Sp1 and Sp3 foci distribution throughout mitosis

Shihua He and James R. Davie*

Manitoba Institute of Cell Biology, University of Manitoba, Winnipeg, Manitoba, R3E 0V9, Canada

*Author for correspondence (e-mail: davie@cc.umanitoba.ca)

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Summary

The mammalian transcription factors Sp1 and Sp3 compete for the same DNA binding sites but play different roles in the regulation of expression of numerous genes. It is known that, in the interphase nucleus, Sp1 and Sp3 are organized into distinct foci. In this study, we show that throughout the mitotic process, while being displaced from the condensed chromosomes and dispersed throughout the cell, Sp1 and Sp3 maintain their separate punctate distributions. In metaphase, both Sp1 and Sp3 foci show a high degree of colocalization with microfilaments, suggesting that F-actin is involved in the organization of Sp1 and Sp3 foci during mitosis. Constant Sp1 and Sp3 levels were observed during mitosis, signifying a recovery of the pre-existing Sp1 and Sp3 population in newly formed nuclei. In late telophase, Sp1 and Sp3 are equally segregated between daughter cells, and their subnuclear organization as distinct foci is restored in a sequential fashion with Sp3 regrouping into the newly formed nuclei prior to Sp1. Both Sp1 and Sp3 return to the nuclei ahead of RNA polymerase II. Our results support a model in which entry of Sp1, Sp3 and RNA polymerase II into the newly formed nuclei is an ordered process.

Key words: Sp1, Sp3, Transcription factors, Mitosis, Cytoskeleton

Introduction

The transcription factors Sp1 and Sp3 are ubiquitously expressed in mammalian cells and participate in regulating the expression of genes involved in almost all cellular processes (Cawley et al., 2004). Despite their binding to the same DNA sites, they have different roles. Unlike Sp1, which is usually an activator of transcription, Sp3 functions as an activator or a repressor, with its function being dependent upon several parameters, including promoter context, state of modification and chromatin structure (Suske, 1999; Bouwman and Philipsen, 2002; Li et al., 2004; Sapetschnig et al., 2004).

Another feature distinguishing Sp1 and Sp3 is their localization at distinct subnuclear sites as evidenced by the non-overlapping foci revealed by the high-resolution fluorescence microscopy analysis of interphase nuclei (He et al., 2005). This lack of colocalization applies to the association of Sp1 and Sp3 with the nuclear matrix, because they occupy different sites (He et al., 2005). This differential subnuclear distribution of the Sp1 and Sp3 foci might have a biological significance. It is conceivable that the differential subnuclear distribution of the Sp1 and Sp3 foci plays a role in deciding the temporal and dynamic occupancy of a promoter by one or the other Sp factor. For example, although Sp1 and Sp3 both bind to the *TFF1* promoter, their binding has been shown to be reciprocally exclusive (He et al., 2005). It is possible that the spatial position of a promoter next to a Sp1 or Sp3 domain influences, which of the two factors will occupy the Sp-binding site and affects gene regulation.

The distinct distribution of Sp1- and Sp3-foci must be maintained and transferred to progeny cells after mitosis. From the few imaging studies that have investigated the mitotic partitioning of regulatory proteins, it appears that the distribution of most transcription factors becomes dispersed through the mitotic cell in prophase and is mostly restored to colocalize with chromatin in telophase. Some transcription factors, for example Oct-1, Fos or TFIIB (Martinez-Balbas et al., 1995; Chen et al., 2002), as well as the chromatin-remodeling factors histone acetyltransferases (HATs) and histone deacetylases (HDACs) (Kruhlak et al., 2001) are clearly translocated away from the condensed chromosomes. Other transcription factors, such as Runx (Zaidi et al., 2003), are predominantly redistributed to an extra-chromosomal space in metaphase and anaphase, except for a subset of foci that remain associated with chromatin throughout mitosis. However, the mitotic displacement of transcription factors cannot be generally assumed because at least two transcription factors, $p67^{SRF}$ (Gauthier-Rouviere et al., 1991) and AP-2 (Martinez-Balbas et al., 1995) were found to stay associated with the condensed metaphase chromosomes. Although the distribution of Sp1 during mitosis has been analyzed in HeLa cells (Martinez-Balbas et al., 1995), it is not possible to know from the data presented whether a subset of Sp1 stays with the condensed chromatin or whether the whole Sp1 population is displaced. The mitotic behavior of Sp3 has not been explored yet.

In this study, we used high-resolution fluorescence microscopy to examine the distribution of Sp1 and Sp3 during the different phases of mitosis. Our results show that Sp1 and Sp3 keep their distinct punctate distribution during mitosis, but they get entirely displaced from the condensed chromosomes and are temporarily localized with the microfilaments. Moreover, there is a temporal order to Sp1 and Sp3 postmitotic reorganization, with Sp3 re-entering the nuclei ahead of Sp1.

Results

Mitotic redistribution of transcription factors Sp1 and Sp3

Our previous in-situ-fluorescence microscopy studies of transcription factors Sp1 and Sp3 showed that they are

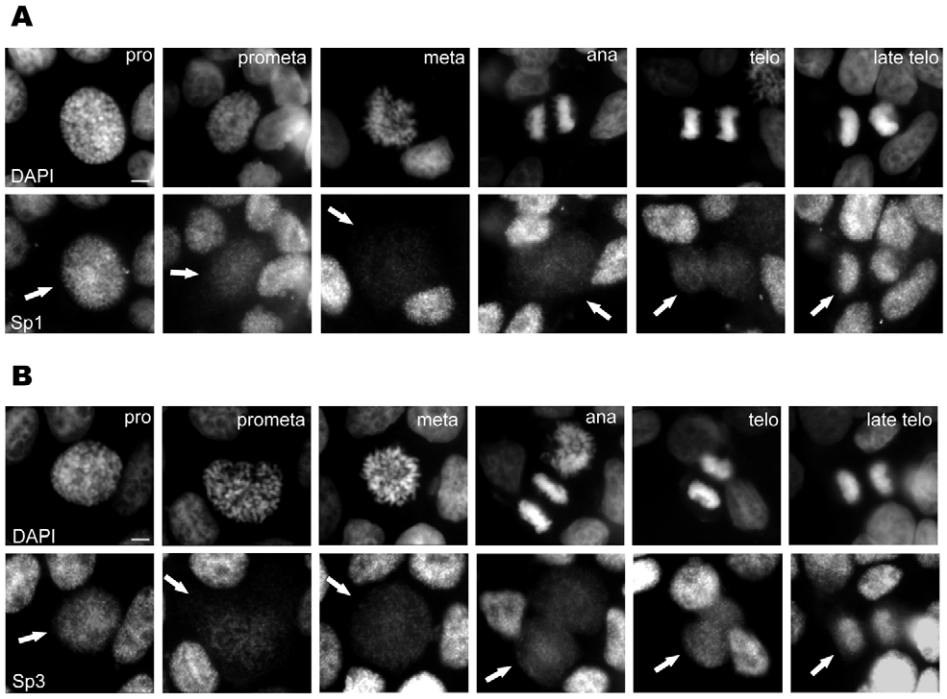

Fig. 1. Temporal distribution of transcription factors Sp1 and Sp3 during mitosis. MCF-7 cells were subjected to indirect immunofluorescence labeling with an antibody against Sp1 or Sp3, and co-stained with DAPI for identification of cell-cycle stage. Cells at various phases of mitosis were digitally imaged. Bar, 5 μm.

distributed throughout the interphase nucleus at distinct foci, occupying the same nuclear space as DNA. However, Sp1 and Sp3 are not dependent on DNA for their nuclear distribution, because this distribution of foci is maintained in nuclear matrices that are devoid of DNA (He et al., 2005). During mitosis, a dispersion of the Sp1 staining from mitotic chromosomes has been reported in HeLa cells (Martinez-Balbas et al., 1995). To verify the redistribution of Sp1 during mitosis in MCF-7 cells and to investigate the fate of Sp3, we analyzed the spatial distributions of Sp1 and Sp3 in MCF-7 cells at different phases of mitosis by using fluorescence microscopy after indirect immunofluorescence labeling of MCF-7 cells grown and fixed on coverslips. These MCF-7 cells constituted a cycle-asynchronized cell population. Cells from each mitotic phase were identified according to their DAPI staining. Fig. 1A shows the dispersion of Sp1 immunolabeling initiating in prometaphase and persisting until telophase, in agreement with a previous report (Martinez-Balbas et al., 1995). By late telophase and/or early G1 phase, the Sp1 staining was mostly restored to the daughter nuclei. Similarly, Sp3 immunostaining was found dispersed throughout the cell from prometaphase until telophase, after which it again coincided with the DAPI staining (Fig. 1B). We also observed that, from prophase to telophase, the bulk of Sp1 and Sp3 was displaced from condensed chromatin or chromosomes (Fig. 1).

Previous studies showed that Sp1 colocalized with HDAC1 and HDAC2 and estrogen receptor α (ERα), albeit at low levels in the MCF-7 nucleus. Similar results were obtained with Sp3. Thus, for comparison, we examined the distribution of these regulatory proteins during mitosis. Fig. 2 shows that the immunolabeling of HDAC2 and ERα started to spread throughout the cell in prometaphase, similar to Sp1 and Sp3. However, the return of HDAC2 staining to its interphase distribution started to occur earlier than for Sp1, Sp3 and ERα. In anaphase, HDAC2 already appeared mostly colocalized with DNA. The temporal reorganization of HDAC1 was similar to that of Sp1 and Sp3 (data not shown). This observed mitotic rearrangement of HDAC1 and HDAC2 is in agreement with published data, although in our study HDAC2 seemed to relocalize with chromatin in an earlier phase (Kruhlak et al., 2001). On the other hand, ERα was slower to re-enter the nucleus, perhaps slower than Sp1 and Sp3, still maintaining a somewhat dispersed staining, only partially colocalized with DNA in late telophase and/or early G1 phase (Fig. 2).

Mitotic partitioning of Sp1 and Sp3

The immunofluorescence intensity of Sp1, Sp3, HDAC2 and ERα in mitotic cells was considerably less than in interphase nuclei (Figs 1 and 2). To elucidate whether this reduction in immunofluorescence signal was solely due to a dilution effect or whether there was a degradation of these proteins occurring during mitosis, we compared the immunofluorescence intensities between cells blocked in G2 phase or mitosis. MCF-7 cells synchronized in G2 and/or M phase on coverslips were immunolabeled with anti-Sp1, anti-Sp3, anti-HDAC2 or anti-ERα antibodies, while co-stained with DAPI to differentiate cells in G2 phase from cells in M phase. The immunofluorescence intensity of cells in G2 phase was compared with that of cells in mitosis. The diagram in Fig. 3A shows that interphase and metaphase levels of Sp1, Sp3, ERα and HDAC2 were similar, indicating the absence of significant mitotic degradation. To verify these results, we performed immunoblot analyses of cellular extracts from MCF-7 cells at various stages of the cell cycle. Fig. 3B,C shows the Sp1, Sp3 or ERα levels relative to those of actin in asynchronized MCF-7 cells (cultured in Dulbecco's modified Eagle's medium, DMEM), and a MCF-7 cell population with 90% of the cells blocked in G0 and/or G1 phases (treated with hydroxyurea, HU) or with 85% of the cells blocked in G2 and/or M phases (colcemid). Sp1, Sp3 and ERα levels appeared constant in the

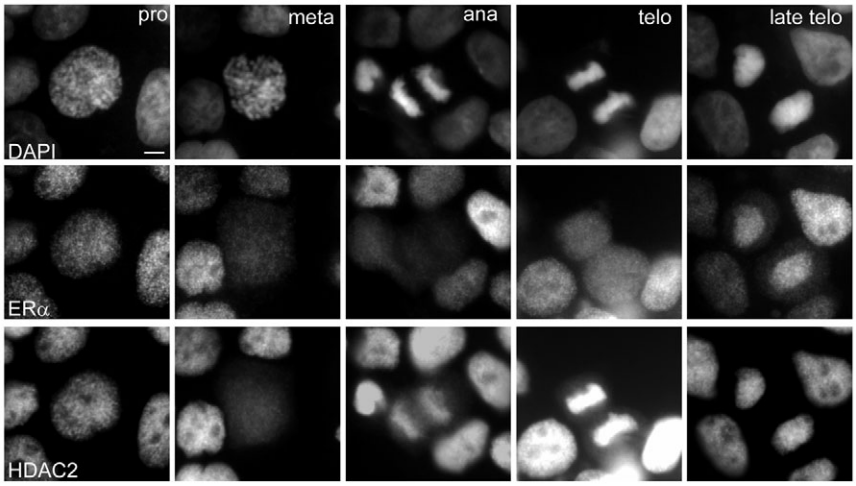

Fig. 2. Temporal distribution of ERα and HDAC2 during mitosis. MCF-7 cells were immunolabeled with antibodies against ERα or HDAC2, and co-stained with DAPI. Cells at various phases of mitosis were digitally imaged. Bar, 5 μm.

three samples, confirming that there was no significant change in the levels of Sp1, Sp3 and ERα in interphase and mitotic cells.

Next, we examined the partition of Sp1 and Sp3 between daughter nuclei, by measuring the fluorescence intensities in two postmitotic nuclei, and comparing the mitotic partition-coefficient of the regulatory proteins with that of the DNA staining with DAPI. We found that Sp1 and Sp3 were equally segregated between daughter nuclei after division (Fig. 4). The same results were obtained for ERα, HDAC1 and HDAC2 (data not shown).

Displacement of Sp1 and Sp3 from mitotic chromosomes as distinct foci

We have previously shown that Sp1 and Sp3 coexist in the interphase nucleus as separate discrete foci (He et al., 2005). To elucidate what happens to these distinct foci during mitosis, we used fluorescence microscopy and image deconvolution to compare their relative spatial distributions in MCF-7 cells throughout mitosis. Asynchronized cycling cells were fixed on coverslips, and cells at different mitotic phases were identified in accordance with their DAPI staining. Fig. 5 shows that, from prophase to telophase, Sp1 and Sp3 remained organized as distinct foci while being translocated away from chromatin. In prophase, when chromatin began to condense, the migration of Sp1 and Sp3 foci to extra-chromosomal spaces was observed. In prometaphase, Sp1 and Sp3 foci started spreading throughout the cell. In late telophase, Sp1 and Sp3 foci were mostly restored to their interphase distribution. Significantly, Sp1 and Sp3 foci remained distinct at all stages of mitosis.

To further define the timing of the Sp3 mitotic displacement relative to nuclear membrane dissolution, we performed

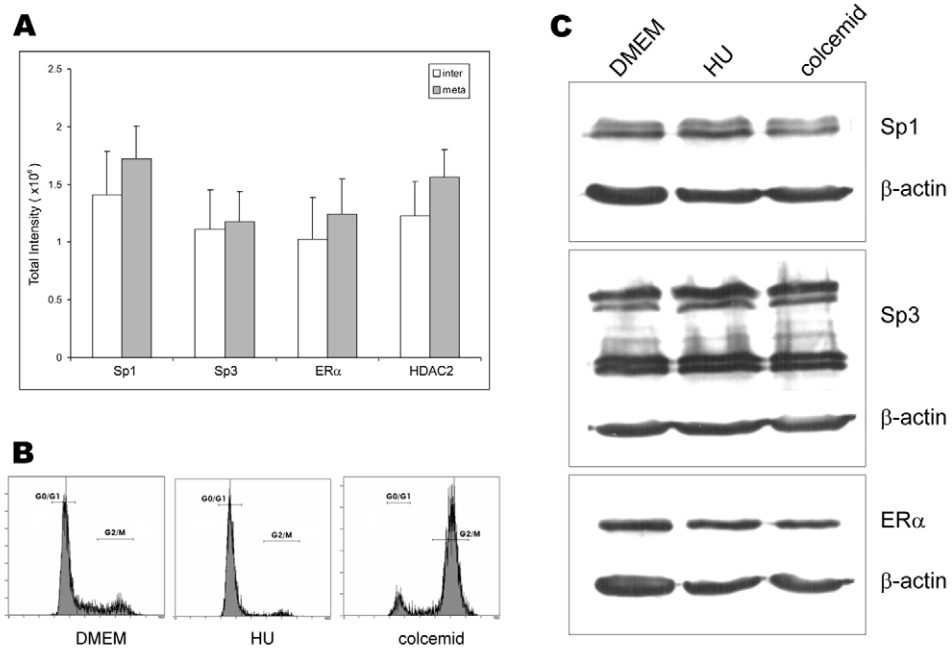

Fig. 3. Quantitative redistribution of Sp1, Sp3, ERα and HDAC2 during mitosis. (A) MCF-7 cells were treated with 0.06 μg/ml colcemid for 16 hours and immunolabeled with anti-Sp1, anti-Sp3, anti-HDAC2 or anti-ERα antibodies, while co-stained with DAPI, to identify the phase of the cell cycle. Cells were digitally imaged. Metaphase cells are recognized by the bright DAPI staining of their condensed chromatin. The pixel intensities of interphase and metaphase cells were quantified from the same image of one slide displaying both types of cells. The total intensities of 30-50 interphase cells and 30-50 metaphase cells were measured with AxioVision 4.1 software. Error bars show the standard deviation (s.d.). (B) The cell-cycle status of different cell populations was characterized by FACS analysis: MCF-7 cells grown in DMEM (DMEM), treated with 1.5 mM hydroxyurea (HU) or treated with 0.06 μg/ml colcemid (colcemid). (C) Immunoblots of cellular extracts of MCF-7 cells in different phases of the cell cycle as shown in (B) were probed with antibodies as indicated.

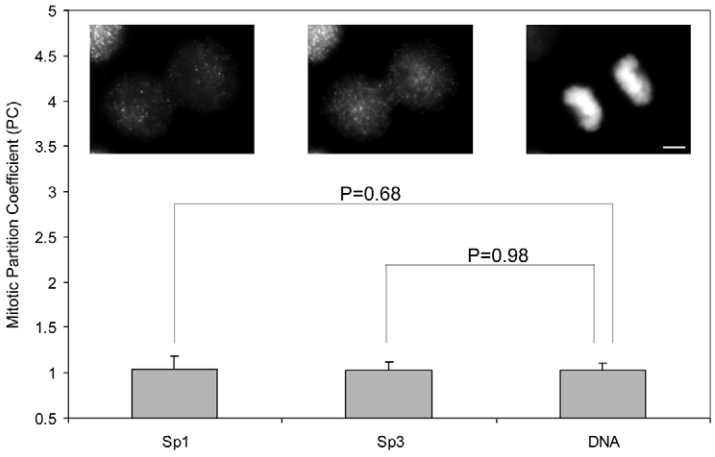

Fig. 4. Equal partitioning of Sp1 and Sp3 between daughter nuclei. MCF-7 cells grown on coverslips were treated with 0.06 μg/ml colcemid for 16 hours, cultured for 3 more hours in fresh medium and immunolabeled with anti-Sp1 and anti-Sp3 while co-stained with DAPI. Bar, 5 μm. A quantitative image analysis was performed to determine the relative levels of Sp1 and Sp3 in the telophase cells. The partition coefficient (PC) indicates the ratio of integrated signal intensities between two daughter nuclei. Student's *t*-test was applied to assess the significance of observed differences.

double-immunofluorescence labeling of Sp3 with lamin A/C and examined MCF-7 cells throughout mitosis by fluorescence microscopy followed by image deconvolution (Fig. 6). In interphase, Sp3 was distributed throughout the nucleus; lamin A/C were concentrated in the nuclear lamina underlying the inner nuclear membrane. In prophase, when the chromatin started condensing, Sp3 started to concentrate in extra-chromosomal space. In prometaphase, Sp3 began pouring out of the nucleus as the lamina ruptured. Lamin A/C reappeared in telophase around the segregated chromosomes and then became visible as part of the lamina surrounding the nuclei. Simultaneously, Sp3 foci began to congregate in the newly formed nucleus.

Sequential entry of Sp1 and Sp3 into daughter nuclei

It was reported that RNA polymerase II (RNAP II) and general transcription factors, including TFIIE, re-entered daughter nuclei after the nuclear envelope and/or lamina assembly (Prasanth et al., 2003). The concurrent repositioning of Sp3 into telophase nuclei with the lamina formation prompted us to ascertain the order in which Sp1, Sp3 and RNAP II re-enter postmitotic nuclei. With this purpose, we performed indirect double-

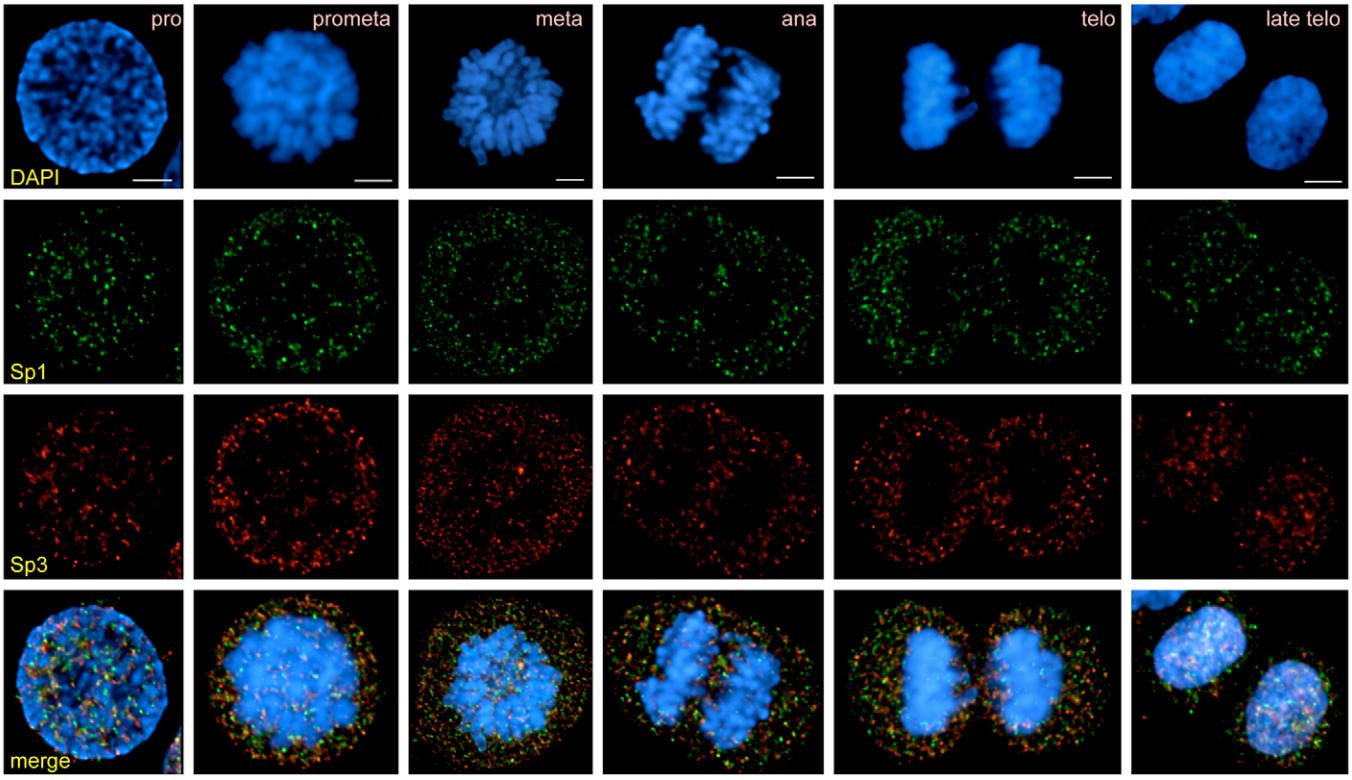

Fig. 5. Distribution of Sp1 and Sp3 throughout the mitosis stages. MCF-7 cells were grown on coverslips in estrogen-complete medium, fixed, and double labeled with anti-Sp1 and anti-Sp3 antibodies. DNA was stained by DAPI. Sp1 and Sp3 distributions were visualized by fluorescence microscopy and image deconvolution as described in Materials and Methods. Single optical sections are shown. Yellow in the merged images signifies colocalization. Bars, 5 μm.

immunofluorescence labeling of asynchronized MCF-7 cells, using antibodies against Sp1 or Sp3 and RNAP II. We used an anti-RNAP II antibody that recognized both hypo-phosphorylated and Ser5-phosphorylated forms of the enzyme. Fluorescence microscopy in the ApoTome mode was used to select and study postmitotic nuclei. Fig. 7 shows that both Sp1 and Sp3 re-enter daughter nuclei before RNAP II, which is in agreement with the concomittant re-entry of Sp3 with the lamina formation. Next, using the same technique, we carried out a Sp1-Sp3 double labeling to find out whether Sp1 and Sp3 returned to the nuclei at the same time. Among the asynchronized MCF-7 cells, we searched for cells that were between telophase and late telophase, specifically cells that happened to be in the time interval when the return of Sp1 and Sp3 to the nucleus had been initiated but not yet completed. Fig. 7 shows that Sp3 labeling colocalized with the DAPI staining of DNA in daughter nuclei at a time when Sp1 staining was still spread throughout the cells. Thus Sp3 clearly returned earlier to the nuclei than Sp1.

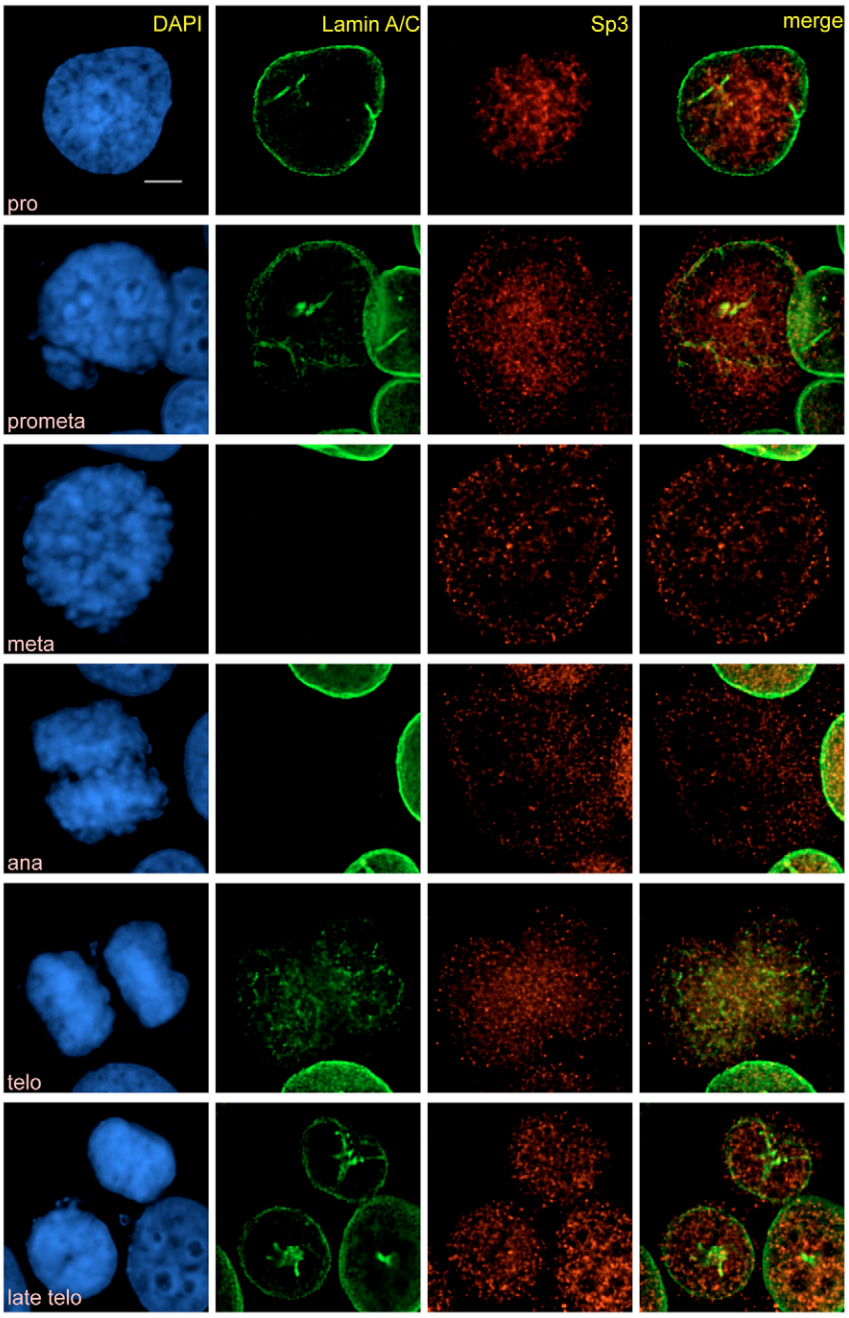

Fig. 6. Temporal mitotic distribution of Sp3 and lamins. MCF-7 cells were grown on coverslips in estrogen-complete medium, fixed, and double labeled with rabbit anti-Sp3 antibodies and mouse monoclonal anti-lamin A/C antibodies. DNA was stained by DAPI. Spatial distribution was visualized by fluorescence microscopy and image deconvolution as described in Materials and Methods. Single optical sections are shown. Bar, 5 μm.

Mitotic association of Sp1 and Sp3 with F-actin

We have previously demonstrated that Sp1 and Sp3 bind different sites of the nuclear matrix in interphase cells (He et al., 2005). In an effort to find out whether another scaffolding structure is involved in keeping Sp1 and Sp3 organized as separate foci during their mitotic transfer, we used immunofluorescence microscopy and image deconvolution to test their association with several cellular components. First, because three inner nuclear membrane proteins (lamin B receptor, and lamina associated polypeptides 1 and 2) have been reported to redistribute into the endoplasmic reticulum membrane during mitosis (Yang et al., 1997; Ellenberg et al., 1997), we performed double immunolabeling of Sp1 and calnexin, a membrane-bound chaperone protein of the endoplasmic reticulum (David et al., 1993). Although the merged images in Fig. 8 show few merged (yellow) foci, most Sp1 foci are distinct and separate from the calnexin staining in metaphase cells. Fig. 8 also shows that Sp1 did not colocalize with intermediate filament proteins, cytokeratin 8 or cytokeratin 18. Conversely, there was a high degree of colocalization between Sp1 and the microfilaments F-actin, with Sp1 foci aligning along the actin fibers (Fig. 8). Colocalization of Sp1 and F-actin was also noticed in the space vacated by chromosomes, whereas such a colocalization was not seen in the case of Sp1 and cytokeratin 8 or cytokeratin 18, with only Sp1 showing as a star-like staining. Similarly to Sp1, Sp3 was not found to colocalize with the endoplasmic reticulum or the intermediate filaments but was found to colocalize with F-actin (data not shown). This suggests that the microfilaments are involved in the organization of Sp1 and Sp3 foci during mitosis.

To find out whether an in vitro interaction of Sp1 and Sp3 with F-actin can be detected, MCF-7 nuclear extract was added to F-actin and the mixture was centrifuged to test whether Sp1 and Sp3 would co-sediment with F-actin. Pellet and

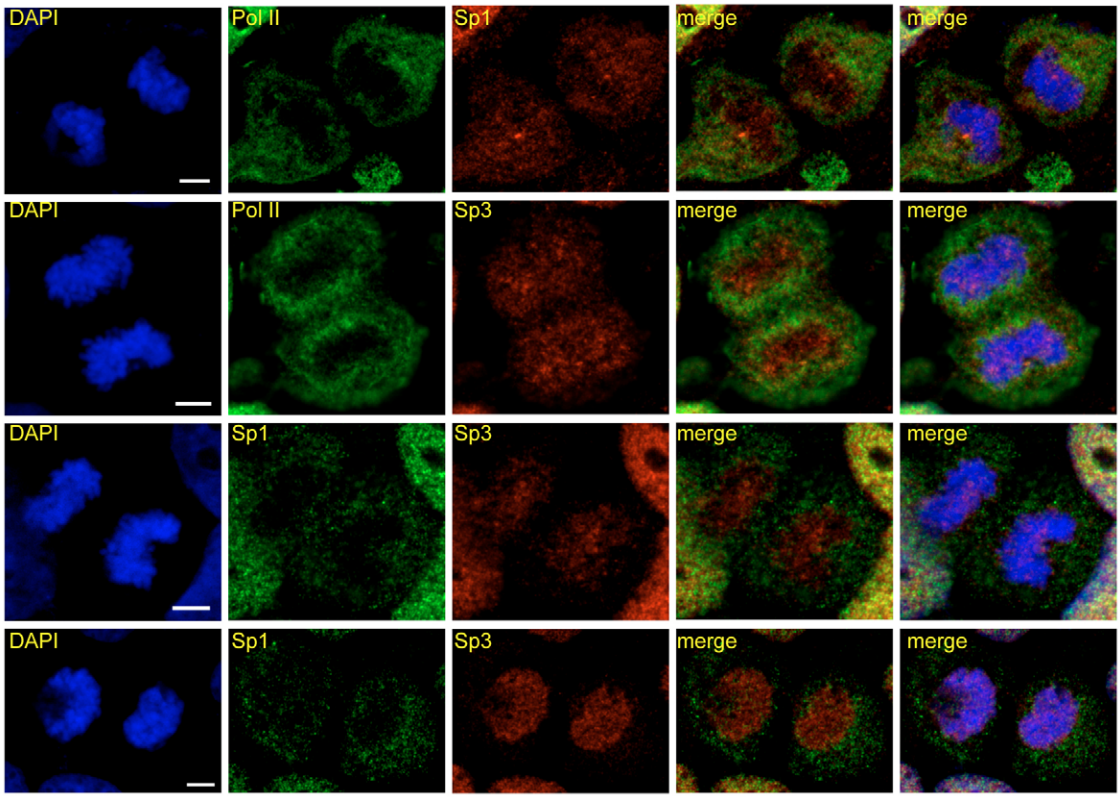

Fig. 7. Sequential re-entry of Sp1, Sp3 and RNAP II into daughter nuclei. MCF-7 cells were grown on coverslips in estrogen-complete medium, fixed, and double labeled with anti-Sp1 or anti-Sp3 and anti-RNAP II antibodies or with anti-Sp1 and anti-Sp3 antibodies. DNA was stained by DAPI. Spatial distribution was visualized by fluorescence microscopy in the ApoTome mode. Bars, 5 μm.

supernatant fractions were resolved by SDS-PAGE and immunoblotted with anti-Sp1 and anti-Sp3 antibodies. Bovine serum albumin (BSA) was added to the mixture as negative control. BSA and actin were visualized with staining with Coomassie Blue. In the presence of F-actin, Sp1 and Sp3 were present in the pellet and supernatant fractions (Fig. 9, lanes 5 and 6), whereas in the control samples containing no actin (Fig. 9, lanes 1 and 2) or non-polymerized G-actin (Fig. 9, lanes 3 and 4), Sp1 and Sp3 were only detected in the supernatant fractions. Hence, Sp1 and Sp3 were only sedimented with the microfilaments. Quantification of the immunoblot signals in the pellet fractions from three independent experiments indicates that 66±14% and 74±13% of Sp1 and Sp3, respectively, are precipitated with F-actin. By contrast, most of the BSA added as negative control and visualized by Coomassie Blue staining was found in the supernatant fractions from the three samples, without actin (Fig. 9, lanes 1 and 2), with G-actin (Fig. 9, lanes 3 and 4) or F-actin (Fig. 9, lanes 5 and 6). Only 9±3% of BSA sedimented in the presence of F-actin. Actin itself was also visualized with Coomassie Blue. As expected, G-actin was only visible in the supernatant fraction (lanes 3 and 4), whereas F-actin was highly enriched in the pellet fraction (lanes 5 and 6), with the level of sedimented actin being 72±0.1%. Thus, our results support the idea that Sp1 and Sp3 associate with polymeric actin.

Discussion

Sp1 and Sp3, which contribute to the regulation of thousands of genes involved in diverse biological processes (Suske, 1999; Li et al., 2004; Cawley et al., 2004), are distributed throughout the interphase nucleus as distinct and separate foci. In this study, we have explored the foci distribution throughout mitosis to understand how this differential subnuclear organization of Sp1 and Sp3 is restored after mitosis. We demonstrate that Sp1 and Sp3 keep their distinct punctate distribution patterns throughout mitosis. Sp1 and Sp3 foci are translocated away from condensed chromatin in prophase, they are dispersed throughout the cell in prometaphase, when the nuclear envelope and lamina are disrupted and they re-enter the nucleus in late telophase while the nuclear envelope and lamina reassemble. The timing of the mitotic displacement and repositioning of Sp1 and Sp3 foci is generally comparable with that of other factors and components participating in transcription initiation (Martinez-Balbas et al., 1995; Kruhlak et al., 2001; Zaidi et al., 2003; Prasanth et al., 2003). However, our observation that Sp1 and Sp3 re-enter the nucleus while the lamina reforms and before RNAP II re-enters, differs from a previous study showing that the RNAP II and general transcription factors, such as TFIIE, re-enter the nucleus only after the nuclear envelope and lamina are assembled (Prasanth et al., 2003). Moreover, Sp3 regroups into daughter nuclei before Sp1, thus, has the opportunity to bind to Sp sites before Sp1 and, depending on promoter context, can act as an activator or a repressor. Furthermore, we observed that already in anaphase, the HDAC2 staining had begun to return to colocalization with DNA. This sequence of events was not observed for HDAC1 in our studies with MCF-7 cells or in previously published studies in Indian muntjac fibroblasts (Kruhlak et al., 2001). The earlier return of HDAC2 to the chromatin might be due to a specific role in chromatin remodeling.

Contrary to a previous report that Sp1 is degraded by the proteasome-dependent system in a cell-cycle-dependent fashion (Grinstein et al., 2002), we found that Sp1 and Sp3

protein levels do not significantly differ throughout the cell cycle. Our finding that Sp1 and Sp3 levels, as well as HDAC2 and ERα levels, remain constant during cell division implies that these regulatory proteins are recycled into daughter nuclei, as it has been established for transcription factor Runx2 (Zaidi et al., 2003) and RNA polymerase II transcription factors TFIIB and TBP – although in the case of the RNA polymerase II transcription factors, a low turnover rate was observed (Prasanth et al., 2003). Current information suggests that the bulk of regulatory proteins are recycled and equally partitioned into progeny nuclei.

Unlike the mitotic behavior of the Runx2 transcription factor (Zaidi et al., 2003), the mitotic displacement of Sp1 and Sp3 foci from condensed chromosomes is complete. No subset of the Sp1 or Sp3 population stays associated with the condensed chromosomes during mitosis. We found no association of Sp1 and Sp3 with the endoplasmic reticulum. Among the two cytoskeleton components that we investigated, the intermediate and microfilaments, we found that it was to the microfilaments that the Sp1 and Sp3 foci localized. This observation suggests that F-actin participates in the mitotic relocation of Sp1 and Sp3 foci. It has recently been demonstrated that in oocytes, an actin network is responsible for delivering the chromosomes to the microtubule spindle (Lenart et al., 2005). Besides, it has been proposed that both meiotic and mitotic spindle assembly generally depends on F-actin (Miller and Bement, 2005). It is conceivable that the microfilaments are also involved in the mitotic shuttle of Sp1 and Sp3 foci. Sp1 and Sp3 foci might maintain their separate identities throughout mitosis as a result of indirect interactions with F-actin being mediated by different proteins. It is possible that a differential association of Sp1 and Sp3 with the microfilaments supports their subsequent differential association with the nuclear matrix in interphase nuclei. Polymeric actin has been shown to be present in the nucleus and is responsible for the observed insolubility of HDAC complexes during nuclear extract preparation (Andrin and Hendzel, 2004; M. J. Hendzel, personal communication). These reports, in conjunction with the in vitro co-sedimentation of Sp1 and Sp3 with F-actin, further suggest a role of polymeric actin in the interphase and mitotic organization of Sp1 and Sp3.

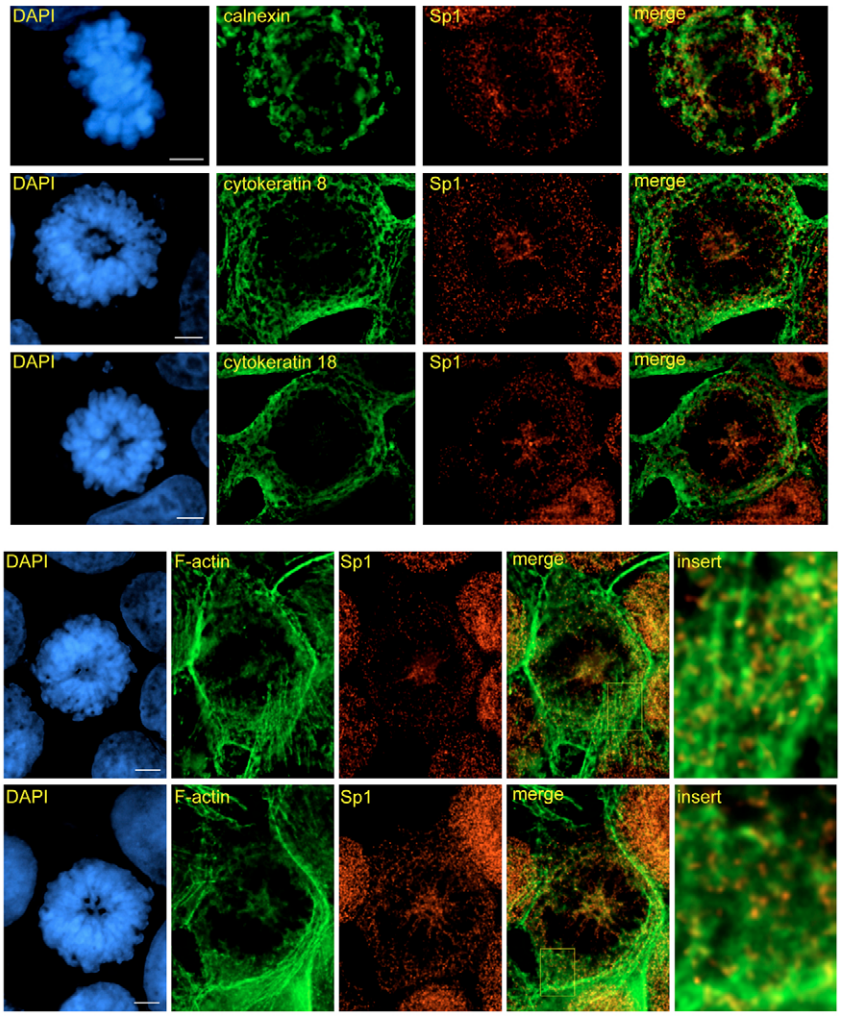

Fig. 8. Mitotic colocalization of Sp1 with F-actin. MCF-7 cells were grown on coverslips in estrogen-complete medium, fixed, and labeled with rabbit polyclonal anti-Sp1 antibodies and mouse monoclonal anti-calnexin or anti-cytokeratin 8 or anti-cytokeratin 18 antibodies. F-actin was labeled by Alexa-Fluor-488-conjugated phalloidin. DNA was stained by DAPI. Spatial distribution was visualized by fluorescence microscopy and image deconvolution as described in Materials and Methods. Single optical sections are shown. Yellow in the merge images signifies colocalization. Bar, 5 μm.

Materials and Methods

Cell culture

The human breast cancer cell line MCF-7 (T5) (ER-positive and estrogen-dependent) was grown under estrogen-complete conditions as described previously (Spencer et al., 2000; Sun et al., 2001). For arrest in G0 and/or G1 phase, MCF-7 cells were treated with 1.5 mM hydroxyurea (HU) for 16 hours, cultured for 13 hours in fresh DMEM medium with 10% FBS and treated again with 1.5 mM HU for 13 hours. For arrest in G2 and/or M phase, cells were treated with 0.06 μg/ml of colcemid for 16 hours. The cell-cycle status was determined by FACS analysis (Chadee et al., 1999).

Indirect immunolocalization and fluorescence microscopy

Indirect immunolocalization was performed as described previously (He et al., 2005). Cells were plated onto poly-L-lysine-coated coverslips in 24-well tissue culture plates. Cells were fixed in 3.7% formaldehyde in PEM buffer (80 mM piperazine-N,N′-bis (2-ethanesulfonic acid), 5 mM EGTA, 2 mM $MgCl_2$, pH 6.8) for 30 minutes on ice. Cells were washed three times for 5 minutes in PEM buffer, quenched with the addition of 75 mM ammonium chloride and 20 mM glycine, and permeabilized with 0.5% Triton X-100 in PEM buffer for 30 minutes. Coverslips were blocked in 5% dry milk in TBST (20 mM Tris-HCl, pH 7.4, 150 mM NaCl, 0.1% Tween 20) for 1 hour at room temperature and incubated with the appropriate primary antibodies overnight at 4°C. The following antibodies were used at the indicated dilution: mouse monoclonal antibodies against estrogen receptor (ER) α (1:50, Novocastra Laboratories Ltd, Newcastle, UK), Sp1 (1:50, Santa Cruz Biotechnology, Santa Cruz, CA), HDAC2 (1:250, Santa Cruz Biotechnology), lamin A/C (1:50, Novocastra Laboratories Ltd), calnexin (1:1000, Affinity BioReagents (ABR), Golden, CO), cytokeratin 8 and cytokeratin 18 (1:250, Zymed Laboratories

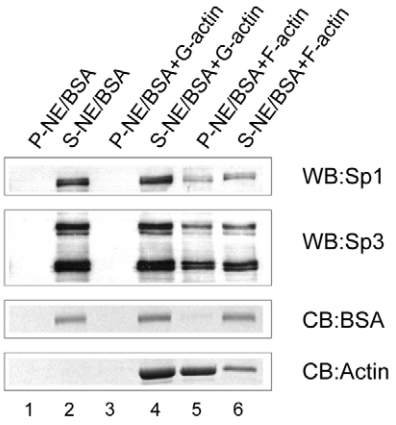

Fig. 9. Co-sedimentation of Sp1 and Sp3 with F-actin. MCF-7 nuclear extract was mixed with G-buffer and polymerization inducer (lanes 1 and 2), G-actin (lanes 3 and 4) or F-actin (lanes 5 and 6). BSA was added as negative control. Mixtures were spun. Pellet (lanes 1, 3 and 5) and supernatant (lanes 2, 4 and 6) fractions were resolved by SDS-PAGE. Sp1 and Sp3 were detected by western blot (WB), whereas BSA and actin were visualized by Coomassie Blue (CB) staining

Inc., CA), RNA polymerase II (clone CTD4H8) (1:2000, Upstate, Charlottesville, VA) and rabbit polyclonal antibodies against Sp1 (1:1000, Upstate), Sp3 (1:500, Santa Cruz Biotechnology), histone deacetylase (HDAC) 1 (1:5000, Affinity BioReagents, Golden, CO) and HDAC2 (1:2000, Affinity BioReagents). Alexa-Fluor-488-conjugated goat anti-mouse IgG (Molecular Probes, Eugene, OR) or Cy3-conjugated goat anti-rabbit IgG (Sigma, St Louis, MO) were used as secondary antibodies. F-actin was labeled with Alexa-Fluor-488-conjugated phalloidin (3 units per coverslip, Molecular Probes, Eugene, OR). DNA was counterstained with 4′6-diamidino-2-phenylindole (DAPI) and the coverslips were mounted onto glass slides using the Prolong Gold antifade reagent (Molecular Probes). Control experiments including epitope-peptide-blocking or primary-antibody-omission demonstrated the specificity of the antibodies used. Digital images were captured and processed with fluorescence microscopy and deconvolution analysis or generation of optical sections with the ApoTome (Carl Zeiss) as indicated.

Quantitative image analysis

Image pixel intensities were measured with the AxioVision 4.1 software. To compare the relative amounts of relevant proteins between interphase and mitotic cells, interphase and mitotic cells were selected from the same slide. To establish the relative protein or DNA distribution between two daughter nuclei following mitosis, the partition coefficient (PC) was determined as indicated previously (Zaidi et al., 2003). Briefly, PC was expressed as the ratio of integrated signal intensities between two daughter cells ($PC=I_x \div I_y$, where I_x and I_y are integrated pixel intensities of each progeny cell). The significance of the observed differences was tested by two-tail paired Student's *t*-test. Differences were considered statistically significant at $P<0.05$.

Immunoblotting

SDS-PAGE and immunoblot analyses were performed as described previously (Samuel et al., 1998).

F-actin co-sedimentation assay

To generate F-actin filaments, G-actin from rabbit muscle (Sigma) was diluted to 0.4 mg/ml in G-buffer (5 mM Tris-HCl pH 8.0, 0.2 mM ATP, 0.5 mM DTT, 0.2 mM $CaCl_2$, 0.002% NaN_3), mixed with polymerization inducer (50 mM KCl, 2 mM $MgCl_2$, 1 mM ATP), and then incubated at room temperature for 1 hour. To prepare MCF-7-cell nuclear extracts, nuclei were resuspended in G-buffer, containing 0.5% NP-40 and proteinase inhibitors, sonicated and centrifuged at 13,000 rpm for 10 minutes in a microcentrifuge. The supernatants were then centrifuged at 100,000 ***g*** for 15 minutes to remove aggregates. The F-actin co-sedimentation assay was performed by adding 10 μl of MCF-7-cell nuclear extract into 90 μl of the F-actin solution generated as above. After incubation for 60 minutes on ice, the samples were spun at 100,000 ***g*** for 60 minutes. Supernatants were removed completely, and pellets were dissolved in 100 μl G-buffer. Aliquots (20 μl) of the dissolved pellets and equal volumes of supernatants were loaded onto SDS-10% polyacrylamide gels, transferred to nitrocellulose membranes, and immunochemically stained. As a negative control, BSA was added into the actin solutions. The polyacrylamide gel was stained with Coomassie Blue to visualize the partition of BSA in the pellet and supernatant fractions.

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