The histone H1 proteins are a heterogeneous group of lysine-rich histones which play a central role in chromatin organization (1-4, reviewed in Ref. 5). The complement of H1 proteins in any one organism derives from the existence of a set of primary amino acid sequence variants (6-11) which are subject to posttranslational modifications (reviewed in Ref. 11). The relative proportions of the H1 subtypes change in a tissue-specific manner (12-14) and vary during development (9, 15, 22, 23) also affect the levels of specific Hls.

Six histone H1 subtypes and histone H5, isolated from chicken erythrocyte nuclei, were visualized on acid/urea polyacrylamide gels. Four of the H1 subtypes have been purified to homogeneity by fast protein liquid chromatography on a strong cation exchange column. The other two subtypes were obtained as enriched fractions from the same fast protein liquid chromatography experiments. Because six chicken H1 genes have been completely sequenced (Coles, L. S., Robins, A. J., Madley, L. K., and Wells, J. R. E. (1987) J. Biol. Chem. 262, 9656-9663), it was possible to assign each of the six H1 proteins to a specific gene after amino acid sequence analysis of peptides derived from the subtypes.

Alignment with Their Respective Genes*

The functional significance of multiple H1 subtypes is not fully understood. It has been reported that the H1 variants are nonrandomly distributed between regions of chromatin with different transcriptional potentials (24-26). In vitro experiments, in which different H1 subtypes have been shown to differ in their ability to condense DNA, correlate with the in vivo results (25, reviewed in Ref. 6).

In the chicken, at least four different H1 subtypes, excluding the erythroid specific H5 histone, have been reported (26-28). The ratios of the H1 subtypes have been shown to vary between tissues (13, 28, 29) and during differentiation of certain cell types (26, 30). Purification of individual H1 subtypes is a prerequisite for studying their effect on chromatin structure in vitro, their role in transcriptional control, and for raising subtype-specific antibodies. Limited purification of individual H1 subtypes has been previously achieved using Amberlite IRC-50 (27) or CMC-25 Sephadex (28), but resolution was poor and chromatography times were extensive. We report here the identification of six H1 subtypes from chicken erythrocytes and the complete purification of four subtypes using the powerful technique of fast protein liquid chromatography (FPLC).

Six chicken H1 genes have now been isolated and sequenced (four of them reported for the first time in the accompanying paper (40), see also Refs. 31 and 32). Peptides from the FPLC-purified H1 subtypes have been sequenced, and the alignment of each subtype with a H1 gene sequence has been achieved.

MATERIALS AND METHODS

Isolation of Lysine-rich Histones from Chicken Erythrocytes—Blood (approximately 50 ml each) was obtained from several 6-month-old roosters by heart puncture and collected into tubes containing 5 ml of heparin (1 mg/ml). Nuclei were prepared (33), and lysine-rich histones (H1 and H5) were extracted from purified nuclei with 5% perchloric acid (34). The acid-soluble histones were dialyzed extensively against water (Milli Q-purified), lyophilized, and weighed.

Fractionation of Lysine-rich Histones by FPLC Chromatography—Initially, the H1 proteins were purified from the lysine-rich mixture (which also contains H5 and high mobility group proteins) by FPLC chromatography on a strong cation exchange column (Pharmacia, Mono S HR 5/5). The column was equilibrated with 50 mM potassium phosphate buffer, pH 6.5. Five to ten mg of protein was loaded on the column and eluted with a gradient of 0-14% guanidinium hydrochloride (GdmHCl) in 50 mM phosphate, pH 6.7, over 15 min. The flow rate was 1 ml/min, and the eluate was monitored at 220 nm. The H1 proteins were pooled, dialyzed against water, concentrated using a Savant Speed-Vac, and used for further fractionation.

The pooled H1 proteins were further fractionated into subtypes on the same FPLC cation exchange column. Four to six mg of protein was loaded onto the column in 50 mM potassium phosphate buffer, pH 6.5, containing 2.8% GdmHCl. Fractionation into subtypes was achieved by elution with a shallow gradient of 4.5-5% GdmHCl over 30 min at a flow rate of 1 ml/min. All subtypes were pooled, dialyzed, and concentrated as described above.

Polyacrylamide Gel Electrophoresis—Purified lysine-rich histones were analyzed on 15% polyacrylamide SDS gels as previously described (35).

The histone H1 subtypes were separated on acid/urea polyacrylamide gels according to the method of Fan and Chalkley (23) with some modifications. The separating gel (200 x 1 mm) was 15% acrylamide, and a 6% acrylamide stacking gel was used. Following pre-electrophoresis overnight at 100 V, the samples (5 mg//track for purified subtypes and 20 mg//track for total Hls) were applied and electrophoresed at a constant voltage of 150 V for 72 h. The gel was stained in 1% Coomassie Blue R-250.

Preparation of H1 Subtype Peptides for Sequencing—Each purified H1 subtype was digested with V8 protease (Endo-Glu-C) from Staphylococcus aureus to generate peptides for sequencing. Digestion of 100-200 μg of protein in 100 μl of 10 mM potassium acetate, pH 4.5, 1

1 The abbreviations used are: FPLC, fast protein liquid chromatography; SDS, sodium dodecyl sulfate; HPLC, high performance liquid chromatography; GdmHCl, guanidinium hydrochloride.

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was carried out at 37 °C for 15 min with 3 μg of protease. The digested samples were loaded directly onto an HPLC reverse phase column (Brownlee Guard Cartridge, C-18 300-Å pore, 10 μm particle size) which was equilibrated with water containing 0.1% trifluoroacetic acid. The elution buffer (B) was acetonitrile (Waters) containing 0.05% trifluoroacetic acid. The peptides were eluted with a gradient of 0-35% B over 35 min. Individual peptide peaks were collected, rechromatographed to ensure purity, and dried (Savant Speed-Vac) for sequencing. Purified peptides were subjected to amino-terminal amino acid sequencing using an Applied Biosystems microsequencer.

RESULTS

Resolution of H1 Subtype Proteins on Acid-urea Polyacrylamide Gels—The histone H1 proteins from chicken erythrocytes have previously been resolved into four subtypes by electrophoresis on acid-urea polyacrylamide gels (26). The modifications made here (see "Materials and Methods") to the Panyim and Chalkley gel system have allowed us to visualize six H1 subtypes from chicken erythroid cells (Fig. 1). These subtypes have been named as illustrated in Fig. 1. Previous reports (26, 27, 30) have shown four H1 subtypes in chickens usually named H1-a to HI-d. Naming the subtypes here, HI-a and HI-a' and HI-c and HI-c' does not indicate a closer relationship between these subtypes, but simply serves as a convenience when comparisons are made to previous reports.

The gel track was scanned with a laser densitometer to determine the ratios of the H1 subtypes present in erythroid cells. A typical densitometer scan is illustrated in Fig. 1. Histone H5 (not illustrated) makes up 60–65% of the lysine-rich histones in erythrocytes. In the estimates of the other H1 subtype ratios, H-1a and H1-a' and H1-c and H1-c' are considered together since they are not sufficiently well separated on the gels to be considered as separate peaks by the densitometer integrator. However, Fig. 1 clearly illustrates the presence of the six subtypes. H1-a/H1-a' comprises 36–38% of the total H1s, with H1-b representing 20%, H1-c/H1-c' 23%, and H1-d 18–19%.

Fractionation of H1 Subtypes by FPLC Chromatography—Nuclei from 50 ml of chicken blood yielded 40–50 mg of crude perchloric acid-soluble protein. The H1 proteins were readily purified from H5 and other contaminants on an FPLC cation exchange column (Fig. 2). Polyacrylamide-SDS gel electrophoresis of the column peaks confirmed that the H1 proteins were free from other contaminants and that the two H1 peaks (2 and 3 in Fig. 2) were enriched for different H1 subtypes. Approximately 30% of the crude acid extract elutes with the H1 peaks.

Further fractionation of the H1 subtypes was achieved using a very shallow GdmHCl gradient to elute the H1 proteins from the column. Optimum conditions for subtype purification, as outlined under "Materials and Methods," were determined. Fig. 3A illustrates a typical profile obtained when 1 mg of purified H1 protein is chromatographed using an elution gradient of 4.5–5% GdmHCl. Five peaks, labeled P1 to P5, were consistently obtained. Further separation of P1 and P2 was achieved using a shallower GdmHCl gradient.

Aliquots from the pooled peak fractions were electrophoresed on 18% acid-urea polyacrylamide gels. Peaks P1, P3, P4, and P5 each contained a pure H1 subtype (Fig. 3B). Table I indicates the protein subtype(s) contained in each of the
**Fig. 3. Purification of histone H1 subtypes by FPLC.** A. 1 mg of histone H1 protein was chromatographed on a cation exchange FPLC column. The protein was fractionated into peaks labeled P1-P5 by elution with a linear gradient (4.5-5%) of GdmHCl in 50 mM potassium phosphate, pH 6.5. - - - - % GdmHCl. B, aliquots from peaks P1-P5 were electrophoresed on an 18% polyacrylamide acid/urea gel for 72 h at 150 V. Lane 1, total H1 protein; lane 2, P1; lane 3, P2; lane 4, P3; lane 5, P4; lane 6, P5. The H1 protein subtypes are labeled H1 a-d, in order of mobility on the gel.

**Table I**
Alignment of purified H1 subtypes with their respective genes

| FPLC peak | H1 subtype | H1 gene |
|-----------|------------|---------|
| P1        | H1-a       | 11L     |
| P2        | H1-a       | 11L     |
|           | H1-a'      | (11R)   |
|           | H1-b       | 03      |
| P3        | H1-c       | 0.10    |
| P4        | H1-d       | 02      |
| P5        | H1-c'      | 01      |

The pure H1 subtype proteins obtained by FPLC column chromatography were digested with V8 protease, and the resulting peptides were separated by HPLC chromatography. The predicted amino acid sequences of the H1 proteins (31, 32, 40) indicate that V8 protease would generate seven peptides from each protein product, except for the product of gene 11L (see Fig. 4) which has an extra glutamate residue.

From the gene sequences, we can predict which peptides will contain the most amino acid changes between subtypes. Therefore, in order to assign a protein subtype to a gene, the peptides whose elution profiles changed from one subtype to another were initially chosen for sequencing. The FPLC peak P2 is a mixture of H1-a, H1-a', and H1-b. H1-a is obtained in pure form in peak P1. The V8 peptides common to P1 and P2 (Fig. 5, A and B) were therefore derived from H1-a. By sequencing the abundant peptides unique to P2 (indicated with *arrows* in Fig. 5A), we could align subtype H1-b with the H1 gene from clone 03. Since H1-a' is present at very low levels in P2, its sequence was not obtained from this material. We have matched five H1 subtypes with H1 genes and can assume that H1-a' is the product of the sixth gene, 11R, but this needs further verification.

Fig. 4 shows the predicted amino acid sequences of the six peaks from the FPLC column. Thus, H1-a, H1-c, H1-c', and H1-d were obtained as pure preparations. Peak P2 contained both H1-a and H1-b, but when P1 and P2 are well separated, H1-b is enriched in the P2 fraction. H1-a' is present in erythrocytes as a minor component and has proven impossible to purify using cation exchange chromatography as it elutes in the P2 fraction.

Assignment of Each H1 Protein Subtype to an H1 Gene—
Six histone H1 subtypes have been identified from chicken erythrocytes, and each subtype can be aligned with the predicted amino acid sequence of a chicken H1 gene. Previous reports have shown four H1 subtypes in both erythrocytes and other chicken tissues (17, 18, 28, 29). These subtypes were generally assigned the names H1-a-d, in order of increasing mobility on acid/urea polyacrylamide gels (23). By modifying the Panyim and Chalkley gel system, we have identified two extra subtypes giving a total of six H1 subtypes in chicken erythrocytes.

The ratios of the major H1 subtype groups in chicken erythrocytes, i.e. a-d, are similar to those previously reported (17, 29). H1-a is the most abundant subtype in erythrocytes, whereas in other fully differentiated chicken tissues, H1-a is present at low levels, and the relative level of H1-c is greatly increased (18, 29, 30). Similar results have been obtained with nondividing chicken cells in culture (36). It has been suggested that H1-c may be functionally homologous to H1" from mammalian tissues (18, 36). Since erythrocytes contain large quantities of the histone H5 which again has been equated with H1" (20, 24, 37), the need for H1-c in this tissue may be replaced by H5. In view of the fact that H1-c can now be resolved into two proteins arising from two independent genes, it would now be possible to distinguish which of these two proteins is responsible for the relative increase in H1-c during differentiation of certain chicken tissues.

The availability of purified subtypes will allow these studies to be refined. We have purified four H1 subtypes to homogeneity on an FPLC cation exchange column, i.e. H1-a, H1-c, H1-c', and H1-d. By sequencing peptides from the pure subtypes and from a fraction enriched for H1-b, we have shown that each subtype protein is the product of a defined chicken H1 gene (40). Purified subtypes could also...
be produced in large quantities in suitable Escherichia coli expression systems.

The assignment of each H1 protein subtype to its respective H1 gene now enables us to investigate the control mechanisms operating on the expression of a set of closely related genes.

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REFERENCES
1. Thoma, F., Koller, T., and Klug, A. (1979) J. Cell Biol. 83, 403–427
2. McGhee, J. D., Rau, D. C., Charney, E., and Felsenfeld, G. (1980) Cell 22, 87–96
3. Thomas, J. O. (1985) in Eukaryotic Genes, Their Structure, Activity and Regulation (MacLean, N., Gregory, S. P., and Flavell, R. A., eds) pp. 9–30, Butterworth & Co., Ltd., London
4. Thomas, J. O. (1984) J. Cell Sci. Suppl. 1, 1–20
5. Reeves, R. (1984) Biochim. Biophys. Acta 782, 343–393
6. Cole, K. D., York, R. G., and Kistler, W. S. (1984) J. Biol. Chem. 259, 13695–13702
7. Von Holt, C., De Groot, P., Schwager, S., and Brandt, W. F. (1984) in Histone Genes, Structure, Organization and Regulation (Stein, G. C., Stein, J. L., and Marzluff, W. F., eds) pp. 65–105, John Wiley & Sons, New York
8. Risley, M. S., and Eckhardt, R. A. (1981) Dev. Biol. 84, 79–87
9. Lennox, R. W., and Cohen, L. H. (1983) J. Biol. Chem. 258, 262–268
10. Cole, R. D. (1977) in The Molecular Biology of the Mammalian Genetic Apparatus (Tw'o, P., ed) pp. 93–104, Elsevier/North-Holland, New York
11. Cole, R. D. (1984) Anal. Biochem. 136, 24–30
12. Bustin, M., and Cole, R. D. (1968) J. Biol. Chem. 243, 4500–4505
13. Kinkade, J. M., Jr. (1969) J. Biol. Chem. 244, 3375–3386
14. Hohmann, P. (1980) Arch. Biochem. Biophys. 205, 198–209
15. Harrison, M. F., and Wilt, F. H. (1982) Exp. Zool. 223, 245–256
16. Maxon, R., Cohn, R., and Kedes, L. (1983) Annu. Rev. Genet. 17, 239–277
17. Appels, R., Wells, J. R. E., and Williams, A. F. (1972) J. Cell Sci. 10, 47–59
18. Winter, E., Levy, D., and Gordon, J. S. (1985) J. Cell Biol. 101, 167–174
19. Hohmann, P., Bern, H. A. A., and Cole, R. D. (1972) J. Natl. Cancer Inst. 49, 355–360
20. Gjerset, R., Gorka, C., Hasthorpe, S., Lawrence, J. J., and Eisen, H. (1982) Proc. Natl. Acad. Sci. U. S. A. 79, 2333–2337
21. Tan, K. B., Borun, T. W., Charpentier, R., Christofalo, V. J., and Croce, C. M. (1983) J. Biol. Chem. 257, 5337–5338
22. Bannow, G. A., and Gorovsky, M. A. (1984) in Histone Genes, Structure Organization and Regulation (Stein, G. C., Stein, J. L., and Marzluff, W. F., eds) pp. 163–173, John Wiley & Sons, New York
23. Panyim, S., and Chalkley, R. (1969) Arch. Biochem. Biophys. 130, 337–346
24. Pehrson, J. R., and Cole, R. D. (1982) Biochemistry 21, 456–460
25. Huang, H.-C., and Cole, R. D. (1984) J. Biol. Chem. 259, 14237–14242
26. Roche, J., Gorka, C., Goeltz, P., and Lawrence, J. J. (1985) Nature 314, 197–198
27. Dupressoir, T., and Sautiere, P. (1984) Biochem. Biophys. Res. Commun. 122, 1136–1145
28. Harborne, N., and Allan, J. (1966) FEBS Lett. 194, 267–272
29. Panyim, S., Bilek, D., and Chalkley, R. (1971) J. Biol. Chem. 246, 4206–4215
30. Berdikov, V. A., Goren, G., Argutinskaya, S. V., Cherepanova, V. A., and Kileva, E. V. (1975) Mol. Biol. (Mosc.) 10, 887–896
31. Cole, L. S., and Wells, J. R. E. (1985) Nucleic Acids Res. 13, 585–594
32. Sugarman, B. J., Dodgson, J. B., and Engel, J. D. (1983) J. Biol. Chem. 258, 9005–9016
33. Dingman, C. W., and Sporn, M. B. (1964) J. Biol. Chem. 239, 3483–3492
34. Johnson, L. D., Driedger, A., and Marks, A. (1964) Can. J. Biochem. 42, 785–811
35. Shannon, M. F., Wigley, P. L., and Wells, J. R. E. (1985) FEBS Lett. 186, 180–186
36. Smith, B. J., Cook, Y., Johns, E. W., and Weiss, R. A. (1981) FEBS Lett. 135, 77–80
37. Cary, P. D., Hines, M. L., Bradbury, E. M., Smith, B. J., and Johns, E. W. (1981) Eur. J. Biochem. 120, 371–377
38. Hannon, R., Bateman, E., Allan, J., Harborne, N., and Gould, H. (1984) J. Mol. Biol. 180, 131–149
39. Marion, C., Roche, J., Roux, B., and Gorka, C. (1985) Biochemistry 24, 6328–6335
40. Cole, L. S., Robins, A. J., Madley, L. K., and Wells, J. R. E. (1987) J. Biol. Chem. 262, 9656–9663