The B1C8 protein is in the dense assemblies of the nuclear matrix and relocates to the spindle and pericentriolar filaments at mitosis

(nuclear structure/cell architecture/mitotic spindle)

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ABSTRACT The B1C8 monoclonal antibody detects a 180-kDa nuclear matrix-specific protein. The protein is a component of the dense, metabolically active bodies or assemblies revealed by resinless section electron microscopy of the nuclear matrix. These assemblies are scattered through the nuclear interior, enmeshed in a complex network of 11-nm filaments. Resinless section electron microscopy of immunogold-stained nuclear matrix preparations shows B1C8 located in many but apparently not all the assemblies. In this regard, the B1C8 antigen resembles previously studied nuclear matrix proteins such as the H1B2 protein. The speckled pattern of nuclear immunofluorescence by B1C8 reflects this labeling of the dense assemblies in the nuclear matrix. Somewhat unusual is the faint staining of cytoplasmic microtubules by B1C8, which appears to be due to a weakly cross-reacting protein. During cell division, the B1C8 antigen redistributed drastically, showing the dispersion of nuclear matrix assemblies at mitosis. Speckles of B1C8 fluorescence first coalesced at prophase within the nuclear interior and then scattered into numerous cytoplasmic speckles by prometaphase. At metaphase, the B1C8 speckled cytoplasmic staining had become even more widely distributed and finely grained. Also, intense labeling appeared at the mitotic pole and on the spindle fibers themselves. The reassembly of B1C8 antigens into larger cytoplasmic speckles began at anaphase and finally, at telophase, most B1C8 labeling redistributed into speckles in the re-forming nuclei.

There is increasing evidence that most, and probably all, nuclear macromolecular metabolism is closely associated with the nuclear matrix. More recent evidence indicates that nuclear events are highly localized into metabolically active centers. DNA synthesis takes place in replication sites (1–3), heterogeneous nuclear RNA (hnRNA) processing, and RNA transport in spliceosomes that process hnRNA (4–6), while nucleoli have long been known as dense bodies that synthesize and process rRNA (7).

We have previously shown that the nuclear matrix is composed of a profuse network of “core filaments” that enmesh many dense bodies of diverse sizes and morphologies (8–10). Conventional electron micrographs of the nuclei show mostly the dense chromatin. Removal of the chromatin reveals an extensive nonchromatin nuclear structure consisting of filaments and dense bodies. While visible in conventional micrographs, only resinless section electron microscopy shows the filaments and dense bodies with great clarity in three dimensions. Immunolocalization with monoclonal antibodies shows the dense bodies to be of complex protein composition and to be the principal sites of nuclear metabolism. Because of their various types, complex composition and variety of functions, we suggest that these dense bodies be termed “assemblies” and will so refer to them here.

Electropherograms show >100 nuclear matrix proteins comprising a unique set found nowhere else in the cell (8). Some nuclear matrix proteins are specific to an individual cell type but many occur in most cell types and appear to be universal (11–13). Having created a library of monoclonal antibodies to nuclear matrix proteins, we are using these to systematically characterize some of the more interesting nuclear matrix proteins. So far, most of these antibodies detect proteins concentrated in the nuclear matrix assemblies and therefore give a speckled immunofluorescence. This paper describes one of these newly characterized nuclear matrix proteins, the B1C8 antigen.

MATERIALS AND METHODS

Cell Culture. MCF-7, a human breast adenocarcinoma cell line (ATCC HTB22), and CaSki, a human cervical carcinoma cell line (ATCC CRL 1550), were maintained in Dulbecco’s modified Eagle’s medium supplemented with 10% fetal bovine serum. The cells were kept in a 37°C incubator with 7% CO2/93% air. HeLa cells (ATCC CCL2) were grown in suspension in minimal essential medium supplemented with 7% horse serum. CaSki cells were synchronized with 4 mM thymidine for 24 hr and released for 16.5 hr for studies of mitosis.

Cell Extraction. The cell fractionation technique has been published (8, 9, 14) and was used here with minor modifications. The cells were washed twice with cold phosphate-buffered saline (PBS) and extracted in cytoskeletal buffer (10 mM Pipes, pH 6.8/100 mM NaCl/300 mM sucrose/3 mM MgCl2/1 mM EGTA/1.2 mM phenylmethylsulfonyl fluoride/2 mM vanadylriboside complex) containing 0.5% Triton X-100 for 7 min at 4°C to remove the soluble proteins. Cytoskeletal proteins were removed in RSB (10 mM Tris-HCl, pH 7.4/10 mM NaCl/3 mM MgCl2/1.2 mM phenylmethylsulfonyl fluoride/2 mM vanadylriboside complex) with 1% Tween 40 and 0.5% sodium deoxycholate at 4°C for 5 min with brief Vortex mixing. Chromatin was removed by double digestion with RNase-free DNase I in digestion buffer (10 mM Pipes, pH 6.8/50 mM NaCl/300 mM sucrose/3 mM MgCl2/1 mM EGTA/1.2 mM phenylmethylsulfonyl fluoride/2 mM vanadylriboside complex) containing 0.5% Triton X-100, first with 5–10 units per 10^6 cells at 37°C for 20 min, and ammonium sulfate stock solution was added slowly to make a final concentration of 0.25 M and incubated at room temperature for 5 min. The sample was deposited by centrifugation and resuspended in digestion buffer; 40 units of pure DNase I per 10^6 cells was added with digestion at 37°C for 40 min. Again, 1 M ammonium sulfate was added to make

Abbreviation: hn, heterogeneous nuclear.

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a final concentration of 0.25 M. This process removed all the chromatin and left the nuclear matrix containing the ribonucleoprotein and nuclear matrix proteins. After centrifugation, both supernatant fractions containing chromatin were pooled together. The pellet was resuspended in cytoskeletal buffer, and a stock of 4 M NaCl was added slowly to make a final concentration of 2 M and incubated at room temperature for 10 min to reveal the core filaments. After centrifugation (3000 rpm in an IEC PR-J centrifuge), the pellet was resuspended in disassembly buffer [8 M urea/20 mM 2-(N-morpholino)-ethanesulfonic acid, pH 6.6/1 mM EGTA/0.1 mM MgCl₂/1 mM phenylmethylsulfonyl fluoride/1% 2-mercaptoethanol]. In immuno-microscopy experiments, chromatin was digested with restriction enzymes Hae III and Pst I (400 units/ml each) for 50 min at 37°C. In some experiments, microtubules were stabilized by adding taxol (5 μg/ml) to the cells and incubating for 5 min at 37°C prior to the extraction. Taxol (5 μg/ml) was also added to all the buffers during the room temperature extraction.

Monoclonal Antibody Production. BALB/c mice were injected at 2-week intervals with 100–400 μg of nuclear matrix proteins prepared from MCF-7 cells with Ribi adjuvant system (Ribi Immunochem). A final booster injection was given without adjuvant 4 days before fusion. Splenocytes from immunized animals were fused with cells from the nonsecreting mouse myeloma line P3X63-Ag8.653. Selection for hybrid cells was done in 96-well plates in culture medium containing aminopterin, hypoxanthine, and thymidine. Clones of surviving cells were screened by ELISA and immunofluorescent staining of MCF-7 cells. The B1C8 hybridoma line was subcloned three times by limiting dilution. The antibody is an IgM.

Immunofluorescence Microscopy. Cells were grown on glass chamber slides. After extraction to different stages, cells were fixed in 3.7% formaldehyde for 20 min either at 4°C or at room temperature, washed with PBS, and blocked in 10% normal goat serum in PBS for 15 min. After incubation with the first antibody for 2 hr at 37°C, samples were washed with PBS and blocked with 3% bovine serum albumin in PBS for 30 min at room temperature. After incubation with rhodamine-conjugated goat anti-mouse immunoglobulins in PBS, samples were washed three times with PBS and mounted with Aqua-Mount (Lerner Laboratories, Pittsburgh). In the double-staining experiments, affinity-purified fluorescein-conjugated goat anti-mouse IgM was used as the second antibody for B1C8, and rhodamine-conjugated goat anti-mouse IgG was used as the second antibody for anti β-tubulin staining.

For microtubule disassembly experiments, Colcemid (0.2 μg/ml) was incubated with the cells for 2.5 hr at 37°C before extraction and fixation.

Gel Electrophoresis and Immunoblotting. Proteins in cell fractions were concentrated by using a Centricon 10 (Amicon). The concentrated proteins from ~4 × 10⁸ cells were separated by SDS/PAGE (15) in 7.5% minigels run in duplicate. The gel was stained with Coomassie brilliant blue R250 and the other gel was transferred to nitrocellulose at 150 mA overnight and then at 600 mA for 1 hr. Tween 20 was not used in blot washing buffers because its use resulted in high backgrounds. The blot was washed once in TBS (25 mM Tris-HCl, pH 8.0/137 mM NaCl/2.5 mM KCl) and blocked in 1% (wt/vol) dry milk (in PBS) for 2 hr at 37°C and then in 10% fetal calf serum at 37°C for 1 hr. After washing with TBS plus 0.5% bovine serum albumin (BSA) three times at room temperature and rinsing once with PBS, B1C8 culture supernatant (1:10) was added and incubated at room temperature with gentle shaking for 2 hr. The blot was washed again with TBS plus 0.5% BSA three times and blocked with 1% dry milk in PBS at room temperature for 30–60 min with gentle shaking. After washing again with TBS plus 0.5% BSA three times and rinsing once with PBS, alkaline phosphatase-conjugated goat anti-mouse IgG and IgM was added for 1 hr at room temperature. The blot was washed with TBS/0.5% BSA at least six times. After rinsing with alkaline phosphatase buffer (100 mM Tris-HCl, pH 9.5/100 mM NaCl/5 mM MgCl₂), the alkaline phosphatase signal was detected by adding nitroblue tetrazolium/bromochlorindolyl phosphate substrate and was stopped by adding 20 mM Tris-HCl (pH 8.0) and 5 mM EDTA.

Electron Microscopy. High-resolution immunolocalization of B1C8 was done by resonance section electron microscopy as described (16).

RESULTS

We selected the B1C8 monoclonal antibody from an anti-nuclear matrix antibody library because of its bright, speckled staining of the interphase nucleus and its interesting behavior at mitosis. The library was constructed by spleen fusion after immunization of a mouse with the purified nuclear matrix proteins of the MCF-7 line of human breast carcinoma cells. Hybridoma clones were screened first by ELISA and then by indirect immunofluorescent staining of MCF-7 cells.

Localization of the 180-kDa B1C8 Polypeptide in the Nuclear Matrix. We separated MCF-7 cells into soluble, cytoskeletal, and nuclear fractions (9). Nuclei were further fractionated to remove chromatin and then washed with 2 M NaCl, leaving the nuclear matrix–intermediate filament scaffold (9). Proteins from each fraction equivalent to equal numbers of cells were analyzed by electrophoresis and then visualized by protein staining or Western blotting. The B1C8 monoclonal antibody clearly detected a protein band at 180 kDa located principally in the nuclear matrix fraction (Fig. 1). A small amount of a corresponding 180-kDa band appeared in the chromatin fraction. This latter band could be associated with chromatin but likely represents a small amount of B1C8 eluted from the matrix by the 0.25 M ammonium sulfate used to remove chromatin after DNase I digestion. The B1C8 protein appears in every human cell type examined so far and is probably universal (data not shown).

Spatial Distribution of B1C8 Antigen in Interphase Nuclei. Immunofluorescent staining with the B1C8 antibody labeled bright speckles throughout the nuclei except for exclusion from the nucleoli (Fig. 2A). Similar speckles are often seen

![Fig. 1. Western blot of B1C8 in the nuclear matrix. CaSkii cells were partitioned into the soluble, cytoskeletal, chromatin, and nuclear matrix fractions as described (9). Protein of each fraction from equal numbers of cells was analyzed by SDS gel electrophoresis, transferred to nitrocellulose membrane, and probed with the B1C8 antibody. The detected antigen was a 180-kDa protein in the nuclear matrix fraction (arrowhead), the only lane shown here. No reaction was detected in the soluble and cytoskeleton fractions and a barely detectable band was present in the chromatin fraction. Numbers on right are kDa.]
movements at mitosis (18). The unique behavior of the B1C8 antigen during mitosis is shown in Fig. 4.

When chromosomes began to condense at prophase (Fig. 4 A and B), the speckled fluorescence of B1C8 appeared to concentrate and fuse into a compact mass at the nuclear center. By prometaphase (Fig. 4 C and D), the fluorescent central mass had begun to disperse, with bright speckles appearing for the first time in the peripheral cytoplasm. The structures detected as speckles are bound to the cytoskeleton; they are not removed by the 0.5% Triton X-100 extraction that removes soluble proteins.

At metaphase, with chromosomes aligned at the metaphase plate, the B1C8 antigen was, in part, finely distributed throughout the cytoplasm while a large amount was on the mitotic spindle with the highest concentration at the spindle poles (Fig. 4 E and F). At anaphase (Fig. 4 G and H), when the daughter chromosomes began to separate, some B1C8 staining remained at the spindle poles while the finely divided cytoplasmic staining had coalesced into larger speckles similar in size to the interphase nuclear speckles. These larger speckles appeared to migrate to the region around the unfolding chromosomes that would become the nuclei of the daughter cells.

Reassembly of daughter nuclei was well advanced by late telophase cytokinesis as shown in Fig. 4 I and J. The B1C8 antigen had largely returned to the nucleus, appearing as bright speckles and some diffuse fluorescence. However, considerable staining remained in the cytoplasm and midbody, much of which would migrate to the nuclei by interphase.

The metaphase labeling with B1C8 is unusual in that both the spindle and pericentriolar region bind the antibody. We examined the labeling in metaphase by using the much higher-resolution immunogold-stained resinless sections as shown in Fig. 5. The B1C8 monoclonal antibody decorated the dense web of pericentriolar filaments, which appear as the pericentriolar "cloud" in conventional electron micrographs, as shown in Fig. 5A. The labeling of spindle microtubules (Fig. 5B) showed the antibody decorating small masses on the spindle microtubules. It is unknown whether these are related to the B1C8-labeled masses of the interphase microtubules.

**B1C8 Cross-Reacts with a Microtubule-Associated Protein.** In contrast to most anti-nuclear matrix antibodies, which stain only the nucleus, B1C8 also stained the cytoskeleton, albeit weakly (Fig. 6A). Double staining with anti-tubulin antibody showed coincidence with B1C8 in the cytoplasm (Fig. 6B). The staining was very sensitive to the temperature

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**Fig. 2.** Immunofluorescence staining of CaSki cells with B1C8 antibody. (A) Triton X-100 extracted cells. Cells on coverslips were extracted with 0.5% Triton X-100 in the near physiological cytoskeleton buffer, fixed with 3.7% formaldehyde, and stained with B1C8 antibody and then with rhodamine-conjugated anti-mouse second antibody. (B) Salt extracted nuclear matrix preparations. The 0.5% Triton X-100-extracted cells from A were further processed. Chromatin was removed by DNase I digestion and 0.25 M ammonium sulfate extraction before a 2 M NaCl wash. The cells were then stained with antibody as in A.

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**Fig. 3.** Immunoelectron microscopy of the nuclear matrix stained with B1C8. CaSki cells in monolayer culture were processed as described in Fig. 2B to obtain the 2 M NaCl-extracted nuclear matrix. The cells were stained with the B1C8 antibody and then with a 10-nm gold bead-conjugated anti-mouse antibody. After fixation with glutaraldehyde, the cells were prepared for resinless section microscopy as described in Materials and Methods and elsewhere (16). L, nuclear lamina; DA, dense assemblies; CF, core filaments; IF, intermediate filaments outside the nuclear lamina.
of extraction; staining by B1C8 disappeared completely when cells were extracted in the cold. Both the pattern and temperature dependence of cytoplasmic staining suggested that the B1C8 monoclonal antibody was labeling microtubules. Disrupting microtubules by prior treatment with Colcemid abolished the cytoplasmic staining of both tubulin (Fig. 6D) and B1C8 (Fig. 6C) with no effect on nuclear staining.

The epitope binding B1C8 on the microtubules is not tubulin but apparently a microtubule-associated protein. The immunogold-stained resinless section electron microscopy in Fig. 6E shows that the antibody did not decorate the microtubules directly but on small associated masses. However, this protein is apparently distinct from the B1C8 nuclear antigen since it is not detected in Western blots of preparations highly enriched in microtubule proteins (data not shown).

**DISCUSSION**

B1C8 is a member of the growing family of anti-nuclear matrix antibodies. Most of these stain nuclei with a speckled pattern, indicating that their antigens are localized in the dense assemblies scattered throughout the filament web of the matrix. We propose that nuclear metabolic functions appear to be concentrated in these dense assemblies.

We will show elsewhere that the B1C8 antigen is associated with the hnRNA splicing machinery and that the antibody immunoprecipitates splicing complexes from nuclear extracts. Thus, the coarsely speckled immunofluorescence staining by B1C8 apparently reflects the selective staining of

**FIG. 5.** Immunoelectron microscopy of B1C8 staining of the metaphase cytoskeleton. Synchronized CaSki cells grown in monolayers were harvested at mitosis. Cells were extracted with Triton X-100 as described in Fig. 2A and stained with B1C8 and a 10-nm gold bead-conjugated second antibody, and resinless sections for electron microscopy were prepared. (A) Pericentriolar filaments. Ct, centriole. (B) Spindle microtubule bundle and attached chromosome. Chr, chromosome; Mt, microtubules.

**FIG. 6.** B1C8 weak staining of cytoplasmic microtubules. CaSki cells on coverslips were extracted at room temperature in cytoskeletal buffer containing 0.5% Triton X-100 and taxol (5 µg/ml) for microtubule stabilization. (A) B1C8 staining. (B) Anti-tubulin staining. (C) B1C8 staining after treatment with Colcemid (0.2 µg/ml). (D) Anti-tubulin staining after treatment with Colcemid (0.2 µg/ml). (E) Resinless section electron microscopy of a cytoplasmic microtubule in a CaSki cell treated and stained as in A but processed for electron microscopy. Mt, microtubule.
splicesomes. As shown by electron microscopy (Fig. 3), B1C8 decorates many of the dense assemblies of the nuclear matrix so that these dense assemblies must correspond to the speckles of immunofluorescence. This agrees with previous findings using the H1B2 anti-nuclear matrix antibody (19) and with FA-12 (20), an anti-hn ribonucleoprotein antibody, which give coarse speckled fluorescence and also selectively stain the large, electron-dense bodies.

In contrast to other anti-nuclear matrix antibodies, B1C8 shows some staining of filaments in the cytoplasm when cells are prepared so as to preserve the microtubules. Disrupting the microtubules by cold extraction or by treatment with Colcemid destroys the cytoplasmic staining. The immuno-gold-stained resinless section electron microscopy in Fig. 6E shows the stained antigen is on small masses associated with the microtubules. This is not completely unprecedented. Kallajoki et al. (21) have reported the existence of a microtubule-associated protein that is also a nuclear protein. A nuclear form of the microtubule-associated protein Tau has also been found (22). However, here the cytoplasmic antigen appears to be different from the nuclear B1C8 protein. The antibody failed to stain Western blots of partially purified microtubule proteins even when these represented 20 times the number of cells per gel lane compared to the Western blot of nuclear matrix protein shown in Fig. 1 (data not shown).

At mitosis, the dense assemblies containing B1C8 apparently disperse since no corresponding structures can be seen in resinless sections of mitotic cells. At metaphase, B1C8 antigen associates with pericentriolar filaments at the spindle pole and with the spindle microtubules themselves. It is possible that some of the spindle staining is from the protein associated with the interphase microtubules, but there seems to be too much spindle fluorescence for that to be the sole source. Another nuclear matrix protein, the 238-kDa NuMA protein, relocates to the mitotic spindle at mitosis (23-26). Like the B1C8 protein, the distribution of NuMA is heavier near the spindle poles. At telophase, B1C8 begins to form a coarse speckled pattern, first in the cytoplasm and then in the newly forming nuclei.

The results here and elsewhere suggest that nuclear metabolism is concentrated in discrete, dense bodies or assemblies of complex composition and function. The assemblies labeled by B1C8 probably correspond to the splicesomes. A corresponding localization has long been known for nuclear rRNA, which is synthesized and processed in the nucleolus, another type of dense assembly. Also, some antibodies stain with a much finer-grained texture, which may correspond to the clustersomes described by Berezney (27).

We now have at least the beginning of the structure-function relation in the interphase nucleus. The filament web of the nuclear matrix positions dense assemblies that contain machinery for RNA metabolism, both hnRNA and rRNA. Very possibly, DNA replication is associated with smaller dense assemblies, a hypothesis that will be tested elsewhere.

There remains the question of where chromatin attaches to the nuclear matrix.

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