Species and Organ Specificity in Very Lysine-rich Histones*

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SUMMARY

Very lysine-rich histones were extracted by aqueous trichloracetic acid from calf thymus, from the thymus, mammary gland, and liver of rabbits, and from chicken livers. The elution profiles of these histones, obtained by chromatography on Amberlite IRC-50 with a shallow guanidinium chloride gradient, revealed that the very lysine-rich histone complement varied from one animal species to another when a single kind of organ was considered, and even varied among different organs of a single species.

The chromatographic resolution of the subfractions of very lysine-rich histones, their amino acid compositions, and their relative mobilities on polyacrylamide gels suggest that the whole very lysine-rich complement of any species consists of a moderately large number of molecular types.

The association of histones with DNA in the nucleus has prompted the study of the role of these basic proteins in the genetic processes of the cell. Among various hypotheses concerning the function of these proteins, the proposition advanced by Stedman and Stedman (1) that histones may interact with DNA in a specific manner, thereby influencing the phenotype of a cell, has stimulated a search for species and tissue specificity among these proteins. This search has yielded meager results.

With the exception of a serine-rich histone from avian erythrocytes (2), all histones obtained to date from a range of tissues extending at least from plants to mammals can be placed in the same three major classes: the very lysine-rich, the slightly lysine-rich, and the arginine-rich (3). Although Fambrough, Fujimura, and Bonner (4) have demonstrated some variation in the proportions of the major histone classes in pea tissues, by and large, when histones are compared from one species to another or from one tissue to another the relative proportions of the major classes are approximately constant. Furthermore, the amino acid compositions and even peptide maps of any one class are similar regardless of the tissue from which the class was isolated (cf. p. 38 of Reference 3; 5).

A search for species and tissue specificity among histones classified in such a gross way has therefore not been very fruitful. All three major classes of histones are known to be heterogeneous, and a total of six major molecular types is widely recognized (cf. p. 57 of Reference 5). Further subfractionation is possible, however, and comparative studies on subfractions within a single class might reveal differences among tissues which were not evident when whole classes were compared. An excellent example of the latter type of comparison was given by Bellair and Mauritzen (6), who reported tentative quantitative differences among the β-histones (which are mainly arginine-rich subfractions) derived from various tissues.

The recent development by Kinkade and Cole (7) of techniques for resolving four subfractions of the very lysine-rich histones of calf thymus opened an especially good opportunity to compare these histones in a series of organs. Furthermore, this class of histone is advantageous for comparative studies because it can be extracted from nucleoprotein in a rather direct specific way, minimizing possible artifacts due to degradation or material losses during the isolation. De Nooij and Westenbrink showed that solutions of trichloroacetic acid would extract reasonably pure very lysine-rich histone from calf thymus (8). Thymocytes are unusual in having a very large nucleus and a small cytoplasm, but we have recently shown that the technique is applicable to rabbit mammary gland (9) which has relatively normal cells in this regard. The extracts obtained proved suitable for chromatographic comparisons with the subfractionation system of Kinkade and Cole (7). These techniques have been used in a search for species and tissue specificity of very lysine-rich histones, with the results presented in this paper.

EXPERIMENTAL PROCEDURE

Quick frozen rabbit thymus (from young rabbits), mammary gland Type I, and livers were obtained from Pel Freeze Biologicals, Inc., Rogers, Arkansas. The calf thymus glands (2- to 8-day-old calves) and chicken livers (from 8-week-old White Rock chickens) were removed within 30 min after the animals were killed, and were frozen on solid CO₂. With the exception of the mammary gland, the very lysine-rich histones were obtained according to the method of De Nooij and Westenbrink (8). To obtain these histones from the mammary gland the above method was slightly modified (9). Trichloroacetic acid was the product of Merck Sharp and Dohme. Guanidine hydrochloride, purchased from Matheson

* This research was supported by United States Public Health Service Grants AM 02691, AM 06482, and GM 31. Support by the Agricultural Experimental Station is also acknowledged.

(Received for publication, April 8, 1968)
Coleman and Bell (technical grade) was purified as described before (9).

Protein was determined by turbidimetry (9). Disc electrophoresis in 15% polyacrylamide gels was done according to the method of Reisfeld, Lewis, and Williams (10) at 10 ma per gel. Hydrolysis (in 6 N HCl at 110° for 22 and 72 hours) and amino acid analyses were performed according to the method of Moore and Stein (11) on a Beckman-Spinco amino acid analyzer.

Ion exchange chromatography on Amberlite IRC-50 (Bio Rex-70; 10 meq per g, dry weight, 200 to 325 mesh range, Bio-Rad Laboratories, Richmond, California) was carried out as described by Kinkade and Cole (7). The following columns were used: 2.2 × 30, 2.2 × 15, and 1.2 × 15 cm. The total volumes of the gradients were 1700, 850, and 250 ml and the flow rates were 10, 5, and 1.30 ml per hour, respectively. Fractions were collected at 30-min intervals.

RESULTS

Previous work (9) established that the technique of De Nooij and Westenbrink (8) for the isolation of very lysine-rich histone was applicable not only to thymus but to mammary gland as well. Furthermore, it was shown that a relatively wide range of trichloracetic acid concentrations extracted these histones from mammary gland quantitatively. Although the more dilute trichloracetic acid solutions extracted nonhistone protein along with the very lysine-rich histone, no contaminants (or degradative products) could be detected in the region of Amberlite IRC-50 chromatograms where very lysine-rich histone appeared. The technique of De Nooij and Westenbrink thus seemed applicable to tissues in general (for the purpose of preparing samples suitable for chromatographic comparisons) without rigorous control of the trichloracetic acid concentration. Very lysine-rich histone was therefore extracted by trichloracetic acid solutions from calf thymus, from the thymus, mammary gland, and liver of rabbits, and from chicken liver. Electrophoresis of these extracts in polyacrylamide gels gave the results shown in Fig. 1. The patterns show essentially only one zone for each preparation, except in the case of chicken liver. The second (faster) electrophoretic zone in this case was apparently not a very lysine-rich histone, since it did not appear in electrophoretic analyses of the chromatographic peaks discussed later. Very likely this was the serine-rich histone found in chicken erythrocytes, and might have come from the liver itself or from the blood contained in the liver. In any case, it is important to note that all the electrophoretic patterns were quite free of the fast running zones characteristic of degradation products (7).

It should also be noted that gel electrophoresis could not distinguish among the very lysine-rich histones of different tissues. This is most dramatically shown in Fig. 1F, where the coelectrophoresis of a mixture of these histones from three tissues is shown to give a single zone.
Fig. 3. Polyacrylamide gel electrophoresis of chromatographic fractions of very lysine-rich histones. 
A and B, 10 to 11 μg of Peak 1 and Peak 2 from rabbit liver; C and D, 7 to 8 μg of Peak 0 and Peak 1 from chicken liver; E, 3.2 μg of Peak 1 from calf thymus and 4.4 μg of Peak 1 from rabbit thymus; F, 4.0 μg of Peak 1 from rabbit thymus; G, 6 μg of Peak 3 from rabbit thymus; H, coelectrophoresis of F and G; I, 8 μg of Peak 1 from rabbit mammary gland; J, 7 μg of Peak 2 from rabbit mammary gland; K, coelectrophoresis of 6.1 μg of Peak 1 and 3.0 μg of Peak 2 from rabbit mammary gland. Migration was from bottom (+) to top (−) at 10 mA per gel. A and B were run for 25 min; C to J were run for 50 min.

Table I

<table>
<thead>
<tr>
<th>Organ</th>
<th>Peaks (in order of mobility)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Calf thymus</td>
<td>1 = 3b &gt; 2 &gt; 3a</td>
</tr>
<tr>
<td>Rabbit thymus</td>
<td>1 &gt; 4 &gt; 2 = 3 &gt; 0</td>
</tr>
<tr>
<td>Rabbit mammary gland</td>
<td>1 &gt; 4 &gt; 2 &gt; 3</td>
</tr>
</tbody>
</table>

* Established previously (7).

Chromatography on Amberlite IRC-50, as shown in Fig. 2, proved more successful in revealing distinct differences when very lysine-rich histones were compared from one organ to another. The chromatographic pattern displayed by the very lysine-rich histone from calf thymus is like that obtained by Kinkade and Cole (7) (for slightly degraded very lysine-rich histone from the same tissue). However, instead of the three main chromatographic peaks observed in the case of calf thymus, the very lysine-rich histone derived from the thymus of the rabbit was resolved into five peaks. On polyacrylamide gels each of the rabbit thymus peaks displayed only a single electrophoretic zone, and upon rechromatography the peaks were eluted as authentic components. That Peak 0 was not a product of degradation was indicated by its presence in the chromatographic pattern of a preparation extracted directly from whole thymus gland with 8% trichloroacetic acid, a modification of the method of De Nooij and Westenbrink which has been shown to avoid significant amounts of degradation products in calf thymus extracts (7). Furthermore, the amount of this peak was constant in all preparations studied (14 preparations) and, unlike most degradation products, its mobility on polyacrylamide gels was slower than the other four main components.

A comparison between the histones of two tissues from a single species is shown in Fig. 2, B and C. The most distinctive feature in the chromatograph from the rabbit mammary histone when compared to that of rabbit thymus histone was the prominence of the peaks that emerged late. Rechromatography confirmed the chromatographic authenticity of these peaks, and electrophoresis failed to resolve the subfractions of rabbit mammary histones into additional components.

The chromatographic patterns of the very lysine-rich histones derived from the two kinds of liver were similar to each other and strikingly different from those derived from the other tissues. The liver histones were fractionated by this system into fewer main components and were eluted at higher guanidinium chloride concentrations. As shown in Fig. 3, these peaks were electrophoretically homogeneous. Similarly, homogeneity was indicated by gel electrophoresis of samples of rabbit liver histones from various places in the chromatogram (Peak 0, the shoulder of Peak 1, and the trailing edge of Peak 2) and also samples from several points on the chromatogram of chicken liver histones. The faster electrophoretic zone seen in Fig. 1A was not found in the chromatographic fractions of chicken liver histone.

Co-chromatography of isolated fractions of the histone from one tissue with either the full complement of histone from another tissue or isolated fractions showed that the peaks numbered 1 from calf thymus, rabbit thymus, and rabbit mammary gland were chromatographically similar. These experiments also showed that Peak 2 of rabbit thymus is eluted between Peaks 1 and 2 of calf thymus and that the main peak of very lysine-rich histones from chicken liver chromatographs near Peak 4 of rabbit thymus.

From Fig. 2 it is apparent that Peaks 1 to 4 from rabbit thymus do not match Peaks 1 to 4 from rabbit mammary gland. The difference in elution volume from Peak 1 to Peak 4 in the rabbit thymus histone (14 chromatograms) was always significantly
greater than the corresponding difference for the mammary gland histones (9 chromatograms).

As in the case of calf thymus, the various peaks from the rabbit tissues had electrophoretic mobilities in polyacrylamide gels which were so nearly equal that they could not be distinguished unless separated peaks were subjected to coelectrophoresis in pairs (Fig. 3). By coelectrophoresis, however, the relative mobilities of the peaks within each tissue were established, (Table I). As in the case of calf thymus, the first major chromatographic peak (Peak 1) from the rabbit thymus and mammary tissues had the highest electrophoretic mobility of all the very lysine-rich histones from that tissue. The mobility of this peak was almost indistinguishable from that of Peak 4 (rabbit thymus and mammary gland), the last to emerge from the IRC 50 column.

Amino acid analyses were carried out on the individual subfractions from all the tissues. Hydrolysis of the rabbit thymus fractions for 22 hours and 70 hours revealed that isoleucine, leucine, and valine are completely released in 22 hours, and so the histones from other tissues were hydrolyzed for only 22 hours prior to amino acid analyses. The compositions, given in Table II, show a striking constancy throughout all fractions and yet differences were seen in levels of arginine, aspartic acid, threonine; glutamic acid, proline, and glycine, and one general trend was detected in valine levels. The very lysine-rich histones from every tissue studied showed a progression from high valine contents to low across the chromatographic pattern from smaller elution volumes to larger (see also Kinkade and Cole (12)). The magnitude of this trend is best appreciated by calculating the number of residues of valine present in each fraction on the assumption that all these histones have the same molecular weight (21,000) as the very lysine-rich histones from calf thymus (13, 14). On this basis the rabbit thymus very lysine-rich histones contain from 14 residues of valine in Fraction 0 to 8 residues in Fraction 4.

### DISCUSSION

The results just presented indicate that the complement of very lysine-rich histone varico in a specific way from one kind of animal to another when a single kind of organ is considered, and even varies from one organ to another in a single kind of animal. Our recent study (9) on the application of the procedure of De Nooij and Westenbrink to the mammary gland showed that a wide range of trichloracetic acid concentrations extracted the very lysine-rich histone fractions in constant proportions and uniform yields. This finding seems to rule out artifacts in the isolation procedure as a cause of the variations seen among these histones, especially when considered along with the fact that there was no chromatographic or electrophoretic evidence of proteolytic degradation. Since rechromatography ruled out chromatographic artifacts, we conclude that the differences seen among these histones are real, endogenous differences.

Although differences among the histones in comparing various species are generally considered slight (3, 15), quantitative (16) and even structural differences (17) have been reported previously. The demonstration of differences between species in the subfractions of very lysine-rich histone in the present work is therefore not surprising, although it may suggest more ex-
tensive variation than sometimes is implied. Of greater significance is the correlation of polyphenotypic expression in a single species with levels of histone subfractions. Correlations comparable to the one presented here have been found for the very lysine-rich histones by Kinkade, and for the β-histones by Delaia and Mauritsen (6). In addition, Fambrough, Fujinura, and Bonner (4) have revealed a correlation between the quantity of very lysine-rich histones and the stage of development in pea seedlings. Although such correlations do not prove that histones are involved in genetic repression, they do make the idea somewhat more attractive.

In addition to establishing a correlation between polyphenotypic expression and histone levels, the present data give some further insight into the multiplicity of histone structures. The chromatograms of very lysine-rich histones from the rabbit thymus clearly show a minimum of five molecular types in just this one major class from a single tissue, and further multiplicity is suggested by amino acid analysis. The compositions of the chromatographic peaks from all three rabbit tissues show an impressive constancy. This makes it seem unlikely that the peaks were significantly contaminated, a conclusion which is further supported by the chromatographic and electrophoretic data. Although the compositions of the subfractions show a high degree of uniformity, it appears nevertheless that no two peaks have quite the same composition with the possible exception of Peak I from rabbit thymus and Peak I from mammary gland. While it is not possible absolutely to rule out distortion of the analyses by trivial contamination, the amino acid analyses make it seem likely that there are at least 10 kinds of very lysine-rich histins in just the three rabbit tissues studied here. Of course, the number might be increased if additional tissues were studied or if the peaks were heterogeneous after all.

If histones are genetic repressors and contain within themselves all the specificity required for that function, then the molecular types of histones ought to be multitudinous. This view can be reconciled to present information on histones by assuming that each major class (and subfraction) is a large family of molecular types. Since most cells in higher organisms have most of their genetic information repressed most of the time, their complement of histones ought not to differ greatly and it would not be expected that the presence or absence of a few molecular types of histone could be detected (against a high background of commonly shared types). It would therefore be understandable that tissue differences have been difficult to reveal. It is, however, difficult to understand how such a large variety of molecular species could be resolved into the few, relatively well defined peaks found in the present chromatograms of the very lysine-rich histones; a more or less continuous spectrum of molecular types would seem more likely. Furthermore, the fairly discrete amino acid composition and tryptic peptide patterns of the very lysine-rich histone subfractions determined in this and previous work (12) would argue against the idea of a very large number of molecular types, the structures of which varied independently. The relatively discrete chromatographic resolution and compositions could be reconciled most easily to the notion of multitudinous histones by supposing that the histones of any subfraction contain a large portion of their structures in common and that the remaining structure varies from one type of histone to another. A common ("invariant") site might be designed to interact with DNA in general, and a variable site might be designed to form a complex with, for example, a particular site on a gene or a particular derepressor (e.g. hormones). If the invariant regions tend to overwhelm the resolving systems (chromatography and electrophoresis), the relatively simple patterns observed would be explained. Then, if the relatively discrete differences of the subfractions in amino acid composition and peptide maps could be considered an illusion due to coincidence of peptides on the maps and the insensitivity of amino acid analyses, the molecular types of histones might be multitudinous after all.

The awkwardness of the argument for multitudinous types of histones weakens the case for a repressor role for these proteins, if all the specificity for repression is assigned to the histone. However, if histone is simply the tool used to bring about repression by some agent specific for particular genes, then the number of kinds of histones required could be reduced to as few as one. In support of this view it has been pointed out that there are few histones in any tissue and that variations in histone structure are very small among tissues studied to date. This could indicate that in the evolution of these structures functional requirements would not permit the survival of much mutation and that structural specificity extends over a major portion of each molecule. While this notion implies a specific function for histones, the number of functions could be small. To reconcile this small number with the concept of histones as genetic repressors, it has been postulated that they acquire the specificity for regulating polyphenotypic expression by combining with another molecular agent (e.g. RNA; see Reference 18), which could direct a histone to a particular gene. If the extreme position were adopted that the entire specificity for repression lies in this molecular director, it might be expected that only one kind of histone would be required in an organism. However, the multiplicity of histones, indicated by their resolution into three to six major classes and especially the present subfractionation of one of these classes, makes it very awkward to assign all the specificity to a director molecule. Indeed, the multiplicity may be compounded by such processes as phosphorylation (19) and acetylation (20) of histones. It seems reasonable, therefore, to look for functional specificity in the histones themselves.

The present data strongly support the notion that there are an intermediate number (i.e. tens or scores rather than a few or thousands) of molecular species among the histones. A modification of the director concept could accommodate this degree of multiplicity by combining the notion that directors carry genetic specificity with the idea that histones possess structures specific for categories of function which might not be directly repressive. Among many categories which might be conjectured are those of the time and manner of derepression. Indeed such categories might explain the relative amounts and complexities already observed in the major classes of histones. The slightly lysine-rich histone is always found in the largest amount, and it seems to be relatively homogeneous; this class might be bound to the majority of genes which is repressed at all times except, perhaps, during cell division, and which therefore requires one type of derepression. The very lysine-rich histones, even when grouped together, occur in much smaller amounts than do the slightly lysine-rich and, as shown in the present work, this class of histone is comprised of a moderately large number of molecular types. Very lysine-rich histones, therefore, might be involved in relatively reversible repression throughout the life of the cell, and their number might well correspond to the number of derepressors (such as hormones, perhaps). It may be significant
that Fambrough, Fujimura, and Bonner (4) showed the proportion of very lysine-rich histone to increase as pea seedlings matured.

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*J. Biol. Chem. 1968, 243:4500-4505.*

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