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Purification and Properties of a 90-kDa Nuclear Actin-Binding Protein (42280)

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Abstract. A 90 kDa actin-binding nuclear protein (ABNP) with a p*I* of 5.2 has been purified from the 0.7 *M* NaCl extracted residue fraction of chromatin prepared from Novikoff hepatoma cell nuclei. This residue fraction was previously shown to contain nuclear actin. Although twice the size, similar in p*I*, and similar in amino acid composition to actin, the tryptic peptide map for ABNP is distinct and contains the appropriate number of tyrosine-containing tryptic peptides for a protein of 90,000 molecular weight. A comparison of the amino acid composition of ABNP with those reported in the literature for gelsolin and villin, using a calculation of $S\Delta Q$ as an indication of relatedness, results in values of 30 and 27, respectively. Actin-binding activity, however, was demonstrated for both crude and gel purified ABNP using a gel-overlay technique that employs ^{125}I-G-actin to detect specific actin-binding proteins.

Nuclei prepared from Novikoff hepatoma cells have been shown to contain a form of actin that is chemically distinct from the cytoplasmic actin isolated from the same cell type (1). A comparison of peptide maps obtained for nuclear, cytoplasmic, and rabbit muscle actins showed that nuclear actin, although unique, more closely resembled the peptide map obtained for rabbit skeletal muscle actin. Since that report, actin has been identified in the nuclear matrix prepared from 3T3 mouse fibroblast nuclei (2) and from bovine lymphocyte nuclei (3). Actin was previously identified as a component of nuclear extracts by its migration on $NaDodSO_4$–polyacrylamide gels (4–6) and immunolocalized in the nucleoli, kinetochore, and centriolar regions and in the mitotic spindle of rat kangaroo cells (7, 8). In addition, Fukui and Katsumaru were able to demonstrate the formation of actin bundles in interphase nuclei of Dictyoselium and HeLa cells following treatment with DMSO[2] (9). More recently, Kumar *et al.* have confirmed our results in *Acanthamoeba castellanii* by isolating a slightly more acidic minor actin species from the nuclei of that organism (10). Furthermore, studies by Scheer *et al.* using microinjected actin antibodies have suggested a functional role for nuclear actin in the regulation of protein-coding gene expression (11). It would appear, therefore, that actin represents an authentic and functional component of the interphase cell nucleus of eucaryotic cells and is not merely sequestered, as needed, from cytoplasmic stores (12).

In the cytoplasm, actin has been shown to be associated with a variety of actin-binding proteins that are involved in the regulation of actin polymerization and crosslinking. These include a number of proteins that fall within the 90,000 to 95,000 molecular weight range. Gelsolin, isolated from lung macrophage cells and described by Yin and Stossel (13), has a molecular weight of 91,000 and confers Ca^{2+} sensitivity to the solvation of crosslinked actin filaments. Villin, a protein with a molecular weight of 95,000, which has been shown to exhibit bifunctional activity, regulates the crosslinking of actin at calcium ion concentrations below 10^{-6} molar, but regulates the

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[2] Abbreviations used: ABNP, actin-binding nuclear protein; ABP-A, actin-binding protein from *Acanthamoeba;* ADF-S, actin-binding and depolymerizing protein from serum; Buffer B, 0.2 m*M* $CaCl_2$/0.2 m*M* ATP/1.0 m*M* $MgCl_2$/0.05% NaN_3/0.1% gelatin/5.0 m*M* NaH_2PO_4/0.2 *M* NaCl/pH 7.4; BPAW, *n*-butanol/pyridine/acetic acid/water; DMSO, dimethyl sulfoxide; EDTA, ethylenediaminetetraacetic acid; EGTA, ethylene glycol bis(β-aminoethyl ether)-*N,N,N′,N′*-tetraacetic acid; HMG, high mobility group; IF, isoelectric focusing; PMSF, phenylmethanesulfonyl fluoride; SDS, sodium dodecyl sulfate; SΔQ, estimate of protein relatedness; TPCK, L-1-(tosylamido)-2-phenylethyl chloromethyl ketone; Tris, tris(hydroxyethyl)aminomethane.

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length of actin filaments by capping actively growing actin chains at calcium ion concentrations higher than 10^{-6} molar (14). Additional proteins that either regulate crosslinking, filament length, or the direction of filament polymerization include the 95-kDa protein from Dictyostelium described by Hellewell and Taylor (15), ABP-A described by Pollard (16), 90-kDa protein described by Wang and Bryan (17), and ADF-S described by Harris and Gooch (18) with molecular weights of 95,000, 90,000, 90,000, and 92,000, respectively. It appeared logical, therefore, to examine the nuclear proteins in the 90,000 to 95,000 molecular weight range for actin-binding activity.

Only one protein (ABNP) with a molecular weight of 90,000 was observed to copurify with nuclear actin from the 0.7 *M* NaCl extracted chromatin residue fraction (1). Interestingly, it appeared in an equal mass ratio to nuclear actin. This protein was purified to homogeneity by preparative polyacrylamide gel electrophoresis. It was chemically characterized in terms of its molecular weight, apparent isoelectric point, amino acid composition and iodinated tyrosine-containing tryptic peptide map. Its properties were compared with those reported for highly purified actin regulatory proteins (13, 14). ABNP was analyzed for actin-binding capacity using the ^{125}I-G-actin overlay assay described by Snabes *et al.* for the detection of specific actin-binding proteins (19) and was shown to bind G-actin.

Materials and Methods. *Isolation of nuclei and chromatin.* Novikoff hepatoma ascites cells, implanted ip 6 days prior to experiments, were collected and washed free of erythrocytes according to the method of Boyle (20). Nuclei, free of cytoplasmic tags and the outer layer of the nuclear envelope, were prepared by the 0.025 *M* citric acid method of Taylor *et al.* (21). All solutions contained 1.0 m*M* phenylmethanesulfonyl fluoride to prevent proteolysis (Pierce Chemical Co., Rockford, Ill.). Chromatin was prepared as described by Marushige and Bonner (22) by washing nuclei with 0.075 *M* NaCl/0.025 *M* EDTA (pH 8) followed by 0.01 *M* Tris–HCl (pH 8).

Extraction of chromatin. Chromatin was extracted twice with 0.7 *M* NaCl/25 m*M* sodium phosphate (pH 8) and collected as a pellet by centrifugation at 16,000*g* for 30 min. The washed chromatin was suspended in 20 vol (v/w) of 5 *M* urea/3 *M* guanidine–HCl/0.5 *M* sodium acetate (pH 8) and allowed to extract for 18 hr at 4°C. The extract was centrifuged at 105,000*g* for 6 hr. The supernatant was removed and dialyzed against two changes of 9 *M* urea/1% β-mercaptoethanol and twice against 9 *M* urea/0.18 *M* acetic acid/1% β-mercaptoethanol. The dialysate was centrifuged at 48,000*g* for 30 min and concentrated to 5 mg/ml over a YM-20 membrane.

Preparative polyacrylamide gel electrophoresis. A 1.0-ml sample (5 mg) was loaded on a 6% polyacrylamide/4.5 *M* urea/0.9 *M* acetic acid slab gel (10 × 7.5 × 0.3 cm) (Ortec Inc., Oak Ridge, Tenn.). After electrophoresis at 120 V for 6 hr, the 90,000 molecular weight band was identified on Amido black stained strips cut from the sides of the gel. The corresponding unstained region of the gel was sliced and electroeluted into dialysis bags (23).

Analytical electrophoresis and amino acid analysis. The molecular weight of ABNP was determined by its migration relative to molecular weight standards (phosphorylase B, 92,500; bovine serum albumin, 66,200; ovalbumin, 45,000; carbonic anhydrase, 31,000; soybean trypsin inhibitor, 21,500; and lysozyme, 14,400) 7.5% polyacrylamide gels containing 0.1% sodium dodecyl sulfate according to the method of Shapiro *et al.* (24). Two-dimensional acetic acid/urea polyacrylamide gel electrophoresis was run as described by Busch *et al.* (25). Two-dimensional analytical IF/SDS gels were run as described by O'Farrel (26) and modified by Hirsch *et al.* (27).

The amino acid composition was determined by hydrolysis with 5.7 *N* HCl for 22 hr at 110°C and subsequent analysis on a Beckman Model 121MB amino acid analyzer (Beckman Instruments, Inc., Palo Alto, Calif.). Tryptophan was determined following hydrolysis with 4 *N* methanesulfonic acid containing 0.2% 3-(2-aminoethyl) indole and neutralization with 3.5 *M* NaOH (28).

^{125}I-Protein labeling and peptide mapping. Protein used for peptide mapping was reduced and carboxymethylated (29) and labeled according to the Iodogen method of Fraker and Speck (30) (Pierce Chemical Co.). ABNP was iodinated in the presence of 8 *M* urea/0.3 *M* Tris (pH 7.5) for 5 min and the reaction was terminated by removal from the Iodogen-

containing tube. Protein (5×10^7 cpm) was digested for 22 hr at 37° in 60 m*M* NH_4HCO_3 (pH 8) with two separate additions of 10 μg of trypsin treated with TPCK, (Worthington Biochemical Corp., Freehold, N.J.) initially and at 6 hr. The digestion was terminated at 22 hr and the sample lyophilized. Peptides (2×10^6 cpm) were spotted on 20 × 20-cm cellulose thin-layer plates (EM Merck, Darmstadt, Germany) and separated by the method of Gracy (31). The first dimension was electrophoresis for 40 min in acetic acid/pyridine/water (7/1/135) at 1000 V. The second dimension was ascending chromatography in *n*-butanol/pyridine/acetic acid/water (15/12/3/10) for 16 hr. Peptides were visualized by autoradiography on RP Royal X-Omat film (Eastman Kodak; Rochester, N.Y.).

Gel overlay technique using ^{125}I-actin. Samples to be analyzed were run on 10% acrylamide gels according to the method of Laemmli (32). Gels were either stained with 0.25% Coomassie brilliant blue R or fixed in 40% methanol and 10% acetic acid at 25°C for 30 min and washed in 10% ethanol for 12 hr (19). Actin was purified from rabbit muscle according to the procedure of Spudich and Watt (33) using the gel filtration (Sephadex G-150) modification of MacLean-Fletcher and Pollard (34). Actin (~50 μg) was converted to the G form and labeled according to the method of Bolton and Hunter to specific activities of 4–11 μCi/μg (35). Gels were equilibrated for 2 hr in Buffer B which contained 0.2 m*M* $CaCl_2$/0.2 m*M* ATP/1.0 m*M* $MgCl_2$/0.05% NaN_3/0.1% gelatin/5.0 m*M* NaH_2PO_4/0.2 *M* NaCl (pH 7.4) or Buffer B without calcium ion; ^{125}I-actin (1.0 μCi/ml) was added and the incubation was continued at 4°C for 16 hr (19). In actin overlay competition assays ^{125}I-actin (1.0 μCi/ml) and G actin (10 μg/ml) were added simultaneously and incubated at 4°C for 16 hr. After washing five times with 250 ml of Buffer B, the gel was dried and autoradiographed.

Results. *Actin-binding nuclear protein purification.* Earlier studies had shown that a 90,000 molecular weight protein (protein CP) was present in the 5 *M* urea/75 m*M* sodium phosphate (pH 8) extract of 0.7 *M* NaCl/25 m*M* sodium phosphate washed chromatin in similar mass amounts to nuclear actin (1). A more selective and complete extraction of the 90,000 molecular weight protein was achieved if the salt washed chromatin was extracted with 5 *M* urea/3 *M* guanidine–HCl/0.5 *M* sodium acetate (pH 8). A two-dimensional gel showing ABNP and other proteins in this extract is presented in Fig. 1. It contains ABNP and protein C14 (36), the 70-kDa monomer of the 139-kDa phosphoprotein dimer described by Kuehn *et al.* (37), as major protein components with smaller amounts of nuclear actin and contaminating nuclear proteins. This extract was subjected to preparative polyacrylamide gel electrophoresis on slab gels containing 0.9 *M* acetic acid and 4.5 *M* urea. The ABNP-containing gel slice was identified by the staining of strips with Amido black after being cut from the sides of the gel and the protein was recovered by electroelution (23). An IF/SDS analytical two-dimensional polyacrylamide gel of the purified protein is shown in Fig. 2. ABNP focused at a pH of 5.2 and ran as a 90,000 molecular weight spot in the SDS-containing second dimension. A similar molecular weight value was obtained when the purified protein was run on an SDS-containing polyacrylamide gel as described by Shapiro (24).

Amino acid composition analysis. Gel purified ABNP was hydrolyzed with 5.7 *N* HCl and with 4 *N* methanesulfonic acid containing 0.2% 3-(2-aminoethyl) indole (28). Although cysteine was not quantitated, traces of cysteine were observed at the appropriate retention time. Tryptophan was quantitated. The amino acid composition of ABNP is reported in Table I. A ratio of 1.6 to 1.0 was calculated for acidic to basic amino acids. This value is quite reasonable for a protein with an acidic p*I* of 5.2 and distinguishes it from the more basic classes of nuclear proteins, i.e., the histones and HMG proteins (38, 39). A calculation of SΔQ values for this amino acid composition and those compositions reported in the literature for other 90,000 molecular weight proteins with actin-binding or regulatory activity was performed as described by Marchalonis and Weltman (40). Sums of 27 and 30 were obtained for the squares of the differences in mole percentage of each amino acid for villin and gelsolin, respectively (13, 14), indicating a strong similarity between those values reported in Table I and those reported for villin and gelsolin.

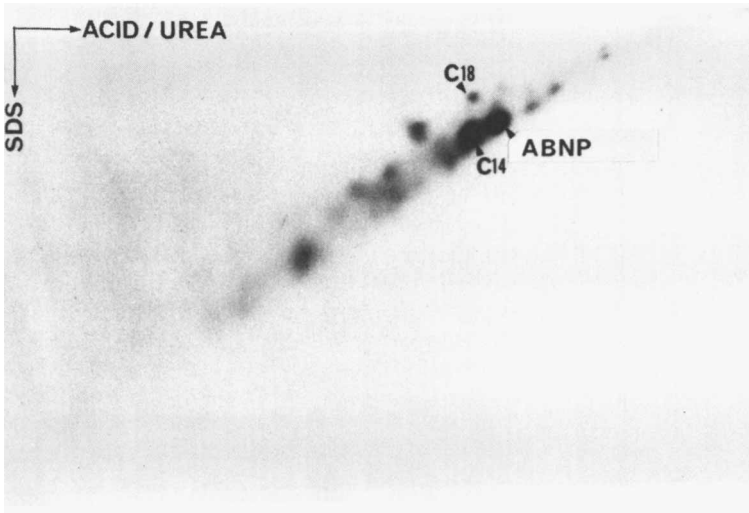

FIG. 1. Two-dimensional polyacrylamide gel of the 5 *M* urea/3 *M* guanidine–HCl/0.5 *M* sodium acetate (pH 8) extract of salt washed chromatin. Approximately 100 μg of chromatin extract was electrophoresed from right to left on a 6% acrylamide gel containing 0.9 *M* acetic acid/4.5 *M* urea (25). The second dimension was an 8% acrylamide gel containing 0.1% SDS/0.01 *M* sodium phosphate (pH 7) run in the vertical direction. The gel was stained for 6 hr with 0.2% Coomassie brilliant blue R.

Tryptic peptide map. The purified ABNP was iodinated by the Iodogen procedure of Fraker and Speck (30) and digested with trypsin that had been treated with TPCK. After electrophoresis at pH 3.6 and ascending chromatography in *n*-butanol/pyridine/acetic acid/water the tyrosine-containing tryptic peptide spots were detected by autoradiography. The autoradiogram is shown in Fig. 3. A total of 21 peptide spots were counted. This represents a good agreement with the maximum theoretical number of tyrosine-containing tryptic peptides possible for a protein of molecular weight 90,000 and a tyrosine mole percentage of 2.8.

Overlay analysis of ABNP. The 90-kDa band corresponding to ABNP was cut from a gel and reelectrophoresed along with the 0.075 *M* NaCl/0.025 *M* EDTA extract of Novikoff hepatoma citric acid nuclei (Fig. 4, lanes A and B). A corresponding set of gel lanes was cut from an unstained gel and subjected to the actin overlay assay of Snabes *et al.* (19) with (Fig. 4, lanes C and D) 0.2 m*M* calcium ion and in the presence of 10 μg/ml cold actin (Fig. 4, lanes E and F). The 90-kDa band corresponding to ABNP was observed to bind ^{125}I-labeled G-actin in both lanes C and D. Similar results to those shown in Fig. 4, lanes C and D, were obtained in the absence of calcium ion (data not shown). Competition with 10 μg/ml cold actin prevented the binding of ^{125}I-G actin. In addition to the 90-kDa ABNP band; four additional bands of lower intensity were detected in Lane C, corresponding in migration to apparent molecular weights of 67, 61, 44, and 39 kDa, which also bound labeled actin. The 44-kDa band in lane C was thought to be endogenous nuclear actin (1).

Discussion. The data presented in this and in an earlier report (1) provide two lines of evidence to support the conclusion that the isolated and partially characterized 90,000 molecular weight protein displays actin-binding activity: (I) ABNP exists in equimass amounts to nuclear actin in the 0.7 *M* NaCl washed chromatin residue fraction and sediments in sucrose density gradients and appears in the excluded volume fraction of a Sepharose CL-6B column in a complex with a stoichiometry similar to that observed in extracts derived from that fraction, and (II) crude and gel purified ABNP bind radiolabeled actin in the ^{125}I-actin overlay assay described by Snabes *et al.* (19).

It should be noted that several bands were detected in the ^{125}I-G-actin gel overlay assay in addition to ABNP (Fig. 4). One of these bands (44 kDa) corresponds to actin. The other three bands (67, 61, and 39 kDa) may

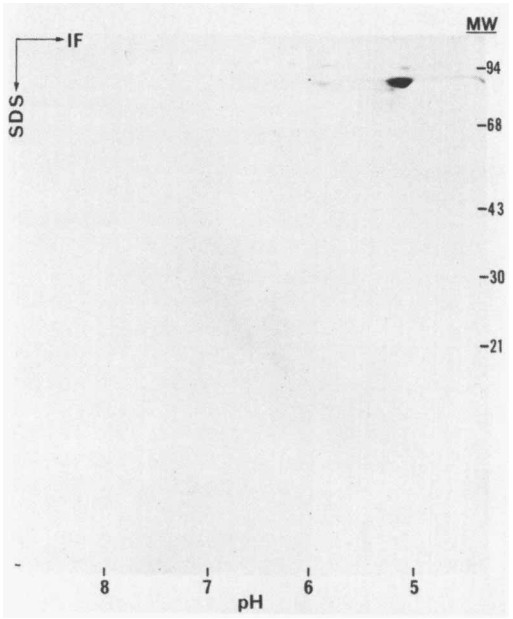

FIG. 2. Two-dimensional IF/SDS gel electrophoresis of purified ABNP. A 20-μg sample of gel purified ABNP was run on a two-dimensional polyacrylamide gel as described by O'Farrell (26) and modified by Hirsch (27). The molecular weight ordinate was constructed from the migration of proteins with known molecular weights: phosphorylase *B*, 92,500; bovine serum albumin, 66,200; ovalbumin, 45,000; carbonic anhydrase, 31,000; soybean trypsin inhibitor, 21,500, and lysozyme, 14,400. The pH gradient was measured with the aid of a micro surface pH electrode; the gel was stained with 0.25% Coomassie brilliant blue R.

represent additional actin-binding proteins, as yet undescribed, that exist within the cell nucleus. Clearly, there are many protein bands in the NaCl–EDTA extract of Novikoff hepatoma that did not bind ^{125}I-labeled G-actin (Fig. 4). Furthermore, all of the ^{125}I-labeled bands were displaced in a competitive overlay assay containing 10 μg/ml unlabeled actin.

Earlier data had suggested that ABNP might merely represent a dimer of nuclear actin. This possibly was eliminated when it was shown that ABNP contained the appropriate number of tyrosine-containing tryptic peptides for a 90-kDa protein. In addition, the tryptic peptide map for ABNP was completely different from the map obtained for nuclear actin (1).

The properties that have been determined for ABNP include a molecular weight of 90,000, an apparent p*I* of 5.2, a ratio for acidic to basic amino acids of 1.6, and a peptide map with the appropriate number of tyrosine-con-

TABLE I. ABNP AMINO ACID COMPOSITION

Amino acids	Mole percentage
Asp	10.7
Thr	5.1
Ser	6.4
Glu	15.7
Pro	4.9
Gly	6.7
Ala	6.5
Val	5.8
Met	2.2
Ile	4.7
Leu	8.6
Tyr	2.8
Phe	3.2
Lys	8.9
His	2.1
Trp	0.3
Arg	5.5
Cys	+

Note. ABNP amino acid composition. Purified ABNP (10 μg) was hydrolyzed in 5.7 *N* HCl for 22 hr at 110°C or with 4 *N* methanesulfonic acid containing 0.2% 3-(2-aminoethyl) indole and amino acid analyzed on a Beckman 121-MB amino acid analyzer. Amino acid values are reported as mole percentages in order to prevent errors associated with molecular weight determination from influencing the values. Cysteine was not quantitated.

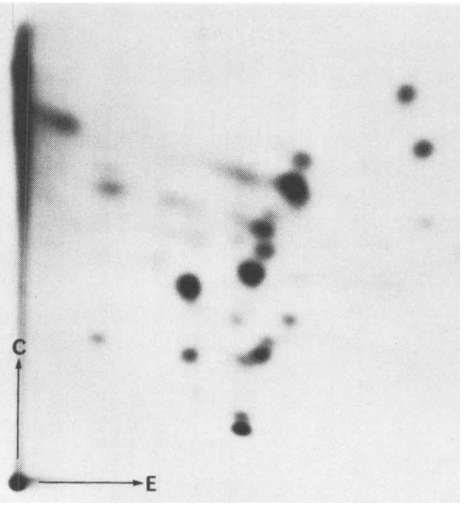

FIG. 3. Two-dimensional ^{125}I-labeled tryptic peptide map of ABNP. Gel purified ABNP was reduced and carboxymethylated and subsequently labeled with ^{125}I by the method of Fraker and Speck (30). Labeled ABNP was digested with TPCK-treated trypsin. Approximately 2 $\times$ 10^6 cpm were spotted on a 20 $\times$ 20-cm cellulose thin-layer plate. Electrophoresis was run at pH 3.6 and 1000 V for 30 min and ascending chromatography in BPAW (15/12/3/10) for 16 hr. ^{125}I-Containing tryptic peptides were detected by autoradiography with RP Royal X-Omat film.

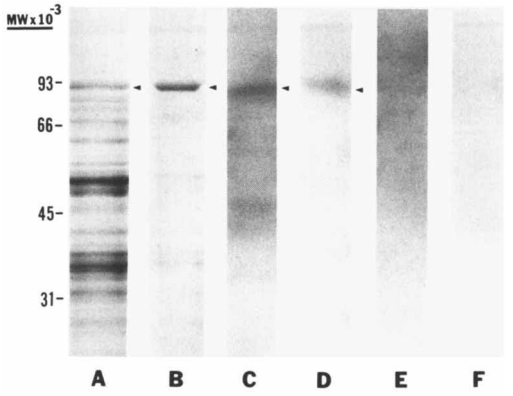

FIG. 4. Polyacrylamide gel electrophoresis and ^{125}-actin overlay assay. The 0.075 *M* NaCl/0.025 *M* EDTA extract of Novikoff hepatoma cell nuclei (A, C, E) and gel purified ABNP (B, D, F) were run on a 10% polyacrylamide gel according to the method of Laemmli (32). Lanes A and B were stained with Coomassie brilliant blue R; lanes C and D were incubated in Buffer B with 1.0 μCi of ^{125}I-actin/ml for 16 hr at 4°C (19) and lanes E and F were incubated in Buffer B containing 1.0 μCi of ^{125}I-actin/ml and G-actin 10 μg/ml for 16 hr at 4°C (19). ^{125}I-Actin binding peptides (lanes C and D) were detected by autoradiography with X-Omat AR film.

taining tryptic peptide fragments. The large amount of glutamic acid in the amino acid composition of ABNP is its only distinguishing feature. Interestingly, it does not contain elevated amounts of glycine, a feature of many RNA binding nuclear proteins (41). Attempts to determine an amino terminal amino acid were negative, indicating that it is probably blocked (data not shown).

One must certainly ask what the function of an actin-binding nuclear protein might be. Studies by Scheer *et al.* have established a close relationship between the states of nuclear actin and the transcription of protein-coding genes (11). Assembly and disassembly of the mitotic apparatus, movement of RNP precursors or their products, and condensation/decondensation of chromatin are all potential functions that could involve regulated motion. The ability to coordinately regulate these activities with the individual phases of the cell cycle by altering the amounts of structural regulatory proteins, or secondary molecules to which they respond, is one set of attractive options for an actin-based system.

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