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Cytostellin distributes to nuclear regions enriched with splicing factors

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SUMMARY

Cytostellin, a ~240 kDa phosphoprotein found in all cells examined from human to yeast, is predominantly intranuclear in interphase mammalian cells and undergoes continuous redistribution during the cell cycle. Here, mammalian cytostellin is shown to localize to intranuclear regions enriched with multiple splicing proteins, including spliceosome assembly factor, SC-35. Cytostellin and the splicing proteins also co-localize to discrete foci (called 'dots'), which are distributed throughout the cell during mitosis and part of G_1. The cytostellin that is localized to these dots resists extraction by Triton X-100, indicating that it is tightly associated with insoluble cell structures. All immunostainable cytostellin reappears in the nucleus before S-phase. Although cytostellin and the splicing proteins co-localize in interphase and dividing cells, cytostellin is not detected in purified spliceosomes, and it associates with six unidentified proteins, forming a macromolecular complex that is biochemically distinct from the proteins that comprise spliceosomes. This macromolecular complex is detected at constant levels throughout the cell cycle, and the level of cytostellin protein remains constant during the cell cycle. Nevertheless, intranuclear cytostellin immunostaining fluctuates markedly during the cell cycle. The monoclonal antibody (mAb) H5 epitope of cytostellin is 'masked' in serum-starved cells, but 60 minutes after serum stimulation intense cytostellin immunoreactivity appears in the nuclear speckles. This rapid induction of cytostellin immunoreactivity in subnuclear regions enriched with many splicing factors, as well as accumulations of RNA polymerase II (Pol II) transcripts, suggests that cytostellin may have a function related to mRNA biogenesis.

Key words: cytostellin, cell cycle, splicing factors

INTRODUCTION

Many of the macromolecules involved in pre-mRNA processing are concentrated in discrete regions of mammalian cell nuclei that are commonly termed nuclear 'speckles' (reviewed by Spector, 1993). Approximately 20-50 speckles are visualized in nuclei immunostained with antibodies directed against a subset of heterogeneous nuclear RNPs (hnRNPs: Fakan et al., 1984), small nuclear RNPs (snRNPs: Spector et al., 1983, 1991; Fakan et al., 1984; Nyman et al., 1986; Verheijen et al., 1986; Habets et al., 1989), and non-snRNP splicing proteins (Fu and Maniatas 1990; Spector et al., 1991; Xing et al., 1993; Carter et al., 1993). In addition, the nuclear speckles can be visualized by fluorescence in situ hybridization (FISH) with antisense snRNA oligonucleotide probes (Huang and Spector, 1992; Carmo-Fonseca et al., 1991), and with oligo(dT) probes, which hybridize to polyadenylated RNA (Carter et al., 1991). Because nuclear speckles contain many proteins and ribonucleoprotein (RNP) complexes that play a direct role in pre-mRNA processing, and they also contain RNAs produced by RNA polymerase II (Pol II)-dependent transcription, it is likely that supramolecular complexes involved in pre-mRNA processing (e.g. spliceosomes) are located in these regions.

Nascent pre-mRNA transcripts appear to be synthesized in the nucleoplasm adjacent to the nuclear speckles (reviewed by Spector, 1993). Electron microscopic studies have shown a rapid incorporation of [^{3}H]uridine in the perichromatin granules adjacent to the nuclear speckles (Fakan et al., 1976; Spector, 1990). In addition, the hnRNP C proteins are localized to the perichromatin granules, but not the interchromatin granules in the center of the speckles (Fakan et al., 1984). Many studies suggest a close spatial relationship between Pol II-mediated transcription and pre-mRNA processing (Beyer and Osheim, 1988; Fakan et al., 1986; Sass and Pederson, 1984; Huang and Spector, 1991). In fact, recent experiments showed that RNA polymerase II, hnRNPs and the splicing factor, SC-35, are coordinately recruited to new sites of gene transcription in vivo (Jiménez-Garcia and Spector, 1993). Taken together, these data suggest that Pol II transcription and pre-mRNA-processing reactions take place at sites in the vicinity of the nuclear speckles. Consistent with this notion, FISH has been used to localize nascent fibronectin transcripts (Xing et al., 1993) and c-*fos* transcripts (Huang and Spector, 1991) to one or two discrete intranuclear sites, each associated with one of the ~20-50 nuclear speckles.

Here we show that cytostellin, a ~240 kDa phosphoprotein present in cells of all eukaryotes from human to yeast (Warren et al., 1992), is localized to nuclear speckles in mammalian cells. Cytostellin co-localizes with multiple splicing proteins, including spliceosome assembly factor (SC35) in interphase cells, but it is not a component of purified spliceosomes. At the onset of mitosis intranuclear cytostellin and SC35 redistribute

to multiple foci (dots), which are widely distributed throughout the cell. Cytostellin is tightly associated with these dots, as indicated by its insolubility in nonionic detergents. Cytostellin and SC35 co-localize to these dots throughout mitosis and part of G_1. Significantly, the cytostellin-containing dots remain outside the nucleus in postmitotic G_1 cells, and they completely disappear from the extranuclear compartment by the beginning of S phase.

In contrast to the splicing proteins, cytostellin's pattern and intensity of intranuclear immunoreactivity fluctuates markedly during the cell cycle; however, the level of cytostellin protein remains constant. Cytostellin's intranuclear immunoreactivity is 'masked' in serum-starved cells, but 60 minutes after serum stimulation, intense cytostellin immunoreactivity appears in the nuclear speckles. The ability of serum stimulation to induce cytostellin immunoreactivity in subnuclear regions containing many splicing factors, as well as accumulations of RNA polymerase II (Pol II) transcripts, suggests that cytostellin may have a function related to mRNA biogenesis.

MATERIALS AND METHODS

Cell culture

Madin Darby canine kidney (MDCK) cells were maintained in Dulbecco's modified Eagle's medium (DME) supplemented with 7% fetal bovine serum (FBS; Warren and Nelson, 1987). HeLa S3 cells were maintained in monolayer culture with RPMI 1640 medium supplemented with 7% FBS and 10 mM glutamine (Gibco BRL, Gaithersburg, MD). Monolayer cultures were maintained at 37°C in a humidified atmosphere containing 5% CO_2. For suspension culture of HeLa S3 cells, RPMI 1640 medium was further supplemented with 10 mM HEPES and grown in spinner flasks (Corning, Fairless Hills, PA) at 37°C at 30 rpm. To study the effect of serum stimulation on cytostellin's pattern of nuclear immunofluorescence, MDCK cells were grown in serum-free DME for 24 hours, and then the medium was changed to DME supplemented with 20% fetal calf serum and 20 μg/ml anisomycin (Huang and Spector, 1991).

Antibodies

The mouse monoclonal IgM antibodies H5 and H14, which specifically recognize cytostellin, were produced as described previously (Warren et al., 1992). The control IgM, TEPC-183 was purchased commercially (Sigma, St Louis, MO). For some experiments, H5 and TEPC were covalently linked to agarose beads using the Aminolink system (Pierce, Rockford, IL). Additional antibodies were generously supplied by the following investigators: anti-SC35 (Fu and Maniatas, 1990) from T. Maniatias, Harvard University; human autoimmune anti-U1 snRNP p70 serum and anti-Sm snRNP monoclonal antibody (mAb) Y12 (Lerner et al., 1981) from Susan Baserga, Yale University; human autoimmune anti-lamin serum from Frank McKeown, Harvard University; anti-U2AF (Zamore and Green, 1991) from Michael Green, University of Massachusetts; anti-PTB and anti-PSF (Patton et al., 1991) from Jim Patton, Vanderbilt University; anti-Ser-Arg domain mAb 104 (Roth et al., 1991) from Mark Roth, University of Washington; and anti-coilin (p80) (Andrade et al., 1993) from E. K. L. Chan, Scripps Research Foundation.

Immunofluorescence microscopy

Immunofluorescence staining was performed as described previously (Warren et al., 1992). In specified experiments cells were permeabilized prior to fixation: briefly, cells grown on coverslips were washed with three changes of PBS, permeabilized by incubation with 1% Triton X-100 in PBS for 10 minutes at 4°C, washed free of detergent with three changes of 1.75% paraformldehyde at 4°C, and fixed by incubation with 1.75% paraformaldehyde for 20 minutes at 4°C. The state of chromosomal condensation was assessed with the DNA binding fluor, 4′,6′-diamidino-2-phenylindole (DAPI) at 5 μg/ml as described previously (Baron et al., 1991). DNA replication during S phase was monitored via incorporation of the fluorescent nucleoside, 5′ bromodeoxyuridine (5′ BrdU) as described previously (Magaud et al., 1988). Immunofluorescence microscopy and photography were performed as described previously (Warren et al., 1992).

Nucleocytoplasmic fractionation

Nuclei were isolated from MDCK cells according to the method of Martelli et al. (1992) with slight modifications. Briefly, MDCK cells were washed with TBS (150 mM NaCl, 20 mM Tris-HCl, pH 7.4), resuspended in 10 mM Tris-HCl, pH 7.8, 1% Nonidet P-40, 10 mM β-mercaptoethanol, 2 mM $MgCl_2$ and 1 mM PMSF, diluted with an equal volume of double-distilled water, and sheared by 20 passages through a 23-gauge needle. (Cell lysis and nuclear preservation were assessed by phase-contrast microscopy.) The nuclear fraction was obtained by washing the 400 ***g*** pellet of the above cell lysate with 10 mM Tris-HCl, pH 7.4, 2 mM $MgCl_2$, 1 mM PMSF, and then lysing the nuclei with 2× SDS-PAGE sample buffer (Warren et al., 1992). The cytoplasmic fraction was obtained by recentrifuging the 400 ***g*** supernatant at 400 ***g*** to remove residual debris. Samples of nuclear and cytoplasmic fractions, each derived from equal cell numbers, were subjected to SDS-PAGE and western immunoblot analysis with mAb H5 as described previously (Warren et al., 1992). The purity of the nuclear and cytoplasmic fractions was judged as follows: (i) the cytoplasmic fraction was analyzed by phase-contrast microscopy, and no nuclei were visualized; (ii) nuclear and cytoplasmic fractions were also immunoblotted with antibodies directed at proteins known to distribute to the cytoplasm and peripheral cytoskeleton (e.g. $p60^{c\text{-}src}$ and spectrin). Over 95% of these proteins were present in the cytoplasmic fraction.

Cell cycle analysis

Counterflow centrifugal elutriation of HeLa S3 cells grown in suspension culture was performed as described previously (Kauffman et al., 1990). Briefly 1.5×10^8 to 2.0×10^8 cells were pelleted at 600 ***g*** and resuspended in 20 ml of PBS, which was subsequently diluted with 20 ml of RPMI with 10% calf serum. Elutriation was performed with a JE-6B elutriation rotor in a J-6B centrifuge (Beckman, Palo Alto, CA) using ice-cold RPMI with 5% calf serum. The sample was loaded at a pump speed of 14 ml/min and passed through a gas trap before entering the rotor, to avoid the introduction of air into the system (Kauffman et al., 1990). Fractions were collected on ice at pump speeds of 40 to 60 ml/min. Cell number of each fraction was determined with a Coulter Counter (Coulter Electronics, Hialeah, FL), then total cell extracts from 2×10^6 cells were prepared by boiling the cell pellet in 2× sample buffer for 5 minutes and subjecting the sample to SDS-PAGE and western immunoblot analysis with mAb H5 as described previously (Warren et al., 1992). The cell cycle profile of each fraction was determined by flow cytometry using an EPICS Profile Analyzer (Coulter Electronics, Hialeah, FL).

Metabolic labeling

Subconfluent monolayers of HeLa cells were obtained by adding 2×10^5 cells to 6-well tissue culture chambers and incubating with RPMI medium supplemented with 7% FBS for 16-24 hours. The growth medium was then removed and monolayers were washed two times with PBS before adding 100 μCi of [^{35}S]methionine (~1,200 Ci/mmol; Amersham) in 1 ml of 95% methionine-free RPMI (GIBCO BRL, Gaithersburg, MD) supplemented with 10% FBS (dialyzed against 50 mM Tris-HCl, 150 mM NaCl) and 5% complete RPMI supplemented with 7% complete FBS. For HeLa (S3) cells grown in suspension culture, 1.5×10^6 cells were plated onto 6-well plates and incubated with RPMI medium supplemented with 7% FBS for 2

hours. The monolayers were washed twice in PBS and incubated with 100 μCi of [^{35}S]methionine as above. After incubating for 3-4 hours at 37°C the medium was discarded and the monolayer was washed three times with PBS. The cells were solubilized with 1 ml of TD buffer (0.5% Triton X-100, 0.5% sodium deoxycholate, 50 mM Tris-HCl, pH 7.5, 250 mM NaCl, 5 mM EDTA, 50 mM sodium fluoride, and 1 mM PMSF) at 4°C and particulate material was removed by centrifugation at 16,000 ***g*** for 30 minutes at 4°C. The solubilized cell extracts were initially incubated for 1-2 hours at 4°C with TEPC-183-agarose beads to reduce nonspecific immunoprecipition. The TEPC-183-agarose beads were removed by centrifugation and the supernatant was then incubated for 6-12 hours at 4°C with either H5-or TEPC-183-agarose beads. The beads were then washed three times with 1 ml of TD buffer before being eluted with 0.1 ml of 2% SDS, 20 mM Tris, pH 7.5, at room temperature for 15 minutes. Finally, 33 μl of 4× SDS-PAGE sample buffer (Warren et al., 1992) was added and the sample was boiled for 3 minutes before being subjected to 8% SDS-PAGE. The gel was subsequently processed for fluorography as described previously (Laskey and Mills, 1975; Warren and Nelson, 1987).

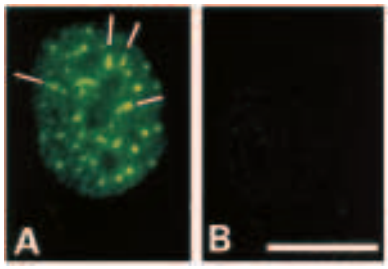

Fig. 1. Immunolocalization of cytostellin in MDCK cells. Cells were extracted with 1% Triton X-100 at 4°C for 15 minutes, fixed with 1.75% paraformaldehyde and subjected to indirect immunofluorescence as described in Materials and Methods. The first antibody was either the IgM mAb H5 (A) or control IgM (B). The second antibody was fluorescein isothiocyanate (FITC)-conjugated goat anti-mouse IgM. Specific nuclear staining is consistently concentrated in approximately 50 dots or speckles but is also present diffusely throughout the nucleus. Some of the speckles appear in linear groups indicated by pins (A). The nucleoli do not stain. Bar, 10 μm.

RESULTS

Cytostellin is concentrated in nuclear speckles enriched with splicing proteins

Monoclonal antibody (mAb) H5, which binds specifically to the ~240 kDa cytostellin protein in immunoprecipitation and immunoblot analyses, was used previously to immunolocalize this protein in interphase nuclei (Warren et al., 1992). The immunostaining is concentrated in approximately 50 dot-like intranuclear structures (speckles), but it is also present diffusely throughout the nucleus. These cytostellin-containing structures are observed in a subpopulation of unsynchronized cells, and are optimally visualized by extracting the MDCK cells with buffers containing 1% Triton X-100 prior to fixation. Apparently, the non-ionic detergent removes much of the diffusely staining component, thereby unmasking the dot-like structures, which retain cytostellin immunoreactivity (Fig. 1). Cytostellin's nuclear localization is confirmed biochemically by immunoblotting nuclear and cytoplasmic fractions of MDCK cells with mAb H5 (Fig. 2, arrowhead). The majority of this ~240 kDa protein is present in the nuclear fraction of MDCK cells. Similar results have been obtained with HeLa cells and various rodent cell lines (unpublished results).

These results prompted us to compare the pattern of cytostellin staining with those of other proteins known to have a punctate intranuclear distribution. One such protein, SC-35, is a 35 kDa protein essential for spliceosome assembly and function (Fu and Maniatis, 1990). SC-35 specifically marks discrete intranuclear regions (speckles) that are enriched with many splicing proteins (for review, see Spector, 1993). Double immunofluorescence studies using mAb H5 (IgM) and monoclonal anti-SC-35 (IgG) show that both of these proteins distribute to speckles of interphase nuclei (Fig. 3). Diffuse cytostellin immunoreactivity is also present (Fig. 3B,D and F). Monoclonal antibody anti-SC-35 stains speckles in all nuclei (Fig. 3A,C and E, arrowheads), but anti-cytostellin mAb H5 stains these speckles in only three of the four nuclei shown (Fig. 3B,D and F, arrowheads). The nucleus shown at the bottom of panels E and F lacks immunoreactive cytostellin dots (Fig. 3F, thick arrow), but it does stain diffusely. The diffuse

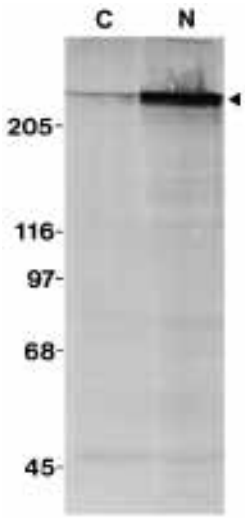

Fig. 2. Nuclear localization of cytostellin in MDCK cells. MDCK cells were lysed in the presence of 0.5% NP-40 (Martelli, 1992). Cell lysis and nuclear preservation were confirmed by phase-contrast microscopy, and then nuclei were isolated from the non-nuclear fraction by centrifugation at 400 ***g***. Nuclear (N) and non-nuclear (cytoplasmic (C)) extracts, each derived from an equal number of cells, were subjected to SDS-PAGE. The separated proteins were transferred to nitrocellulose and immunoblotted with mAb H5 followed by alkaline phosphatase-conjugated goat anti-mouse IgM. The arrowhead indicates the 240 kDa cytostellin band.

and punctate intranuclear cytostellin staining pattern resembles the staining pattern of snRNPs in the pre-mRNA splicing class (see Spector, 1993), but unlike the snRNPs and SC-35, cytostellin immunoreactivity is highly variable among nuclei of cycling cells (see below).

The close spatial relationship between SC-35 and cytostellin observed in interphase speckles is retained when these two proteins redistribute to widely dispersed positions in the cell periphery of dividing cells. This is illustrated by a pair of MDCK cells undergoing cytokinesis (Fig. 4A, mAb αSC-35; B, mAb H5; arrowheads). SC-35 and cytostellin co-localize in

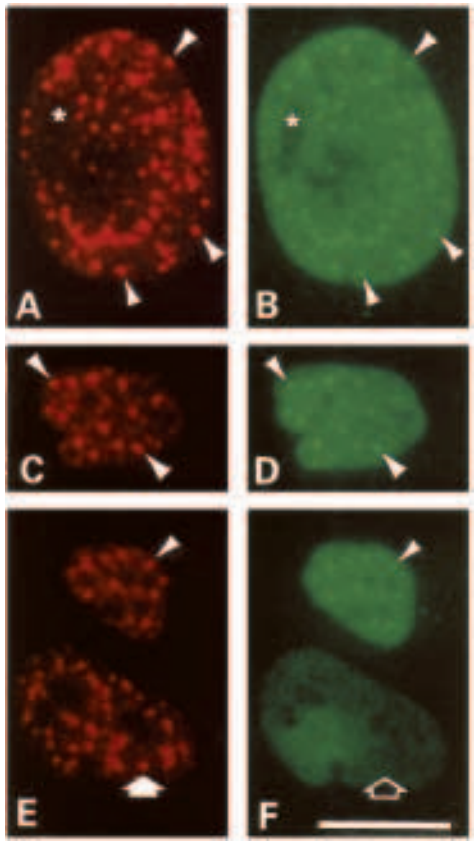

Fig. 3. Cytostellin and spliceosomal protein SC35 are concentrated in nuclear speckles. MDCK cells were fixed with 1.75% paraformaldehyde, permeabilized with 0.5% Triton X-100 and double-immunostained with an IgG mAb against SC35 (A,C and E) and an IgM mAb H5 (B,D and F) followed by rhodamine-conjugated goat anti-mouse IgG and FITC-conjugated goat anti-mouse IgM. Arrowheads indicate nuclear dots (nuclear speckles), which co-immunostain both antibodies. SC35 is concentrated in the nuclear speckles of all nuclei (A,C and E), but cytostellin is concentrated in nuclear speckles of a subset of nuclei. Some nuclei have diffuse cytostellin immunostaining, but lack dot-like immunostaining (E,F, thick arrows). Diffuse cytostellin immunoreactivity is present in all nuclei shown here, but some nuclei completely lack cytostellin immunoreactivity (see below). Asterisks indicate nucleoli. Bar, 10 μm.

these discrete dots at all stages of mitosis, and the SC-35 immunoreactivity also resists extraction with 1% Triton X-100 (unpublished observations). Monoclonal antibody Y12 (Lerner et al., 1981), which binds to the spliceosomal snRNPs B, B′ and D, also immunostains these dots in metaphase and anaphase cells; and to a lesser degree, cells in telophase (unpublished data). The presence of SC-35, snRNPs and cytostellin in the mitotic/G_1 dots suggests that the supramolecular organization of the intranuclear speckles is at least partially retained during cell division while the dots are bound to multiple sites in the cell periphery.

Cytostellin and SC-35 are tightly associated with dot-like insoluble cell structures in mitotic and postmitotic cells

Most nuclear proteins reassemble in the nucleus during telophase (Newport and Forbes, 1987); however, some

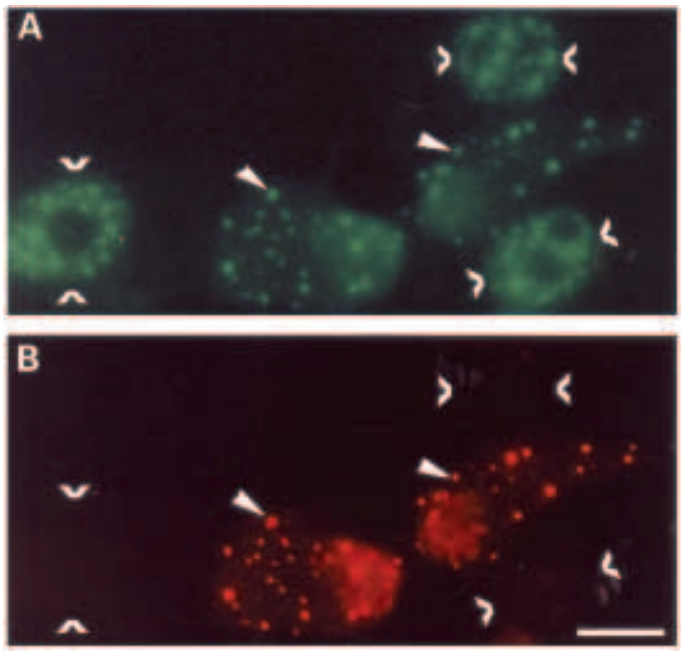

Fig. 4. Cytostellin and SC-35 co-localize in dots of mitotic cells. Cells were fixed with 1.75% paraformaldehyde, permeabilized with 0.5% Triton X-100 and double stained with anti-SC35 (A) and mAb H5 (B). Anti-SC35 is visualized with FITC-conjugated second antibody and H5 with rhodamine-conjugated second antibody as shown in Fig. 3. A pair of cells undergoing cytokinesis is shown in the lower middle of the field. Extranuclear dots stained both by anti-SC35 and mAb H5 are indicated by arrowheads. Note that SC35 is present in nuclear speckles in three nuclei (A, bracketed by carets), but that these nuclei do not stain with mAb H5 (B, bracketed by carets). Bar, 10 μm.

proteins such as SC35 (Spector et al., 1991) and hnRNP proteins (Piñol-Roma and Dreyfuss, 1991) remain outside the nucleus in daughter cells, after the nuclear lamina has reassembled. Microscopically visible cytostellin-containing dots also remain outside the nucleus, as shown by a postmitotic cell pair co-immunostained with antibodies directed at lamins A and C (Fig. 5E) and mAb H5 (Fig. 5D). All of the immunostainable lamin has been reassembled into the nuclear lamina in these nascent nuclei, whereas abundant cytostellin dots are still present in the cell periphery. The cytostellin-containing dots remain outside the nucleus beyond the point shown in Fig. 5, since extranuclear cytostellin-containing dots are readily detected in a subpopulation of interphase cells, identified by larger, more smoothly contoured nuclei (Fig. 6); these interphase cells frequently appear in pairs, suggesting that they have recently undergone cell division (unpublished observations).

The discrete microscopic appearance of the cytostellin-containing dots suggests that this fraction of cytostellin is incorporated into insoluble structures. To determine the solubility properties of cytostellin in the dots, MDCK cells were extracted with 1% Triton X-100, fixed and immunostained with mAb H5 (Fig. 5A-C). This concentration of detergent extracts soluble cytoplasmic and membrane-associated MDCK cell proteins, leaving an insoluble residue comprising cytoskeletal proteins (Nelson and Veshnock, 1986; Morrow et al., 1991). Cytostellin immunoreactivity resists this detergent

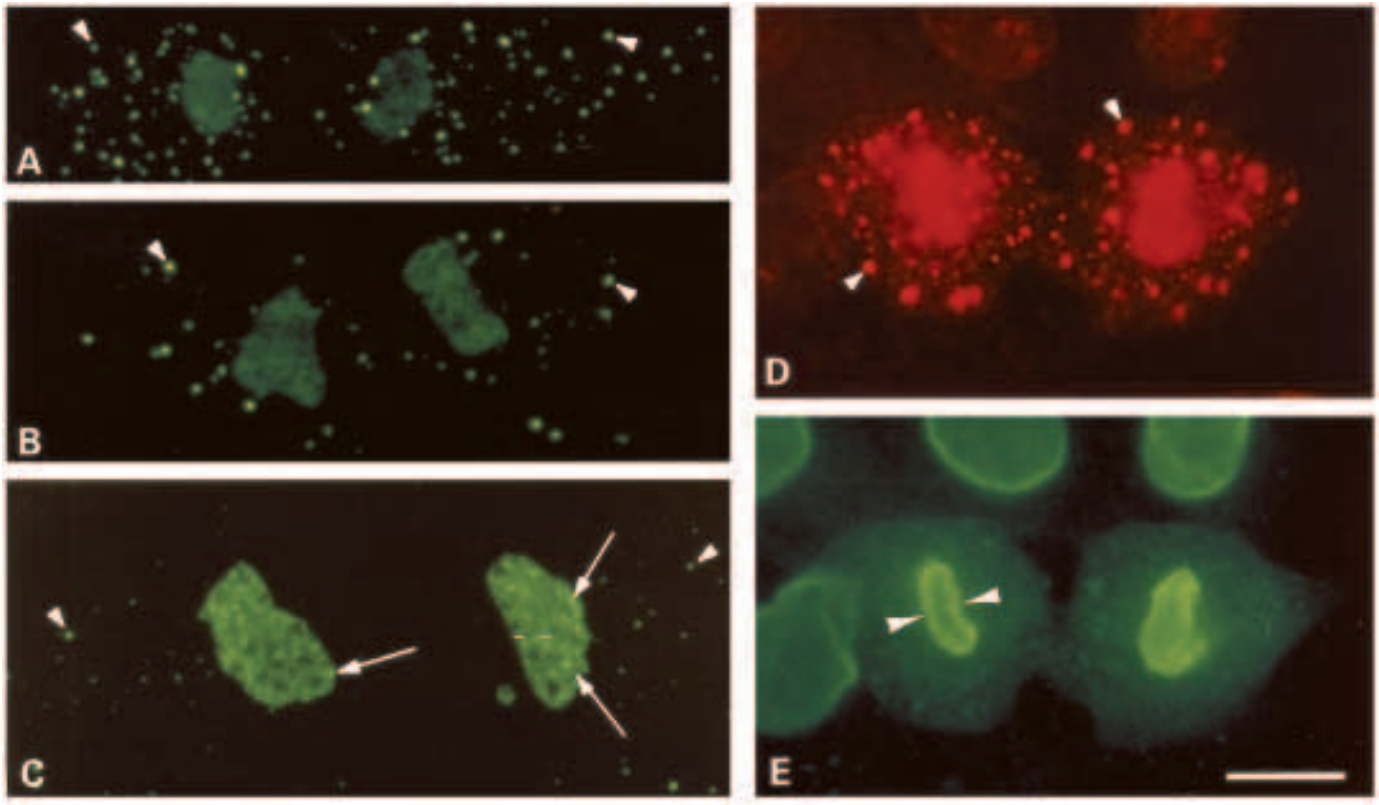

Fig. 5. Cytostellin-containing dots remain bound to insoluble cell structures in the cell periphery at the end of mitosis. Daughter cell pairs (A,B,C) at different stages of cytokinesis were extracted with 1% Triton X-100 for 15 minutes at 4°C prior to fixation with 1.75% paraformaldehyde and then stained with mAb H5 as in Fig. 1. This fraction of dot-associated cytostellin is outside the reassembling nuclei, and resists 1% Triton X-100 extraction, indicating a tight cytoskeletal association. In (D) and (E) daughter cells were double-stained with mAb H5 (D) and an antibody to nuclear lamins A and C (E). The mAb H5 was visualized with rhodamine-conjugated goat anti-mouse IgM, and the lamins with FITC-conjugated goat anti-human IgG. Arrowheads in (A-D) indicate cytostellin-containing dots that remain outside the reassembling nuclei. The arrows in (C) indicate cytostellin in nuclear speckles. Arrowheads in (E) indicate a nascent nucleus, which has fully incorporated lamin into the nuclear lamina. Although the daughter cells have reassembled their nuclear laminae, cytostellin-containing dots remain outside the nucleus. Bar, 10 μm.

extraction, indicating that the cytostellin-containing dots are associated with insoluble cell structures, probably the peripheral cytoskeleton (Fig. 5A-C). Cell pairs at different stages of cytokinesis are presented here, but similar results were obtained at all stages of mitosis and in early G_1 cells (unpublished data). Note that as the number of extranuclear cytostellin dots diminishes from Fig. 5A to C, the intensity of nuclear staining increases. The large arrows in Fig. 5C show that cytostellin appears in the nuclear speckles of nuclei that have recently reassembled. This suggests translocation of cytostellin to the nucleus, although it is also possible that this pool of cytostellin is degraded, and that the nucleus is replenished by newly synthesized cytostellin imported to the nucleus during G_1.

Cytostellin redistributes to the nucleus prior to S-phase

All immunostainable cytostellin is intranuclear by the beginning of S-phase, as shown by the absence of extranuclear cytostellin-containing dots in all cells that incorporated 5′ bromodeoxyuridine (5′ BrdU) following a 15 minute pulse (Fig. 7). Note that the pattern and intensity of anti-5′ BrdU immunostaining (Fig. 7A) varies among cell nuclei, suggesting that cells in different periods of S-phase have been labeled with 5′ BrdU (Nakayasu and Brezney, 1989). Because cytostellin-containing dots are outside the nucleus in daughter cells, and the cell periphery is devoid of cytostellin-containing dots by the beginning of S-phase (Fig. 7B), it is concluded that cytostellin redistributes to the nucleus during G_1.

Nuclear cytostellin immunoreactivity changes during cell cycle

A heterogeneous pattern of intranuclear immunoreactive cytostellin is characteristic of cycling MDCK, HeLa and rodent cells, and many proliferating cell populations stained in situ (unpublished data), and it is revealed by two anti-cytostellin mAbs (H5 and H14; Warren et al., 1992), which bind to separate cytostellin epitopes (unpublished results). In most unsynchronized proliferating cell populations in vitro, 10-15% of the nuclei have diffuse cytostellin immunoreactivity and discrete speckles (e.g. Fig. 3B and D), nearly 50% of the nuclei stain diffusely but lack speckles (e.g. Fig. 3F), and many nuclei do not stain (e.g. Fig. 4B). These observations suggest that the pattern and intensity of nuclear cytostellin immunoreactivity changes during the cell cycle.

In G_1 daughter cells cytostellin is clearly detectable in

nuclear speckles; particularly if the daughter cells are extracted with non-ionic detergents prior to fixation (Fig. 5C, arrows; also see Fig. 6). Thus, during early G_1 a fraction of cytostellin is in dots in the extranuclear compartment, while another fraction has relocated to the nucleus. However, nuclear cytostellin immunoreactivity becomes undetectable in MDCK cells that have been growth-arrested by confluency or serum deprivation (Fig. 8B, arrowheads). The same cell nuclei stain intensely with mAb Y12, which is directed against snRNPs of the Sm group (Fig. 8C; arrowheads). In contrast, a rare mitotic (prophase) cell stains intensely with mAb H5 and mAb Y12 (Fig. 8B and C; open arrows), and many nuclei of actively growing MDCK cells stain well with mAb H5 (Fig. 8A). Growth-arrested MDCK cell nuclei also stain with mAb anti-SC-35 (unpublished observations), and nuclei that stain with anti-SC-35, but not with mAb H5, can be readily identified in unsynchronized cell populations (Fig. 4B, carets). Thus, cytostellin's mAb H5 epitope is undetectable in growth-arrested nuclei, but multiple splicing proteins are easily stained.

Two patterns of cytostellin immunoreactivity are detected in S-phase cells: some nuclei have weak, diffuse immunoreactivity (Fig. 7B, arrowheads), while others stain more intensely (Fig. 7B, carets). The variable pattern of 5′ bromodeoxyuridine (5′ BrdU) immunoreactivity in Fig. 7A probably indicates cells at different positions of S-phase (Nakayasu and Brezney, 1989), but the sequence of changes in cytostellin immunoreactivity during S-phase and their temporal relationship with different stages of chromosome replication remain to be determined. Finally, previous studies showed that intranuclear cytostellin immunoreactivity intensifies markedly at G_2/M transition, coincident with chromosomal condensation (Warren et al., 1992), a phenomenon illustrated by the mitotic cell in Fig. 8B (see open arrow). Cytostellin undergoes similar cell cycle-specific changes in nuclear immunoreactivity in several types of human and rodent cells in vitro (unpublished observations).

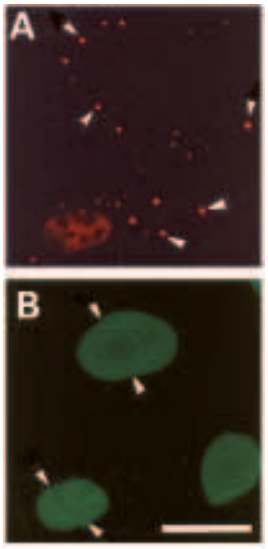

Fig. 6. Cytostellin-containing dots are extranuclear in a subset of interphase MDCK cells. MDCK cells were fixed with 1.75% paraformaldehyde and double-immunostained stained with mAb H5 and anti-lamin antibodies as in Fig. 5D and E. The nuclei shown in Fig. 5B are nascent, as indicated by their irregular contours and relatively small size (Fig. 5E, arrowheads), but the nuclei shown in Fig. 6 have smooth contours and are relatively larger (B). Arrowheads in (A) extranuclear cytostellin dots. Arrowheads in (B) nuclei of the cells shown in (A) stained with anti-lamin antibodies. Extranuclear cytostellin-containing dots are frequently identified in pairs of interphase cells, suggesting that the cells are daughters in G_1. Bar, 10 μm.

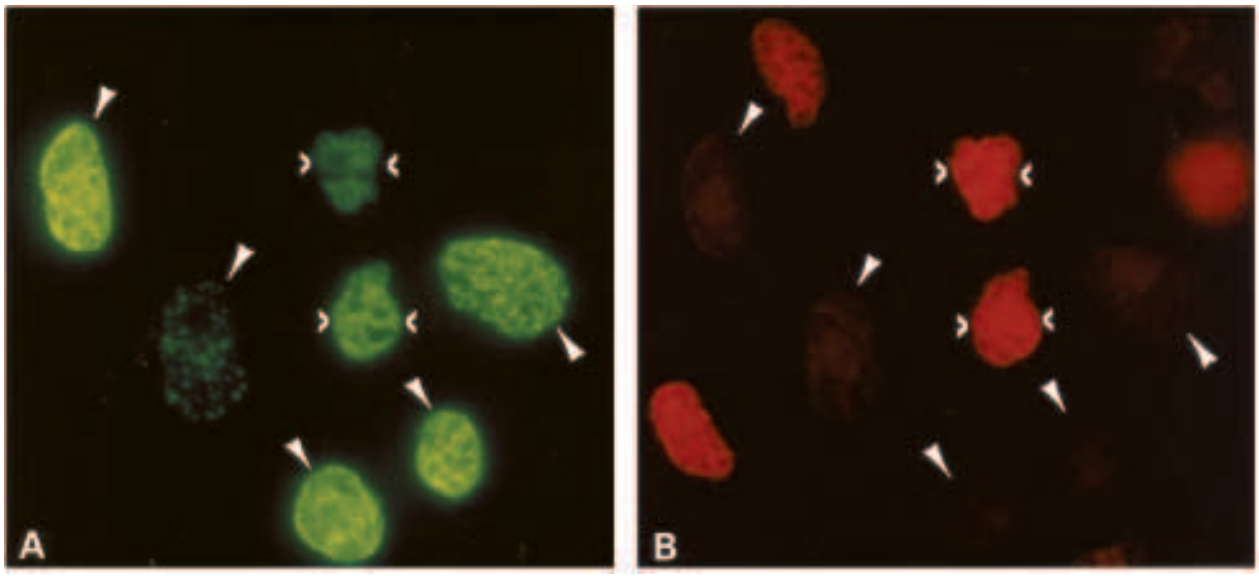

Fig. 7. Subcellular localization of cytostellin in S-phase cells. MDCK cells were incubated in complete medium containing 5′ bromodeoxyuridine (5′ BrdU) for 15 minutes, washed twice in PBS, fixed with 1.75% paraformaldehyde, permeabilized with 0.5% Triton X-100, and immunostained with anti-BrdU (A) or the anti-cytostellin mAb H5 (B) as described (Magaud et al., 1988). Anti-BrdU was visualized with FITC-conjugated goat anti-mouse IgG FITC and cytostellin rhodamine-conjugated goat anti-mouse IgM. Arrowheads indicate several replicating (S-phase) cells, which have incorporated 5′ BrdU into DNA (A), but which stain faintly or not at all with anti-cytostellin (B). The pair of cells bracketed by carets have incorporated 5′ BrdU (A) and also demonstrate a diffuse staining pattern with anti-cytostellin (B). Several cells staining diffusely with anti-cytostellin mAb H5 (B) did not incorporate 5′ BrdU (A) and therefore are not S-phase cells.

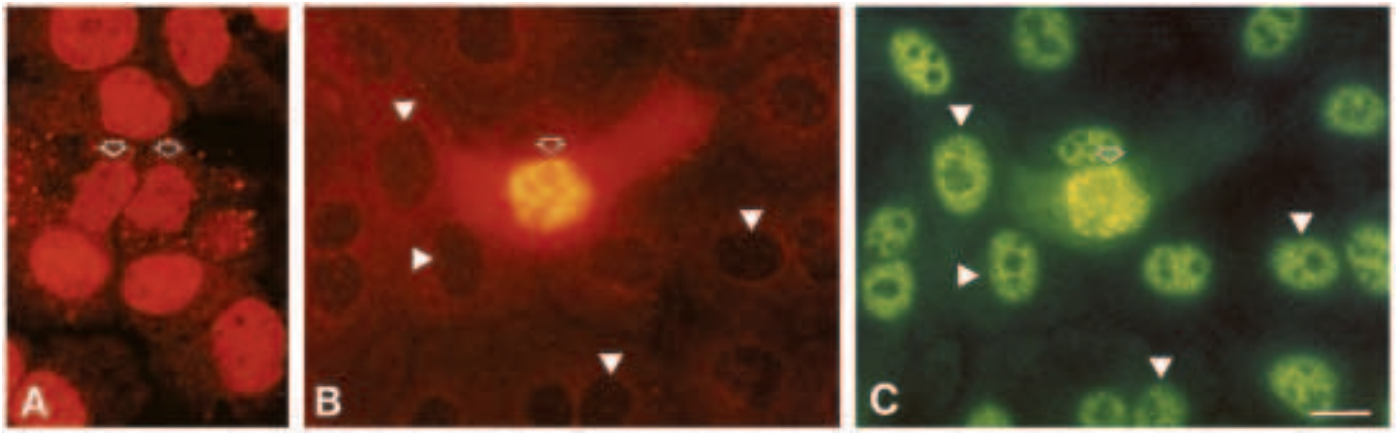

Fig. 8. Serum deprivation leads to diminished intranuclear cytostellin immunoreactivity. MDCK cells were grown in complete medium containing 7% FBS (A) or in serum-free DME for 24 hours prior to fixation (B and C). Next, the cells were fixed in 1.75% paraformaldehyde, permeabilized with 0.5% Triton X-100 and double-stained with mAb H5 (B) and mAb Y12 (C). mAb H5 was visualized with rhodamine-conjugated goat anti-mouse IgM and Y12 with FITC-conjugated goat anti-mouse IgG. Arrowheads indicate nuclei in which anti-cytostellin staining is absent but snRNP staining with Y12 is very intense (B and C). In (A) a telophase cell pair is indicated (open arrows). In (B) and (C) a prophase cell is shown with intense nuclear cytostellin and snRNP staining (open arrows). Bar, 10 μm.

Serum stimulation induces cytostellin immunoreactivity to appear in the intranuclear speckles

The above results show that cytostellin's nuclear immunoreactivity is affected by the cell cycle stage and growth state, but do not reveal conditions that induce the dot-like cytostellin staining in nuclear speckles, which is seen in a subpopulation of unsynchronized cells (Figs 1 and 3). Little or no cytostellin immunoreactivity is detected in the nuclei of serum-starved cells (Fig. 8B). We tested the idea that the appearance of intranuclear CS immunoreactivity is influenced by the growth conditions. To do this, growth-arrested cells were treated with either 20% FBS (Fig. 9A) or serum-free medium (Fig. 9B). Clearly, the serum induces intense cytostellin immunoreactivity within 60 minutes (Fig. 9A). The cytostellin immunoreactivity is concentrated in multiple speckles that closely resemble the punctate intranuclear staining observed in 10-15% of unsynchronized cells (Fig. 1). This serum-induced 'burst' of cytostellin immunoreactivity in the speckles coincides spatiotemporally with the transcriptional activation of Pol II genes, which takes place in the early growth response to serum growth factors (see Discussion). The rapid appearance of immunoreactive cytostellin in the speckles may indicate the recruitment of cytostellin to sites of Pol II transcription and pre-mRNA processing, or 'unmasking' of cytostellin molecules already in the nuclear speckles (see below).

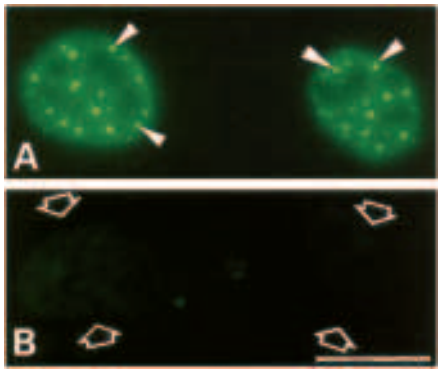

Fig. 9. Serum stimulation induces intense nuclear cytostellin immunoreactivity in speckles. MDCK cells were maintained in serum-free DME for 24 hours and stimulated with medium containing 20% FBS for 60 minutes (A), or in medium lacking FBS (B). Cells in both panels were fixed and immunostained with mAb H5 followed by FITC-conjugated goat anti-mouse IgM. Arrows in (B) mark very weakly staining (left) and non-staining (right) nuclei in the serum-deprived cells. Arrowheads in (A) indicate two representative nuclei that stain intensely with mAb. Nearly all of the serum-stimulated cells had intense dot-like cytostellin immunoreactivity, a striking appearance rarely observed in mixed populations of MDCK cells grown under normal conditions (DME with 7% FBS). There is also increased diffuse cytostellin immunoreactivity. Bar, 10 μm.

The level of cytostellin protein remains constant during the cell cycle

Cytostellin's fluctuating immunoreactivity may be due to periodic synthesis and degradation of cytostellin. To address this possibility, cytostellin protein content was determined in cells at each stage of the cell cycle. HeLa cells show a heterogeneous pattern of nuclear immunofluorescence very similar to MDCK cells, and they can be easily separated by centrifugal elutriation into fractions enriched with cells in the G_1, S and G_2/M stages of the cell cycle (Kaufmann et al., 1990). Thus, equal numbers of HeLa cells at various positions in the cell cycle were solubilized and subjected to immunoblot analysis with mAb H5 (Fig. 10). The results show clearly that the level of cytostellin protein is relatively constant throughout the cell cycle. Similar results were obtained using pharmacologically synchronized cells (unpublished observations).

Monoclonal antibody H14 binds to a different cytostellin epitope, but stains nuclei of unsynchronized cells with a heterogeneous pattern similar to H5 (unpublished results). This and the above results suggest that cytostellin's fluctuating immunoreactivity is due to periodic masking and unmasking of cytostellin's H5 and H14 epitopes in the nucleus as cells

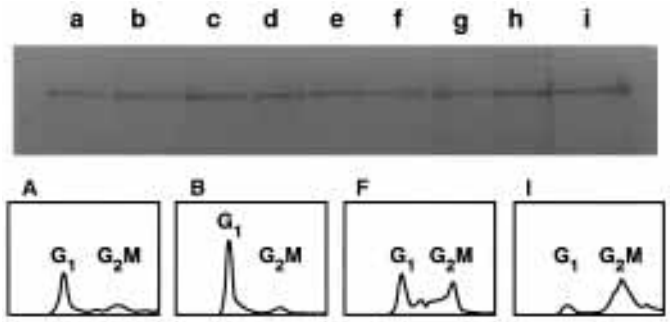

Fig. 10. Cytostellin level remains constant during the cell cycle. HeLa S3 cells grown in suspension culture were fractionated via centrifugal elutriation to yield pools of cells enriched for different phases of the cell cycle (see Materials and Methods). Samples containing 2×10^6 unfractionated cells (lane a) or 2×10^6 fractionated cells (lanes b-i) were solubilized and subjected to SDS-PAGE and western immunoblot analysis with mAb H5. The corresponding flow cytometry profiles of unfractionated cells (A) and representative elutriation fractions (B,F and I) are indicated below the immunoblot.

progress through the cell cycle. The H5 epitope might be masked by episodic changes in cytostellin's phosphorylation state, conformation, or by transient intermolecular interactions that prevent mAb H5 from binding to cytostellin. These possibilities are being investigated.

Cytostellin is associated with six proteins throughout the cell cycle

The co-localization of cytostellin and SC-35 at various stages of the cell cycle prompted us to ask whether cytostellin interacts biochemically with SC-35 or other proteins localized to speckles and spliceosomes. To detect proteins that associate with cytostellin, HeLa cells were labeled with [^{35}S]methionine, extracted with a buffer containing 0.5% Triton X-100/0.5% deoxycholate and the extract was immunoprecipitated with mAb H5. The immunoprecipitates were boiled in sample buffer and subjected to SDS-PAGE and fluorography (Fig. 11). mAb H5 immunoprecipitates cytostellin (arrowhead) and a complex of several proteins. Five (p40, p45, p55, p66 and p80) are invariably present (Fig. 11A and B); a sixth (p190) appears in some experiments (Fig. 11A) and not in others (Fig. 11B). The TEPC 183 myeloma protein (control IgM) precipitates several unidentified proteins that are distinct from the cytostellin-associated proteins, including faint bands at ~45 and ~55 kDa, which co-migrate with cytostellin-associated proteins (Fig. 11A). However, the ~45 and ~55 kDa bands are reproducibly more intense in the mAb H5 lane as illustrated in a separate experiment (Fig. 11B).

Complexes immunoprecipitated with mAb H5 were transferred to nitrocellulose and blotted with antibodies directed at several proteins located in nuclear speckles, spliceosomes and coiled bodies (Andrade et al., 1993). The following antibodies did not recognize the cytostellin-associated proteins: mAb SC-35, mAb Y12 (anti-snRNP B, B′ and D), anti-U2AF (Zamore and Green, 1991), anti-PTB and anti-PSF (Patton et al., 1991), anti-U1 snRNP p70 (Black et al., 1985), anti-coilin (Andrade et al., 1993) and mAb 104, which is directed at multiple proteins that contain Ser-Arg repeats and which localize to nuclear speckles (Roth et al., 1990). In addition, cytostellin is not detected in crude or purified spliceosome fractions immunoblotted with mAb H5 (D. B. Bregman, S. L. Warren and Robin Reed, unpublished results), and none of the cytostellin-associated proteins co-migrate on 2-D gels with proteins recognized previously as components of purified spliceosomes (Bennet et al., 1992; also unpublished results, D. B. Bregman and Dr Robin Reed, Harvard Medical School). Thus, cytostellin and the six associated proteins form a complex that appears to be distinct from spliceosomal proteins and several other proteins previously identified in nuclear speckles.

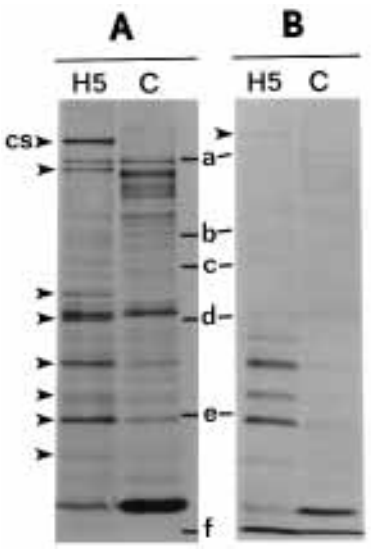

Fig. 11. Immunoprecipitation of cytostellin and associated proteins. HeLa S3 cells were grown in suspension culture (A) or on tissue culture plastic (B) before pulsing with 100 μCi/ml [^{35}S]methionine in 6-well tissue culture chambers for 2.5 hours. Labeled cells were solubilized with TD buffer, clarified by centrifugation and immunoprecipitated with mAb H5 or mAb TEPC-183 linked to agarose beads. The beads were washed three times with TD buffer, and the immunoprecipitated proteins were solubilized and subjected to 8% SDS-PAGE and fluorography (see Materials and Methods). H5, proteins immunoprecipitated by mAb H5; C, proteins immunoprecipitated by mAb TEPC 183; CS, the cytostellin band in (A). The cytostellin band in (B) is indicated by an arrowhead at ~240 kDa. Protein bands that co-immunoprecipitate with cytostellin are indicated by arrowheads on left margin of (A). Most of these bands are present in (B), which was derived from a separate experiment. Molecular mass standards are indicated as follows: a, myosin (205 kDa); b, β-galactosidase (116 kDa); c, phosphorylase (97 kDa); d, bovine serum albumin (66 kDa); e, ovalbumin (45 kDa) and f, carbonic anhydrase (29 kDa).

Cytostellin's episodic immunoreactivity and continuous redistribution during the cell cycle prompted us to ask if these six proteins associate with cytostellin transiently or continuously during the cell cycle. Thus, HeLa cells were separated by centrifugal elutriation into fractions enriched with cells in the G_1, S and G_2/M stages of the cell cycle, and each fraction was pulse-labeled with [^{35}S]methionine for 2 hours, extracted with a buffer containing 0.5% Triton X-100/0.5% deoxycholate and immunoprecipitated with mAb H5. The complexes were subjected to polyacrylamide gel electrophoresis and processed for fluorography. At each stage of the cell cycle, mAb H5 immunoprecipitated a complex comprising cytostellin and the six proteins nearly identical to the complex presented in Fig. 11A, confirming that cytostellin protein levels remain

constant, and that cytostellin is associated with these cytostellin-associated proteins throughout the cell cycle (data not shown).

DISCUSSION

This study shows that a highly conserved protein, cytostellin, co-localizes with the splicing factor, SC-35, at various stages of the cell cycle in mammalian cells. Interestingly, these two proteins also co-localize in amphibian lampbrush chromosomes, both in the loops (the site of nascent transcripts) and in the B-type snurpisomes, which share many properties with speckles in somatic cell nuclei (personal communication, Joseph Gall, Department of Embryology, Carnegie Institution; also see Gall, 1991). Despite the close spatial relationship between cytostellin and SC-35, cytostellin does not appear to be a component of spliceosomes. Cytostellin was not detected in crude and purified spliceosome preparations; none of the cytostellin-associated proteins co-migrated with components of purified spliceosomes on 2-D gels (D. B. Bregman, S. L. Warren and Robin Reed, unpublished observations); and antibodies directed at multiple spliceosomal proteins did not immunoblot the cytostellin-associated proteins revealed in Fig. 11. Moreover, in many types of differentiated cells cytostellin is located in extranuclear compartments that lack splicing machinery (unpublished observations). Finally, transformation of cells by the v-*src* tyrosine kinase induces a fraction of cytostellin molecules, but not SC-35, to relocate to extranuclear sites associated with the peripheral cytoskeleton (unpublished data). Taken together, these data suggest that cytostellin and its associated proteins form a complex that is distinct from spliceosomes.

Nevertheless cytostellin, SC35 and other splicing proteins undergo an indistinguishable mitotic redistribution from the nuclear speckles to the extranuclear dots, and they all reappear in the speckles of interphase cells. The mechanism underlying the co-redistribution of these proteins is unknown. One possibility is that at the onset of mitosis, cytostellin and the splicing proteins that comprise the nuclear speckles rapidly disassemble, disperse throughout the cell, and subsequently reassemble in discrete sites associated with the peripheral cytoskeleton (dots). A similar process might explain their nuclear redistribution during G_1. Alternatively, cytostellin and the splicing proteins might continuously co-localize during their redistribution from the intranuclear speckles to the extranuclear dots. According to this idea, at the onset of mitosis each intranuclear speckle is subdivided into many cytostellin/SC35-containing dots, which redistribute as intact supramolecular structures to sites throughout the cell. However, the cytostellin-containing dots may not be considered to be *bone fide* particles until they are isolated and shown to possess the biophysical properties of particles. The hypothesis that the cytostellin-containing dots are supramolecular particles would be further supported by the direct visualization of mitotic and postmitotic movement of the dots, using time-lapse cinematography.

Perhaps the most interesting finding is that serum stimulation induces the rapid appearance of cytostellin immunoreactivity in the nuclear speckles. Similar changes in cytostellin's intranuclear immunoreactivity have been demonstrated in PC12 pheochromocytoma cells stimulated with nerve growth factor, and in melanocytes stimulated with endothelin and hepatocyte growth factor (N. Eisenberg, R. Halaban. S. Ribisi and S. L. Warren, unpublished results). In the MDCK cells, cytostellin's mAb H5 epitope is masked in serum-starved cells, because it becomes available to the antibody if the cells are extracted by Triton X-100 prior to fixation and immunostaining (unpublished results), and the level of cytostellin protein remains constant throughout the cell cycle. Serum-induced cytostellin immunoreactivity begins to appear in MDCK cell nuclei within 45 minutes and it reaches a maximum at ~60 minutes (unpublished observations); furthermore, this change takes place in absence of protein synthesis. Although the cytostellin immunoreactivity appears most intense in the speckles, it is also evident in a diffuse distribution in the nuclei of serum-stimulated cells (Fig. 9).

Serum growth factors induce transcriptional activation of many Pol II genes in less than an hour after stimulation (for reviews see Hunter and Karin, 1991; Whiteside and Goodburn, 1993; Treisman, 1990), so we suspect that the appearance of cytostellin immunoreactivity in the speckles may coincide with the onset of new Pol II transcription in the nucleoplasm adjacent to the nuclear speckles. The localization of cytostellin to the loops of amphibian lampbrush chromosomes, sites rich in nascent Pol II transcript, suggests that cytostellin can associate with proteins and/or nucleic acids that function in Pol II transcription and pre-mRNA processing. The function of cytostellin remains unknown, but its localization to sites of Pol II transcription and pre-mRNA processing, and the ability of growth factors to induce rapid changes in its immunoreactivity at these sites, suggest that cytostellin may play a role in mRNA biogenesis.

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