4	1	2

(a) Compositions taken from sequences.

(b) References reported in Table X.

Figure 17: Percent amino acid sequence difference versus percent amino acid composition difference for histones of known sequence for which there is available immunological distance data (Table XII).

The values for chicken H1 and H5, goose H5 and trout H1 are included (●). The line of best fit was calculated to be $y = 0.714x + 2.11$ with a correlation coefficient of $r = 0.984$.

TABLE XIII : Summary of Statistical Comparison of Amino Acid Composition for all Histones of Known Sequence for which there is published Immunological Distance Values. (Table X)

$$DI = \frac{X_A - X_B}{2}, \quad S \quad Q = (X_A - X_B)^2 \quad \text{and} \quad D = 0.01 \cdot S \Delta Q^{1/2} \quad \text{where } X_A \text{ and } X_B \text{ represent}$$

the percent composition of amino acid X in proteins A and B respectively, DI is the difference index, D is the compositional divergence and S Q is the index of Marchalonis and Weltman (1971); Cornish-Bowden, 1980. Tabulated values below the critical values represent near certainty of a weak or strong relationship between the two proteins.

Lysine-Rich Histones:					Critical Values for	
Protein A Chicken H5	Protein B Chicken H1	Goose H5	Trout H5	Trout H1	200 Amino Acids	
					Weak	Strong
DI	19.8	4.35	13.9	21.7	15.2	10.1
S Δ Q	232.56	8.45	75.6	246.4	93.0	42.0
D	0.1525	0.0291	0.0869	0.1570	0.0964	0.0648
Relationship	none	strong	weak	none		

Core Histones:					Critical Values for	
Protein A Calf H2A	Protein B Human H2A	Chicken H2A	130 Amino Acids	Weak	Strong	
					130 Amino Acids	
DI	1.6	2.9	18.9	12.5		
S Δ Q	2.56	3.84	144	70		
D	0.0160	0.0196	0.120	0.0806		
Relationship	strong	strong				
	1	2				

TABLE XIII (Cont'd)

Core Histones:		Protein B	Trout H2B	Drosophila H2B	Human H2B	Yeast H2B	Critical Values	
Protein A	Protein B	Chicken H2B					140 Amino Acids	Weak Strong
Calf H2B								
DI	6.5	4.0	7.9	4.4	10.25	19.6	12.9	
SΔQ	17.98	7.24	25.8	13.34	52.01	155	79.0	
D	0.0424	0.269	0.0508	0.0365	0.0721	0.124	0.0837	
Relationship	strong	strong	strong	strong	strong	strong	strong	
	3	1	4	2	5			

Core Histones:		Protein B	Critical Values	
Protein A	Protein B	Yeast H3	140 Amino Acids	Weak Strong
Calf H3				
DI	7.3	18.1	12	
SΔQ	28.38	133	60	
D	0.0533	0.115	0.0775	
Relationship	strong			

Core Histones:		Protein B	Chick H4	Critical Values	
Protein A	Protein B	Human H4		100 Amino Acids	Weak Strong
Calf H4					
DI	0.95	0.95	21.4	14.1	
SΔQ	1.81	1.81	186	84.0	
D	0.0135	0.0135	0.136	0.0917	
Relationship	strong	strong	strong	strong	
	(identical)				

II. Inhibition Studies

Because immunological distance measurements failed to give any insight into the serologically reactive components of the antibody populations elicited in this study, inhibition experiments were attempted in order to find antibody populations that were specific for the mid-globular domain of chicken H5. Use of this naturally occurring domain reduced the possibility that determinant regions would be disrupted by enzymatic or chemical cleavage. Hydrodynamic and scattering criteria, circular dichroism, NMR and molar enthalpy of melting measurements (Aviles et al, 1978) have shown this peptide to retain all the helicity and features of tertiary folding that are observed in the intact chicken H5 molecule. Therefore conformational determinants should be shared with the native protein.

It was not surprising therefore that this globular domain peptide was capable of fixing complement. This complement-fixing ability of the globular domain was not due to protein aggregation because lysine-rich histones and their peptides do not show any tendency to aggregate in solution (Bradbury et al, 1975). The fact that the fragment 32-189 of chicken H5 did not fix complement in Mura's guinea pig anti-H5 (1980) may reflect the loss of residues 22-31 and perhaps some resultant conformational alteration of the globular domain. In contrast, when

Mazen in a similar study(unpublished observations) used peptides derived from chicken H5 with rabbit anti-H5 + RNA, both peptides 32-189 and 59-189 were capable of fixing complement by forming multivalent complexes with antibody.

In peptide inhibition studies using the two peptides derived from calf or rat thymus H1 by NBS cleavage, the longer peptide containing middle and C-terminal domains(equivalent to two-thirds of intact H1) was capable of binding complement(Hekman & Sluyser, 1973; Bustin & Stollar, 1973b). The C-terminal peptide derived from calf thymus H2B by CNBr cleavage(62 residues) was also capable of fixing complement(di Padua Mathieu & Stollar, 1980). In all these studies the shorter N-terminal peptide(less than one-third of intact molecule) was incapable of fixing complement, the C-terminal peptides fixed less complement than the intact molecules, and the complement-fixing ability of the intact protein could not be restored by mixing the peptide fragments. In contrast, three peptides derived by CNBr cleavage of chicken H3(Sapeika et al, 1976), respectively 90, 30 and 15 residues, were unable to fix complement alone but an equimolar mixture restored 97% of the complement-fixing ability of the intact molecule. These examples of inconsistencies in results from various sources underline the importance of the contribution played by protein conformation in antibody production and recognition. They also attest to the variability of

antibody populations within test serums.

III. Enzyme-Linked Immunosorbent Assay and Lysine-Rich Histone Sequence Homology

Many interpretive as well as technical problems arose from the application of microcomplement fixation to the study of the homologies of lysine-rich histones. The relationship between immunological distance and percent amino acid sequence difference was shown not to be valid. Not only was complement fixation a poor indicator of sequence homologies among lysine-rich histones but also the ability of the mid-globular domain peptide to fix complement raised uncertainties in the interpretation of results. Titers of antiserums and cross-reactions were not consistent from day to day and experimental conditions involved stringent requirements of pH, temperature and ionic strength. Any contaminating metals, amines, chelating agents, organic solvents or enzymes could destroy the hemolytic activity of complement and high antiserum or antigen concentrations led to anticomplementarity (Levine & Van Vunakis, 1969). The mechanism of "complement fixation" was too complicated, since it required multivalent antigen and bivalent antibody-forming complexes of unknown composition which subsequently bound complement, followed by detection with

a poorly understood secondary reaction, i.e. red blood cell lysis.

Another technique was therefore sought that would detect the reaction between a monovalent peptide or hapten and one bivalent antibody molecule and involved reactions whose mechanisms were better understood. The enzyme-linked immunosorbent assay (ELISA) fulfilled these requirements. The technique takes advantage of the histones' natural property of binding to plastic keeping the antigen in the solid phase. Multivalency of the antigen is not a requirement, primary and secondary antibodies are readily reacted with the antigen, and unbound antibody is easily removed in solution.

It has been reported that to distinguish between mutant hemoglobins, quantitative complement fixation required molar concentrations of reactants of 10^{-9} - 10^{-8} mole/l, whereas for radioimmunoassay molar concentrations of reactants was 10^{-9} - 10^{-11} (Reichlin & Noble, 1977). ELISA has been shown to be as sensitive as radioimmunoassay (Engvall & Perlmann, 1972).

Both solid-phase radioimmunoassay and ELISA have been adapted for use in histone studies and are found to be 100 times more sensitive than microcomplement fixation (Romani et al, 1980; Romac et al, 1981). A quantitative distinction between two Friend leukemia virus H2A variants differing by two amino acids was detected reciprocally by corresponding antisera with solid-phase

radioimmunoassay in test tubes and a wide range of IgG concentrations ($1:10^3$ - $1:10^4$), whereas only low concentrations ($1:8400$) of serum revealed the difference in complement fixation assay (Blankstein et al, 1980).

Solid-phase radioimmunoassay in tubes was used to detect the possibility of cross-reaction due to monovalent determinants between fragments 1-58 and 63-125 of calf thymus H2B as compared to the possibility of cross-reaction due to multivalent determinants using microcomplement fixation (di Padua Mathieu & Stollar, 1980). No cross-reaction was found with either method. Solid-phase radioimmunoassay in microtiter plates was used to compare the antigenicity of 2 classes of HMGs from calf thymus and duck erythrocytes. The relatedness derived from radioimmunoassay was different from that derived from complement fixation results (Romani et al, 1979). The cross-reaction of Xenopus H1 with an antiserum raised against Xenopus H1^S (Brown et al, 1981), reported as "Xenopus H5", was not apparent at high serum dilution in complement fixation tests but did show using radioimmuno- and enzyme-linked assays (Moorman et al, 1980).

ELISA has been used to detect free histones as well as histones complexed in the nucleosome (Romac et al, 1981) and for the localization of epitopes in H4 using peptide inhibition (Muller et al, 1983). These workers have compared the inhibition of direct binding to the inhibition of complement fixation and found that the

competitive ELISA assay is less sensitive than microcomplement fixation. The molar ratios for inhibitors in microcomplement fixation was $10^3:1$, whereas for ELISA it was $10^5:1$, but the reactivity of the peptides in the two methods did not change. Although quantitation was found to be less reliable when direct binding of peptides to microtiter plates was compared with results from competitive inhibition of ELISA (Foster, unpublished results), other workers have obtained the same results from both methods (Romani et al, 1980; Muller et al, 1983).

My results, using competitive inhibition of ELISA with intact chicken H5 or the globular domain peptide of H5, gave no indication of a serologically reactive localization of antibodies to the mid-globular domain except in two early antibody populations. The use of ELISA has confirmed the microcomplement fixation results that the use of RNA complexed with lysine-rich histone as immunogen and/or the production of antibodies in different species has no effect on the recognition by antibody of specific antigenic regions of the antigen molecules. In fact when these antibodies were used to detect the structural homology among lysine-rich histones of known sequence, it was found that lysine-rich histones within a species were more closely related than within subclasses. Using antibodies raised against rat thymus H1 subfractions, Bustin & Stollar (1973a) showed that rat liver H1 subfractions cross-reacted in the complement

fixation test better than any calf thymus H1 subfractions; i.e. species-specificity was of a higher order than subclass-specificity. Perhaps there is a species-specific, shared determinant region on the lysine-rich histones that these antibody populations recognize. Since most species variation is in the N-terminal arm and the globular domain is highly conserved among all subclasses (Yaguchi et al, 1979; Pehrson & Cole, 1981), this species-specific determinant would probably be located in the C-terminal arm. When the globular domain of chicken H5 was used to elicit polyclonal antibodies in rabbits, no cross-reaction with chicken H1 was found (Allan et al, 1982) perhaps because the C-terminal arm had been eliminated from the immunogen. Srebrevia et al (1983) have reported production of antibodies in rabbits using chromatographically pure mouse liver H1⁰ complexed with RNA as immunogen which displayed more species-specificity than subtype-specificity. They suggest that this was due to the use of histone-RNA complexes as immunogen, but this relationship was not evident in this study.

All sequenced lysine-rich histones contain one phenylalanine residue situated near the C-terminus of the mid-globular domain (approximately amino acid 100), around which are clustered up to 15 homologous residues, with only conservative amino acid substitutions. Antisera unable to distinguish between either species or subclasses of lysine-rich histones could therefore contain large

populations of antibodies to this conserved lysine-rich histone sequence. Reports of a monoclonal antibody specific for H1, H1⁰ and H5 (Smith et al, 1984) is most probably reacting with this epitope.

If antibodies are to be used to analyze the sequence or structural homologies among lysine-rich histones, this cross-reactivity will have to be removed. Attempts in this study to purify antibodies specific only to chicken H5 by affinity chromatography were unsuccessful. Preparation of specific antibodies to the mid-globular domain either by immunization, affinity chromatography or monoclonal antibody technique might reduce this cross-reactivity.

The species variation between subclasses of lysine-rich histones resides in the N-terminal arm (Yaguchi et al, 1979). Although probably only the sequence-conserved globular domain attains stable tertiary structure in free lysine-rich histones, the primary structural differences between lysine-rich histone subtypes in the N-terminal arms affect that conformation (Smerdon & Isenberg, 1976). Common antigenicity depends upon conformational as well as sequence homology (Bradbury et al, 1972). Not only must the whole molecule of the heterologous antigen retain the same conformation as the immunogen, but also domains within from the heterologous antigen must retain identical conformation for homology studies to be valid. The effect

of amino acid substitution elsewhere in the molecule and its subsequent affect on the conformation of these domains must be understood.

Thus antibodies, specific for the conserved mid-globular domain of lysine-rich histone from one particular species and very sensitive to slight conformational perturbations, may not recognize globular domains from other species as homologous because of the conformational changes induced by sequence variation in the N-terminal arm of the heterologous histone. This may explain why antisera in this study cross-reacted very well with the globular domain of chicken H5 but did not recognize the same highly conserved region from goose H5 as homologous.

Consideration must also be given to the fact that conformational differences may not arise only from amino acid variation in the N-terminus but also from ionic environment or physical manipulation. Histone protein adsorbed on plastic, covalently bound to the resin of an affinity column or blotted to nitrocellulose may have little resemblance to the conformation of the immunogen. The discussion of the importance of antigen conformation in cross-reactions in relation to the microcomplement fixation results is equally relevant to the ELISA results.

Table XIV summarizes the comparison of percent amino acid sequence difference from histones of known sequence with all other methods of comparing histone homologies; i.e. percent composition differences and the relationship derived from the graph of percent composition difference(y) versus percent sequence difference(x) ($y=0.7x$), Cornish-Bowden relationships derived from amino acid compositions, and the relationships derived from the microcomplement fixation data and immunological distance values. From this information and the results obtained from the ELISA study, it is evident that the degree of cross-reactivity does not necessarily correspond to the degree of sequence homology.

The use of immunological techniques may only establish the evolutionary relatedness among the histone proteins and may not provide a good indication of sequence homology. Certainly the antisera in this study have confirmed that lysine-rich histones evolved from a common ancestor (Yaguchi et al, 1979) and perhaps it can be postulated that evolutionary divergence of the lysine-rich histones occurred after speciation, although not all antisera confirmed this.

If cross-reactivity is indicative of structural relatedness, and if structural homology reflects

TABLE XIV: Summary of Methods Describing the Relatedness of Histones

Histones	Percent (a) Sequence Difference	Percent (b) Composition Difference	Percent (c) Sequence Difference	(f) Cornish Bowden	Immunological Distance	Percent (d) Sequence Difference	Percent (e) Sequence Difference
Lysine-rich:							
Chicken H5	0	0	0	-	0	0	0
Goose H5	14	14	20	strong	12	2	5
Trout H5	-	35	50	weak	118	24	69
Trout H1	67	50	71	none	140	28	82
Chicken H1	71	56	80	none	71	14	42
Core:							
Calf H2A	0	0	0	-	0	0	0
Human H2A	1.5	3.1	4	strong(1)	0	0	0
Chicken H2A	2.9	4.7	7	strong(2)	0	0	0
Calf H2B	0	0	0	-	0	0	0
Human H2B	4.8	8.8	13	strong(3)	4	1	2
Chicken H2B	4.8	4.8	7	strong(2)	4	1	2
Trout H2B	14	7.2	10	strong(1)	50	10	29
Drosophila H2B	13.6	15.2	22	strong(4)	50	10	29
Yeast H2B	33	21.5	31	strong(5)	25	5	15
Calf H3	0	0	0	-	0	0	0
Yeast H3	11.1	14.8	21	strong	44	9	26
Calf H4	0	0	0	-	0	0	0
Chicken H4	0	0	0	identical	15	5	9
Human H4	1	2	3	strong(2)	0	0	0

(a) From published sequences.

(b) From published compositions.

(c) Calculated from graph of % composition difference = 0.7(% sequence difference).

(d) Calculated from published relationship of immunological distance = 5(% amino acid sequence difference). (Prager & Wilson, 1971)

(e) Calculated from the graph of immunological distance = 1.7(% amino acid sequence difference for histones).

functional homology, then it can be said that all of the lysine-rich histones in this study perform the same function; i.e. structural involvement with polynucleosomal DNA packaging, however no distinction can be made between the function of H1 and the H1 satellites because very little difference was found between their patterns and levels of cross-reactivity. These results do not preclude structural relatedness and therefore specialized function of the H1 satellites but only indicate that caution must be exercised when interpreting immunochemical results in terms of homologous structure and function. To establish a clear evolutionary and functional relationship among these multi-domain proteins, data from the sequence of every domain is required. The conserved central domain of H1, H5 and H1^O/H1^S could have a very similar function and their N- and C-terminal domains, less-conserved, may be responsible for any differences in function among them (Barbero et al, 1982).

The results of this study have shown that the cross-reactivity of antisera do not give a good indication of percent amino acid sequence difference between homologous histone proteins and their peptides. If antibodies to lysine-rich histones are to be used as reliable probes in homology studies, the findings of this study must be applied and controlled for experimentally, if the results of future experiments are to be reliably interpreted. Inclusion of other biochemical data along

with evidence derived from a variety of immunological techniques such as ELISA, protein blotting and monoclonal antibody production would help support homology claims. It is relevant therefore, to reassess current literature with the results of this study in mind.

In a study of the serological detection of homologies of mouse liver H1^O with chicken H5 and calf thymus H1 (Mura & Stollar, 1981), the species-specificity of the antiserum was removed by affinity chromatography and its absence demonstrated using microcomplement fixation and radioimmunoassay. Blotting experiments showed that antibodies to chicken H5 cross-reacted strongly with mouse liver H1^O and antibodies to calf thymus H1 cross-reacted with chicken H1, mouse H1 and weakly with mouse H1^O. They therefore conclude that mouse H1^O shares more homology with H5 than with H1. However, the serological specificity of the anti-CH5 antiserum to the globular domain was not demonstrated, as it was in a later immunoelectron microscopy study (Frado et al, 1983) and therefore the homology of chicken H5 with H1^O in this specific region was not demonstrated.

Shimada et al (1981) have used microcomplement fixation results as well as electrophoresis, amino acid analysis, N-terminal determination and tryptic peptide mapping to characterize the homology between an H1^O-like protein (H1^S; Brown et al, 1981) found in bull frog erythrocytes and both chicken H5 and bovine liver H1^O. An

antiserum raised against bull frog H1^S cross-reacted only with bovine H1^O and not chicken H5, but an antiserum raised against chicken H5 did not cross-react with bovine H1^O or bull frog H1^S. They conclude that H1^S is more like H1^O than H5 and that the sequence homology between H5 and H1^O previously indicated from partial sequence analysis(Pehrson & Cole, 1981) was very limited. My results indicate that based on the immunochemical data alone, this may not be a valid interpretation, and the results obtained may be peculiar only to a particular antiserum dominated by antibodies against limited determinants.

Antibodies to the globular domain of chicken H5 cross-reacted with H1^O but not H1, and early antisera against the globular domain of H1^O did not cross-react with either intact chicken H5 or H1 or their globular domains(Yasuda et al, 1984). Again, this result may be peculiar to these antibody populations and may not necessarily indicate, as the authors propose, that the globular domain of H1^O has immunological determinants absent from H1 and H5. In fact they state that a later bleed of this antiserum did cross-react with both the globular domain from chicken H5 and bovine H1^O. They do, however, confirm convincingly using immunochemistry, that the H1^O-like protein(Shimada et al, 1981;H1^S-Brown et al, 1981) from bullfrog erythrocytes is more like bovine liver H1^O than chicken H5.

Using ELISA, antibodies to the globular domain of chicken H5 cross-reacted with ox liver H1⁰ and not with calf thymus or chicken H1, but a weak cross-reaction with the globular domain of chicken H1 was detected in protein blotting experiments(Allan et al, 1982). Immunochemical studies on the occurrence of H1⁰-related protein in trout liver and chicken liver(Srebrevna & Zlatanova, 1983; Srebrevna et al, 1983) are hard to interpret due to "results not shown", but they have not included proper controls to show antibody cross-reaction with either trout liver H1, trout RBC "H5" or chicken liver H1 casting suspicion on their results.

Using antisera specific for the globular domains of H5/H1⁰, Smith et al (1984) have surveyed the higher and lower eukaryotes for the distribution of H1⁰- and H5-like protein structures in the hopes of finding universality of the H1 satellite class. This widespread occurrence would provide good evidence that the function of this class of histones is indeed involved in control of gene expression. Although they do confirm that H1D and H1E from Xenopus laevis are H1⁰-like proteins, they fail to find evidence for an H1⁰- or H5-like protein in lower invertebrate eukaryotes(a crustacean, two fungi and a plant). However they may have chosen their experimental subjects inappropriately in that Artemia salina and wheat germ are embryonic tissues, which may not contain H1 satellites because they are not yet terminally differentiated while

the two fungi Saccharomyces cerevisiae and Physarum polycephalum are actively synthesizing nucleic acids. It should be noted that Yasuda et al(1984) have immunochemically detected an H1^O-like protein in Physarum and Côté et al(1982) have reported an H1-like protein from Physarum with an amino acid composition similar to H1^O.

The universality of H1 satellite class of lysine-rich histones may only become evident as thorough biochemical as well as immunochemical studies are undertaken. Evidence for the existence of this class of histones in invertebrates is already accumulating(Alfageme et al, 1974; Barbero et al, 1982). Negative results based primarily on antibody-reactivities(Smith et al, 1984) do not substantiate conclusively the lack of occurrence of H1 satellites in lower eukaryotes and therefore the suggestion that H1 satellites are not involved in the repression of DNA synthesis or transcription.

Appendix I. Standardization of Conjugate for ELISA

The secondary antibody-enzyme conjugate, protein A-alkaline phosphatase was prepared in the lab by Peter Foster using the method of Engvall(1978). The optimum dilution of conjugate was determined using the direct ELISA(Methods III, C(i)) in which a constant concentration of antigen in the well was reacted with either 2-fold or 5-fold dilutions of conjugate. The conjugate titer used was chosen as the inflection point of the upper plateau on a graph of optical density versus antiserum dilution for various conjugate dilutions.

APPENDIX II

Summary of Immunological Distance Values for the Lysine-Rich Histones:
Chicken H5 and H1, Trout H5 and H1(a)

Antiserum (b)	Antigens	Index of Dissimilarity	Immunological Distance	Average I.D.	% Amino Acid Sequence Difference (c)	Amino Acid Difference (d)
Anti-CH5	CH5/CH1	1.81	26	71	14	71
Anti-CH1	CH1/CH5	14.4	116			
Anti-CH5	CH5/TrH1	25	139	140	28	67
Anti-TrH1	TrH1/CH5	26	141			
Anti-CH1	CH1/TrH1	24	138	140	28	38
Anti-TrH1	TrH1/CH1	26	141			
Anti-CH5	CH5/TrH5	10.9	104	118	24	-
Anti-TrH5	TrH5/CH5	21.5	133			
Anti-CH1	CH1/TrH5	72	186	186	36	-
Anti-TrH1	TrH1/TrH5	4.6	67	67	42	-

(a) Calculated from microcomplement fixation results of Peter Foster.

(b) All antisera raised in guinea pigs without RNA.

(c) Theoretically derived using the formula $y=5x$ (Prager & Wilson, 1971).

(d) From published sequences: CH5-Briand et al, 1980; CH1-Sugarman et al, 1983; TrH1-MacLeod et al, 1977.

APPENDIX III

Summary of Competitive ELISA Results with Homologous
and Heterologous Lysine-Rich Histones^(a)

Antiserum	Inhibitors				
	No I	CH5	CH1	TrH5	TrH1
G.P. ^(b) Anti-CH5	0 ^(c)	100	22	0	0
Anti-CH1	0	0	100	0	0
Anti-TrH5	0	60	47	100	20
Anti-TrH1	0	24	150	80	100
G.P. Anti-CH5	0	100	48	7	1
Anti-CH1	0	21	100	45	56
Anti-TrH5	0	30	42	100	26
Anti-TrH1	0	0	94	17	100
G.P. Anti-CH5 ^(d)	0	100	75	27	0
Anti-CH5 ^(d)	0	100	66	24	19
Anti-CH5	0	100	45	5	0
Rab. ^(e) Anti-CH5	0	100	43	12	3
Anti-CH5 ^(d)	0	100	27	3	22
Anti-CH5 ^(d)	0	100	85	53	0
Anti-CH5 ^(d)	0	100	54	24	3
Anti-CH5 ^(d)	0	100	70	4	24
Anti-CH5 ^(d)	0	100	47	0	0
Anti-CH5 ^(d)	0	100	52	0	11
Anti-CH5 ^(d)	0	100	70	0	26

(a) Calculated from results of Peter Foster.

(b) Antisera elicited in individual guinea pigs (G.P.).

(c) Reported as percent inhibition.

(d) Immunogen + RNA.

(e) Antisera elicited in individual rabbits (Rab.).

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