

Immunolocalization of actin in intact and DNA- and histone-depleted nuclei and chromosomes of *Allium cepa*

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ABSTRACT

The presence of actin in eukaryotic nuclei and chromosomes, and especially in higher plant nuclei and chromosomes, has not been well established. We detected actin in meristematic cells of *Allium cepa* with indirect immunofluorescence technique and observed bright fluorescence in the intact nuclei and chromosomes, indicating that actin is present in the nuclei and chromosomes of the higher plant. We labeled sections of the meristematic cells of *A. cepa* with immunogold technique, gold particles were found over the whole nuclei and a number of gold particles were concentrated in condensed chromatin and nucleoli, confirming the results of the immunofluorescence observations. We treated the nuclei and chromosomes of *A. cepa* with DNase I and 2M NaCl and obtained DNA- and histone-depleted nuclei and chromosomes. Indirect immunofluorescence tests showed that the DNA- and histone-depleted nuclei and chromosomes reacted positively with the anti-actin antibodies. These results demonstrate that actin exists not only in intact nuclei and chromosomes, but also in DNA- and histone-depleted nuclei and chromosomes of the plant. In addition, our immunofluorescence tests indicate that tropomyosin is present in the nuclei and chromosomes of *A. cepa*.

Key words: *Actin, Allium cepa, chromosomes, nuclei.*

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INTRODUCTION

Actin is a contractile protein which was first isolated from vertebrate muscular cells. A large volume of evidence have indicated that actin occurs as an important cytoskeletal protein in both muscular and non-muscular cells of eukaryotes[1]. Since the 70's, biochemical analysis of animal and lower plant cells have showed that actin is one of the non-histone proteins of the nuclei and chromatin[2-12], and many functions have been attributed to nuclear actin including the condensation of chromosomes[13], nuclear transportation[14], and transcription and processing of RNA[15]. Recently, immunofluorescence microscopy, immunoelectron microscopy, confocal imaging and Western blot analysis have been combined to localize actin in the nuclei and chromatin of animal cells[16-19]. However, the study of actin in the nuclei and chromosomes of higher plants has been limited.

Some previous biochemical studies suggested that actin is present in the nuclear matrix of mammalian cells[14, 20]. In contrast, there was a report that actin-like polypeptide revealed by SDS-PAGE in the chromosome scaffold of bovine liver cells is the contamination of the cytoplasmic components[21]. Since many studies used isolated nuclear matrices and chromosome scaffolds, the question of adventitious association of actin with the nuclear matrices and chromosome scaffolds would be difficult to answer. In order to confirm the presence of actin in these infra-nuclear and infra-chromosome structures, it is necessary to localize actin in situ.

In this paper, immunofluorescence and immunogold techniques were used to demonstrate actin in intact nuclei and chromosomes of meristematic cells of *Allium cepa*. We treated the nuclei and chromosomes with DNase I and 2 M NaCl, obtained the DNA- and histone-depleted nuclei and chromosomes, and immunolocalized actin in these treated nuclei and chromosomes in situ. In addition, tropomyosin localized was also in the nuclei and chromosomes with immunofluorescence technique.

MATERIAL AND METHODS

Preparation of specimens for immunofluorescence tests

Bulbs of *Allium cepa* were grown at 26 °C. When roots were 1.5 cm long, root tips were cut off, fixed in 45% acetic acid for 30 min, washed in double distilled water and post-fixed in 4% paraformaldehyde (in 0.01 M PBS, pH 7.4) for 1 h. Then the root tips were washed in double distilled water, digested in a mixture of 0.5% cellulase and 0.5% pectinase for 4 to 6 h, hypotonized in double distilled water for 1 h, and squashed in 45% acetic acid on slides. After coverslips were removed in liquid nitrogen, specimens were viewed with a phase contrast microscope and the selected ones stored in PBS.

In order to remove histones from the nuclei and chromosomes, some of the specimens were pretreated with 2 M NaCl (30 min, 4 °C) according to the protocols described by Berezney and Coffey[22] and Paulson et al[23]. They were washed in double distilled water, digested in 40 µg/ml DNase I for 1 h at room temperature and then washed in PBS. Both pretreated and non-pretreated specimens were used for immunofluorescence tests.

Immunofluorescence tests

Specimens were washed in PBS containing 0.5% Triton X-100, incubated with rabbit anti-actin antibodies (Sigma, A2668) or rabbit anti-tropomyosin antibodies (Sigma, T3651) (1:100 diluted with PBS) for 1 h at 37 °C, and washed in PBS and in PBS containing 0.5% Triton X-100. Specimens were then incubated with goat anti-rabbit IgG conjugated to FITC (Gibco BRL, 9817SA) for 1 h at 37 °C (1:200 diluted with PBS), washed in PBS and in double distilled water, covered with 90% glycerin and observed with an Olympus BH2 microscope.

In order to obtain reliable results for immunofluorescence tests, another protocol was used in this study. The protocol was proposed by Moens and Earnshaw[24] and as follows: Specimens were rinsed in mix (PBS containing 0.05% Triton X-100, 0.25% gelatin, 0.005 M EDTA) for 10 min, blocked in holding buffer (PBS containing 0.05% Triton X-100, 0.3% BSA) for 10 min. They were then incubated with the rabbit anti-actin antibodies (1:40 diluted with holding buffer) for 1 h at 37 °C and overnight at 4 °C. Specimens were washed in PBS and in mix, blocked in holding buffer, and incubated with goat anti-rabbit IgG antibodies conjugated to FITC for 1 h at 37°C. They were finally washed in PBS and in double distilled water, covered with 90% glycerin and observed with a Nikon UFX microscope.

Controls included omission of the primary antibodies. These controls were run concurrently with each experiment and were invariably negative.

Immunogold labeling and electron microscopy

Root tips were fixed in a mixture of 2% glutaraldehyde and 4% paraformaldehyde (in PBS) for 2 h, washed in PBS and in double distilled water, dehydrated in an ascending series of ethanol (25%, 50%, 75% and 90%, 1 h for each) and kept in 90% ethanol for 12 h. They were then infiltrated in infiltration mixture 1 (Lowicryl K4M:90% ethanol, 1:2, 12 h) and mixture 2 (K4M:90% ethanol, 2:1, 12 h), followed by infiltration in pure K4M (room temperature, 24 h; 0 °C, 24 h; -20 °C, 24 h). Specimens were placed in capsules and polymerized under UV light (-35 °C, 24 h; room temperature, 24 h).

Sections were cut on a Reichert-Jung ultramicrotome at a thickness of 70-80 nm and mounted on uncoated nickel grids (300 mesh). Grids were pretreated in a saturated NaIO₄ solution for 45 min and in 0.1 N HCl for 10 min, washed in PBSTG (PBS, 0.2% Tween-20, 15 mM glycine) and blocked in BSA (1% BSA in PBSTG, pH 7.4) for 15 min. They were then incubated in anti-actin antibody (1:25 diluted with 0.1% BSA) for 1 h at 37 °C and overnight at 4 °C. After washing in PBSTG (pH 7.4, then pH 8.0), they were incubated in a solution of protein A-10 nm colloidal gold (Sigma, 1:20 diluted with 0.1% BSA) for 1 h, washed in PBSTG (pH 8.0, then pH 7.4) and double distilled water. The specimens were observed at 75 KV with a Hitachi-600 electron microscope.

Control grids were exposed to 0.1% BSA instead of the antibodies. Nonspecific binding was not observed.

RESULTS

Immunofluorescence tests of actin

Fluorescence microscopic observations upon non-pretreated specimens clearly showed that both intact nuclei and intact chromosomes of *A. cepa* were labeled with rabbit anti-actin antibodies (Figs 1 and 2). The interphase nuclei (arrow in Fig 1) were composed of fine fibrils which gave off bright fluorescence. At prophase, fluorescent fibrils that represented prophase chromosomes were thicker and brighter than that at interphase, and the fibrils twined with each other to form a network that retained the contour of the nucleus (arrowhead in Fig 1). At metaphase, structures with typical morphology of metaphase chromosomes showed intense labeling,

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and longitudinal substructures representing sister chromatids in these metaphase chromosomes could be easily distinguished (Fig 2).

Observations on the specimens pretreated with 2 M NaCl and DNase I further indicated that the DNA- and histone-depleted nuclei and chromosomes were also labeled intensely with the anti-actin antibodies (Figs 7-11). The nuclear antigen detected was found to be distributed throughout the DNA- and histone-depleted interphase nucleus which still showed the shape of intact nuclei (Fig 7). A network consisting of fluorescent fibrils was clearly seen in the prophase nucleus (Fig 8), the labeling signals showed that nuclear actin was mainly situated in the DNA- and histone-depleted prophase chromosomes. The labeling pattern of the DNA- and histone-depleted metaphase chromosomes (Fig 9) was similar to that of intact chromosomes (Fig 2). At anaphase, the DNA- and histone-depleted chromosomes were labeled and situated in one of the mitotic poles, they merged with each other to form a block and chromosome arms protruded from the periphery of the block (Fig 10). In the DNA- and histone-depleted telophase cell, two daughter nuclei sent forth bright fluorescence (Fig 11), showing much intenser labeling than that of the surrounding cytoplasm. It is worth indicating that the same labeling pattern was found in the specimens prepared with the two protocols for immunofluorescence.

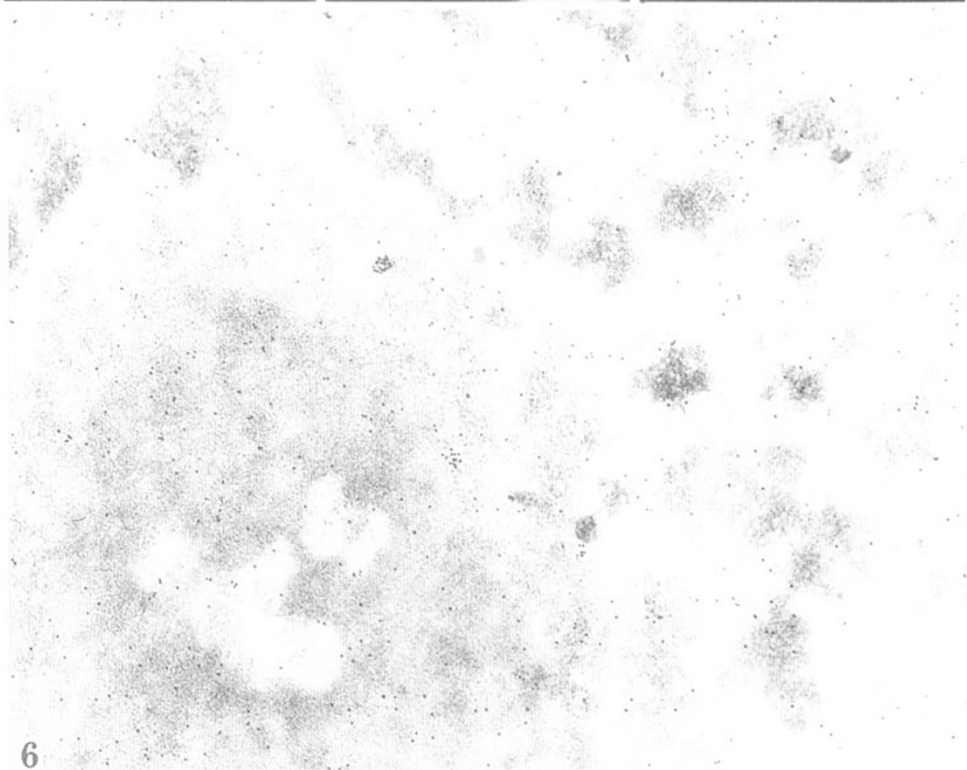
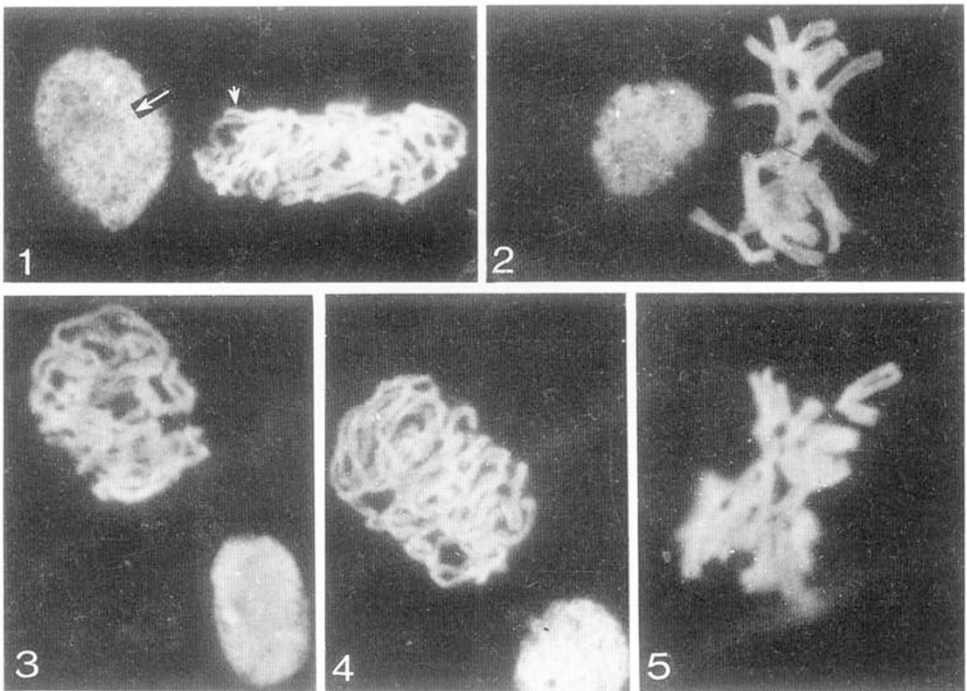
Immunogold labeling of actin

Immunogold technique was used to confirm the presence of actin in nuclei of *A. cepa*. After reaction with the anti-actin antibodies and protein A-gold, sections of meristematic cells of *A. cepa* showed general labeling throughout the whole nucleus

▷ **Fig 1, 2.** Micrographs showing intact nuclei and chromosomes of *Allium cepa* labeled with rabbit anti-actin antibodies and FITC-conjugated goat anti-rabbit IgG antibodies. The interphase nuclei gave off prominent fluorescence, indicating the presence of actin in the interphase nuclei (arrow in Fig 1). The prophase nucleus contained fibrils which corresponded to the prophase chromosomes, and the fibrils showed labeling of actin and twined with each other to form a fluorescence network within the prophase nucleus (Fig 1). Metaphase chromosomes were intensely labeled (Fig 2). Note that the longitudinal substructures representing the sister chromatids in the metaphase chromosomes were distinguishable (Fig 2)

Fig 3- 5. Micrographs showing nuclei and chromosomes of *Allium cepa* labeled with rabbit anti-tropomyosin antibodies and FITC-conjugated goat anti-rabbit IgG antibodies. The intact interphase nuclei and prophase chromosomes sent forth bright fluorescence, demonstrating the existence of tropomyosin (Fig 3). The specimens were treated with DNase I and 2 M NaCl to remove DNA, histones and most of nonhistones from the nuclei and chromosomes. The DNA- and histone-depleted interphase nuclei and prophase chromosomes (Fig 4) as well as metaphase chromosomes (Fig 5) showed intense labeling of tropomyosin.

Fig 6. Electron micrograph showing a nucleus of *Allium cepa* which was labeled with anti-actin antibodies and protein A-10 nm colloidal gold. Large amounts of the gold particles were distributed over the nucleolus and condensed chromatin, and a few gold particles were found in the interchromatin domains.



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(Fig 6). The total number of gold particles and their density in different domains in the nuclei of both the labeled specimens and control ones were obtained (Tab 1) from analysis of the EM photographic plates by using IBAS image processing system, and the statistics of the several photographic plates may occasionally show a small difference from that observed in a single photograph. Analysis of distribution of gold particles revealed that a number of gold particles were concentrated upon condensed chromatin and nucleolar regions, only a few gold particles were located in interchromatin domains. The average density of gold particles in nucleoli was $34.17/\mu\text{m}^2$, and that in condensed chromatin was $23.37/\mu\text{m}^2$, while the density of gold particles in interchromatin domains was only $6.16/\mu\text{m}^2$ which was similar to that of the controls ($4.37/\mu\text{m}^2$, Tab 1).

Immunofluorescence tests of tropomyosin

The anti-tropomyosin antibodies directed against rabbit tropomyosin labeled both intact nuclei and chromosomes and DNA- and histone-depleted nuclei and chromosomes of *A. cepa* (Figs 3-5, 12). Prominent fluorescence was observed in intact interphase nuclei and prophase chromosomes (Fig 3), and also found in DNA- and histone-depleted interphase nuclei and prophase chromosomes (Fig 4). At metaphase and anaphase, DNA- and histone-depleted chromosomes showed similar typical labeling patterns (Figs 5 and 12), like that of actin in intact ones.

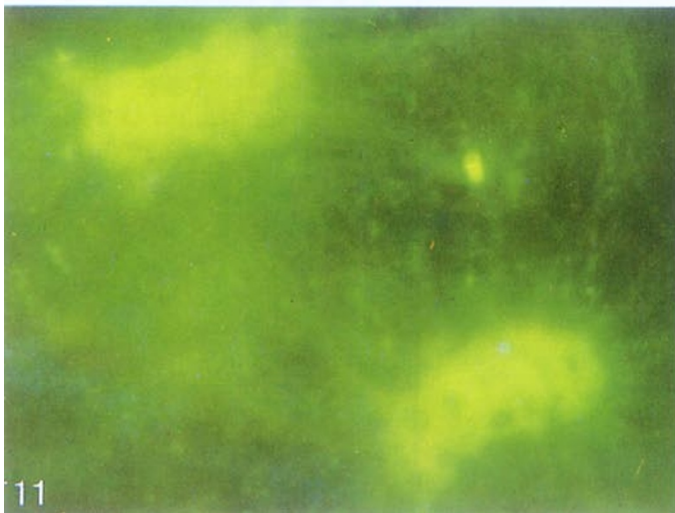
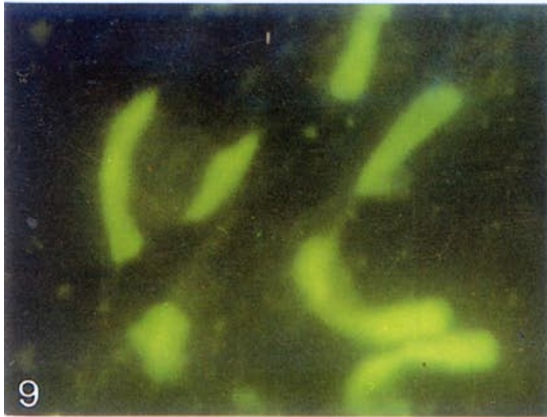
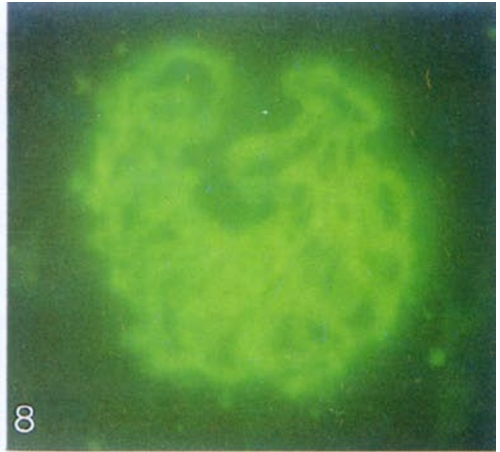
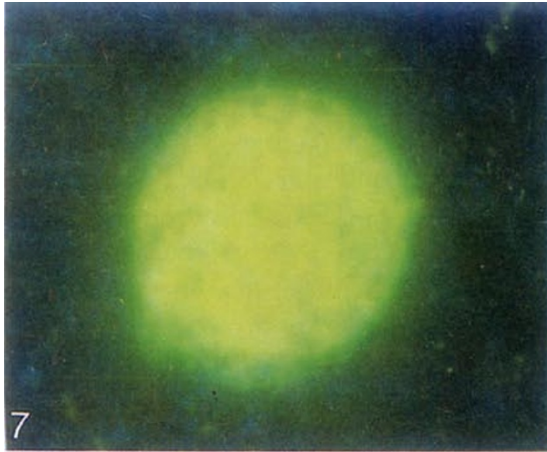
DISCUSSION

Actin is a constituent of nuclei and chromosomes

Actin was first isolated from vertebrate muscular cells and has been proved to be

- ▷ **Fig 7-11.** Micrographs of DNA- and histone-depleted nuclei and chromosomes of *Allium cepa*. Specimens were treated with DNase I and 2 M NaCl to remove DNA, histones and most of the nonhistones from the nuclei and chromosomes, and then labeled with rabbit anti-actin antibodies and FITC-conjugated goat anti-rabbit IgG antibodies. The DNA- and histone-depleted interphase nuclei (Fig 7), prophase chromosome fibrils (Fig 8) and metaphase chromosomes (Fig 9) showed positive reaction to actin, and the labeling patterns were similar to those of the intact nuclei and chromosomes as shown in Figs 1, 2). Fig 10 showing the labeled DNA- and histone-depleted chromosomes at anaphase, the chromosomes were gathered together in one of the mitotic poles. Note that some chromosome arms protruded from the periphery of the merged block. Fig 11 showed the two daughter nuclei in one telophasic cell which has been depleted of DNA and histones. Note that the daughter nuclei gave much intenser labeling of actin than that of the surrounding cytoplasm.

Fig 12. Fig 12 showing the DNA- and histone-depleted anaphase chromosomes of *A. cepa* labeled with tropomyosin. Note the protrusion of chromosome arms from the merged block of chromosomes.



vvTab 1. Density of colloidal gold particles in nuclei of *A. cepa*¹

Specimens	No. of		Experimental specimens				Control ²	
	Nucleolus		Chromatin		Interchromatin domain		Control ²	
	Number of gold particles	Area (μm^2)	Number of gold particles	Area (μm^2)	Number of gold particles	Area (μm^2)	Number of gold particles	Density
1	164	4.81	55	2.66	14	3.01	13	2.29
2	214	5.34	45	2.24	21	20.10	26	4.58
3	181	5.46	59	2.39	24	24.72	28	4.93
4	68	2.30	51	2.35	16	21.72	37	6.25
5	117	3.71	68	2.82	20	24.12	20	3.52
6	53	1.71	70	2.42	27	28.91		
7	2.14	5.30						
Σ	1011	28.63	348	14.88	122	19.71	124	
$M \pm SD$		34.17 ± 3.98		23.37 ± 2.99		6.16 ± 1.10		4.37 ± 1.42

1. The root tips of *Allium cepa* were fixed in 2% glutaraldehyde and 4% paraformaldehyde, dehydrated in an ascending series of ethanol and embedded in Lowicryl K4M. Thin sections were labeled with anti-actin antibodies and protein A-10 nm colloidal gold, observed with Hitachi-600 electron microscope. Using IBAS imaging processing system, the statistics of total number of gold particles and their density in different domains of nuclei of the labeled specimens and control one was obtained.
2. The analysed area of each sample of the control was $5.68 \mu\text{m}^2$.

present as an important cytoskeletal protein in both muscular and non-muscular cells of eukaryotes[1]. Biochemical analysis has shown that actin occurs in isolated nuclei of slime mold[2, 5], of amphibian oocytes[7, 8, 11], of mammalian cells[3, 4, 6] and of cells of some other species (reviewed by References 17, 19). By using immunogold labeling and electron microscopy, Armbruster et al[16] localized actin in nuclei of Chinese hamster ovary cells. Recently, Milankov and Boni[17] demonstrated by immuno-confocal microscopy and ultrastructural immunogold labeling, that interphase nuclei of dorsal root ganglion neurons and of PC12 cells contain actin. Parfenov et al[19] further revealed at electron microscopic level that actin filaments are present in the nuclei of frog oocytes. Although many reports are found in the literature concerning actin in animal nuclei, the presence of the protein in nuclei and chromosomes of higher plants has not been well established.

In the present paper, meristematic cells of *A. cepa* were labeled with rabbit anti-actin antibodies and FITC-conjugated goat anti-rabbit IgG antibodies and observed with fluorescence microscopy. Both the nuclei and chromosomes sent forth bright fluorescence, indicating that the nuclei and chromosomes contain actin. Sections of the meristematic cells were labeled with the anti-actin antibodies and protein A-10nm colloidal gold and observed with electron microscopy. The gold particles were found over the nuclei, and a great deal of gold particles were concentrated on condensed chromatin and nucleoli, confirming that actin is a constituent of the nuclei of *A. cepa*. All these results are consistent with the recent observations upon actin component in nuclei and chromosomes of *Vicia faba*[25]. These results from higher plants, together with previous biochemical and immunocytochemical evidence from animals and lower plants, suggest that the existence of actin in nuclei and chromosomes is an universal phenomenon of eukaryotic cells.

Actin is associated with DNA- and histone-depleted nuclei and chromosomes

Previous reports have indicated that when mammalian cell are digested with DNase I to remove most of the DNA and extracted with high concentrations of salt (usually 2 M NaCl) to remove histones, a fibrillar network of non-histone proteins remains in the region of the nucleus which has been termed as nuclear matrix. The DNA- and histone-depleted chromosome which retains the shape of the metaphase chromosomes is termed the chromosome scaffold[22-23, 26-33]. The first described component of proteins of the chromosome scaffold is DNA topoisomerase II[34-35], but the multiple protein components of the nuclear matrix and chromosome scaffold have not been fully characterized.

As here mentioned above, actin has been proved to be one non-histone protein in the nuclei and chromosomes of animal and lower plant cells[2, 3, 5, 16-19], and the data presented here further indicate that actin is also a constituent of nuclei and chromosomes of a higher plant *Allium cepa*. However, opinions upon whether actin occurs in the nuclear matrix and chromosome scaffold is still controversial. Capoco

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et al[20] reported that SDS-PAGE analysis shows a 43kD polypeptide in the nuclear matrix of HeLa cells and suggested that the polypeptide is possibly actin. Nakayasu and Ueda[14] indicated, on the basis of results of SDS-PAGE and Western blot, that four actin components are associated with the nucleus and nuclear matrix of mouse L5178Y cells. Pieck et al[21] revealed a 43kD polypeptide in SDS-PAGE analysis of chromosome scaffolds isolated from bovine liver cells but they considered it as a contamination of the cytoplasmic components. Recently, Zeng et al[36] demonstrated that actin is a constituent of the nuclear matrix and chromosome scaffolds of a lower plant *Physarum polycephalum*.

In this paper, meristematic cells of *A. cepa* were treated with DNase I and 2 M NaCl to remove DNA and histones from the nuclei and chromosomes. After reaction with the anti-actin antibodies, fluorescence localizing the antigen was observed in the DNA- and histone-depleted nuclei and chromosomes. After comparing the fluorescence densities of non-pretreated and pretreated preparations and of preparations at different stages (interphase, prophase, metaphase, anaphase and telophase), two interesting points emerged. One is that non-pretreated (intact) nuclei and chromosomes and pretreated (DNA- and histone-depleted) nuclei and chromosomes show the similar fluorescence densities when they were labeled according to the same protocols. The another one point is that the antibody-directed antigen detected in the DNA- and histone-depleted preparations is mainly located in the chromosomes through the whole mitotic cycle i.e., from interphase to telophase. At interphase and prophase, the fluorescence is focused on the fibrils that represent the interphase and prophase chromosomes; at metaphase, anaphase and telophase, the brighter fluorescence is also displayed by chromosomes of these stages. All these observations lead to the conclusion that actin is not only present in intact nuclei and chromosomes but also present in the DNA- and histone-depleted nuclei and chromosomes. Although there is no indisputable biochemical data to indicate that actin is a constituent of the nuclear matrix and chromosome scaffold of *A. cepa*, our immunofluorescence results do provide new evidence supporting the point of view that actin is associated with the DNA- and histone-depleted nuclei and chromosome of the higher plant.

Tropomyosin is present in nuclei and chromosomes

Tropomyosin is one of actin-binding proteins, which usually coexists with actin in a wide range of cytoplasmic structures and stabilizes actin filaments by binding along the length of the filaments[1]. Douvas et al.[3] reported that tropomyosin is one of non-histone proteins of rat liver chromatin. In the present paper, immunofluorescence observations indicate that tropomyosin occurs in both intact and DNA- and histone-depleted nuclei and chromosomes. In addition, the distribution of tropomyosin during the whole mitotic cycle is similar to that of nuclear actin. Based on these results, it is tempting to suggest that tropomyosin also functions as an actin-binding protein in the nuclei and chromosomes.

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