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SHORT NOTE

Identification and Definition of Nucleolus-Related Fibrillar Bodies in Micronucleated Cells

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Small nucleolus-related bodies which occur in the nucleoplasm of "micronuclei" lacking nucleolar organizers have been studied by immunofluorescence microscopy. These bodies stained specifically with three different antibodies directed against proteins that are normally associated with the dense fibrillar component of functional nucleoli, but not with antibodies specific for certain proteins of the granular component or the fibrillar centers. Our data show that, in the absence of rRNA genes, the various constituent proteins characteristic of the dense fibrillar component spontaneously assemble into spherical entities but that the subsequent fusion of these bodies into larger structures is prevented in these micronuclei. The similarity between these nucleolus-related bodies of micronuclei and the prenucleolar bodies characteristic of early stages of nucleologenesis during mitotic telophase is discussed. © 1988 Academic Press, Inc.

Micronucleated cells represent a useful experimental model system for studies of the function and expression of genomic subsets during interphase. In contrast to normal cells, where the total genome is contained in a single nucleus, micronucleated cells comprise numerous small "nuclei" each containing only a subfraction of the genome, often corresponding to an individual chromosome (e.g., [4, 13]). Micronuclei are transcriptionally active and export newly synthesized RNA molecules to the common cytoplasm, although apparently at a somewhat reduced rate [14]. Only those micronuclei which contain a chromosome carrying a nucleolus organizer region (NOR) are able to form normal nucleoli and hence to produce rRNAs [11, 14, 16]. In micronuclei lacking rRNA genes, spheroidal structures resembling certain nucleolar substructures ("nucleolus-like bodies") have been detected which differ from normal nucleoli by their smaller size and purely fibrillar nature, as demonstrated by electron microscopy [11, 16] (for review see [9]). A relationship between these nucleolus-like bodies and normal nucleoli was suggested by the reactivity of both structures with ammoniacal silver, Azur B, and a nucleolar human autoantibody of unknown specificity [11, 16].

Recently various antibodies to defined nucleolar constituents have been described and their reactivity with morphologically distinct nucleolar components

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determined (for review see [19, 25]; for further Refs. see Table 1). In the present immunocytochemical study we have used such antibodies in order to analyze the relationship between functional nucleoli and these nucleolus-related bodies in more detail.

Materials and Methods

Cells. Cells from kidney epithelia of *Xenopus laevis* (line A6) and rat kangaroo (PtK_2) as well as RVF-SMC cells (an established cell line derived from vascular smooth muscle of rat vein) were grown in monolayers as described [5–7]. Micronucleation was induced by addition of colchicine (0.2 μg/ml) to the cultures for 24–48 h [11, 16].

Antibodies. The following antibodies were used: (a) Rabbit antibodies against RNA polymerase I from rat hepatoma [21, 22], (b) human autoimmune serum (S18) against RNA polymerase I [17], (c) murine monoclonal antibody 72B9 against a M_r ~37,000 protein of the dense fibrillar component related to the U3 RNP complex [18], (d) human autoimmune serum (Scl C) recognizing a protein of the dense fibrillar component identified as fibrillarin and probably related to the antigen of monoclonal antibody 72B9 [18, 19], (e) murine monoclonal antibody No-114 against a structural protein (M_r ~180,000) of the dense fibrillar component of *Xenopus* nucleoli [23], (f) murine monoclonal antibody RS1-105 recognizing ribosomal protein S1 located in the granular component [12] and (g) murine monoclonal antibody No-185 against the nonribosomal protein NO38, a member of the nucleoplasmin family and homologous to the mammalian protein B23 (for review see [2]) which is localized in the granular component of *Xenopus* nucleoli [24]. The immunofluorescence staining patterns of interphase and mitotic cells obtained with these antibodies are summarized in Table 1.

Immunofluorescence microscopy. Cells grown on coverslips were fixed in methanol (−20°C) for 10 min, dipped into acetone (−20°C) for a few seconds, and air dried. Cells were then processed for double-label immunofluorescence. After a 10-min incubation with the first antibody, coverslips were washed for 10 min in PBS (137 m*M* NaCl, 2.7 m*M* KCl, 7 m*M* Na_2HPO_4, 1.5 m*M* KH_2HPO_4) and incubated for 10 min with secondary antibodies labeled with FITC (1:20; Dianova, Hamburg, FRG).

TABLE 1

Reactivities of the nucleolar antibodies used according to their immunofluorescence staining pattern in interphase and mitotic cells

Antibody	Interphase	Mitosis	Ref.
pol I, rabbit IgG to RNA polymerase I	FC[a]	NOR	[22]
S18, human IgG to RNA polymerase I	FC	NOR	[17]
72B9, monoclonal IgG to a M_r ~34,000 protein	DFC	Chromosome surfaces	[18]
Scl C, human antibodies to fibrillarin	DFC	Chromosome surfaces	[18, 19]
No-114, monoclonal IgG to a M_r ~180,000 protein	DFC	Diffuse in cytoplasm	[23]
RS1-105, monoclonal IgM to ribosomal protein S1	GC	Chromosome surfaces	[12]
No-185, monoclonal IgG to NO38, a protein related to both nucleoplasmin and nucleolar protein B23	GC	Chromosome surfaces	[24]

[a] Abbreviations used: FC, fibrillar center; DFC, dense fibrillar component; GC, granular component; NOR, nucleolar organizer region.

After being washed in PBS, cells were labeled with another nucleolar antibody and washed with PBS followed by Texas Red-conjugated antibodies (1 : 200; Dianova, Hamburg, FRG). For visualization of nuclei DNA was stained with diamidinophenylindole (DAPI). Cross-reactivity of secondary antibodies could be excluded in control experiments. Photographs were taken with a Zeiss microscope equipped with epifluorescence optics and appropiate filter set.

Results

Prolonged exposure of cell lines from different species (rat, rat kangaroo, and *X. laevis*) to colchicine produced micronucleated cells as shown by phase-contrast microscopy (Fig. 1 *a*) and staining with the DNA-specific dye DAPI (Figs. 1 *b–d*). Only some of the micronuclei contained large, fully developed, and clearly visible nucleoli (Fig. 1 *a*, arrows). When reacted with antibodies to RNA polymerase I which bind to the fibrillar centers (Table 1; see also [22]), these large nucleoli exhibited a strong fluorescence (Figs. 1 *a'–c'*). In contrast, micronuclei lacking typical nucleoli did not bind RNA polymerase I antibodies to a noticeable extent (Figs. 1 *a'–c'*). Fully developed nucleoli were also positive with antibodies binding to other nucleolar components. Thus, monoclonal antibodies 72B9 and No-114, both recognizing constitutents of the dense fibrillar component, immunostained the large nucleoli as did antibodies to ribosomal protein S1 (RS1–105) and the nonribosomal protein NO38 (No-185) which both bind to the granular component of nucleoli (Figs. 1 *a''–c''*; see also Table 1). With the antibodies directed against ribosomal protein S1 the cytoplasm of micronucleated cells fluoresced uniformly due to the abundant cytoplasmic ribosomes (Fig. 1 *c''*; see also [12]).

Antibodies specific for the dense fibrillar component stained, in addition to the fully developed nucleoli, small spheroidal entities scattered, in varying numbers, throughout the nucleoplasm of micronuclei lacking nucleoli (Figs. 1 *a''*, *b''*). These fluorescent nucleoplasmic dot-like structures were usually restricted to those micronuclei devoid of nucleoli and negative with antibodies to RNA polymerase I. When probed with antibodies to ribosomal protein S1 and the nonribosomal protein NO38, i.e., markers for the granular component, the nucleoplasmic entities did not fluoresce (Fig. 1 *c''*; results for antibody No-185 are not shown).

When double-label immunofluoresence microscopy was performed on micronucleated *Xenopus* A6 cells with human autoantibodies (Scl C) and monoclonal murine antibodies (No-114) which recognize different protein constituents of the dense fibrillar component, the resulting pattern of fluorescence was identical (Figs. 1 *d'*, *d''*), indicating that the nucleolus-related nucleoplasmic bodies included certain proteins similar to, if not identical with, those of the dense fibrillar component of functional nucleoli.

Discussion

Our present immunocytochemical data provide evidence that the nucleolus-related bodies occurring in micronuclei devoid of NORs share a number of proteins and structural properties with the dense fibrillar component of normal nucleoli (for a review of nucleolus architecture and nomenclature of its components see [8]). Three different antibodies reacting specifically with proteins of the

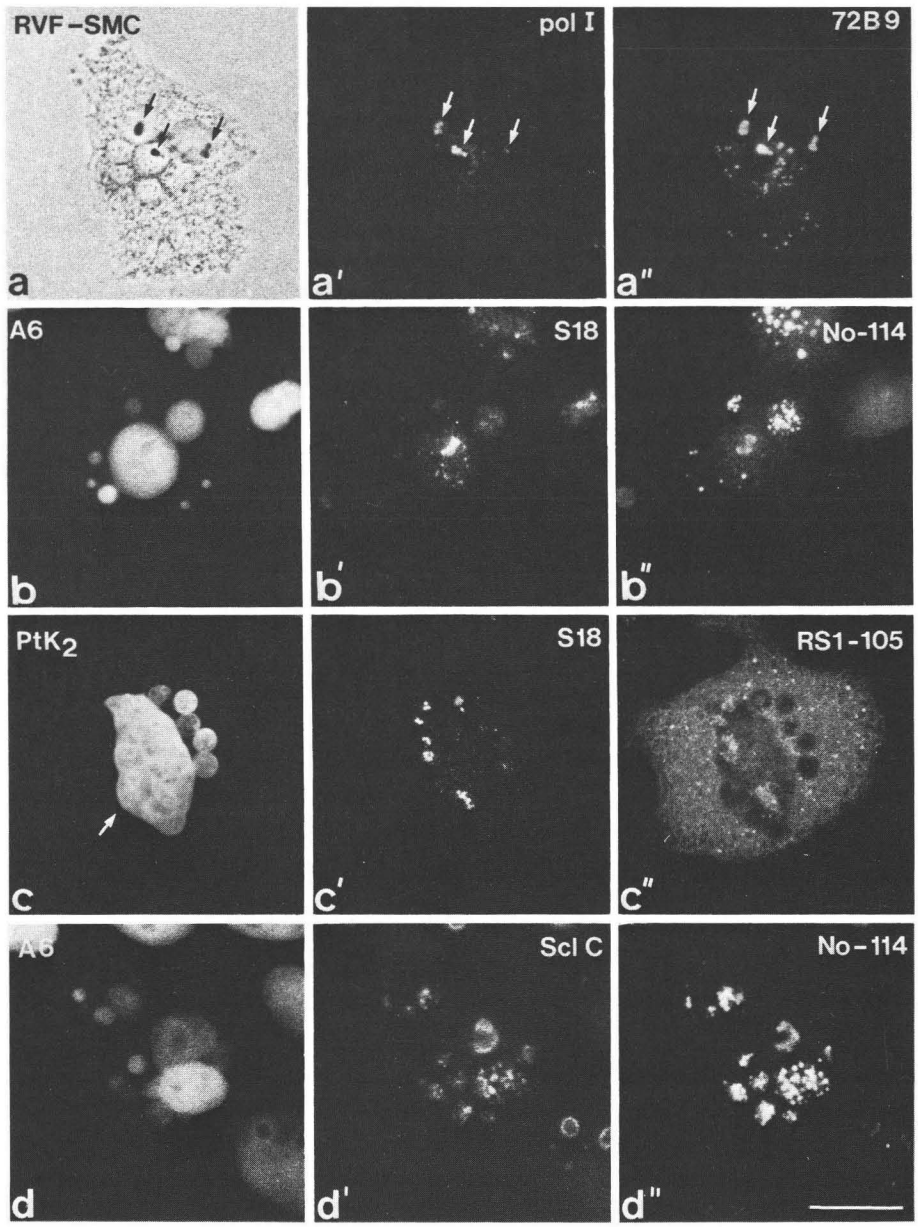

Fig. 1. Double-label immunofluorescence microscopy of micronucleated rat RVF–SMC (*a–a″*), *Xenopus laevis* A6 (*b–b″, d–d″*), and PtK_2 (*c–c″*) cells with antibodies to various nucleolar proteins (middle and right panels; the specificity of the antibodies is summarized in Table 1). The cells are also shown by phase-contrast microscopy (*a*) or after DNA staining with DAPI (*b–d*). Note that only some of the micronuclei contain nucleoli (indicated, e.g., by the arrows in *a*) which are stained with antibodies to RNA polymerase I (arrows in *a′*; one of the three micronuclei is slightly out of focus which reduces the intensity of nucleolar fluorescence) and antibodies reacting with the dense fibrillar component (72B9; arrows in *a″*). Not infrequently, relatively large nuclei containing several nucleoli occur next to a group of micronuclei within a single cell (e.g., arrow in *c*). For further details see text. Bar, 20 μm.

dense fibrillar component of normal nucleoli, i.e., human scleroderma autoantibody Scl C, murine monoclonal antibodies 72B9 and No-114 (see Table 1), also immunostain the nucleolus-related bodies scattered throughout the nucleoplasm of micronuclei which are unable to form definite nucleoli. In contrast, antibodies reacting with constituents of other nucleolar components such as the granular component or the fibrillar centers did not stain these nucleolus-related bodies. These results are fully compatible with earlier ultrastructural studies which revealed a purely fibrillar nature of these structures [11, 16].

Our present findings now enable us to relate these bodies in micronucleated cells to the "prenucleolar bodies" (PNBs) occurring during telophase of mitotic cells as the primary assembly structures during nucleologenesis (for review see [3]). Normally, PNBs are transient structures which rapidly fuse at the NORs to contribute to the reconstitution of nucleoli. However, when transcription of the rRNA genes is blocked by microinjection of antibodies to RNA polymerase I into dividing cells, coalescence of the multiple PNBs is prevented and they remain as separate entities in the nucleoplasm of the daughter cells [1]. Similar effects can also be produced by exposure of animal and plant cells to transcription inhibitors such as actinomycin D and cordycepin (e.g., [3, 20]). In our view, such experimentally prolonged PNB stages are comparable to the situation present in micronuclei lacking NORs. PNBs and nucleolus-related bodies of micronuclei may also correspond to the finely filamentous nuclear aggregate bodies that occur as "homogeneous nucleolar precursors" (HNPs) in early stages of embryogenesis before association with rDNA and the onset of rDNA transcription [28]. Obviously, the assembly of the multiple PNBs and HNPs into larger structures and functional nucleoli depends on the presence of rDNA and transcriptional activity (for a detailed discussion see also [1]).

Anucleolate *X. laevis* embryos represent another experimental system which has received much attention in studies of the effects of the deletion of rRNA genes on nucleolar organization. However, the situation found in the O-nu embryos seems to be more complex and is not fully comparable to that of micronuclei lacking nucleolus organizers. As described in detail by Hay and Gurdon [10], O-nu cells either contain multiple small "pseudonucleolar bodies" with morphological features similar to those of PNBs or larger "pseudonucleoli." Both structures have been shown to be positive with certain autoantibodies obtained from a scleroderma autoimmune serum [26]. This coexistence of multiple small nucleolus-related bodies and larger nucleolus-sized structures with common antigens in anucleolate cells of *Xenopus* is reminiscent of the situation observed after injection of limiting amounts of RNA polymerase I antibodies into mitotic cells [1]. Under such conditions of partial suppression of the transcriptional activity of the rRNA genes, fusion of some of the PNBs took place [1]. It should be remembered that anucleolate mutants of *X. laevis* have been shown to contain some small amounts of rDNA-related sequences [26, 27], although their transcription has not been demonstrated.

Our results do not support the view that PNBs represent a kind of preformed "miniature nucleoli" containing the whole spectrum of nucleolar proteins [15]. In

the PNBs [1] as well as the nucleolus-related bodies of micronucleated cells (this study) we could locate only those proteins which are specific for the dense fibrillar component of definite nucleoli. This finding substantiates our earlier notion that the material comprising the dense fibrillar component of functional nucleoli can spontaneously assemble into discrete spherical structures, independent of the transcriptional machinery [1]. Micronuclei might provide a valuable experimental system to further dissect the individual processes and analyze the factors involved in nucleolar assembly.

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