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Comparison of the Major Polypeptides of the Erythrocyte Nuclear Envelope

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The three most abundant nonhistone polypeptides (molecular weights 75,000, 71,000 and 61,000) of the avian erythrocyte nucleus have previously been isolated in the nuclear envelope fraction. They have been separated by sodium dodecylsulfate-polyacrylamide gel electrophoresis and peptide-mapped after limited enzymatic digestion. Three enzymes – chymotrypsin, papain and Staphylococcus aureus protease –were used. Results obtained with each enzyme indicate strong similarities between the three nuclear envelope polypeptides. The amino acid compositions of the two most abundant polypeptides (P75 and P71) have been determined and found to be similar. Further, they readily yield large fragments upon brief alkaline hydrolysis. For both P75 and P71 the degree and the pattern of alkaline fragmentation are almost identical. A 61,000-dalton polypeptide which appears to be P61 is obtained from P75 and P71 by mild acid hydrolysis. These results establish the close chemical similarity of these predominant polypeptides in the erythrocyte nucleus and suggest that they serve related functions.

Key words: nuclear envelope polypeptides, chemical and enzymatic digestion

INTRODUCTION

The avian erythrocyte nucleus has a number of properties which make it a useful system for correlating nonhistone proteins with nuclear structure. Erythrocyte nuclei yield relatively simple nonhistone polypeptide electropherograms with much of the protein in the MW 61,000–75,000 range [1, 2]. Further, the predominant polypeptides of this nucleus [3, 4] are located in the nuclear envelope fraction and, indeed, appear to be nuclear pore complex-fibrous lamina fraction components (compare with refs 5–8) because they are of appropriate molecular weight and are not dissolved by detergent washes [9, 10]. This relative insolubility reflects strong, specific protein-protein interactions and has been confirmed in the chicken erythrocyte by polypeptide cross-linking studies using both bifunctional imidates [11] and disulfide bond formation by oxidation of intrinsic sulfhydryls [10]. In particular the two predominant polypeptides occur in regular oligomers,

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each yielding homogeneous polymers when cross-linked in the envelope [10]. These polypeptides probably serve a common function in eukaryotes because homologous polypeptides occur in rat liver and in HeLa cell nuclei [12].

Further characterization of these polypeptides could provide insight into nuclear architecture, particularly in the perinuclear region. Unfortunately, the relative insolubility of these polypeptides and their large and similar molecular weights (75,000 and 71,000) make their isolation and characterization difficult. A promising approach to the study of such proteins has been to fragment them with proteolytic enzymes active in the presence of sodium dodecylsulfate (SDS). Fragments characteristic of each digested polypeptide were identified by SDS-polyacrylamide gel electrophoresis [13]. We have separated three polypeptides – P75, P71 and P61 (identified by approximate molecular weight) by electrophoresis and extracted the polypeptides from gel slices. Mild acid [14] and alkaline hydrolysis, enzymic digestion, and amino acid analysis indicated a close sequence similarity between these three polypeptides.

MATERIALS AND METHODS

Materials

Acrylamide was obtained from Bio-Rad; N,N′-methylenebisacrylamide, ammonium persulfate, 2-mercaptoethanol, and N,N,N′,N′-tetramethylethylene diamine were obtained from Eastman Kodak; Coomassie brilliant blue R, deoxyribonuclease I, and Tris were obtained from Sigma Chemical Co. Sodium dodecylsulfate (BDH specially pure) was obtained from Gallard-Schlesinger Co. Triethanolamine was obtained from Fisher Scientific Co. Triton X-100 was obtained from J.L. Baker Chemical Co. All other chemicals were reagent grade. Chymotrypsin (Type I-S) and papain (Type III) were obtained from Sigma Chemical Co., and staphylococcus V8 protease from Miles Laboratories.

Protein controls for alkaline hydrolysis were purchased from commercial sources. Horse plasma transferrin (pooled) was purchased from U.S. Biochemicals Corp. Bovine liver glutamate dehydrogenase (crystalline) was obtained from Boehringer Mannheim. Bovine serum albumin (Fraction V), ovalbumin (grade V), conalbumin (Type II), soybean trypsin inhibitor (Type 1-S), cytochrome c (Type III) and myoglobin (Type II) were obtained from Sigma Chemical Co. Protein was dissolved in distilled water and dialyzed against sample buffer over night.

Preparation of Nuclear Fraction

Isolation of chicken erythrocyte nuclei and of a nonionic detergent-washed nuclear envelope fraction has been described [10]. This procedure has been modified slightly by the addition of another DNase treatment and high salt extraction after the detergent wash. These additional steps tend to eliminate histone contamination.

Analytical Methods

Protein samples in 4% SDS were heated in a boiling water bath for 2–3 minutes and subsequently dialyzed over night against 10 mM sodium phosphate (pH 7.2)/ 0.1% sodium dodecylsulfate. Protein was stored in this buffer at −20°C. Protein was determined by the method of Lowry et al [15]. Electrophoresis was performed in cylindrical gels according to the method of Shapiro et al [16]. Gels were stained, destained, and scanned spectrophotometrically as described previously [3].

Recovery of Polypeptides

Polypeptides were extracted from unstained gels. The protein to be purified was first reduced for 20 minutes in 5.0% 2-mercaptoethanol prior to separation. After the electrophoretic step, the bands containing P75, P71, and P61 were visualized in cold 100 mM KCl [17], sectioned out, washed in distilled water, and crushed in syringes. The crushed material from 11 gel slices was extracted with 3.0 ml of 10 mM sodium phosphate (pH 7.2)/5 mM sodium azide/0.10% sodium dodecylsulfate/1.0% 2-mercaptoethanol at 37°C. Extraction times varied from 5 to 15 h. Polyacrylamide was removed by low-speed centrifugation and washed with 2.0 ml of extraction buffer. The extracts were combined and filtered through fine glass wool.

Amino Acid Analysis

P75 and P71 were purified by two electrophoresis steps (a total of 7 h at 4.7 V/cm). Samples of extracted P75 and P71 used for amino acid analysis are shown in Figures 1b and 1c. The remainder of the extracts was lyophilized. The lyophilized samples were washed as described by Bray and Brownlee [18] prior to performic acid oxidation [19] and hydrolyzed for 22 h at 110°C with 5.7 N HCl prior to analysis on a Beckman 121 M amino acid analyzer.

Digestion of Extracted Polypeptides

Both single and mixed samples of extracted polypeptides were routinely examined by analytical electrophoresis. Individual polypeptides were exposed to proteolytic digestion by each of three enzymes: chymotrypsin, Staphylococcus aureus V8 protease, and papain. Each extracted polypeptide was incubated over night in a 37°C water bath with amounts of enzyme as indicated in figure legends. Reactions were terminated by electrophoresis on analytical gels.

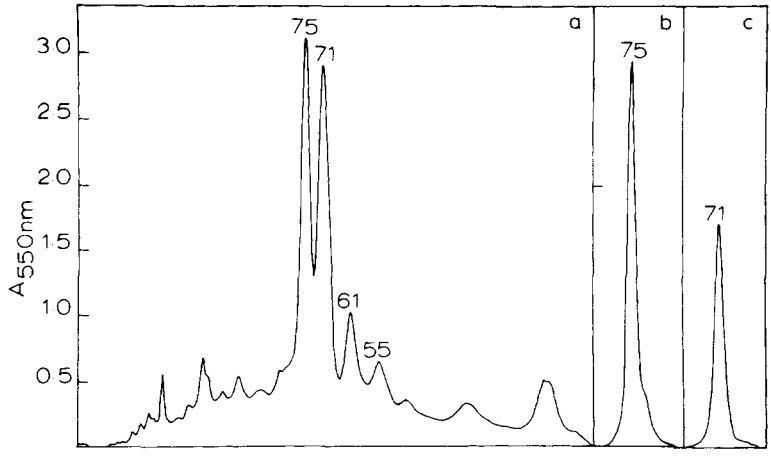

Fig. 1. Electropherogram of the Triton-insoluble nuclear envelope protein from chicken erythrocytes. The polypeptides (15 μg) were prepared, separated, and stained as described under Methods (a). The numbers represent the molecular weights of the associated peaks in thousands. P75 (b) and P71 (c) were isolated as described under Methods and hydrolyzed for amino acid determination (see Table I).

Peptide Mapping

Analysis of peptides generated by enzymatic proteolysis was performed on 15% polyacrylamide gels containing 0.5 M urea [20]. The digests were subjected to electrophoresis for 4 h at 5 mA per gel. Staining and destaining have been previously described [3].

RESULTS

Only a few polypeptides constitute much of the Triton-insoluble protein of the avian erythrocyte nuclear envelope fraction. These are identified by their approximate molecular weights in Figure 1: P75, P71, P61, and P55. The P71 band is broadened by a small amount of P68, a polypeptide which occurs in small and variable amounts in preparations from chicken erythrocytes (compare Shelton [14] and Shelton and Cochran [10]). For isolation of polypeptides, gels were loaded with amounts of protein ranging from that shown in Figure 1 to approximately twice as much. In Figures 1b and 1c, examples of P75 and P71 isolated under the most stringent conditions are shown. These samples were subjected to two electrophoretic steps before extraction. Their amino acid compositions are presented in Table I and are similar. Preliminary proteolytic digestions had suggested some homology between P75, P71, and P61 [21].

TABLE I. Amino Acid Composition of Chicken Erythrocyte P75 and P71*

Amino acid	Background		Nanomoles P75		P71		Mole percent P75	P71
Lys	0.29	0.30	0.54	0.54	0.81	0.81	4.0	3.7
His	0.07	0.06	0.10	0.18	0.24	0.25	1.2	1.3
Arg	0.08	0.04	0.54	0.52	0.79	0.77	7.8	5.2
Asx	0.27	0.28	0.91	0.82	1.67	1.58	9.8	9.7
Thr	0.11	0.11	0.38	0.33	0.81	0.72	4.2	4.8
Ser	0.25	0.24	0.82	0.79	1.66	1.58	9.3	9.9
Glx	0.30	0.30	1.33	1.31	2.62	2.55	16.9	16.5
Pro	–	–	0.27	0.22	0.53	0.46	4.2	3.6
Gly	0.46	0.45	1.01	1.02	2.08	2.08	9.1	11.7
Ala	0.20	0.18	0.73	0.72	1.27	1.26	9.0	7.8
Cys	0.29	0.29	0.29	0.39	0.29	0.29	0.8	[a]
Val	0.12	0.12	0.35	0.34	0.80	0.81	3.8	5.0
Met	–	–	0.24	0.25	0.39	0.36	4.2	2.7
Ile	0.09	0.09	0.26	0.27	0.63	0.63	3.0	3.9
Leu	0.19	0.19	0.86	0.84	1.54	1.53	11.0	9.7
Tyr	0.06	0.06	0.15	0.14	0.39	0.40	1.5	2.5
Phe	0.10	0.07	0.11	0.17	0.35	0.41	0.8	2.1

*Experimental details are presented under Methods. Duplicate determinations were performed on all samples. Background was measured in extracts from an acrylamide gel which received all treatments. Duplicate determinations were averaged and values were corrected for background before calculations of mole percent. Nanomole values are provided as a measure of experimental variation.

[a]It is unlikely that P71 is devoid of cysteine because of its formation of dimers sensitive to disulfide reduction [10, 12]. The zero value probably results from the high-background cysteine, the small amount in these polypeptides, and the limited amount of purified protein available.

Enzymic Digestion

In view of these results, a more extensive mapping study was undertaken. For these studies up to 30 μg of protein was loaded on each preparative gel and polypeptides were extracted after one electrophoretic step. An analysis of the material is presented in Figure 2. P75 and P71 reveal only traces of cross-contamination. Mixtures of the three extracts yielded the expected three bands with the correct mobilities. Digestion products were examined on 15% polyacrylamide. Polypeptide controls for these digestions were incubated over night without enzyme (Fig. 2). In some experiments the controls contained small amounts of degradation products but these did not correspond with enzyme products (results not shown). In this experiment sufficiently more P61 extract was used to give an intensity of bands approximately equal to P75 and P71.

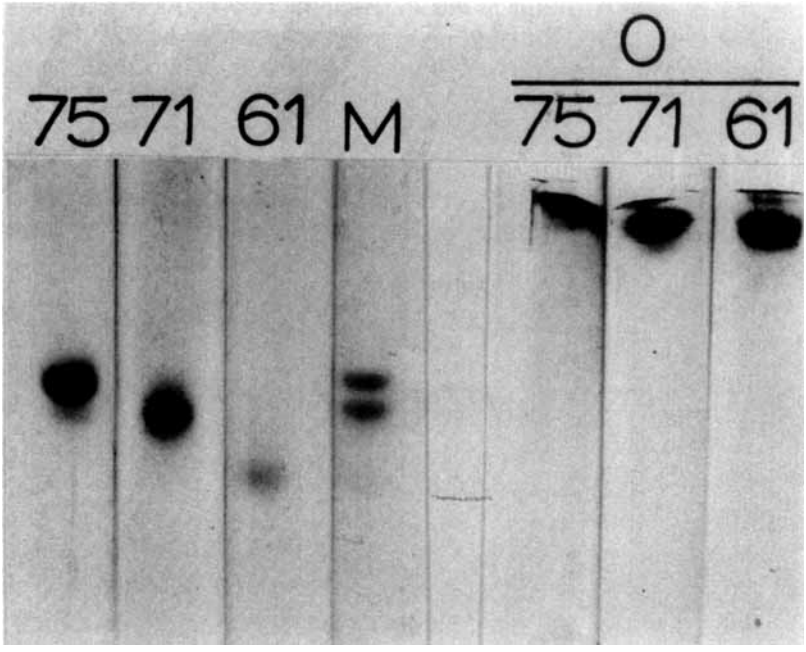

Fig. 2. Enzymatic digestion control polypeptides. Sample protein (25–30 μg) was applied to 5% polyacrylamide gels and the polypeptides separated by electrophoresis for 3.5 h at 4.7 V/cm. After the electrophoresis, the polypeptides were extracted as described under Methods. The extracted polypeptides were first examined by electrophoresis on 5% polyacrylamide gels. In the first four gels the gels are identified by the approximate molecular weight of the polypeptide (75, 71, and 61) or by M where the samples were mixed on a single gel. In this preparation P75 and P71 each contain a trace of the other. Peptides obtained by enzymic digestion were analyzed on 15% polyacrylamide gels as described under Methods. Controls were incubated without enzyme and are presented above. The number above the horizontal line indicates the quantity of proteolytic enzyme (0 in this figure) and the numbers below the line identify the polypeptide subjected to digestion. As will be seen in Figures 3–5, appearance of protein through most of the gel was enzyme-dependent. In some experiments the control polypeptides suffered some degradation as evidenced by slightly faster minor bands, but these did not compromise the digestion products presented in Figures 3–5. It should be noted that P61 (gel under 61) partially resolves into two bands in the 15% gels.

Results of papain digestions are presented in Figure 3. The enzymic products from each of the three polypeptides were nearly identical. Four product bands were obtained from P75. The largest of these may appear as a trace from P71. The other three bands can be detected in the proteolytic products from P75, P71, and P61. Papain (50 and 100 pg) alone gave no visible bands. Of the three enzymes used, papain gave the most complete digestion. Common digestion products were also obtained with Staphylococcus aureus protease (Fig. 4). These included a major product (large arrow) and several minor products (small arrows). Control gels ruled out the contribution of protein bands by the protease.

Chymotrypsin digestion (Fig. 5) yielded two common major fragments from each polypeptide (large arrows). Several minor fragments migrating below the arrows also appeared to be common to P75 and P71 (small arrows). Undigested material and some aggregated protein is detectable above the arrows. Additional chymotrypsin (5 μg) increased the products and decreased the starting bands and aggregates. The two lowest-molecular-weight products from P71 migrated more slowly than comparable bands from P75 (vertical bars). The same behavior was observed with insulin, which was included as an internal standard in some experiments (results not shown). Because of this anomaly, the bottom, strongly staining peptides obtained from P61 by digestion with 2 μg chymotrypsin may not be unique but rather reflect the altered migration. Only faint bands are attributable to digested chymotrypsin.

Chemical Cleavage

In the course of examining the erythrocyte nuclear polypeptides, the effect of heat at different pH's was investigated (Fig. 6). Polypeptide electropherograms typical of the material

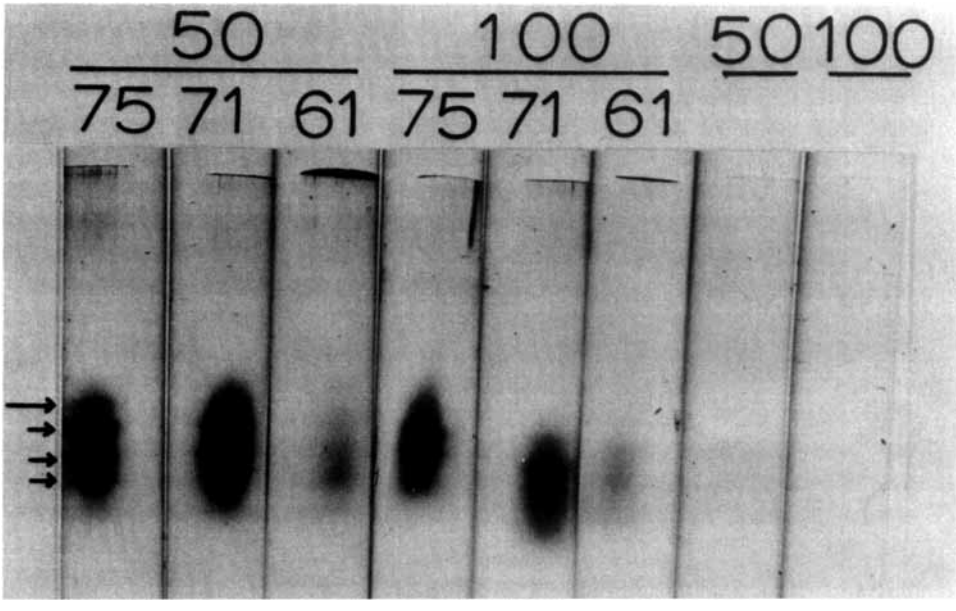

Fig. 3. Papain digestion of P75, P71, and P61. Polypeptides prepared as described under Figure 2 were used in this experiment. The papain (pg) used in each set of digestions is shown over the horizontal bars. Papain incubated alone showed little staining. Three polypeptide products (small arrows) and possibly a fourth (large arrow) were common to the three nuclear polypeptides.

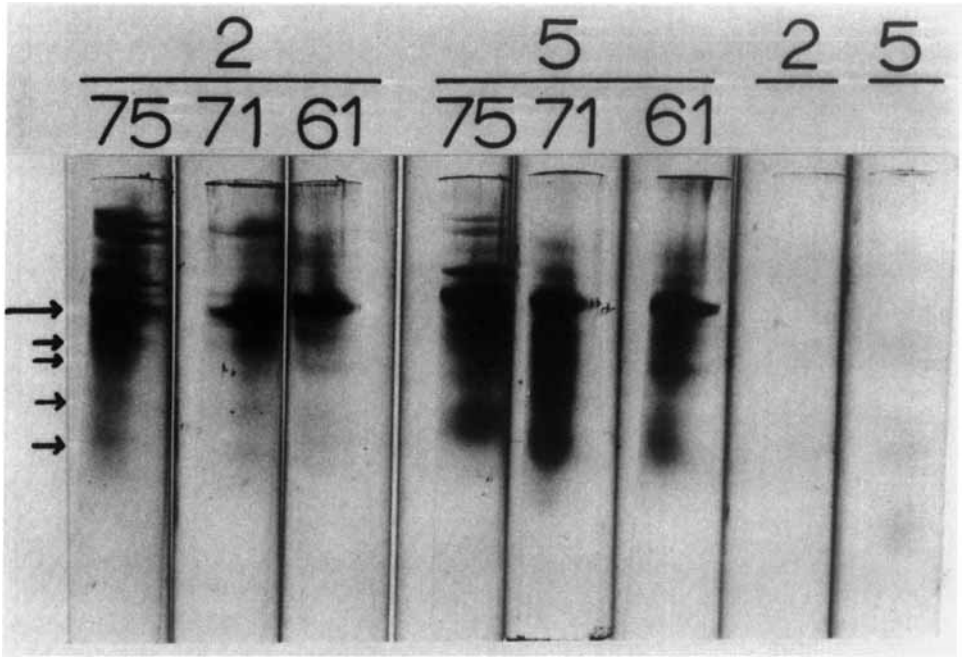

Fig. 4. Staphylococcus aureus V8 protease digestion of P75, P71, and P61. All conditions were described under Figure 2 except for the protease concentration, which was in micrograms. All three polypeptides yielded a common major product (large arrow) and four common minor products (small arrows).

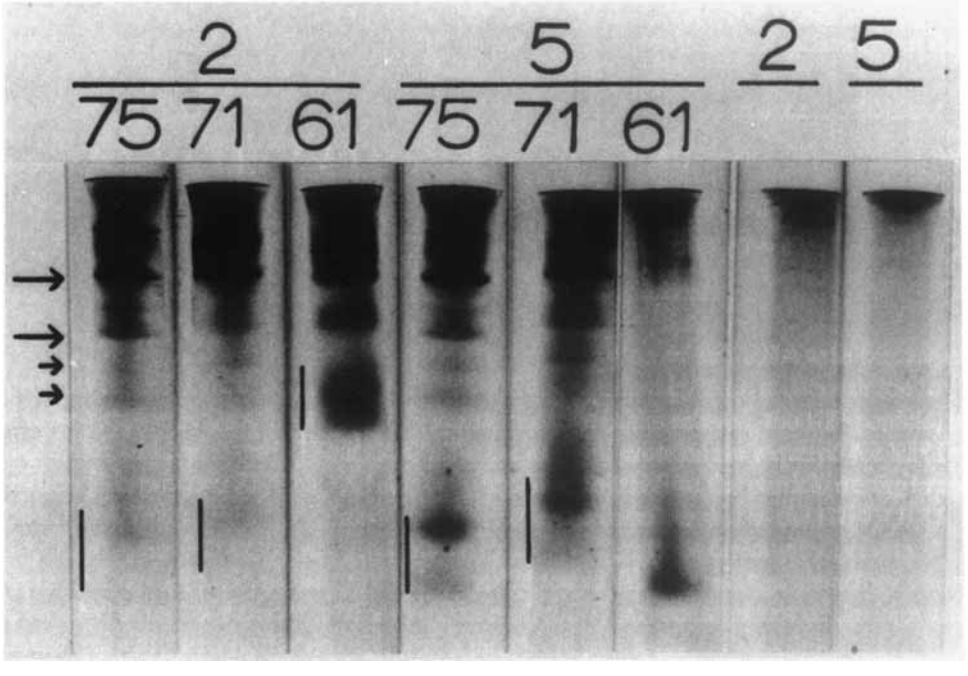

Fig. 5. Chymotryptic digestion of P75, P71, and P61. All conditions were the same as those described under Figure 4. Common major products are indicated by the largest arrows. Two minor products from P75 and P71 are indicated by small arrows. Two bands occur beside the vertical bars. These may be common bands. Migration in this region varied in the chymotrypsin digestion studies. Insulin added as an internal standard also showed anomalous migration (results not shown).

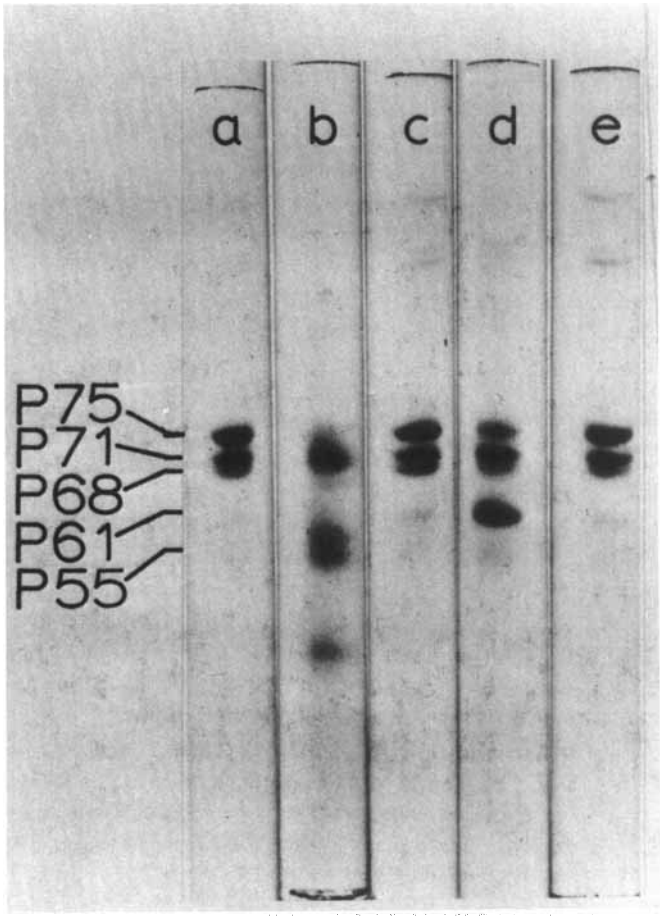

Fig. 6. Effect of pH and heat on the stability of the oligomeric nuclear polypeptides. Protein in 10 mM sodium phosphate (pH 7.1)/0.1% sodium dodecyl sulfate was mixed with one-tenth volume 1.0 M sodium phosphate at pH's of either 3.5, 7.2 or 11.0 and then incubated for 6 h at either room temperature or 55°C. Samples were analyzed by electrophoresis on 5% acrylamide gels. The pH and temperature of treatment for each electropherogram were a) 11.0 and room temperature, b) 11.0 and 55°C, c)7.2 and 55°C, d) 3.5 and 55°C, and e) no treatment. The characteristic polypeptides found in untreated samples are identified by their approximate molecular weights in thousands.

isolated in the nuclear fraction from erythrocytes is present under a, c, and e. In Figure 6, it can be seen that heating at a moderately acid pH caused a major increase in P61 (electropherogram d; see also Shelton [14]). In contrast, heating at pH 11 caused a diminution of P75 and P71 and an increase in material at and below P55 (electropherogram b). These conversions were dependent on both heat and alkali (compare control electropherograms a, c, and e).

The alkaline cleavage reaction was tested at higher temperatures for shorter times. At 100°C, the conversion proceeded within minutes and yielded the same products. P61 appeared to be susceptible also (Fig. 7). In alkaline buffers below pH 11 the conversions were markedly less apparent (pH 10) or did not occur (pH's 8 and 9) within 2 minutes (results not presented).

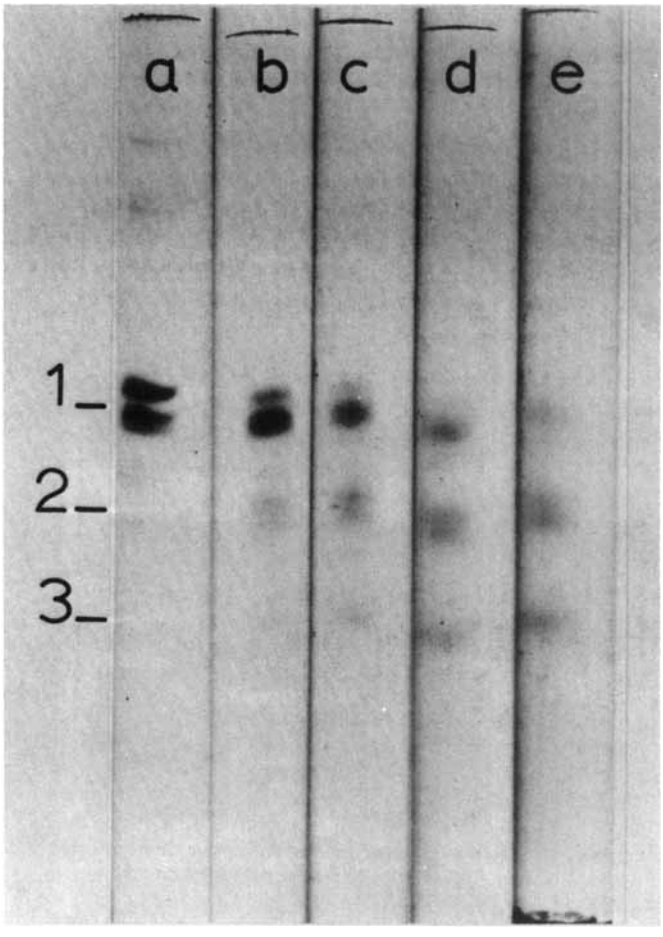

Fig. 7. Time course of breakdown at pH 11.0 and 100°C. Protein was mixed with one-tenth volume 1.0 M sodium phosphate (pH 11.0) as described under Figure 6 and divided among five sealed tubes. One was retained as a zero-time control a) and the other four were dropped into a boiling water bath. These were removed sequentially after 2 b), 4 c), 6 d), and 10 e) min. Samples were analyzed by electrophoresis on 5% acrylamide gels. The numbers indicate the region of P75, P71, and P68 (1), the region of the two closely spaced polypeptides produced by alkali (2), and a third product (3).

The brief alkaline hydrolysis procedure was utilized to test the relatedness of P75 and P71. Protein was extracted before hydrolysis, as described under Materials and Methods. P75 and P71 are compared in Figure 8. The reactivities of the two polypeptides were virtually identical. For each, the two bands under arrow A were apparent within 1 minute and the third major product under arrow B, which was barely discernable at 1 minute, could be easily detected by 3 minutes.

It was of interest to determine how other proteins would respond to this treatment because alkaline hydrolysis is not commonly used for selective cleavage. The results of treating four different proteins for various times are presented in Figures 9 and 10. Of these proteins, only transferrin was as sensitive to alkali as the nuclear polypeptides (Fig. 9). Serum albumin, glutamate dehydrogenase, and ovalbumin were moderately reactive, yielding charac-

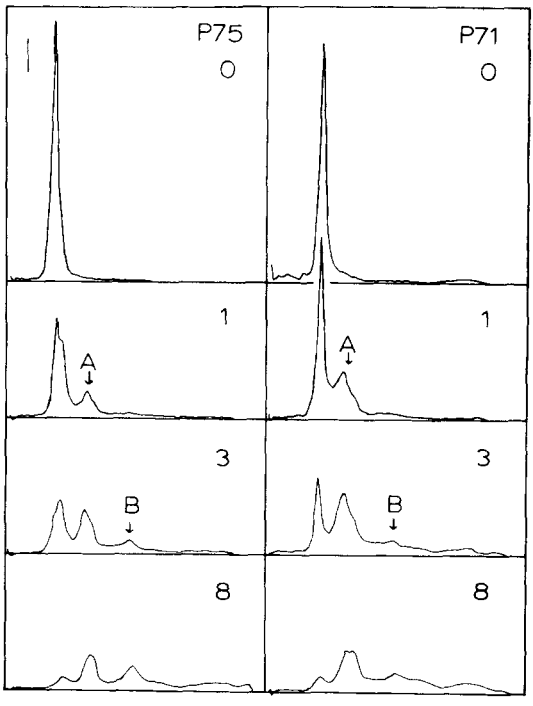

Fig. 8. Electropherograms of polypeptides P75 and P71 after heating at pH 11. P75 and P71 were extracted from gels and dialyzed against 10 mM sodium phosphate (pH 7.1)/0.1% sodium dodecyl sulfate for 6 h. Samples of each (0.9 ml) were mixed with 0.1 ml 1.0 M sodium phosphate (pH 11). Samples (0.2 ml) of each alkaline solution were added to heated tubes in a boiling water bath. Times of heating, in minutes, are indicated in each panel. Treatment was stopped by plunging the tubes into an ice bath. The cooled samples were made 10% in glycerol and the entire sample transferred to 8.5% polyacrylamide gels for electrophoretic analysis. The vertical bar indicates 0.1 A_{550} nm. Major products arising by 1 min are indicated by arrow A; those arising by 3 min, arrow B.

teristic fragmentation patterns within 10 minutes (Figs. 9 and 10). Treatment for 30 minutes led to further breakdown in most instances, but the fragmentation patterns tended to lose useful details. The addition of mercaptoethanol (pH adjusted to 11.0) produced more distinct product bands (compare b and c, Fig. 9). Conalbumin had a comparable sensitivity to these proteins while myoglobin, cytochrome C, and trypsin inhibitor were relatively insensitive (results not shown).

These proteolytic and alkaline digestion studies indicate considerable sequence homology between P75, P71, and P61. These results with P75 and P61 appear to confirm an observation that P61 can be formed from P75 by mild acid hydrolysis [14]. However, it was previously concluded that P71 resisted hydrolysis under the same conditions although P68, a band isolated in some avian preparations, was comparable to P75 in sensitivity. Assuming that peptide material of MW 14,000 is removed from P75 by acid treatment, the sensitive bond(s) must exist in P71 if all P71 sequences occur in P75. (The 4,000 difference between P75 and P71 is too small to include bonds which could yield a 61,000-dalton product from P75.) Accordingly, P71 acid sensitivity was reinvestigated and P68 was carefully excluded from the sample. The results (Fig. 11) indicate that P71 has acid sensitivity. The results were confirmed with polypeptides extracted from gel slices (results not shown).

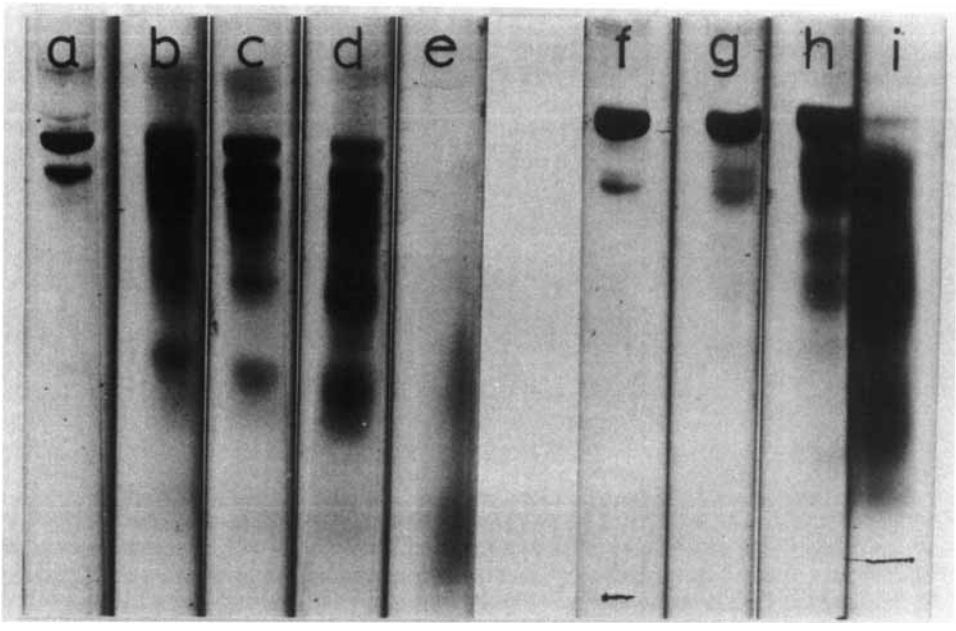

Fig. 9. Alkaline cleavage of transferrin and serum albumin. Horse plasma transferrin (gels a–e) and bovine serum albumin (gels f–i) were examined for alkali sensitivity by boiling for the following times at pH 11: b,c,g) 3 min; d,h) 10 min; e,i) 30 min. The zero-time controls (a,f) contained 5 μg protein, gel g 8.3 μg protein, and all others 12.5 μg protein. In gel b the protein was heated in the absence of 2-mercaptoethanol. The other heated samples were 1% in 2-mercaptoethanol during the heating step.

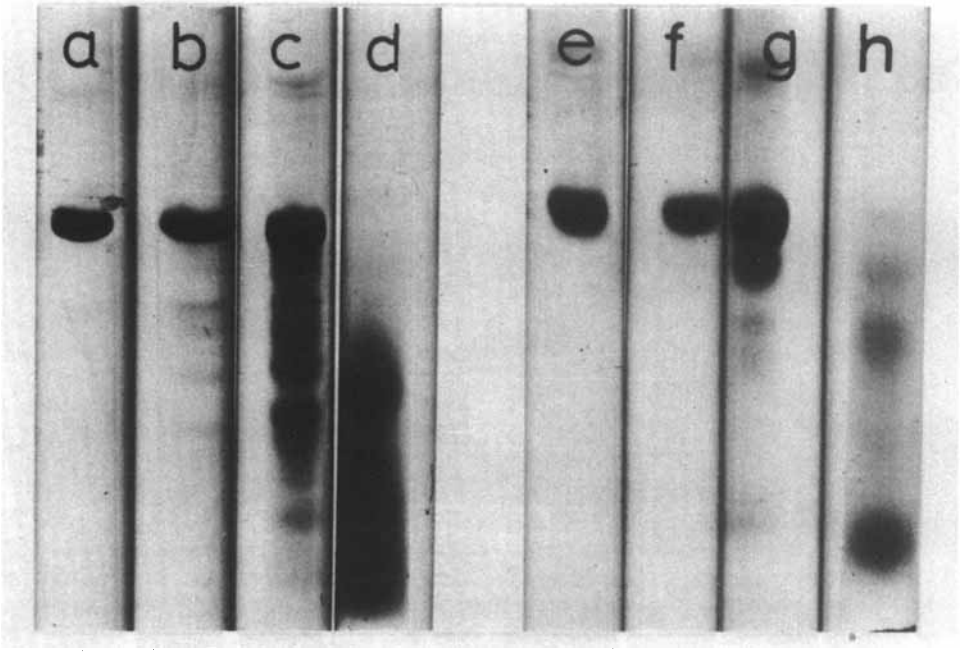

Fig. 10. Alkaline cleavage of glutamate dehydrogenase and ovalbumin. Glutamate dehydrogenase (a–d) and ovalbumin (e–h) were treated as described for serum albumin under Figure 9.

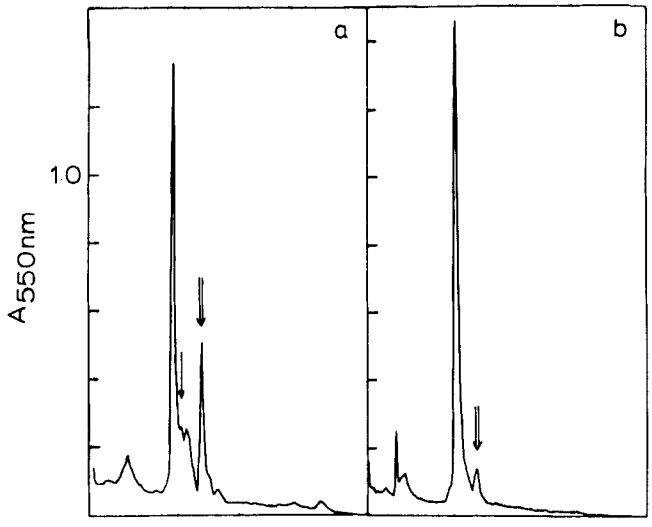

Fig. 11. Acid sensitivity of P75 and P71. Gel slices containing P75 (a) and P71 (b) from 23 μg of sample protein were obtained as described under Figure 2 (all steps prior to extraction), cleared in distilled water for 10 min, washed in 10 mM sodium phosphate (pH 3.5)/0.1% sodium dodecylsulfate, and then boiled in 0.5 ml of the same buffer for 10 min. The polypeptide content of each gel slice was then examined by electrophoresis on 7.5% polyacrylamide gels for 3.5 h at 4.7 V/cm. Unaffected P75 occurs as the major peak in (a). The single arrow in (a) indicates the migration position of P71. The predominant hydrolysis product is indicated by the double arrow. P71 is the predominant peak in (b). A small amount of hydrolysis product is indicated by the double arrow in (b). The peaks indicated by double arrows in both panels migrated identical distances. In both (a) and (b) a small amount of dimeric material, which probably formed during the potassium chloride visualization step, occurred between the main band and the gel top position.

DISCUSSION

The predominant polypeptides of the avian erythrocyte nuclear envelope fraction have been compared by several procedures. Limited proteolysis with three different enzymes indicate that P75, P71, and P61 share regions of sequence homology. In each study some common products were obtained among the more prominent products. Some differences were also revealed, particularly among the higher-molecular-weight products of chymotrypsin and Staphylococcus aureus protease digestion. In assessing these differences it must be noted that the original polypeptides differ in molecular weight and thus the products of minimal digestion are likely to reflect these differences. Alternatively, the differences might arise because of the absence of sequence homology.

The similarity of P75 and P71 is supported by studies of their sensitivity to mild alkali. Both reacted almost completely within 8 minutes and yielded fragments that were quite similar. Each was distinguishable from control proteins. These results are most readily explained if P75 and P71 are chemically related, that is, if they share similar primary structures. The acid-dependent conversion of P75 and P71 to a polypeptide of the same electrophoretic mobility as P61 further suggests the relatedness of the three polypeptides. However, the in vitro mechanism of the acid cleavage is unknown. The acid sensitivity could be due either to a common sensitive peptide bond or to a postsynthetic modification of the polypep-

tides. An important question which arises from both the enzymatic and the chemical studies is whether the similar polypeptides, P75, P71, and P61, arise from the processing of a single gene product or whether they are products of different genes. Possibly, although this does not seem likely, the small differences in electrophoretic migration represent unusually stable shape changes assumed by the same polypeptide.

Similar results, in preliminary form, have been obtained by fingerprinting major polypeptides from the rat liver nuclear matrix [22], a related fraction. Further, immunologic studies of three rat liver nuclear pore complex–fibrous lamina polypeptides indicate that they cross-react [8, 23]. Thus there seems to be little doubt that related polypeptides predominate in the insoluble nuclear envelope fraction.

P75, P71, and P61 do not appear to be products of proteolytic degradation which occurs during isolation. Jackson has obtained polypeptide electropherograms for the chicken erythrocyte nuclear polypeptides similar to that presented in Figure 1 using a different isolation procedure and both phenylmethylsulfonyl fluoride and tetrathionate as proteolytic inhibitors [4]. We obtain the same electropherograms when phenylmethylsulfonyl fluoride is included in the isolation medium (results not shown).

There appear to be more than three related polypeptides in these nuclear fractions. P68 is a polypeptide found in small amounts in some chicken preparations [14] but it is a major polypeptide in similar fractions from rat liver and HeLa cells [12]. Its mild acid sensitivity [14] is similar to P75 and it yields the same proportions of monomer, dimer, and higher polymers as P75 when polypeptides are cross-linked in the HeLa nuclear envelope [12].

The biologic significance of similar polypeptides occurring in the insoluble nuclear envelope fraction is unknown. The simplest explanation is that they have related functions. It should be noted, however, that P75 and P71, cross-linked by disulfide bond formation in isolated nuclear envelope fractions, form only homogeneous polymers and each also yields different amounts of dimer and higher polymers [10]. These observations seem to indicate that there are functional differences between the closely related polypeptides.

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