INTRODUCTION

In recent years, the functional organization of the interphase nucleus has emerged as a field of intense research activity. Data have accumulated indicating that a variety of nuclear operations occur at discrete sites within the nucleus, thereby implying the existence of an underlying organized structure (for review see Spector, 1993). The nuclear matrix (operationally defined as the insoluble structural framework remaining after cells are treated with detergents, nucleases and high salt solutions) has indeed been implicated in key cellular processes such as DNA replication and transcription as well as RNA processing and transport (Berezney and Coffey, 1975; Jackson and Cook, 1985; McCready et al., 1980; Nakayasu and Berezney, 1989; Razin et al., 1985; Reuter et al., 1984; Robinson et al., 1982; Thorburn et al., 1988; Zeitlin et al., 1987). Electrophoretic analyses have shown that the nuclear matrix is composed of a complex set of proteins, some of which are cell-type specific whereas others appear to be ubiquitous (Fey and Penman, 1988; Stuurman et al., 1990). However, little is known about specific components that take part in the physical partitioning of the multitude of nuclear functions.

Particular attention has recently been directed to the subnuclear location of pre-mRNA transcription and processing, and to their relationship to the nuclear matrix. Early ultrastructural studies have shown that transcription of pre-mRNA occurs at perichromatin fibrils, which are ribonucleoprotein (RNP) structures located at the border of condensed chromatin at the periphery of the nucleus and contain newly synthesized heterogeneous nuclear RNA (hnRNA) (Fakan, 1978; Puvion and Moyne, 1981). The presence in these structures of poly(A) RNA, recently revealed by electron microscope in situ hybridization (Visa et al., 1993), and of antigenic determinants of hnRNPs and small nuclear RNPs (snRNPs) (Fakan et al., 1984; Puvion et al., 1984), provided further evidence that they correspond to newly synthesized hnRNPs. The processing of nuclear pre-mRNA also appears to be a highly compartmentalized operation. Splicing takes place in spliceosomes, which are composed of the snRNP particles U1, U2, U4, U5, U6 and other splicing factors (Lamond, 1993; Lührmann et al., 1990; Zieve and Sauterer, 1990). The structural organization of splicing snRNPs has been documented by several immunofluorescence studies using antibodies to various components of the spliceosome (Lerner et al., 1981; Nyman et al., 1986; Reuter et al., 1984; Spector, 1984) as well as in situ hybridization experiments with snRNA probes (Carmo-Fonseca et al., 1992; Huang and Spector, 1992). These studies have revealed

SUMMARY

It was previously demonstrated that monoclonal antibody CC-3 binds to a phosphorylation dependent epitope present on a 255 kDa nuclear protein (p255). We show here that in interphase cells, p255 distributes to typical nuclear speckles that correspond to the localization of spliceosome components as revealed by antibodies to the m^G cap of snRNAs or to the non-snRNP splicing factor SC-35. Immunofluorescence and immunoblot studies indicated that p255 is resistant to extraction with non-ionic detergents, nucleases and high ionic strength buffers and may thus be defined biochemically as a nuclear matrix phosphoprotein. To determine the nature of the association of p255 with the nuclear structure, its distribution was studied at different stages of the cell cycle and after the cells were treated with nucleases or heat shocked. We found that the antigen diffused into the cytoplasm during metaphase but was reorganized into cytoplasmic speckles during anaphase-telophase transition, where it colocalized with SC-35. Nuclear matrix preparations that were digested with DNases and RNases showed that interphasic p255 still localized to nuclear speckles even though snRNA and snRNP antigens were removed. Heat-shocked cells labelled with monoclonal antibody CC-3 exhibited more rounded and less interconnected speckles, identical to those decorated by anti-SC-35 antibody under such conditions. These results indicate that p255 and SC-35 are present in the same nuclear structures, to which they are more tightly bound than the snRNP antigens. They further suggest that both proteins are implicated in spliceosome assembly or attachment.

Key words: nuclear matrix, spliceosome, phosphoprotein, p255, monoclonal antibody CC-3
that the spliceosome components are concentrated in a limited number of typical nuclear speckles forming a latticework within the interphase nucleus. At the electron microscopic level, this speckled pattern represents regions enriched in interchromatin granules and perichromatin fibrils (Fakan et al., 1984; Puvion et al., 1984; Specter, 1993; Specter et al., 1983). In contrast to the latter, interchromatin granules have little or no labeling in their interior following [3H]uridine incorporation (Fakan and Bernhard, 1971; Fakan and Nobis, 1978), implying that these structures contain RNA species with slow turnover rates and suggesting that they are sites of splicing factor assembly and/or storage (Spector, 1993). However, recent studies have shown that interchromatin granule clusters contain significant amounts of poly(A) RNA and may thus be involved in RNA transport or sorting (Visa et al., 1993), if not in the splicing operation itself (Carter et al., 1991, 1993; Wang et al., 1991).

In recent years, an impressive number of nuclear proteins, mostly revealed by antibodies to nuclear matrix or spliceosomal components, have been shown to colocalize with the spliceosomes in a speckled distribution and/or to associate with the clusters of interchromatin granules (Brancolini and Schneider, 1991; Bregman et al., 1994; Cleverenger and Epstein, 1984; Compton et al., 1992; Fu and Maniatis, 1990; Nickerson et al., 1992; Smith et al., 1989; Specter et al., 1987; Turner and Franchi, 1987; Wan et al., 1994). Most of these proteins exhibit distinct characteristics, such as the specificity of their expression, the nature of their association with the insoluble nuclear structures, their involvement or not in splicing, their distribution at mitosis and their intrinsic biochemical properties. It is likely that the complete characterization of these proteins and of other unidentified components, as well as the study of their interactions, will provide a more detailed picture of nuclear matrix function. Here we show that p255, an evolutionarily conserved nuclear antigen that we previously identified indirectly via the monoclonal antibody CC-3 (Thibodeau and Vincent, 1991), is a bona fide nuclear matrix phosphoprotein, which is concentrated in the speckled domains. We find that the association of this protein with the nucleus is distinct from that of the snRNP components but is similar to that of the non-snRNP splicing factor SC-35 (Spector et al., 1991), with respect to its behavior during mitosis or after RNase digestion and heat shock.

MATERIALS AND METHODS

Cell cultures

Chick embryo fibroblasts, Chinese hamster ovary (CHO) cells, NIH 3T3 mouse fibroblasts, African green monkey COS-7 cells and human breast adenocarcinoma MCF-7 cells were grown on glass coverslips at 37°C with 10% CO2 in Iscove’s modified Dulbecco’s medium (IMDM) supplemented with 10% fetal bovine serum (FBS). Potoroo kidney cells (Ptk2) were grown under the same conditions except that the medium was supplemented with 20% FBS. Xenopus cells XI 177 were grown at 23°C in Dulbecco’s modified Eagle’s medium (DMEM) supplemented with 8 mM Hepes and 10% FBS. For heat shock experiments, CHO cells grown on glass coverslips were transferred to Petri dishes containing pre-warmed medium (45°C) and the dishes were immersed in a 45°C water bath for 15 minutes.

Antibodies

mAb CC-3 (IgG 2a) was obtained after immunization of a Balb/C mouse with pharyngeal regions isolated from 72 hour chick embryos in a search for molecules with restricted patterns of expression during development (Thibodeau et al., 1989). The CC-3 hybridoma cell line was grown in IMDM supplemented with 10% FBS and the culture supernatant was used undiluted as a source of CC-3 antibody for immunofluorescence and immunoblotting experiments. Monoclonal antibody Ab-1 (IgG1) against 2,2',7-trimethylguanosine (m7G-cap) was purchased from Oncogene Science (Uniondale, NY). Anti-snRNP mAb Y12 (IgG2a) (Lerner et al., 1981) and mAb SC-35 (IgG1) (Fu and Maniatis, 1990) were generous gifts from J. A. Steitz and T. Maniatis, respectively. Conjugated antibodies to mouse immunoglobulin subclasses were purchased from Caltag Laboratories (San Francisco, CA).

Cell extractions

For immunofluorescence studies, cells were grown on coverslips, washed in PBS and extracted on a monolayer by gently adding and removing extraction solutions. For electrophoretic analyses, cells were extracted in suspension, with centrifugation steps (600 g, 3 minutes) between treatments. The procedure used was essentially that described by He et al. (1990) for the preparation of RNA-containing nuclear matrix. Cells were first extracted in cytoskeleton buffer (10 mM Pipes, pH 6.8, 100 mM NaCl, 300 mM sucrose, 3 mM MgCl2, 1 mM EGTA, 0.5% Triton X-100, 4 mM vanadyl riboside complex, 1 mM PMSE) for 3 minutes at 4°C. After a brief wash in PBS, DNA was digested with 25 U/ml RNase-free DNase I (Boehringer Mannheim, Laval, Québec) for 30 minutes at room temperature in digestion buffer (10 mM Pipes, pH 6.8, 50 mM NaCl, 300 mM sucrose, 3 mM MgCl2, 1 mM EGTA, 0.5% Triton X-100, 4 mM vanadyl riboside complex, 1 mM PMSE). Chromatin was then removed by three 10-minute washes with 0.25 mM ammonium sulfate in digestion buffer to yield the nuclear matrix/intermediate filament structure (He et al., 1990). From this stage on, the buffers were supplemented with 2 mM o-vanadate as phosphatase inhibitor. An additional high salt treatment was applied by washing the nuclear matrix with 2 M NaCl in digestion buffer (3x 5 minutes), and RNA was digested with 100 µg/ml RNase A and 40 U/ml RNase T1 in digestion buffer (without vanadyl riboside complex) for 60 minutes at room temperature.

In some experiments, the nuclear matrix was prepared according to Brancolini and Schneider (1991) with slight modifications. Briefly, cells grown on coverslips were washed in PBS and extracted with TSKM buffer (25 mM Tris-HCl, pH 7.4, 100 mM NaCl, 50 mM KCl, 5 mM MgCl2, 1 mM PMSE) containing 0.5% Nonidet P-40 (NP-40) for 3 minutes at 4°C. Cells were then incubated in TSKM supplemented with 25 U/ml RNase-free DNase I, 100 µg/ml RNase A and 40 U/ml RNase T1 for 60 minutes at room temperature. Chromatin was extracted with 30 mM Tris-HCl, pH 7.4, 10 mM MgCl2, 1 mM PMSE and 0.4 M (NH4)2SO4 and cells were finally rinsed with TSKM and PBS prior to fixation and staining.

Immunofluorescence microscopy

Intact cells and cells at different stages of nuclear matrix preparation were fixed on coverslips with cold methanol (20 minutes, -20°C). Similar results were obtained when cells were fixed for 30 minutes in 4% paraformaldehyde in PBS and permeabilized for the same time with 0.5% Triton X-100. Incubations with the primary antibodies were done at room temperature for 45 minutes. After washing in PBS, antibody binding sites were revealed by goat anti-mouse IgGs labelled with fluorescein. For double labelling experiments, monoclonal antibody CC-3 (IgG2a) was mixed either with anti-m7G-cap or SC-35 (both IgG1) for the first incubation and the reactions visualized with FITC-conjugated goat anti-mouse IgG2a and Cy3-conjugated goat anti-mouse IgG1. In some experiments, DNA was stained by incubating the coverslips in propidium iodide (0.1 µg/ml) for one minute. Immunofluorescence observations were performed on a Zeiss Axioptoph microscope equipped with epifluorescence optics.
Electrophoresis and immunoblotting

Analytical SDS-PAGE was performed on a MiniProtean II apparatus (Bio-Rad) using 5-15% polyacrylamide gradient gels (acylamide:bis, 30:0.15) to resolve high Mr proteins as described previously (Ho-Kim et al., 1991). Samples were solubilized in electrophoresis sample buffer (Laemmli, 1970) and heated for 3 minutes at 95°C prior to loading. Gels were stained with 0.1% Coomassie Blue R-250. Immunoblotting of proteins from SDS-PAGE was done as previously described (Cossette and Vincent, 1991). The blots were saturated with PBS containing 2% BSA for 1 hour at 37°C and reacted with monoclonal antibody CC-3 for 45 minutes at 37°C. After extensive washes in PBS containing 5% powdered milk, the blots were incubated in the same conditions with 125I-labelled goat immunoglobulins against mouse IgG heavy chains. Washed immunoblots were exposed to Fuji RX films. In some experiments, nitrocellulose sheets were treated with alkaline phosphatase prior to antibody reaction, as described previously (Thibodeau and Vincent, 1991).

RESULTS

Localization of p255 in snRNA-containing nuclear speckles

Monoclonal antibody CC-3 was selected for its strong immunoreactivity with mitotic cells (Thibodeau and Vincent, 1991). In interphase cells, however, CC-3 reactivity was confined to nuclear granules and mediated, as demonstrated on immunoblots, by a 255 kDa protein (p255). The reactivity of p255 with CC-3 was shown to be dependent on the presence of a phosphorylated epitope, and both p255 and its phospho-dependence on CC-3 recognition were conserved in most animal cells (Thibodeau and Vincent, 1991). When mammalian cells were stained with CC-3, the fluorescence was typically concentrated in nuclear speckles apparently connected by less intensely labelled nucleoplasmic material (Fig. 1c-g). Immunostaining of amphibian and avian cells gave a less prominent speckled pattern but the reactivity was confined to a fine nuclear punctate pattern (Fig. 1a and b). Nucleoli were consistently negative in these experiments. This dot-like distribution pattern of reactivity was strikingly reminiscent of that described for fluorescence microscopy studies using anti-snRNPs and anti-snRNAs antibodies (Nyman et al., 1986; Reuter et al., 1984; Spector and Smith, 1986; Spector et al., 1991; Verheijen et al., 1986) as well as antisense snRNA oligonucleotide probes (Carmo-Fonseca et al., 1991a,b; Huang and Spector, 1992). This prompted us to proceed with double immunolabelling experiments on CHO cells with CC-3 and a monoclonal antibody which recognizes the snRNA-specific m3G cap structure. We found that both antibodies decorated the same intranuclear speckles occupying a significant portion of the nucleoplasm excluding the nucleoli (Fig. 2a and b). As illustrated in Fig. 2c, the speckled patterns were coincident while the anti-m3G cap antibody showed a more diffuse nuclear staining. When cells were briefly extracted with NP-40 in TSKM (see Materials and Methods) before fixation and immunolabelling (Fig. 2d-f), the colocalization of both antigens was more evident, presumably due to partial loss of the nuclear components diffusely stained by both antibodies. Most, if not all, interphase cells showed p255 distribution confined to nuclear speckles. Some cells displayed weak CC-3-staining, which most probably represents late telophase cells that had not returned to the interphase pattern (see below). Identical results were obtained with various cell lines (data not shown).

p255 is a nuclear matrix component

The resistance of p255 to extraction with non-ionic detergents suggested that this polypeptide could interact with insoluble nuclear structures. To investigate this possibility, CHO cells were submitted to in situ sequential fractionation in order to produce nuclear matrix preparations, using the method of He

Fig. 1. Presence of the nuclear protein p255 in various cell types. Xenopus cells Xl 177 (a), chick embryo fibroblasts (b), CHO (c), PtK2 (d), COS-7 (f) and MCF-7 (g) cells were fixed in methanol at −20°C for 20 minutes and subjected to indirect immunofluorescence with monoclonal antibody CC-3 as described in Materials and Methods. In mammalian cells (c-g), fluorescent staining is concentrated in nuclear speckles but also diffusely distributed throughout the nucleoplasm, except for the nucleolar region. In Xenopus and chicken cells (a,b), the dot-like structures are less prominent. Bar, 10 µm.
et al. (1990). The fate of p255 was monitored at each step in the procedure. Cells were extracted with Triton X-100, treated with DNase I and reextracted first with 0.25 M ammonium sulfate to remove chromatin and then with 2 M NaCl to reveal the core filaments of the nuclear matrix (He et al., 1990). Fig. 3 shows immunostaining for p255 after each extraction step. As can be judged by propidium iodide staining, DNA was thoroughly removed during the procedure (Fig. 3b,d,f,h,j). Some CC-3-mediated diffuse fluorescence was apparently removed with Triton X-100, yielding a discrete nuclear punctate pattern (Fig. 3c). After DNA digestion and chromatin elution with 0.25 M ammonium sulfate, there was a marked shrinkage of the nuclei (Fig. 3e and f) and the residual structures were barely visible by phase contrast microscopy (not shown). The fluorescence intensity nevertheless remained approximately the same, but the speckles were less apparent due to their incorporation into the contracted nuclear remnant. Further extraction with 2 M NaCl (Fig. 3g) did not significantly alter the pattern or the intensity of fluorescence, despite the fact that this treatment has been shown to remove 35% of the total proteins remaining after chromatin removal (He et al., 1990). This observation suggested a tight association of residual p255 with the nuclear matrix scaffold, a view that was further substantiated by the observation that RNA digestion with RNases A and T1 did not markedly modify CC-3 fluorescence (Fig. 3i). After such treatment, labelling could not be observed with anti-m7G cap antibody or with the monoclonal anti-Sm Y12 antibody (data not shown).

Fig. 4 shows the Coomassie Blue-stained gel (A) and the corresponding p255 immunoblot (B) of the nuclear subfractions analyzed on a high-porosity acrylamide gel, which provides good resolution of high molecular mass proteins. Samples from an equivalent number of cells were loaded in each well to facilitate the visualization of the progressive removal of nuclear proteins and to estimate the relative content of p255 in each fraction. The intensity of most Coomassie Blue stained bands decreased during cell fractionation (Fig. 4A). The efficiency of the procedure was assessed by the enrichment of a few polypeptide bands in the 50-70 kDa region of the gel (see lanes 3, 4 and 5 in Fig. 4A) that could correspond to nuclear lamins and intermediate filament proteins previously described as the predominant protein species remaining after RNA digestion (Fey and Penman, 1988; He et al., 1990). In contrast to the majority of proteins, the intensity of immunoreactive p255 was only slightly reduced during fractionation. Most of it resisted the extraction procedures (Fig. 4B, lanes 2-5), although partial solubilization was observed during chromatin digestion and subsequent high-salt washes (Fig. 4B, lanes 7 and 8). These experiments complemented the fluorescence studies and further suggested that a significant portion of nuclear p255 was tightly associated with the nuclear matrix and that this interaction was RNA-independent. The band corresponding to p255 could not be identified on Coomassie Blue or silver stained gels, implying most probably that it is a minor component of the nuclear matrix. As we have already shown that p255 recognition by monoclonal antibody CC-3 in whole

Fig. 2. Colocalization of p255 and snRNA in the nuclear speckles. CHO cells were either fixed in cold methanol (a-c) or briefly extracted with 0.5% NP-40 prior to fixation (d-f) and double-immunostained with monoclonal antibody CC-3 (a and d) and anti-m7G cap antibody (b and e). The same nuclear speckles are labelled by both antibodies, as best illustrated by the yellow colour of the dots on the merged images (c and f). In non-extracted cells, the speckled pattern is more evident with CC-3 (a) than with anti-m7G cap antibody (b). After NP-40 treatment (d-f), colocalization on the speckles is more evident although snRNA still appears more widely distributed in the nucleoplasm (e). Bar, 10 µm.
Localization of CC-3 antigen in mitotic cells

We have previously demonstrated that monoclonal antibody CC-3 reacts more strongly with mitotic cells (Thibodeau and Vincent, 1991). Indeed, this antibody was originally selected from a library of monoclonals for its ability to localize mitotic foci on histological sections (Thibodeau et al., 1989; Thibodeau and Vincent, 1991). In most cultured cells, the
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Fig. 4. Immunoblots of cell residues and extracted proteins after sequential fractionation of CHO cells to prepare nuclear matrix. (A) Coomassie Blue stained 5-15% SDS-PAGE gel. (B) Immunoblot analysis with monoclonal antibody CC-3. Untreated (lanes 1) or extracted cells (lanes 2-5) were solubilized directly in electrophoresis buffer whereas removed material (lanes 6-9) was dialyzed and lyophilized before solubilization in sample buffer. Each well was loaded with the protein residue from 1.5x10^5 cells. Lanes 2, cells extracted with 0.5% Triton X-100 in cytoskeleton buffer; lanes 3, residual structures obtained after DNA digestion and chromatin elution with 0.25 M ammonium sulfate; lanes 4, 2 M NaCl extracted structures; lanes 5, residual structures after RNA digestion. Lanes 6 to 9 represent the proteins extracted with: lanes 6, Triton X-100; lanes 7, DNase I and 0.25 M ammonium sulfate; lanes 8, 2 M NaCl; and lanes 9, RNases A and T1 treatment. In contrast to the majority of proteins, most p255 resisted to the extractions (B, lanes 2-5), but partial solubilization was observed during chromatin digestion and subsequent high-salt washes (B, lanes 7 and 8). Molecular masses of the α (240) and β (220) spectrin subunits, phosphorylase a (97), bovine serum albumin (66), ovalbumin (45) carbonic anhydrase (31), trypsin inhibitor (22) and lysozyme (14) used as molecular mass markers, are given in kDa.

extremely intense reactivity of CC-3 at metaphase and the rounding up of the cells usually prevented a clear visualization of the immunostained mitotic cell constituents (for example, see the out-of-focus mitotic cell in Fig. 3a). Using PtK2 cells, the immunoreactivity at metaphase and anaphase was shown to be distributed diffusely throughout the cytoplasm and to localize more intensely to the mitotic spindle and the centrosomes (Thibodeau and Vincent, 1991). During anaphase-telophase transition, CC-3 reactivity became concentrated in cytoplasmic speckles and in the midbody. Fig. 6 (a-c) shows a telophase CHO cell double-labelled with CC-3 and anti-m3 G cap. It can be seen that CC-3-stained structures are organized into cytoplasmic speckles still surrounded by some diffuse fluorescence (Fig. 6a) whereas snRNA antigen (Fig. 6b) is localized in the prospective daughter nuclei, whose contours were already detectable in phase contrast (Fig. 6c). Like the interphase nuclear speckles, the mitotic dots were resistant to extraction with non-ionic detergents (data not shown) and were quickly suspected to correspond to the mitotic clusters containing the non-snRNP splicing factor SC-35 (Spector et al., 1991). Double-labelling CHO mitotic cells with CC-3 and anti-SC-35 antibodies revealed that both proteins colocalized in these dots (Fig. 6d-f), as they also did in interphase nuclei (see below).

Nuclease and heat shock sensitivity of CC-3 antigen localization
Spector and colleagues (1991) have demonstrated that SC-35 and snRNPs colocalized within interchromatin granules and perichromatin fibrils but that the localizations of both antigens have different RNase and heat shock sensitivities. When labelled with αSm antibodies, RNase A-digested cells showed greatly reduced or totally absent reactive speckles whereas no change was observed in the distribution of SC-35 (Spector et al., 1991). We prepared nuclease-treated nuclear matrix for double-labelling with CC-3 and either anti-SC-35 or anti-m3 G cap antibodies. We used the procedure described by Brancolini and Schneider (1991), in which nuclear shrinking was less evident, at least in our hands. Under these conditions, we found that no change was observed in p255 and SC-35 localization, and that both proteins were still colocalizing (Fig. 7a-b) while snRNA was no longer detectable (Fig. 7d). snRNP immunoreactivity, as monitored by monoclonal antibody Y12, was also greatly reduced by the treatment (data not shown). Therefore,

Fig. 5. Phosphodependence of p255 recognition by monoclonal antibody CC-3. RNase-treated nuclear matrix residues were electrophoresed and blotted as described in Materials and Methods. Nitrocellulose strips were reacted with CC-3 after incubation in the absence (lane 1), or in the presence of 15 U/ml alkaline phosphatase (lane 2). The strip shown in lane 3 has been incubated with the enzyme in the presence of 50 mM NaPO4. Dephosphorylation of the nuclear matrix protein p255 completely abolished its immunoreactivity to monoclonal antibody CC-3. A minor immunoreactive band migrating faster than p255 probably represented a degradation product induced during sample preparation, since its presence was detected sparsely (see also Thibodeau and Vincent, 1991). Molecular masses of spectrin α- and β- subunits used as markers are given in kDa.
like SC-35, but unlike snRNPs, p255 localization in the speckled regions is not dependent upon the presence of RNA. Heat shock was shown to provoke a uniform distribution of snRNP antigens throughout the nucleoplasm and to induce SC-35-rich clusters to become more rounded and isolated from each other (Spector et al., 1991). Heat-shocked CHO cells double-labelled with CC-3 and either anti-SC-35 or anti-m3 G cap antibodies exhibited comparable behavior (Fig. 8). While snRNA staining changed from a punctate to a relatively uniform pattern (Fig. 8d), the speckles enriched in CC-3 antigen appeared more compact and less interconnected (Fig. 8a and c) and appeared perfectly superimposible on the αSC-35-labelled structures (Fig. 8b).

**DISCUSSION**

It was previously demonstrated that, in mammalian interphase cells, monoclonal antibody CC-3 immunoreactivity was organized in a speckled pattern in the nucleus and was conferred by a phosphorylated epitope produced by a single high $M_r$ polypeptide (p255) (Thibodeau and Vincent, 1991). During mitosis, the reactivity first increased in the prophase nucleus and dispersed within the cytoplasm after nuclear membrane breakdown, where it localized mainly to the mitotic apparatus. When analyzed on immunoblots, a new set of M-phase specific high $M_r$ polypeptides were revealed by CC-3 (Thibodeau and Vincent, 1991). At present, we do not know how these mitotic proteins are related to the interphasic p255, but it can be speculated that most represent phosphorylation variants of the molecule, since the major mitosis-specific bands migrate more slowly than (>300 kDa) or in the vicinity of p255, and their recognition by CC-3 is also phosphate-dependent. The possibility cannot be excluded, however, that M phase-specific antigens unrelated to p255 may become unmasked at mitosis.

The aim of the present study was to determine the nature and the precise localization of the nuclear protein p255. We have shown that p255 is distributed as nuclear speckles in interphase cells of various species and cell types where it colocalizes with snRNAs. We have also shown that p255 is resistant to extraction with non-ionic detergents, nucleases and high ionic strength buffers, and may thus be defined biochemically as a nuclear matrix component. This behavior was best illustrated either by immunofluorescence studies or by immunoblotting...
using the sequential in situ fractionation procedure introduced by Pennman and coworkers (Capco et al., 1982; Fey et al., 1986; He et al., 1990). Although a fraction of p255 was removed during the extractions, it was abundantly retained with the CHO cell nuclear matrix. Furthermore, RNase digestion left most of the p255 with the remaining insoluble residue, which contained a minor fraction of the total cellular proteins and only traces of RNA and DNA. Immunoblotting of these nuclear residues with CC-3 confirmed, as we previously observed using total cell extracts (Thibodeau and Vincent, 1991), that p255 binding by the antibody was phosphorylation dependent. Therefore, p255 is a phosphoprotein tightly associated with the nuclear matrix that colocalizes with snRNA.

During the past few years, several antigens associated with the nuclear matrix were shown to distribute with clusters of interchromatin granules (Brancolini and Schneider, 1991; Bregman et al., 1994; Clevenger and Epstein, 1984; Compton et al., 1992; Fu and Maniatis, 1990; Nickerson et al., 1992; Smith et al., 1989; Spector et al., 1987; Turner and Franchi, 1987; Wan et al., 1994). One of the most characterized, SC-35, is a 35 kDa non-snRNP factor required for splicing and spliceosome assembly (Fu and Maniatis, 1990). Like p255, its nuclear distribution is similar to that of snRNP antigens but SC-35 was shown to display differences in its behavior during mitosis and in the nature of its association with the nuclear structure (Spector et al., 1991). We found that CC-3 antigen colocalized with SC-35 at different stages of the cell cycle and that the sensitivity of both antigens to nucleases and heat shock were identical in every respect. Thus, CC-3 reactivity was detected as diffuse staining during metaphase but reorganized into cytoplasmic speckles during the anaphase-telophase transition, while snRNA localized in the future nuclei. Double labelling mitotic cells with CC-3 and α-SC-35 demonstrated a perfect spatio-temporal coincidence between the markers. So, even if the nature of the mitotic antigens recognized by CC-3 and their relation to interphasic p255 have not been established, it is worth noting that CC-3-mediated reactivity colocalizes with SC-35 in interphase as well as in mitotic cells. This observation suggests that the cell cycle variation observed in CC-3 immunoreactivity involves antigens that are related, if not biochemically, at least cytostructurally.

Nuclear matrix preparations that were digested with RNases showed that p255, like SC-35, still localized to nuclear speckles even though snRNA was no longer detectable and snRNP antigens had been mostly removed. Spector et al. (1991) have shown that after heat shock, snRNP antigens redistributed uniformly in the nucleoplasm whereas the SC-35-rich clusters appeared to be more rounded and less interconnected than in control cells. Heat-shocked cells labelled with CC-3 exhibited similar intensely fluorescent compact and rounded speckles and we found that both markers were colocalized. Collectively, these data suggest that p255, SC-35 and the snRNP antigens are present in the same nuclear domain, into which the two former are more tightly bound than the latter. In this respect, SC-35 and p255 could participate in the structural core of the speckles and, as previously suggested (Spector et al., 1991), be involved in the assembly and attachment of the spliceosomal components to the nuclear matrix.

This structure could include other nuclear matrix proteins as well. Considering the high $M_r$ of p255, it is relevant to note that a few large nuclear matrix proteins have been identified recently, most of them being preferentially localized to nuclear speckles. This is the case of the B1C8 protein, which has been identified using a monoclonal antibody from a nuclear matrix antibody library selected for its speckled staining of the interphase nucleus and its behavior at mitosis, which, incidentally, resembles that of the CC-3 antigen (Wan et al., 1994). The B1C8 antigen is a 180 kDa protein and is therefore probably different from p255, which in our electrophoretic system consistently migrates more slowly than the spectrin α-subunit (240 kDa). Cytostellin, a conserved 240 kDa phosphoprotein, was also shown to distribute to nuclear regions enriched with splicing factors (Bregman et al., 1994) and to share common properties with p255. In contrast to CC-3, however, anti-cytostellin monoclonal antibodies H5 and H14 (binding to different epitopes) stain speckles in a fraction of the cells, as the immunoreactivity appeared to be affected by the cell cycle and the growth state (Bregman et al., 1994). Since the level of the protein remains constant, it was proposed that the fluctuating immunoreactivity of cytostellin was due to periodic masking and unmasking of epitopes, possibly related to the phosphorylation state of the molecule. It is too early to speculate on a possible relation between p255 and cytostellin but we are currently endeavouring to identify the p255 associated proteins, if any. Preliminary immunoprecipitation experiments using different antibodies indicated that p255 at least partially sediments with spliceosome components. As cytostellin is associated with six proteins, forming a complex distinct from the spliceosome (Bregman et al., 1994), we should be able to rapidly clarify this issue.
The colocalization of p255 with SC-35 at different stages of the cell cycle, the identical sensitivity of both antigens to nucleases and heat shock, as well as the phosphorylation-dependent nature of the CC-3-epitope, raise the possibility that p255 might be related to the SR family of proteins, to which SC-35 belongs (Fu and Maniatis, 1992). SR proteins are essential splicing factors sharing structural features including an RNA binding domain and a serine/arginine-rich domain that is responsible for their targeting to nuclear speckles (Birney et al., 1993; Zahler et al., 1992). Each member of the family is able to substitute for any other to complement splicing-deficient extracts (Zahler et al., 1992). SR proteins are recognized by a monoclonal antibody (mAb 104) specific to a shared phosphorylated epitope (Roth et al., 1990, 1991). Their apparent sizes on SDS-PAGE (30 to 75 kDa) exclude any confusion with p255, even though high $M_r$ mAb 104-immunoreactive proteins have been described in Drosophila Kc cells (180 kDa) and in Xenopus oocytes (100-150 kDa). To our knowledge, no proteins larger than 200 kDa have been shown to react with mAb 104 in mammalian cells. In summary, we have identified a nuclear matrix phosphoprotein whose association with the nuclear structures is distinct from that of the snRNP components but very similar to that of the non-snRNP splicing factor SC-35. Studies are under way to characterize p255 and its epitope, and to explore the possibility that p255 is involved in spliceosome assembly or attachment and/or in the process of splicing.

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