Evidence for a Species of Nuclear Actin Distinct from Cytoplasmic and Muscle Actins†

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ABSTRACT: Nuclear actin (protein BJ) has been isolated from the chromatin of Novikoff hepatoma ascites cells and purified to homogeneity by selective extraction, Sepharose CL-6B chromatography, and preparative polyacrylamide gel electrophoresis. A comparison of nuclear and cytoplasmic actins from Novikoff hepatoma cells and rabbit muscle actin was made by amino acid analysis, isoelectric focusing/sodium dodecyl sulfate–polyacrylamide gel electrophoresis, and two-dimensional peptide mapping procedures. By these criteria, all of the proteins compared are actins, but each is chemically distinct. It was concluded, therefore, that nuclear actin is similar to, but not identical with, cytoplasmic actin isolated from Novikoff hepatoma cells. A striking similarity in peptide charge and migration as shown by peptide map analysis was observed for nuclear and rabbit skeletal muscle actins. This may indicate that nuclear actin has the capacity for contractile function. In addition, the actins synthesized in Novikoff hepatoma cells may result from more than two structural genes.

When chromatin-associated nonhistone proteins are extracted and separated by two-dimensional polyacrylamide gel electrophoresis, one can resolve as many as 500 individual nuclear proteins (Peterson & McConkey, 1976; Takami & Busch, 1979). Only a few of these nonhistone proteins have been characterized biochemically or functionally. Actin was originally identified in muscle cells and as a major cytoplasmic protein in most nonmuscle cells (Clarke & Spudich, 1977). It is involved in cell movement and maintenance of cell shape. Actin was identified as a component of nuclear extracts by its migration on $NaDodSO_4$–polyacrylamide[1] gels (LeStourgeon et al., 1975; Douvas et al., 1975; Pederson, 1977). Until recently, the nuclear presence of actin was thought to represent the equilibration of cytoplasmic actin between the nucleoplasm and the cytoplasm (Goldstein et al., 1977). Others have suggested that the presence of actin in nuclear fractions is merely due to contamination and the "sticky" nature of nonhistone proteins (Comings & Harris, 1976; Pederson, 1977). However, Fukui & Katsumaru (1979) detected the presence of nuclear actin in situ by demonstrating the production of nuclear actin bundles in dictyostelium and HeLa cells after treatment with Me_2SO [see LeStourgeon (1978) for more information on nuclear actin]. In this paper, we report evidence that the actin associated with chromatin (protein BJ) is similar to cytoplasmic and muscle cell actins, but it has distinct and characteristic peptides. Therefore, nuclear actin does not result from cytoplasmic contamination or from an equilibration of cytoplasmic actin.

Materials and Methods

Preparation of Nuclei. Novikoff hepatoma ascites cells, implanted intraperitoneally 6 days prior to experiments, were collected and washed free of erythrocytes by washing in 140 mM NH_4Cl/10 mM Tris (pH 7.2) according to the method of Boyle (1968). Nuclei were prepared by the 0.025 M citric acid method (Taylor et al., 1973) and suspended in 0.88 M sucrose containing 3.3 mM Ca^{2+}, layered over 1.1 M sucrose/3.3 mM Ca^{2+}, and centrifuged at 1100*g* for 30 min. All solutions contained 1 mM PMSF (Pierce Chemical Co., Rockford, IL). Nuclei prepared by this procedure have been shown to be free of cytoplasmic contaminants (Taylor et al., 1973).

Chromatin Extraction. Chromatin was prepared by washing nuclei with 0.075 M NaCl/0.025 M EDTA (pH 8.0) followed by 0.01 M Tris-HCl (pH 8) as described by Marushige & Bonner (1966). The chromatin was washed twice with 0.6 M NaCl/25 mM sodium phosphate (pH 8.0). The second wash was incubated for 2 h at 4 °C prior to centrifugation. The washed chromatin was centrifuged at 16000*g* for 30 min. The washed chromatin was extracted with 10 volumes of 5 M urea/75 mM sodium phosphate (pH 8.0) and centrifuged at 16000*g* for 30 min. The supernatant was decanted and saved, and the pellet was resuspended in 5 M urea/75 mM sodium phosphate (pH 8.0) and extracted for 18 h at 4 °C. The 5 M urea extracts were pooled and centrifuged at 105000*g* for 4 h. The supernatant fraction was concentrated by ultrafiltration by using a PM-30 membrane (Amicon Corp., Lexington, MA), and 100 mg was applied to a Sepharose CL-6B (Pharmacia Inc., Piscataway, NJ) column (5 × 90 cm) equilibrated with 5 M urea/75 mM sodium phosphate (pH 8.0). The absorbance of the excluded volume fraction was detected by a model UA-5 absorbance monitor (ISCO, Lincoln, NE) at 254 nm and pooled.

Polyacrylamide Gel Electrophoresis. Samples were digested with RNase A, 60 units/mL (Worthington Biochemical Corp., Freehold, NJ), for 48 h at 4 °C and dialyzed twice against 10 volumes of 9 M urea/1 mM PMSF/1% β-ME, and finally against two changes of 9 M urea/0.18 M acetic acid/1% β-ME. The extract was concentrated by ultrafiltration to 5 mg/mL, and 1.0 mL was loaded on a 6% polyacrylamide and 4.5 M urea/0.9 M acetic acid slab gel (10 × 7.5 × 0.3 cm; Ortec Inc., Oak Ridge, TN) and electrophoresed at 120 V for 5 h or 50 V for 12 h. The band corresponding to actin was identified on Amido Black stained side strips and was isolated as previously described (Knecht & Busch, 1971; Goldknopf et al., 1975). Two-dimensional analytical polyacrylamide gel electrophoresis gels were run by the method of Orrick et al. (1973) as modified by Busch et al. (1974). IF/$NaDodSO_4$ analytical gels were run by the method of O'Farrell (1975)

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[1] Abbreviations used: β-ME, β-mercaptoethanol; BPAW, *n*-butanol/pyridine/acetic acid/water (15:12:3:10); Me_2SO, dimethyl sulfoxide; EDTA, ethylenediaminetetraacetic acid; IF, isoelectric focusing; PMSF, phenylmethanesulfonyl fluoride; $NaDodSO_4$, sodium dodecyl sulfate; TLC, thin-layer chromatography; TPCK, L-1-(tosylamido)-2-phenylethyl chloromethyl ketone.

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as modified by Hirsch et al. (1978).

Novikoff Hepatoma Cytoplasmic Actin Isolation. Novikoff hepatoma cytoplasmic actin was isolated by a modification of the method of Pardee & Bamburg (1979). Novikoff hepatoma ascites cells were suspended 1:2 (w/v) in dissociation buffer [2 mM Tris (pH 8.2)/0.2 mM Na_2ATP/0.2 mM $CaCl_2$/0.5 mM β-ME] and homogenized with a Tissumizer (Tekmar Inc., Cincinnati, OH) until the cells were disrupted. This suspension was stirred at 4 °C for 30 min and centrifuged at 108000*g* for 2 h. The supernatant was pooled, concentrated by ultrafiltration to 10% of its original volume, and loaded on a Sephadex G-150 (Pharmacia Inc., Piscataway, NJ) column (25 × 130 cm) equilibrated in the dissociation buffer. The actin-containing fractions were pooled and concentrated by ultrafiltration to 5 mg/mL. The ATP concentration was increased to 1.0 mM, KCl to 0.1 M, and $MgCl_2$ to 10 mM. The pH was adjusted to 7.2, and the actin was polymerized for 2 h at 37 °C. The "assembled" actin was centrifuged at 105000*g* for 2 h at 25 °C. The gelatinous actin pellet was resuspended in dissociation buffer, dialyzed 3 times against 100 volumes of the dissociation buffer, and centrifuged at 100000*g* for 2 h at 4 °C.

Molecular Weight Determination, Amino Acid Composition, and Amino-Terminal Amino Acid Determination. Actins were hydrolyzed with 5.7 N HCl at 110 °C for 22 h and analyzed on a Beckman Model 121-MB amino acid analyzer (Beckman Instruments, Inc., Palo Alto, CA). Tryptophan was determined by hydrolysis with 4 N methanesulfonic acid containing 0.2% 3-(2-aminoethyl)indole (Moore, 1972) (Pierce Chemical Co., Rockford, IL). Molecular weights were determined on sodium dodecyl sulfate and sodium phosphate containing 7.5% polyacrylamide gels run according to the method of Shapiro et al. (1967). The amino-terminal amino acid determination was done by the dansyl procedure of Weiner et al. (1972) and the phenyl isothiocyanate procedure of Edman & Begg (1967). Rabbit muscle actin was purchased from Sigma Chemical Co. (St. Louis, MO).

Peptide Mapping. The protein sample was reduced and carboxymethylated in preparation for peptide mapping according to the method described by Kibbelaar et al. (1979). The protein was dissolved in 1 mL of 8 M urea/0.3 M Tris (pH 8.0)/0.3% β-ME and reduced for 3 h at 37 °C. The sample was cooled to 25 °C, and a 1000-fold molar excess of iodoacetamide (Sigma Chemical Co., St. Louis, MO) was added. After 30 min in the dark, the reaction was terminated by the addition of 50 μL of β-ME and dialyzed against 0.5% NH_4HCO_3 for 18 h at room temperature. The sample was digested at 37 °C with two separate additions of 2% (w/w) TPCK/trypsin (Worthington Biochemical Corp., Freehold, NJ) at $T = 0$ and at 6 h. Digestion was terminated at 22 h, and the salt was removed by repeated lyophilization. The final product was suspended in 30% acetic acid (20 μg/μL). Peptides were analyzed by the method of Gracy (1977). The digestion product (100 μg) was spotted on cellulose TLC plates (20 × 20 cm) (EM Merck, Darmstadt, Germany) and electrophoresed in acetic acid/pyridine/water (7:1:135) (pH 3.6) for 25 min at 1000 V. Ascending chromatography was completed in 16 h with *n*-butanol/pyridine/acetic acid/water (15:12:3:10). The peptides were visualized after treatment with 0.025% (w/v) fluorescamine (Hoffmann-La Roche Inc., Nutley, NJ) in acetone (Stephens, 1978) and photographed under UV illumination.

Results

Purification of Nuclear Actin. Chromatin prepared from citric acid nuclei (Marushige & Bonner, 1960; Taylor et al., 1973) was extracted with 0.6 M NaCl/25 mM sodium phosphate (pH 8.0). Many high molecular weight nonhistone proteins were released as shown by two-dimensional polyacrylamide gel electrophoresis (Figure 1). Nuclear actin (protein BJ) was not detected in this pattern. The 5 M urea/75 mM sodium phosphate (pH 8.0) extract of the salt-washed chromatin (Figure 2) was substantially enriched in nuclear actin (protein BJ) relative to the other proteins present. When the 5 M urea extract was subjected to gel filtration on a Sepharose CL-6B column equilibrated in the same buffer (Figure 3), the analytical two-dimensional polyacrylamide gel electrophoresis pattern (Figure 4) showed that nuclear actin (protein BJ) was substantially enriched in the excluded volume fraction and that proteins of similar electrophoretic mobility were removed. This fraction was used for preparative acetic acid/urea–polyacrylamide gel electrophoresis isolation of nuclear actin (Knecht & Busch, 1971; Goldknopf et al., 1975). The isolated product was shown to migrate as a single elongated spot on a two-dimensional IF/$NaDodSO_4$ gel (Figure 5).

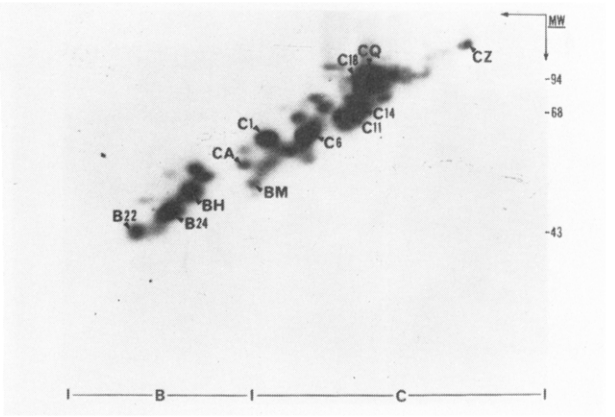

FIGURE 1: Two-dimensional polyacrylamide gel of the 0.6 M NaCl/25 mM sodium phosphate (pH 8) extract of chromatin. Approximately 250 μg of 0.6 M NaCl/25 mM sodium phosphate extract was run on a 6% acrylamide, acid/urea first-dimensional gel. The second dimension was an 8% acrylamide/$NaDodSO_4$ phosphate slab. The gel was stained for 6 h with 0.2% Coomassie brilliant blue R.

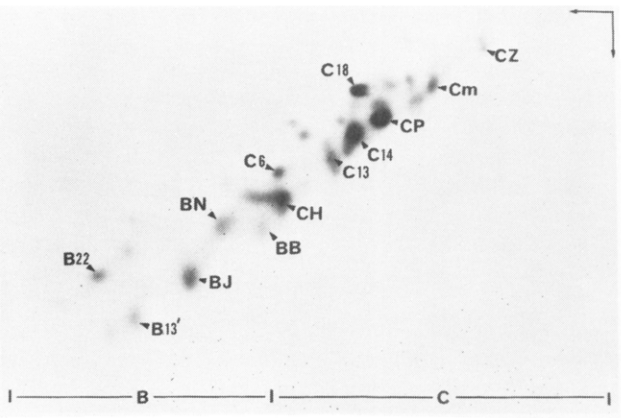

FIGURE 2: Two-dimensional polyacrylamide gel of the 5 M urea/75 mM sodium phosphate (pH 8) extract of salt-washed chromatin. See Figure 1 for conditions.

Analysis of Nuclear Actin. The molecular weight of nuclear actin (protein BJ) was 43 000 when its electrophoretic mobility was compared to that of standard proteins of known molecular weight (Figure 5). The difference of 1000 between the molecular weight of muscle actin (sequence data) and the molecular weight of nuclear actin ($NaDodSO_4$–polyacrylamide gels) is not considered to be significant. Amino acid analysis

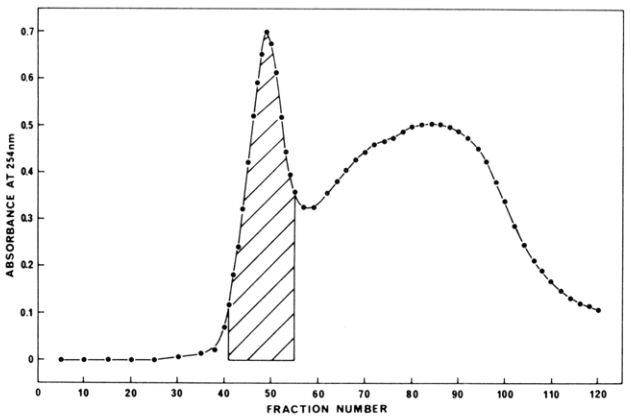

FIGURE 3: Sepharose CL-6B profile for the 5 M urea/75 mM sodium phosphate (pH 8) extract of salt-washed chromatin. Approximately 100 mg of protein was loaded on a 5 × 90 cm column. The conditions are described under Materials and Methods.

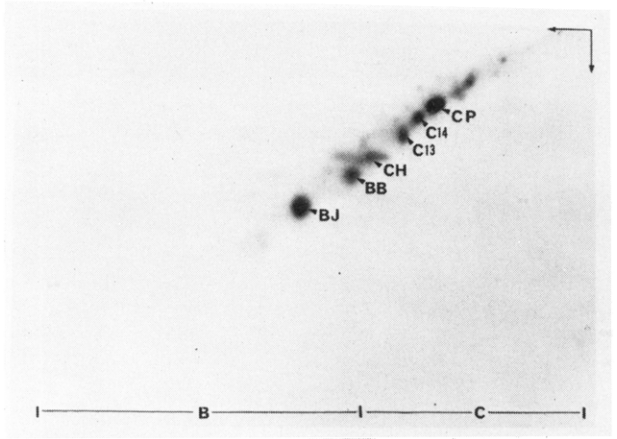

FIGURE 4: Two-dimensional polyacrylamide gel of the Sepharose CL-6B excluded volume fraction. See Figure 1 for conditions.

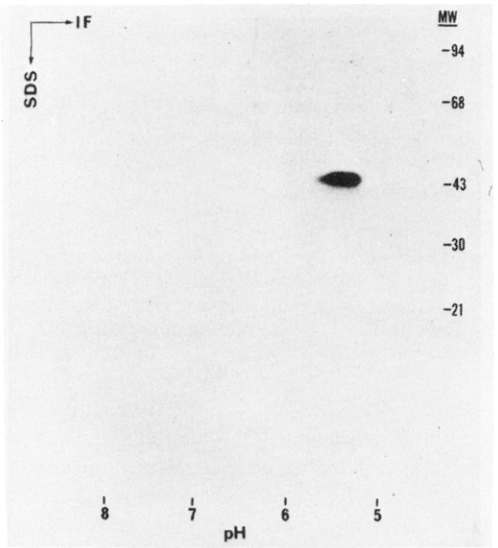

FIGURE 5: IF/NaDodSO$_4$ gel analysis of highly purified nuclear actin (protein BJ). Gels were O'Farrell type gels (1975) as modified by Hirsch et al. (1978). A protein load of 20 μg was analyzed on this gel. The molecular weight ordinate was constructed with the following proteins of known molecular weight: phosphorylase B, 94 000; bovine serum albumin, 68 000; ovalbumin, 43 000; carbonic anhydrase, 30 000; soybean trypsin inhibitor, 21 000; lysozyme, 14 300 (Bio-Rad, Richmond, CA).

(Table I) showed that nuclear actin had a large number of aspartic and glutamic acid residues which resulted in an acidic to basic amino acid ratio of 1.5 (Table II). Nuclear actin

Table I: Amino Acid Compositions

amino acid	number of residues: muscle actin[a]	nuclear actin	cytoplasmic actin
Asx	34	36	34
Thr	27	23	25
Ser	22	25	23
Glx	39	47	44
Pro	19	21	20
Gly	28	31	29[b]
Ala	29	31	31
Cys	5	+	+
Val	21	22	23
Met	16	12	16
Ile	30	24	27
Leu	26	32	31
Tyr	16	13	15
Phe	12	14	13
Lys	19	25	21
His	9	10	9
Trp	4	2	5
Arg	18	20	19

[a] Elziga et al. (1973). [b] Adjusted for contamination.

Table II: Biochemical Characteristics

property	muscle[a] actin	nuclear actin	cytoplasmic actin
mol wt	42 000	43 000	43 000[b]
p*I*	5.3–5.5	5.3–5.5	5.6
acidic/basic	1.7	1.5	1.6
N terminal	blocked	blocked	
total no. of peptides	36	39	39

[a] Clarke & Spudich (1977). [b] Novikoff hepatoma cytoplasmic actin experimentally determined.

(protein BJ) contained two tryptophan residues and detectable amounts of cysteine. Isoelectric focusing of nuclear actin resulted in a single molecular weight species with an isoelectric point range of 5.3–5.5 (Table II, Figure 5). The amino terminus was blocked inasmuch as an amino-terminal amino acid was not detected by either the dansylation procedure of Weiner et al. (1972) or the phenyl isothiocyanate procedure of Edman & Begg (1967) under conditions that provided positive results for similar amounts of myoglobin.

Isolation and Analysis of Cytoplasmic Actin. In order to provide a better comparison for the isolated nuclear actin, cytoplasmic actin was purified from Novikoff hepatoma cells by the polymerization/depolymerization method of Spudich & Watt (1971) as modified by Pardee & Bamburg (1979). The resulting protein migrated as a single spot when analyzed by two-dimensional polyacrylamide gel electrophoresis. The molecular weight was 43 000, and the isoelectric point was 5.6. The amino acid composition of cytoplasmic actin (Table I) was characterized by large amounts of glutamic and aspartic acid like protein BJ. Interestingly, cytoplasmic actin contained five residues of tryptophan, more closely resembling muscle actin. The literature values for muscle actin are included in Tables I and II for comparison (Elziga et al., 1973; Clark & Spudich, 1977).

Peptide Mapping. The tryptic peptide maps of rabbit muscle actin, cytoplasmic actin, and nuclear actin are shown in Figure 6A–C. On the basis of the number of lysine and arginine residues (Table I), the theoretical yield for the different actins is 38–46 tryptic peptides. The total number of peptides identified for each of the three proteins is listed in Table II, which is in fairly good agreement with the theoretical numbers. The three peptide maps contain 20 peptides (num-

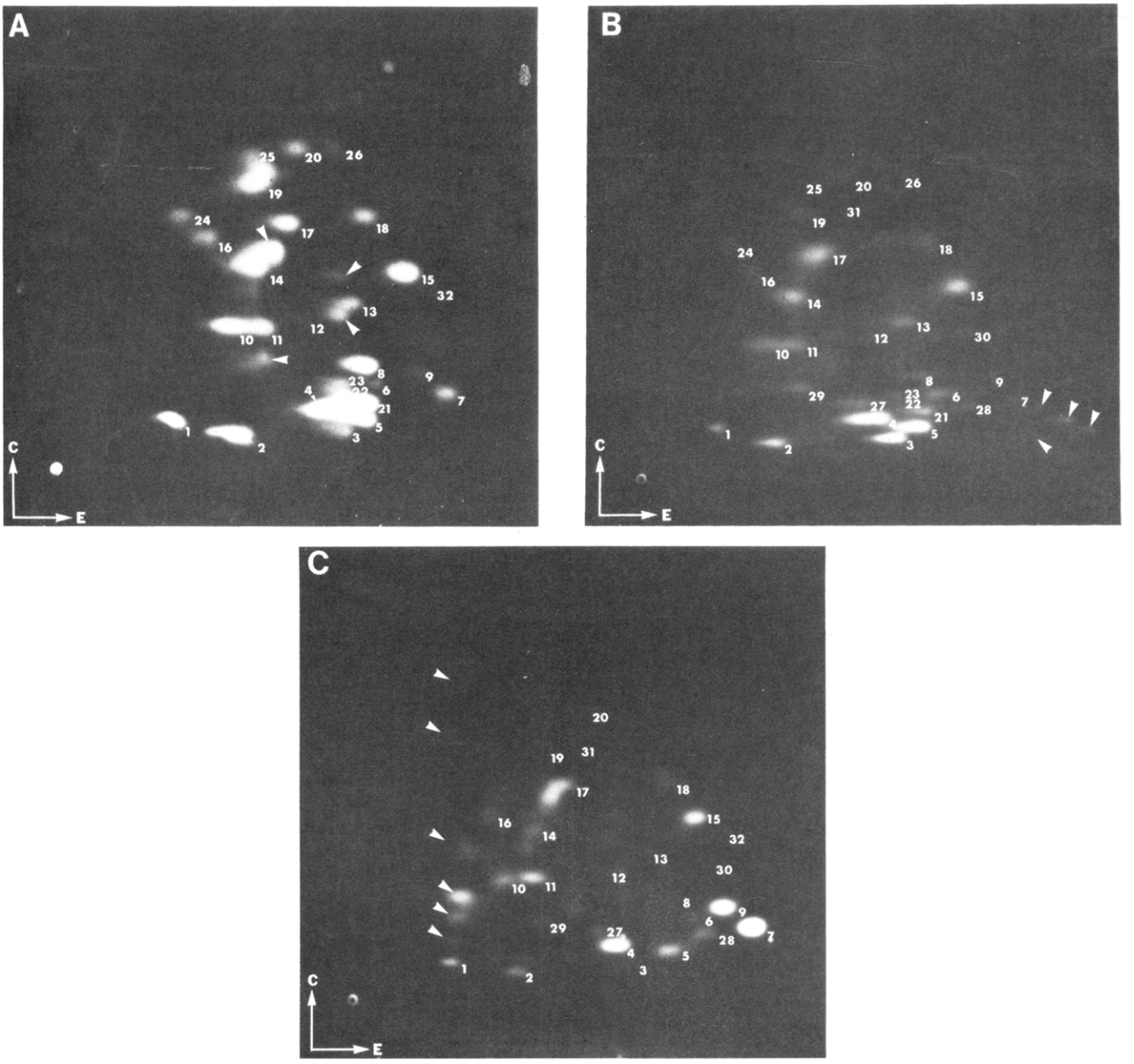

FIGURE 6: Two-dimensional peptide maps of actin tryptic hydrolysates. Map A is rabbit muscle actin, map B is nuclear actin (BJ), and map C is Novikoff hepatoma cytoplasmic actin. Tryptic hydrolysates ($\sim$100 μg) were spotted on cellulose thin-layer sheets and subjected to pH 3.6 electrophoresis in the horizontal direction and ascending BPAW (15:12:3:10) chromatography in the vertical direction. Peptides were detected by spraying the maps with 0.025% fluorescamine in acetone and viewing under UV illumination. The maps shown are representative of patterns obtained a minimum of 3 times derived from two different digestion experiments. Peptides present in two or more of the actins were selected for numbering to simplify the appearance of the patterns and to stress points of homology. The white arrowheads indicate those peptides which are unique to a particular actin species.

bered 1–20, Figure 6A–C) which are common to all of the actins analyzed. In addition to these 20 common peptides, there was one common peptide (peptide 32) shared by rabbit muscle actin and cytoplasmic actin. There were five common peptides (peptides 27–31) shared by cytoplasmic actin and nuclear actin. Each peptide map contained tryptic peptides which were characteristic to that individual actin. The major unique peptides are indicated by the white arrowheads in Figure 6A–C.

Discussion

Nuclear actin was extracted with urea from salt-washed chromatin and isolated in a high state of purity by preparative polyacrylamide gel electrophoresis. The amino acid composition was characterized by a large number of acidic amino acids and the presence of tryptophan which is characteristic of many nonhistone proteins (Busch et al., 1975). Preparative polyacrylamide gel electrophoresis has been used in our laboratory to successfully isolate other nuclear proteins in high purity for chemical characterization, i.e., A24 and C14 (Goldknopf et al., 1975; James et al., 1977). Because of the similarity of the amino acid composition of nuclear actin (protein BJ) to skeletal muscle actin, Novikoff hepatoma cytoplasmic actin was isolated by the method of Pardee & Bamburg (1979) to serve as a control for the subsequent comparisons.

Tryptic peptide maps of the three actins, muscle actin, nuclear actin (protein BJ), and cytoplasmic actin, were compared. There were 20 peptides common to all of the actins. Four areas of striking similarity were identified: peptides 1 and 2, peptides 3–9, the "Y" region (peptides 10, 11, 14, 16, 17 and 19), and peptides 15 and 18. A group of peptides with

slow migration in the electrophoresis dimension was characteristic to cytoplasmic actin while a group of rapidly migrating peptides which moved beyond peptide 7 was repeatedly observed in nuclear actin maps.

Each map contained between 36 and 39 peptides (Table II), which is in good agreement with the expected number of peptides based upon the amino acid composition and the molecular weight. Since protein BJ had 65% peptide homology to muscle actin, an identical molecular weight, and a similar amino acid composition and isoelectric point, it was concluded that protein BJ was an actin.

It has been speculated that nuclear actin is in equilibrium between the cytoplasm and the nucleoplasm (Goldstein et al., 1977) or a cytoplasmic contaminant of the isolation procedure (Comings & Harris, 1976). The peptide maps of the nuclear actin (protein BJ) and the cytoplasmic actin clearly have enough similarities to conclude that both molecules are actins. The distinct peptides argue that these are not the same molecules, i.e., that nuclear actin is a distinct species of actin. The presence of multiple actins within a cell was suggested by Palmer & Saborio (1978), who reported multiple forms of rat brain actin, depending upon the age of the animal. Vandekerckhove & Weber (1978) and Tobin et al. (1980) have reported that there are two or more actin genes in a cell.

A comparison of isolated actins based upon the elongated p*I* spot of Figure 5 is reasonable in light of the sequence information available to date for cytoplasmic actins. Cytoplasmic actins (β and γ) are thought to arise from two structural genes (Vandekerckhove & Weber, 1978) and have been shown to differ only in their amino-terminal peptide sequences by amino acid substitutions at positions 1, 2, 3, and 10.

The characteristic peptides identified in this study for nuclear, cytoplasmic, and muscle actins suggest that more than two genes may account for the actin structures present in Novikoff hepatoma cells. This idea draws additional support from the observation that nuclear actin (BJ) more closely resembles α actin than the combination of β and γ actins which contributes to the cytoplasmic actin map.

It is interesting to reflect upon the similarity between the nuclear and muscle actin peptide maps, which may be indicative of a contractile role for nuclear actin. Among the potential roles a nuclear contractile protein could serve are the transport of precursors (preribosomal particles, HnRNPs, etc.) from various regions of the nucleus or nucleolus to the cytoplasm and the steps involved in chromatin condensation, i.e., for formation of "heterochromatin" (LeStourgeon et al., 1975; Goldstein et al., 1977; Rungger et al., 1979). It is important to consider the possibility that the differences observed in this study between the cytoplasmic and nuclear actins of a hepatoma may have been amplified, to some extent, by posttranslational modifications or by the process of dedifferentiation which has occurred in this specific tumor cell line. The distinctions between actins in normal tissues may be much more subtle.

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