Major nonhistone proteins of rat liver chromatin: Preliminary identification of myosin, actin, tubulin, and tropomyosin

(contraceptible proteins/endogenous protease digestion of chromatin)

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ABSTRACT  Two major nonhistone polypeptides from rat liver chromatin have been identified as myosin and actin. Preliminary observations indicate that three other chromatin polypeptides of molecular weights 50,000, 34,000, and 32,000 are tubulin and heavy and light tropomyosins, respectively. A sixth component of molecular weight 65,000 which has been purified and electrophoresed as a single band on sodium dodecyl sulfate-polyacrylamide gels may be composed in part of protease-digested myosin. These six polypeptides together account for as much as 38% of the nonhistone protein mass of chromatin in this tissue.

Much emphasis has been given to the possible role of nonhistone chromosomal proteins as regulators of gene expression. Because of their heterogeneity (1) and tissue and species diversity (2, 3), tradition makes the nonhistones unlikely candidates for major structural assignments in chromosomes. Despite their heterogeneity, however, more than half the chromosomal nonhistone mass in vertebrates may be composed of as few as 12-18 major polypeptides (3, 4). An even smaller number of polypeptides (roughly 3 or 4) can be considered very major, represented by as many as 10^6 copies per haploid genome (1). It is therefore not inconceivable that the very abundant nonhistones may, like the histones, perform major structural tasks in chromosomes.

Attempts to characterize and assign functions to individual nonhistones have been impeded by at least two major obstacles: First, nuclei contain a protease, preferentially associated with chromatin (5, 6), which, if allowed to act, modifies the pattern of nonhistone polypeptides seen on gels (6, 7). The need to irreversibly inactivate this protease at early stages of chromatin preparation has not been sufficiently appreciated. Second, there is no general agreement on the extent to which nonhistone chromatin proteins may be contaminated by extrachromosomal proteins because there are no widely accepted criteria for discriminating between true chromosomal proteins and contaminants. The establishment of such criteria can be aided by comparison of the proteins found in isolated chromatin with those found elsewhere in the cell. We have therefore isolated and characterized a number of major nonhistone chromosomal proteins of rat liver chromatin.

Among the proteins we have isolated from rat liver chromatin are five which have the properties respectively of actin, tropomyosin, myosin, and breakdown products thereof. We also have evidence for the presence of tubulin in chromatin. Because actin is a major cytoplasmic protein in slime molds and in slime mold chromatin (8), extranuclear protein contamination of chromatin is an important issue. We therefore have compared chromosomal proteins prepared from purified nuclei with those which are prepared from nucleoplasm and whole tissue homogenates. Special attention has been paid to artifacts that might arise from protease digestion of chromatin during purification.

METHODS

Chromatin Preparation and Diisopropylfluorophosphate Treatment. Chromatin from frozen or fresh livers (in batches of 16 livers) was prepared according to Bonner et al. (9) and treated with 8 mM diisopropylfluorophosphate (DFP) to inactivate proteases. Following DFP treatment, 0.2 mM ethyleneglycol bis(β-aminoethyl ether)-N,N' -tetraacetic acid (EGTA) was added to all buffers to protect the DNA from nuclease digestion.

Chromatin from Nuclei. Nuclei were isolated from 10 fresh livers by centrifugation of homogenized tissue through a cushion of 2.3 M sucrose in TKM (0.05 M Tris-HCl, pH 7.5, 0.025 M KCl, 0.005 M MgCl2) buffer (10). The nuclei were resuspended in 21 ml of 8 mM DFP, 10 mM Tris-HCl, pH 7.5. Stirring for 10 min at room temperature followed by a second addition of DFP lysed the nuclei. The crude chromatin was separated from nucleoplasm by centrifugation at 4° at 5000 rpm for 10 min in the Sorvall SS-34 rotor. The supernatant from the first 10 mM Tris-HCl wash after lysis was combined with the nucleoplasm. Two additional washes were performed, discarding the supernatants. The resulting nuclear chromatin was dialyzed against King and Laemmli sample buffer for gel electrophoresis (11) (2.5% sodium dodecyl sulfate, 0.065 M Tris-HCl, pH 6.8) to dissociate proteins from DNA. The latter was removed by centrifugation for 20 hr at 50,000 rpm at 4° in a Beckman Ti 50 rotor.

Nonhistone Protein Preparation. Nonhistone proteins were isolated according to procedures to be described in detail elsewhere. Chromatin was purified through sucrose gradients (9), proteins were extracted in 4 M NaCl, DNA was removed by centrifugation (12), and nonhistones were separated from histones by ion exchange chromatography on Bio-Rex 70 (12,*). Alternatively, chromatin was extracted with 1 M KCl, which removes contractile proteins from chromatin.

Chromatography of Nonhistones. The NaCl-dissociated nonhistones were dialyzed to 0.06 M phosphate buffer, pH 6.8, and applied to a hydroxyapatite column (1 cm^3/100 µg of protein) equilibrated with the same buffer at 4°. This buffer was then used to elute the contractile-like proteins from the column. Under these conditions the other nonhistone proteins remain bound to the column. The eluted proteins were lyophilized and resuspended in 300–500 µl

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Abbreviation: DFP, diisopropylfluorophosphate.

*A. S. Douvas, C. A. Harrington, and J. Bonner, manuscript in preparation.

Biochemistry: Douvas et al.

Table 1. Percent of nonhistones accounted for by six major polypeptide peaks

<table>
<thead>
<tr>
<th>Protein (by molecular weight)</th>
<th>% of 4 M NaCl dissociated*</th>
<th>% of non-dissociated*</th>
<th>% of total†</th>
</tr>
</thead>
<tbody>
<tr>
<td>200,000</td>
<td>0.4</td>
<td>2.2</td>
<td>1.1</td>
</tr>
<tr>
<td>65,000</td>
<td>4.1</td>
<td>6.6</td>
<td>5.1</td>
</tr>
<tr>
<td>50,000</td>
<td>7.4</td>
<td>21.1</td>
<td>12.9</td>
</tr>
<tr>
<td>45,000</td>
<td>5.4</td>
<td>11.2</td>
<td>7.6</td>
</tr>
<tr>
<td>34,000</td>
<td>3.7</td>
<td>10.3</td>
<td>6.3</td>
</tr>
<tr>
<td>32,000</td>
<td>2.6</td>
<td>7.9</td>
<td>4.8</td>
</tr>
<tr>
<td></td>
<td>23.6</td>
<td>59.3</td>
<td>37.8</td>
</tr>
</tbody>
</table>

* Percentages were figured by computer resolution of scans and area calculation, and by manual curve fitting and weighing area under curves. The two methods were in agreement within 3% for 4 M NaCl dissociated proteins, within 1% for total, and identical for the nondissociated fraction.

† Approximately 80% of the chromosomal nonhistones are NaCl dissociable and 40% remain associated with the DNA. % of total was calculated from these proportions.

King and Laemmli buffer. They were separated by filtration on Sephadex G-200 superfine or G-75 gels equilibrated at room temperature and developed with King and Laemmli buffer. Approximately 0.5 mg of protein was applied to each column. The proteins emerging from the column could be applied directly to the gels for electrophoresis.

Polyacrylamide Disc Gel Electrophoresis and Analysis.

Gel electrophoresis in sodium dodecyl sulfate was performed according to King and Laemmli (11). Application of this technique to chromosomal nonhistones and details on the use of molecular weight markers have been described elsewhere (13). At least two molecular weight markers were included in each run. The mean amount of protein applied to each gel ranged from 100 µg for complex to 20 µg for relatively pure samples. Gels were stained with Coomassie brilliant blue, then scanned at 600 nm with a Gilford 2000 spectrophotometer.

RESULTS

Polypeptides of molecular weights 200,000, 65,000, 50,000, 45,000, 37,500, 34,000, and 32,000 are major components of the soluble (dissociable by 4 M NaCl) nonhistone chromatin protein fraction when it is prepared from frozen livers and treated with DFP to prevent proteolysis (Fig. 1). Chromatin from purified nuclei (isolated from fresh tissue) contains all of these polypeptides but relatively less of the polypeptides of 200,000 and 50,000 molecular weight (Fig. 1). The nuclei are purified in the presence of 10 mM MgCl₂ before addition of DFP, a condition that favors proteolysis (14). The 200,000 dalton polypeptide is also typically absent from chromatin isolated from frozen livers without DFP treatment. Evidence that its absence is due to proteolysis is presented in Fig. 3D. Others have also observed that large nonhistones are selectively degraded by endogenous protease(s) (7). The data of Fig. 1 show that the 45,000 dalton protein is a major component of both nuclear chromatin and chromatin made from frozen tissue. This protein is either a minor component or absent in nucleoplasm.

Although the six polypeptides described above are partially extracted by 4 M NaCl, even more of each is not extracted. A material balance is given in Table 1. The data of Table 1 show that the six make up 38% of the mass of nonhistone chromosomal proteins. While we cannot assume that the material in any one gel band is homogenous, evidence is presented below that the 200,000 and 45,000 dalton polypeptides are each at least 90% single biochemical species.

Chromatography of soluble nonhistones on hydroxyapatite in 0.06 M phosphate buffer, pH 6.8, yields an eluate enriched in polypeptides of molecular weights 200,000, 45,000, 34,000, and 32,000 (Fig. 2). These polypeptides coelectrophoresed with myosin and actin isolated from rat muscle by the procedures of Seraydarian et al. (16) and Buller et al. (17) and with the tropomyosin contaminants in the actin preparation. The presence of polypeptides that coelectrophorese with actin and myosin prompted us to subject our soluble nonhistone fraction (obtained by extracting chromatin with either 4 M NaCl or 1 M KCl) to conditions that precipitate muscle actomyosin: dialysis to 1 M KCl, 20 mM Tris-HCl pH 7.0, 10 mM 2-mercaptoethanol followed by dialysis to 0.025 M KCl, 15 mM EDTA, 20 mM Tris-HCl pH 7.0, 20 mM 2-mercaptoethanol to precipitate "actomyosin." Before precipitation, muscle myosin was added to the 1 M KCl extract, which, relative to the 4 M NaCl extract, is impoverished in the endogenous 200,000 dalton protein. The same treatment with some modification was applied to two other chromosomal fractions: First, treatment of chromatin in 7 M urea results in selective extraction of ≥90% of a 45,000 dalton protein*. This extract was dialyzed against the 1 M KCl-containing buffer; rabbit muscle myosin was added to a 1:1 weight ratio; and the protein mixture was dialyzed against the 0.025 M KCl-containing buffer. Second, non-DFP-treated chromatin was extracted with 1 M KCl. Typically this fraction contains no detectable 200,000 dalton protein. Muscle myosin was added to a 2:1 weight ratio and dialysis was performed against the 0.025 M KCl buffer.

![Fig. 1. Comparisons of the electrophoretic patterns of chromatin proteins from purified nuclei, 4 M NaCl extractable nonhistones, and nucleoplasmic proteins.](image-url)
results of these treatments are shown in Fig. 3A–D. The salt- and urea-soluble nonhistones treated with exogenous myosin yielded “actomyosin” precipitates which when subjected to sodium dodecyl sulfate-gel electrophoresis were found to contain 200,000 and 45,000 dalton polypeptides (the same molecular weights as myosin and actin subunits). The precipitation resulted in nearly complete removal of these components from the supernatants. The “actomyosin” precipitate from the 1 M KCl extract of chromatin (with added muscle myosin) was subjected to conditions that dissociate muscle actomyosin (1 M KCl) and yield myosin-free fibrous actin to fibrous actin (addition of 5 mM MgCl$_2$, 5 mM ATP, and reduction of the KCl concentration to 0.6 M). The actomyosin precipitate dissolved and subsequently yielded a fibrous complex composed of the 45,000 dalton polypeptide subunits (Fig. 3B). This complex contained other polypeptides of chromosomal origin, amounting to $\geq$20% of the mass.

Repeated depolymerization and polymerization of the complex resulted in selective removal of these polypeptides, as shown in Fig. 3B.

Because actomyosin complexes from both muscle and nonmuscle sources (e.g., slime mold cells) exhibit ATPase activity, we assayed this activity in the complex isolated from rat liver chromatin. The results are shown in Table 2. The chromosomal preparation has the same specific activity as crude actomyosin from slime mold (14) and although only roughly one-third of the preparation is accounted for by actin plus myosin its calcium-activated specific activity is twice that of muscle myosin (Table 2). The chromosomal actomyosin complex resembles that from slime mold in that the ratio of myosin to actin is lower than that in muscle, and its magnesium-activated ATPase activity (not shown) is lower than its calcium-activated activity (18).

The sodium dodecyl sulfate gel scan of the crude actomyosin precipitate from soluble nonhistone is strikingly similar to that of muscle actomyosin which is contaminated with tropomyosin and a trace of myosin (Fig. 2). The electrophoretic mobilities of the three chromosomal (45,000, 34,000, and 32,000 daltons) polypeptides and those of muscle actin
and the two muscle tropomyosin subunits are identical. This and the observation that hydroxypatite-purified chromosomal actin contains the 34,000 and 32,000 dalton polypeptides (tropomyosin) as contaminants suggest that these three polypeptides may be functionally associated in chromatin as in muscle.

We have attempted to precipitate “actomyosin” from nonhistone proteins extracted from non-DFP treated chromatin. These attempts were made with 1 M KCl extracts of chromatin, with and without exogenous myosin from rabbit muscle. Both types of attempts failed, and therefore only the experiments with added myosin are here described. Dialysis of (non-DFP-treated) chromatin proteins with muscle myosin to 0.025 M KCl caused a precipitate to form, and the muscle myosin and 45,000 dalton protein to disappear from the supernatant (Fig. 3D). However, neither protein was recovered intact in the precipitate. Attempts to solubilize the precipitate in 1 M KCl yielded a heterogeneous soluble fraction, and an insoluble polypeptide of molecular weight 65,000 daltons (Fig. 3D). It appears that all of these polypeptides are breakdown products which result from digestion of myosin and the 45,000 dalton polypeptide (actin) by nuclear protease. The 65,000 dalton polypeptide has been isolated, and its amino-acid composition is compared below to that of myosin (Table 3).

An alternate scheme for purification of actin and a second 65,000 dalton polypeptide was found in dialysis of the whole DFP-treated nonhistone fraction to 0.025 M KCl. The actin precipitates. The supernatant is passed over hydroxyapatite in 0.06 M phosphate buffer, pH 6.8. A protein of 65,000 daltons runs through. The 65,000 dalton protein is further purified by filtration over Sephadex G-75 in King-Laemmli buffer. The precipitated actin is purified by filtration over G-200 in the same buffer. This procedure yields actin free of tropomyosin, and free also of a 50,000 dalton protein which either copolymerizes with or is attached to actin (Fig. 3B). Evidence that this polypeptide is tubulin is presented below.

Actin prepared as described above runs as a single band in sodium dodecyl sulfate polyacrylamide gels. The first 65,000 dalton protein (I) is the major component of the 0.025 M KCl precipitate from non-DFP-treated proteins with exogenous myosin (Fig. 3D). It is apparently therefore a myosin fragment. The second (65,000 II) does not precipitate when treated with crude actin (Fig. 3A). In addition to their solubility differences, the amino-acid compositions of the two 65,000 dalton polypeptides are different. The amino-acid composition of I (but not of II) resembles that of rabbit muscle myosin, as is true of myosin fragments (19, 20). The absence of an endogenous 200,000 dalton polypeptide in non-

### Table 2. ATPase activity of crude “actomyosin” precipitate

| Specific activity (µmol P i /mg per 5 min) | Crude actomyosin | Exp. 1 | 0.188 | Exp. 2 | 0.182 | Rat muscle myosin | 0.089 |

### Table 3. Amino-acid compositions of two 65,000 dalton proteins and muscle myosin

<table>
<thead>
<tr>
<th>Amino acid</th>
<th>Rabbit muscle myosin, mole %</th>
<th>65,000 I, mole %</th>
<th>65,000 II, mole %</th>
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<tbody>
<tr>
<td>Asp</td>
<td>9.8</td>
<td>9.3</td>
<td>10.4</td>
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<tr>
<td>Thr</td>
<td>5.1</td>
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<tr>
<td>Ser</td>
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<td>4.8</td>
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<td>Leu</td>
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<td>9.0</td>
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<tr>
<td>Tyr</td>
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<td>2.6</td>
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<tr>
<td>Phe</td>
<td>3.4</td>
<td>3.0</td>
<td>3.7</td>
</tr>
<tr>
<td>His</td>
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<td>1.9</td>
<td>2.1</td>
</tr>
<tr>
<td>Lys</td>
<td>10.6</td>
<td>9.5</td>
<td>5.3</td>
</tr>
<tr>
<td>Arg</td>
<td>5.0</td>
<td>5.5</td>
<td>6.1</td>
</tr>
</tbody>
</table>

*From Lowey and Cohen (19).

DFP-stabilized chromatin and the presence of a prominent 65,000 dalton polypeptide may indicate conversion of the former to the latter by proteolysis. Even in DFP-treated chromatin some of the 200,000 dalton protein may have been converted to fragments of 65,000 daltons and smaller before treatment.

The possibility that tubulin may be found in chromatin was suggested by the presence of two polypeptides of molecular weight 55,000 and 50,000 (the same size as α and β tubulin, respectively). The amounts and proportions of the 55,000 and 50,000 dalton components relative to each other vary with different preparations. Garrard et al. (1) find approximately equal amounts of the 55,000 and 50,000 dalton polypeptides. We consistently find less of the 55,000 dalton protein in chromatin prepared from frozen livers, but equal amounts in chromatin from fresh tissue. The ability of tubulin to bind to colchicine provides, under appropriate conditions, a specific assay for its presence (21). [14C]Colchicine binding activity was therefore measured in protein samples from fresh and from frozen chromatin. There was approximately 2.5-fold more of the 50,000 dalton protein in fresh chromatin relative to total protein than in frozen material. A 2-fold difference in the respective colchicine binding activities was observed. Bovine serum albumin and proteins from an 0.5 M NaCl extract of chromatin (which does not dissociate the 50,000 dalton component)* had no colchicine binding activity.

A 37,500 dalton polypeptide especially prominent in the nucleoplasm (Fig. 1) is a basic nonhistone which coelectrophores with a basic polypeptide of the same size isolated from mouse ascites ribonucleoprotein (RNP) particles (22, 28). This polypeptide is found only in the template active fraction of DNase II digested chromatin (23).

### DISCUSSION

Two of the polypeptides which we have isolated from rat liver chromatin (200,000 daltons and 45,000 daltons) exhibit properties characteristic of the contractile proteins myosin and actin. They have the same molecular weights on sodium dodecyl sulfate-polyacrylamide gels as do rat muscle myosin.
and actin. In addition: (a) the 45,000 dalton chromatin protein selectively precipitates out from a complex mixture of nonhistones either with exogenous myosin or with the 200,000 dalton chromatin protein and under conditions that precipitate actomyosin in vitro. (b) The precipitated complex has ATPase activity. (c) The complex is dissociated in 1 M KCl and the 45,000 dalton protein can be polymerized under conditions that polymerize g-actin to f-actin. These properties lead us to identify the 200,000 and 45,000 dalton chromatin polypeptides as myosin and actin, respectively. Positive identification of these two proteins as the same biochemical species as actin and myosin from muscle requires analyses of amino acid compositions and tryptic fragments (for variation in muscle and nonmuscle actin, see ref. 27).

The full complement of contractile proteins in skeletal muscle includes actin and nonhistones, tropomyosin in addition to actin and myosin. The molecular weight of actin is 1.8 X 10^5 (24) and tropomyosin has been separated into three fractions with molecular weights of 40,000, 22,000, and 17,000 (25). Although there are polypeptides corresponding to these molecular weights in rat liver chromatin nonhistones, we have not demonstrated their identities. The purification of hydroxyapatite of two polypeptides of molecular weights 32,000 and 34,000 with chromosomal actin led us to suspect that tropomyosin may also be present. The molecular weights of the two unequal tropomyosin subunits on sodium dodecyl sulfate-polyacrylamide gels range from 32,000 for the light to 47,000 for the heavy component, depending on the buffer systems (26). In the King-Laemmli system used here the tropomyosin contaminants of muscle actin ran as 32,000 and 34,000 dalton polypeptides, as did the corresponding proteins from chromatin. In the absence of Ca++, tropomyosin prevents the binding of actin to myosin and therefore inhibits actomyosin ATPase (25). The crude chromatin "actomyosin" complex which is contaminated by the 34,000 and 32,000 dalton polypeptides has a low-Mg++-activated ATPase activity and a high-Ca++-activated ATPase activity, again suggesting the presence in the complex of tropomyosin.

The presence of actin in chromatin from purified nuclei but not in nucleoplasm (Fig. 1) is consistent with the observation that this protein is not easily dissociated from chromatin (11). An exception is found when chromatin is not DFP treated. In non-DFP-treated chromatin there is a considerable amount of loose actin which can be removed by washing in 10 mM Tris-HCl or low concentrations of NaCl. The effect on actin appears to be selective. This observation suggests that actin in chromatin may be organized into fibers, accessible at their distal ends to the surrounding medium. Proteolytic cleavage may release the ends of the fibers into the medium.

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