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Is DNA Topoisomerase IIβ a Nucleolar Protein?

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Abstract We have carried out immunofluorescence labelling of two human cell types, HeLa cells and peripheral blood lymphocytes, prepared by several different fixation/permeabilization protocols using a variety of antibodies against DNA Topoisomerase II (Topo II). We have found that the distribution of Topo IIα was overall similar during interphase and mitosis to that previously reported, regardless of antibody and of sample preparation. On the other hand, the interphase distribution of Topo IIβ was quite variable, depending both on the antibody and on the method used to prepare the sample. Our interpretation of the data is that, like Topo IIα, Topo IIβ is primarily a nucleoplasmic protein, but that unlike Topo IIα, small amounts are also associated with intranucleolar chromatin. © 1996 Wiley-Liss, Inc.

Key words: Topo IIα, Topo IIβ, interphase, mitosis, mitogenic stimulation, nucleoplasm, nucleolus, lymphocytes, HeLa, immunofluorescence

DNA topoisomerases catalyze the interconversion of different topological forms of DNA, with the type II enzymes (Topo II) passing one intact DNA duplex through a transient double-strand break in another. Genetic studies have shown that Topo II is essential for cell growth and is required for chromosome segregation in yeast, which has only a single type II topoisomerase [Poljak and Käs, 1995].

It is now known, however, that in mammalian cells there are two isotypes of Topo II, α and β, that are immunologically and pharmacologically distinct [Chung et al., 1989; Drake et al., 1989], and are encoded by separate, single-copy genes [Tsai-Pflugfelder et al., 1988; Tan et al., 1992]. Topo IIα appears to function in mitotic chromosome condensation and segregation, as well as playing a structural role throughout the cell cycle [Poljak and Käs, 1995]. Immunofluorescence labelling has shown that, as is consistent with its role, Topo IIα is distributed throughout the nucleoplasm during interphase and is associated with chromosomes during mitosis [Sumner, 1996, and references therein]. The precise function of Topo IIβ is unknown, and immunolocalization studies are contradictory. In one series of studies, antibodies against Topo IIβ were reported to stain only the nucleolus [Negri et al., 1992; Zini et al., 1992]. On the other hand, another report, using some of the same antibody preparations, identified both Topo II isotypes in the nucleoplasm as well as in the nucleolus [Petrov et al., 1993].

In our continuing studies of nuclear reorganization during lymphocyte mitogenic activation, we have been investigating the possible role of Topo II in the massive chromatin restructuring that takes place in this process [Roy et al., 1992; Daev et al., 1994]. For some of these studies, we obtained several antibodies against each Topo II isotype, as well as one antiserum that detects both isotypes, and have used them for immunofluorescence labelling of human lymphocytes during mitogenic activation, as well as of HeLa cells during the cell cycle. The results confirm a nucleoplasmic localization for Topo IIα but raise the question as to whether Topo IIβ is a nucleolar protein.

MATERIALS AND METHODS

Cell Culture

HeLa cells were cultured as previously described [Chaly and Chen, 1993], and were seeded onto coverslips one day before fixation.

Lymphocytes from peripheral blood of healthy donors were isolated on Ficoll gradients as previously described [Setterfield et al., 1983]. For resting cell samples, cells were immediately settled onto poly-L-lysine-coated coverslips and

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fixed, as previously described [Roy et al., 1992; Daev et al., 1994]. For stimulated cell samples, concanavalin A (Con A) was added at 10 μg/ml and the cultures were returned to the incubator for 48 h. Cells were then layered onto coverslips as above and fixed.

Fixation and Permeabilization

Several fixation/permeabilization protocols were used, as follows.

All steps were carried out at room temperature unless noted otherwise. Samples were washed in phosphate-buffered saline (PBS) after each step.

Method 1. This is the method we use routinely [Chaly et al., 1984; Roy et al., 1992; Daev et al., 1994]. Briefly, cells are fixed in freshly prepared 3% paraformaldehyde in PBS for 5 min and permeabilized in 0.2% Triton X-100 in PBS for 20 min.

Method 2. Cells were fixed in freshly prepared 4% paraformaldehyde in PBS for 1 min and permeabilized in 0.5% Triton X-100 for 5 min, as previously described [Petrov et al., 1993].

Method 3. Cells were fixed in freshly prepared 2% paraformaldehyde in PBS for 15 min and permeabilized at 4°C in 0.2% Triton X-100 in PBS for 20 min, as previously described [Spector and Smith, 1986].

Method 4. Cells were fixed and permeabilized in 100% acetone for 1 min, as previously described [Negri et al., 1992].

Immunofluorescence Labelling

For single immunolabelling, samples were incubated sequentially for 45 min each in PBS-diluted primary and secondary antibodies, counterstained in 4,6-diamidino-2-phenylindole (DAPI) at 0.5 μg/ml in PBS, and mounted in Vectashield (Vector Labs, Burlingame, CA). All secondary antibodies were obtained from Jackson Immunochemicals (West Grove, PA). For double labelling experiments, antibodies were also applied sequentially, as previously described [Daev et al., 1994].

Primary antibodies. Mouse monoclonal IgG antibodies 1F6 (1:5) and 8F8 (1:25), against Topo IIα and Topo IIβ, respectively, were provided by G. Astaldi Ricotti (Consiglio Nazionale Delle Ricerche, Pavia, Italia) [Negri et al., 1992]. Rabbit polyclonal anti-peptide serum affinity 22 (1:10) against Topo IIα, serum 20 (1:25) and serum 21 (1:10) against Topo IIβ, and affinity 29 (1:10) against both Topo IIα and β were provided by F. Drake (SmithKline-Beecham Pharmaceuticals, King of Prussia, PA) [Chung et al., 1989]. Mouse monoclonal anti-peptide IgGs 8D2 and 5A7, against Topo IIα and Topo IIβ, respectively, were provided by A. Kikuchi (Mitsubishi Kasei Institute of Life Sciences, Tokyo, Japan) [Kimura et al., 1994]. Anti-fibrillarin was a human autoimmune serum provided by R. Ochs (Scripps Institute, La Jolla, CA) [Ochs et al., 1985].

Secondary antibodies. Rabbit and human antisera were detected with fluorescein isothiocyanate (FITC)- or Cy 3-conjugated donkey anti-rabbit or -human IgG (H + L) (1:200). Mouse monoclonals were detected with FITC-conjugated goat anti-mouse IgG (H + L) (1:200). In control samples processed without primary antibody, cells were barely detectable against the background (not shown).

Microscopy

Samples were examined with a Zeiss (Thornwood, NY) Photomicroscope III equipped with a Plan-Neofluar X63, N.A. 1.25, objective, or with a Zeiss Axiophot equipped with a Plan-Neofluar X100, N.A. 1.25 objective, and photographed onto Ilford XP2-400 film.

RESULTS AND DISCUSSION

The fixation/permeabilization procedures tested were: a protocol used routinely in our laboratory (Method 1); the protocol used by Petrov et al. [1993] for localization of the Topo II isotypes (Method 2); a protocol described in Spector and Smith [1986] that has been used extensively to investigate nuclear antigen localization (Method 3); and the protocol used by Negri et al. [1992] in their initial anti-Topo II characterizations (Method 4).

Our observations in this study were carried out on two human cell types. Peripheral blood lymphocytes are an example of a primary cell culture system, and the resting cells represent an in vivo example of growth-arrested cells. Furthermore, the response of these cells to mitogen has been extensively characterized at the morphological, biochemical, and molecular levels [e.g., Setterfield et al., 1983; Crabtree, 1989; Brown et al., 1990]. On the other hand, HeLa cells were chosen because they also are of human origin, represent a permanent cell line, and, moreover, are the cell type used to localize the Topo II isotypes in the studies of Negri et al. [1992] and Zini et al. [1992].

Labelling With Anti-Topo IIα Antibodies

The anti-Topo IIα antibodies we have chosen are as follows: Affinity 22 (Aff 22), a rabbit polyclonal anti-peptide serum [Chung et al., 1989]; mouse monoclonal IgG 1F6, produced against purified Topo II [Negri et al., 1992]; and an anti-peptide mouse monoclonal IgG, 8D2 [Kimura et al., 1994].

With Aff 22, Methods 1, 2, and 4 gave similar nucleoplasmic and non-nucleolar labelling of HeLa interphase nuclei (Fig. 1a–c'). However, labelling was finely granular after Method 1 (Fig. 1a, a'), slightly coarser after Method 2 (Fig. 1b, b'), and sparse and even more granular after Method 4 (Fig. 1c, c'). A similar diffuse, non-nucleolar labelling of the nucleoplasm by Aff 22 was also observed in lymphocytes prepared by Method 1 [Chaly et al., 1996]. Using similar rabbit antisera and CHO cells prepared by Method 2, Petrov et al. [1993] detected Topo IIα by immunofluorescence and immunoelectron microscopy throughout the nucleoplasm, but also in the nucleoli. Using the same immunofluorescence procedures on HeLa cells, we have been unable to detect nucleolar labelling.

Labelling of HeLa with monoclonals 1F6 (Fig. 1d, d') and 8D2 (Fig. 1f, f') after Method 1 was similar to but less punctate than with Aff 22 in cells prepared by the same method.

After acetone fixation, i.e., Method 4, monoclonal 1F6 weakly labelled the nucleoplasm but strongly labelled the cytoplasm of HeLa cells (Fig. 1e, e'). In contrast, using another monoclonal anti-Topo IIα, 6G2, and Method 4 on HeLa, Negri et al. [1992] observed a finely punctate label throughout the nucleoplasm, exclusive of the nucleolar domain.

All the antibodies labelled mitotic chromosomes in samples prepared by all methods, though with slightly different patterns. An example of a prometaphase cell prepared by Method 1 and labelled with 8D2 is shown in Figure 1g, g'. This antibody appears to label the midline of each chromatid, with centromeres and, to a lesser extent, telomeres labelled more brightly. We have previously shown in HeLa cells and in human peripheral blood lymphocytes prepared by Method 1, however, that Aff 22 labels the entire body of the chromosomes diffusely and with equal intensity [Chaly et al., 1996]. A commercial monoclonal anti-Topo IIα has been reported to produce a labelling pattern of mitotic chromosomes similar to that in Figure 1g in a variety of cell types [Sumner, 1996]. This study also showed that the labelling pattern could vary considerably depending on the fixation and permeabilization procedures used. Sumner [1996] suggests that the different staining patterns of mitotic chromosomes with anti-Topo II antibodies reported in the literature may arise from differences in sample preparation.

Our study further shows, however, that different anti-Topo IIα antibodies may display characteristic differences in labelling pattern of mitotic chromosomes even in samples prepared in the same way. These differences in labelling clearly do not arise due to differences in antigen preservation or in antigen accessibility. Our results imply, therefore, that the different antibodies recognize subsets of Topo IIα, perhaps produced by post-translational modifications, which are distributed non-uniformly within the mitotic chromosome. Some combination of sample preparation and the particular antibody used may similarly explain the differences in interphase labelling patterns observed in the various studies described above.

Labelling With Anti-Topo IIβ Antibodies

The anti-Topo IIβ antibodies chosen were the following: Serum 20 and Serum 21, rabbit polyclonal anti-peptide sera [Chung et al., 1989]; mouse monoclonal anti-peptide antibody 5A7 [Kimura et al., 1994]; and mouse monoclonal 8F8 produced against isolated topoisomerase [Negri et al., 1992].

Labelling of HeLa interphase nuclei with Serum 20, Serum 21, and monoclonal 5A7 was almost exclusively diffusely nucleoplasmic after all four Methods (Fig. 2). All the antibodies also stained a few small spots within nucleoli that likely correspond to regions of intranucleolar DNA. These spots were observed most consistently with Serum 20 in cells prepared by Methods 1 (Fig. 2a, a'), 2 (Fig. 2b, b'), and 3 (Fig. 2c, c'). None of the antibodies labelled chromosomes in mitosis (e.g., Fig. 2i, i').

In an immunofluorescence study of Topo II organization in human peripheral blood lymphocytes using serum 20 and sample preparation Method 1, we also observed a diffuse nucleoplasmic labelling pattern in all of the cells throughout mitogenic activation [Chaly et al., 1996]. Furthermore, confocal microscopy of lymphocytes double-labelled with antibodies to the nucleolar antigen, fibrillarin, showed that serum 20 did not label nucleoli in resting lymphocytes. Also, in stimulated cells, serum 20 la-

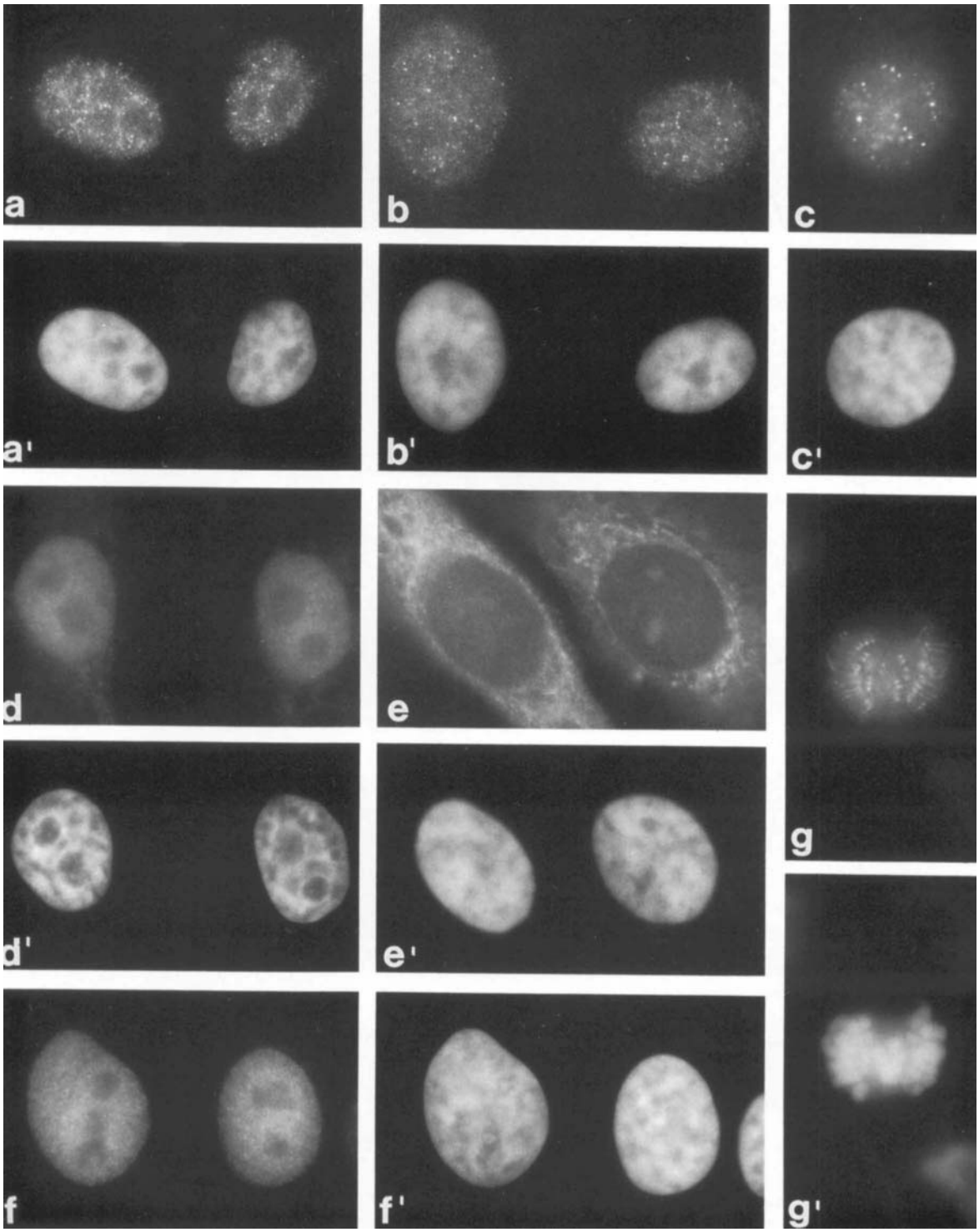

Fig. 1. Immunofluorescence staining of HeLa cells prepared by Method 1 (**a, a', d, d', f, f', g, g'**), Method 2 (**b, b'**), or Method 4 (**c, c', e, e'**). Cells were labelled with Aff 22 (a,b,c), monoclonal 1F6 (e), and 8D2 (f,g), and counterstained with DAPI (a'–g'). ×1,050.

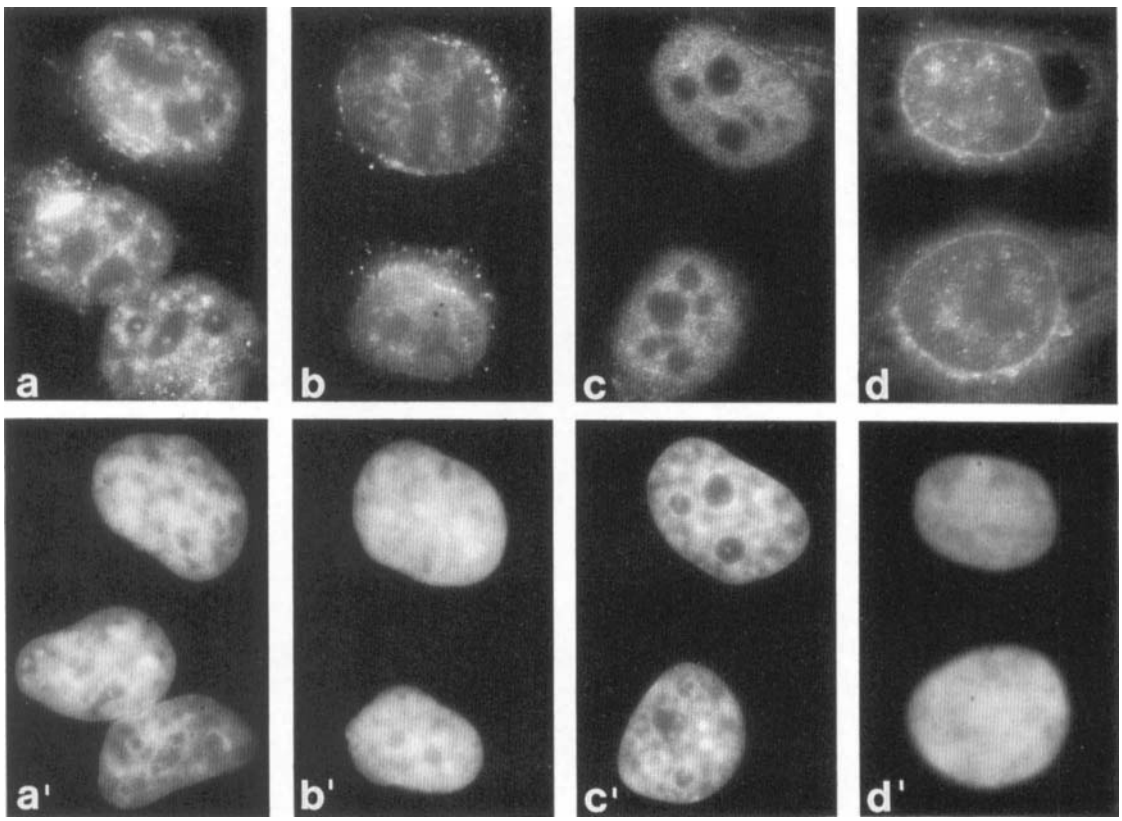

Fig. 2. Immunofluorescence staining of HeLa cells prepared by Method 1 (**a, a', e, e', h, h', i, i'**), Method 2 (**b, b', f, f'**), Method 3 (**c, c', g, g'**), or Method 4 (**d, d'**). Cells were labelled with Serum 20 (a,b,c,d), Serum 21 (e,f,g), or monoclonal 5A7 (h,i), and counterstained with DAPI (a'–i'). ×1,050.

belled a limited number of small spots within nucleoli (similar to those seen in Fig. 2) that coincided with, but were much smaller than, the regions labelled by anti-fibrillarin [Chaly et al., 1996].

However, both Negri et al. [1992] and Petrov et al. [1993], using a similar rabbit polyclonal antiserum and the acetone (Method 4) or Method 2 fixations, respectively, reported nucleolar and nucleoplasmic immunofluorescence labelling. Petrov et al. [1993] also detected Topo IIβ in both nuclear compartments by immunoelectron microscopy with the Serum 20 used in our study. Perhaps the same factors that influence the labelling pattern detected for anti-Topo IIα may also be affecting the labelling pattern produced by these anti-Topo IIβ antibodies.

In contrast, monoclonal 8F8 produced very intense labelling of HeLa interphase nucleoli by the two methods tested, Methods 1 (Fig. 3a, a') and 4 (Fig. 3e, e'). Using another similar monoclonal anti-Topo IIβ, and preparing HeLa cells by acetone fixation (Method 4), Negri et al. [1992] reported exclusively nucleolar labelling, which we also observe with this fixation method. However, in samples prepared by Method 1, we consistently detected additional labelling throughout the nucleoplasm (Fig. 3a, a'). In a recent study, Zweyer et al. [1995] report exclusively nucleolar labelling by 8F8 in methanol-fixed HL-60 cells. However, inspection of the micrographs clearly shows that 8F8 also gives diffuse nucleoplasmic label in their preparations.

At prophase in HeLa samples prepared by the two Methods, the nucleolar labelling became distorted and fragmented in parallel with chromosome condensation and nucleolar disruption (Fig. 3b, b', f, f'). At metaphase, monoclonal 8F8 labelling outlined the chromosomes against a diffuse labelling of the cytoplasm (Fig. 3c, c', g, g'). As chromosomes began to decondense in late telophase, 8F8 labelled several aggregates in each reforming nucleus and also diffusely labelled the nucleoplasmic regions. These aggregates closely resemble the pre-nucleolar bodies, containing nucleolar proteins such as fibrillarin, that have been shown to represent early stages

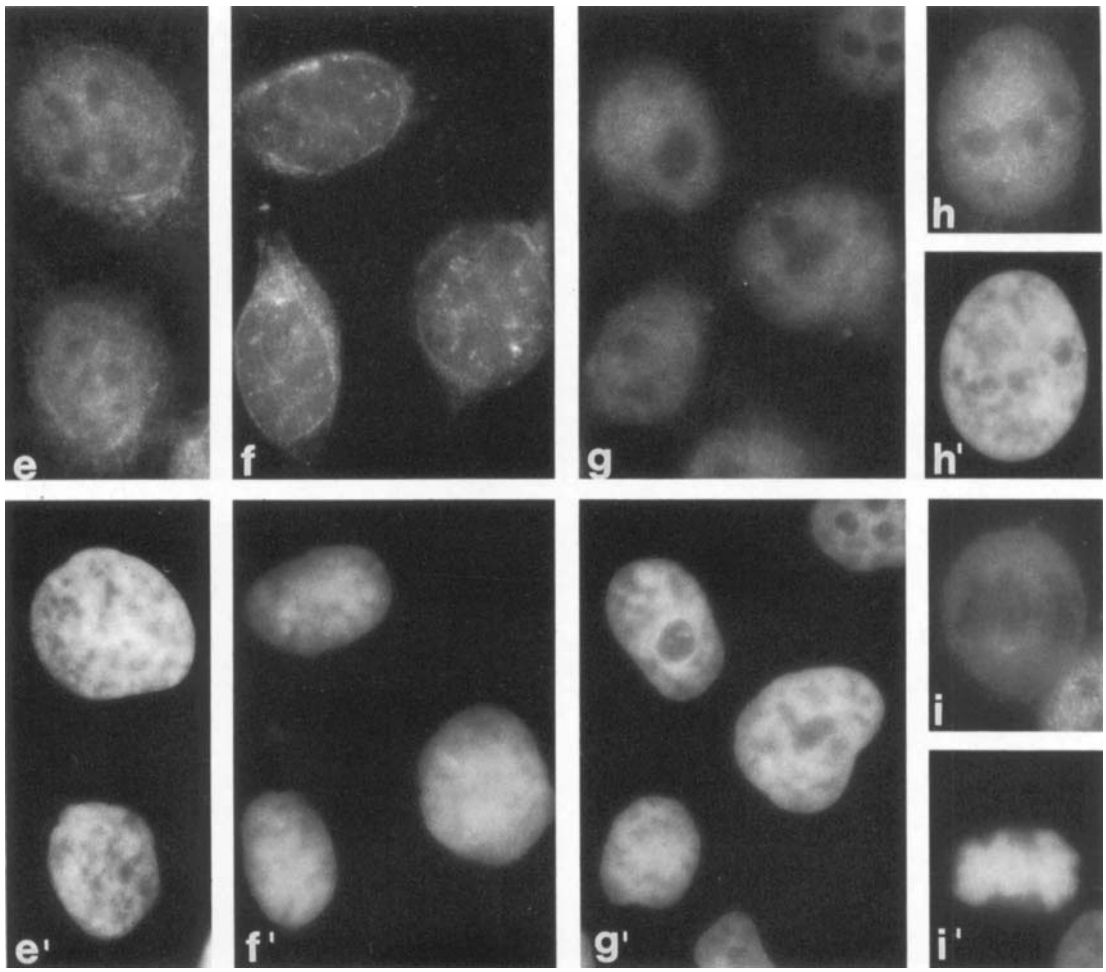

Fig. 2. (Continued.)

in post-mitotic nucleolar reassembly [Scheer and Weisenberger, 1994, and references therein].

Figure 4 shows the 8F8 staining pattern of resting (Fig. 4a, a′) and stimulated (Fig. 4b, b′, c, c′) peripheral blood lymphocytes prepared by Method 1. Only nucleoli were labelled in these cells: single, small ones in resting cells, and multiple, larger ones in stimulated cells. Mitotic cells were essentially unlabelled (Fig. 4c, c′).

The reasons for the discrepancy between the labelling patterns with monoclonal 8F8 and the patterns observed with the other anti-Topo IIβ antibodies are not obvious. All of the antibodies we have used have been clearly shown by various biochemical and immunochemical assays to detect the Topo IIβ isotype specifically. For discussion, see Interpretation and Conclusions below.

Immunolabelling With Antibody Affinity 29

Affinity 29 (Aff 29) is a rabbit polyclonal anti-peptide serum that recognizes both Topo II isotypes [Chung et al., 1989]. Areas of the nucleus that excluded DAPI staining identified the location of nucleoli in most samples.

Labelling of interphase HeLa cells with Aff 29 was highly variable (Fig. 5). All cells in all samples displayed diffuse nucleoplasmic label to varying extents. Diffuse labelling was prominent in samples prepared by Method 1 (Fig. 5a, a′, b, b′) and Method 3 (Fig. 5d, d′), sparser after Method 2 (Fig. 5c, c′), and greatly reduced after acetone (Method 4) fixation (Fig. 5e, e′). This is similar to the fixation-related differences in labelling of HeLa nuclei we observed with the Topo IIα-specific antiserum, Aff 22 (Fig. 1a,b,c). As well, mitotic chromosomes were clearly labelled by this antibody in all samples (e.g., Fig. 5f, f′), as would be expected for a serum with reactivity to Topo IIα.

In addition, however, nucleolar domains were also labelled to some extent in all samples.

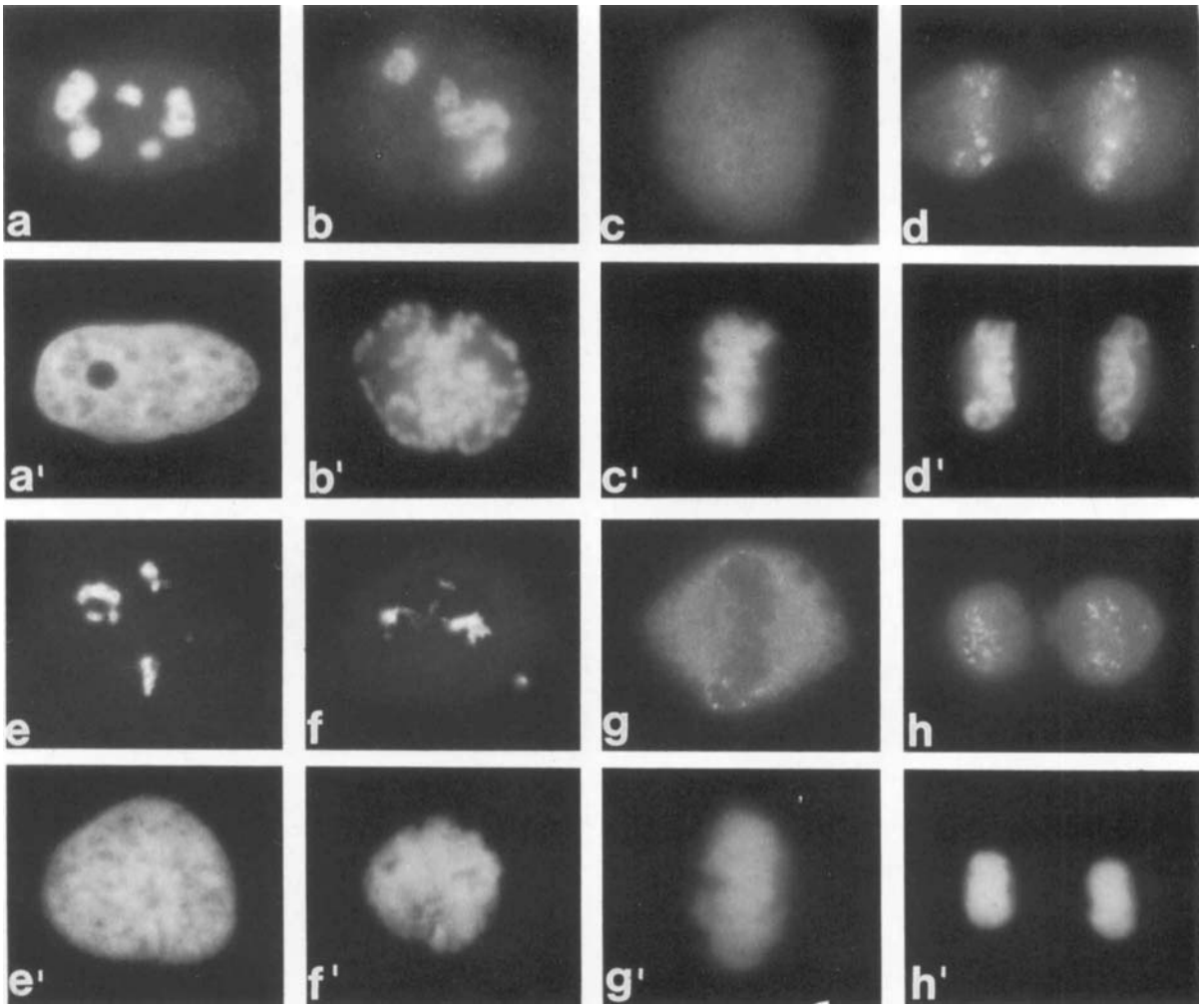

Fig. 3. Immunofluorescence staining of interphase (**a, a', e, e'**) and mitotic (**b, b', c, c', d, d', f, f', g, g', h, h'**) HeLa cells prepared by Method 1 (**a, a', b, b', c, c', d, d'**) or Method 4 (**e, e', f, f', g, g', h, h'**). Cells were labelled with monoclonal 8F8 (a–h) and counterstained with DAPI (a'–h'). ×1,050.

Nucleolar labelling was predominant in all cells in acetone-fixed (Method 4) samples (Fig. 5e), and very prominent in all cells of samples prepared by a brief (1 min) paraformaldehyde fixation (Method 2) (Fig. 5c). In samples fixed for 5 min in paraformaldehyde (Method 1), nucleoli in some nuclei were unlabelled (Fig. 5b), were brightly labelled (Fig. 5a), or were labelled with the same intensity as the nucleoplasm (Fig. 5a,b). Finally, in samples fixed in paraformaldehyde for 15 min (Method 3), nucleoli exhibited a few small spots, as with Serum 20 (Fig. 2a–c), or were diffusely labelled as intensely as the nucleoplasm (Fig. 5d).

In lymphocytes fixed by Method 1, the nuclei displayed a diffuse nucleoplasmic fluorescence in resting (Fig. 6a′, a″) as well as in stimulated (Fig. 6b′, b″, c′, c″) cells. Nucleoli are difficult to identify by DAPI staining in lymphocytes, and were detected by double-labelling with anti-fibrillarin (Fig. 6a–c). They were not labelled by Aff 29 (Fig. 6a′–c′). Mitotic chromosomes were uniformly labelled (Fig. 6c′, c″), as in HeLa cells.

Again, the explanation for the variability in staining with Aff 29 is not clear and is discussed below.

Interpretations and Conclusions

Our data indicate that Topo IIα is not a nucleolar protein, is distributed throughout the nucleoplasm, and is chromosome-associated during mitosis. This distribution is consistent with its proposed role as a structural protein of the interphase nuclear matrix and the mitotic chro-

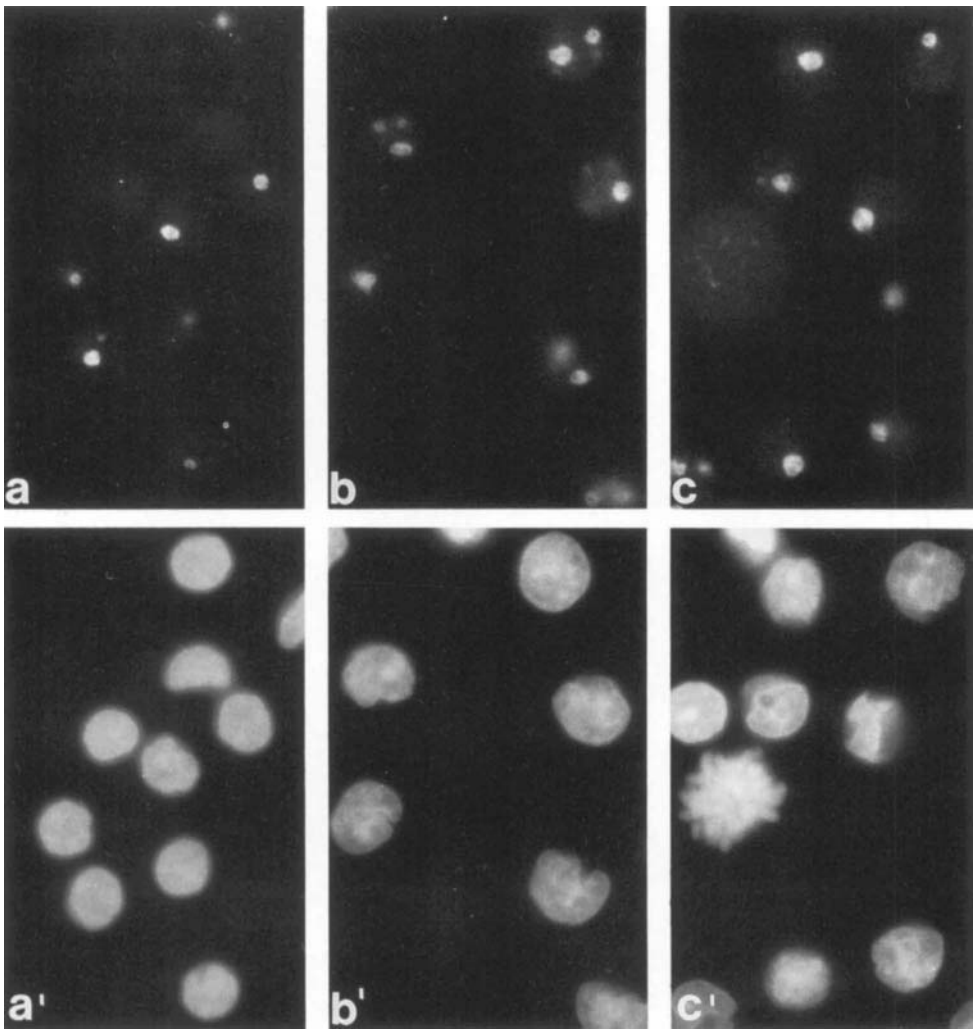

Fig. 4. Immunofluorescence staining of resting (**a, a'**) and stimulated (**b, b', c, c'**) peripheral blood lymphocytes prepared by Method 1. Cells were labelled with monoclonal 8F8 (a–c) and counterstained with DAPI (a'–c'). ×1,050.

mosome scaffold, and with its known function in chromosome condensation and segregation [see Poljak and Käs, 1995, for a recent review].

On the other hand, the data concerning the localization of Topo IIβ fall into three classes.

1. *Affinity 29.* In lymphocytes, Aff 29 did not label nucleoli, and showed a nucleoplasmic staining pattern indistinguishable from that with Serum 20 by conventional fluorescence microscopy [Chaly et al., 1996]. In HeLa cells, variable extents of nucleolar labelling and of nucleoplasmic labelling were observed with Aff 29. We think it is significant that the extent of nucleolar labelling decreased as the quality of nuclear preservation was improved by using paraformaldehyde and by extending the duration of the paraformaldehyde fixation. Furthermore, the extent of nucleolar labelling seemed overall to be inversely correlated with the extent of nucleoplasmic labelling. These observations suggest that the nucleolar labelling is largely due to redistribution of the Topo II isotypes during sample preparation.
2. *Monoclonal 8F8.* Unlike any of the other antibodies tested, 8F8 labelled nucleoli brightly regardless of cell type and sample preparation, and showed diffuse nucleoplasmic staining only in samples fixed with paraformaldehyde. We consider the nucleoplasmic labelling to be Topo IIβ-specific staining, analogous to the nucleoplasmic labelling produced by the other Topo IIβ-

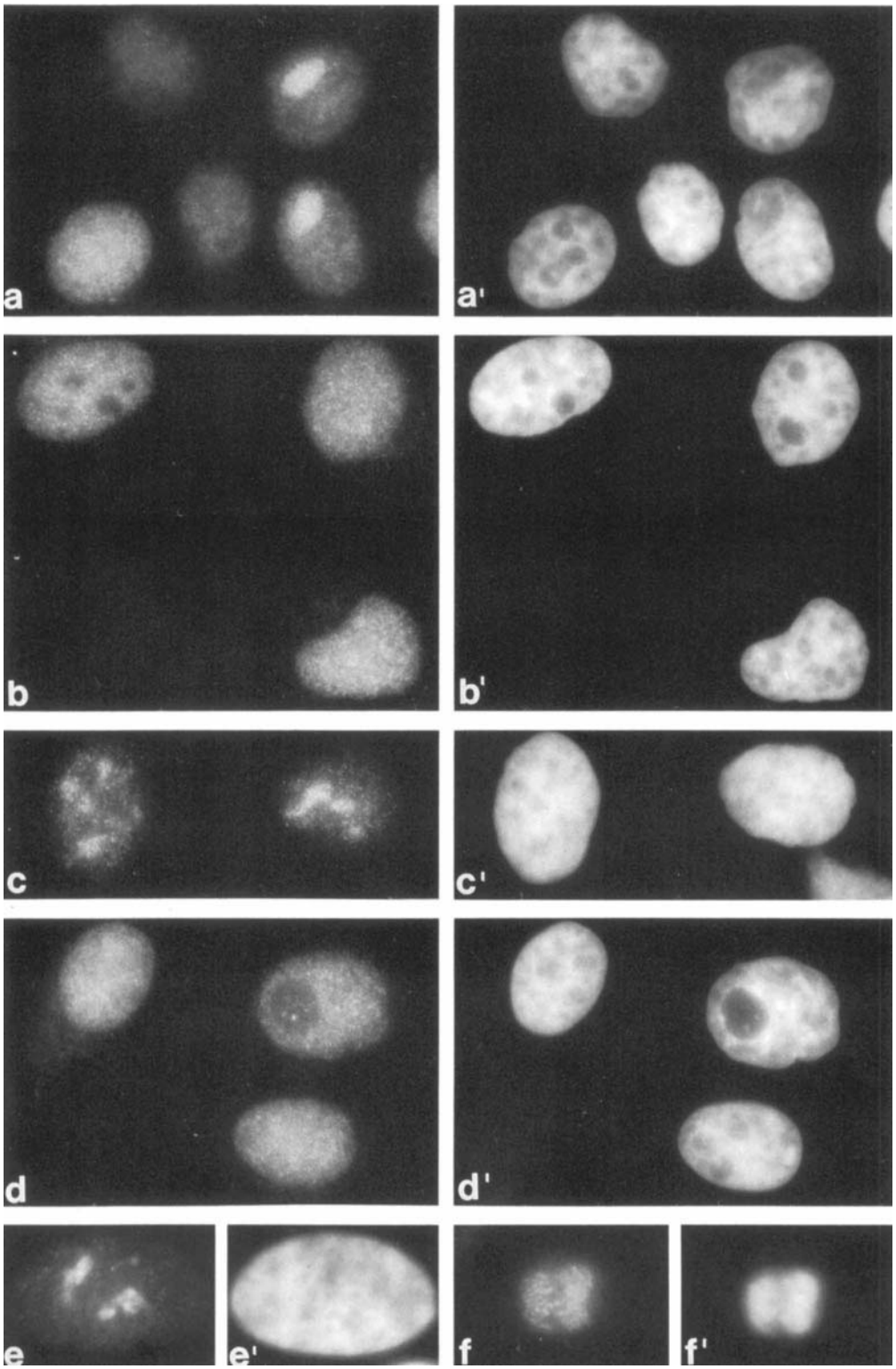

Fig. 5. Immunofluorescence staining of HeLa prepared by Method 1 (**a, a', b, b'**), Method 2 (**c, c'**), Method 3 (**d, d'**) or Method 4 (**e, e', f, f'**). Cells were labelled with Aff 29 (a–f) and counterstained with DAPI (a'–f'). ×1,050.

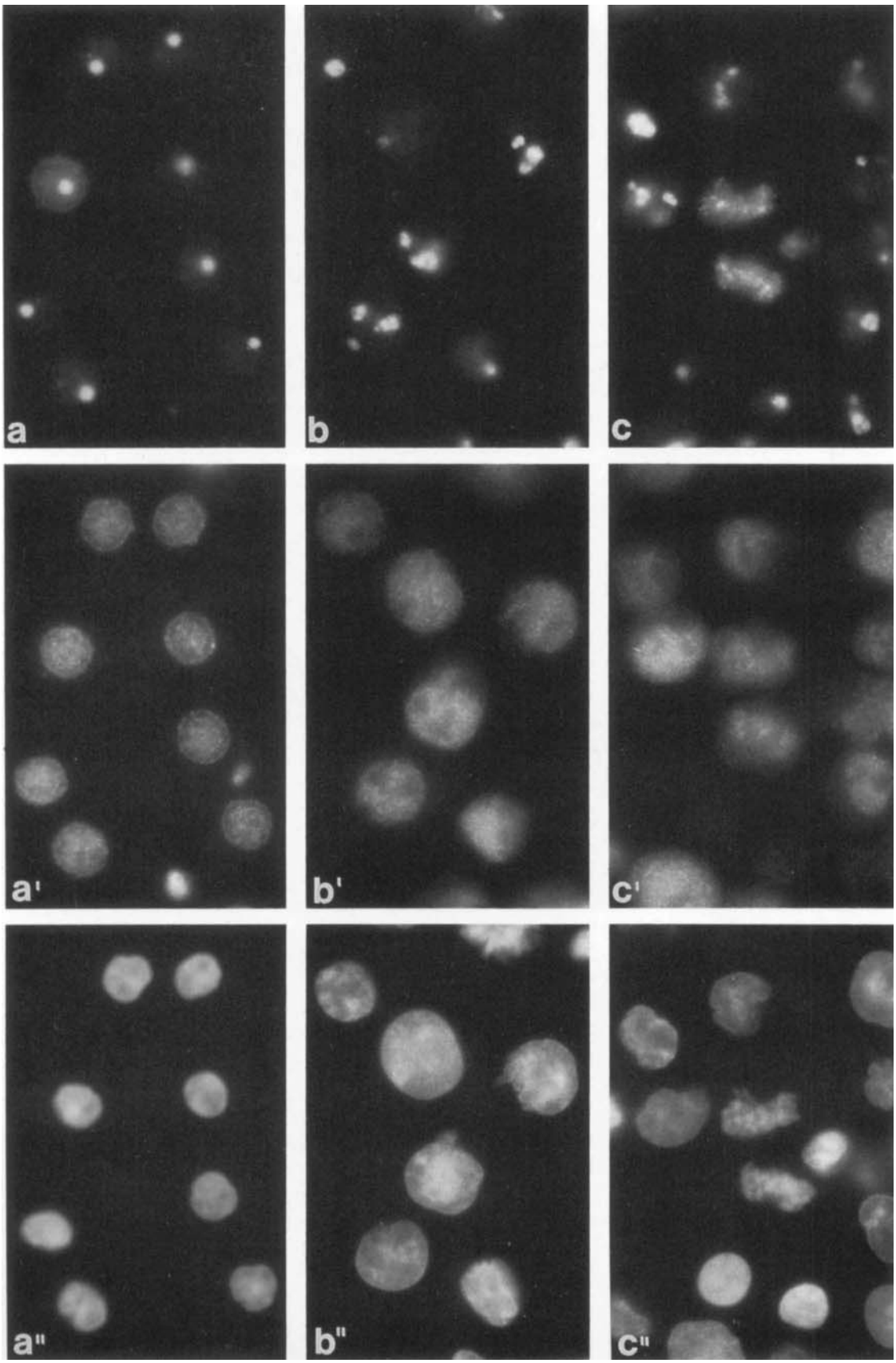

Fig. 6. Double immunofluorescence staining of resting (a–a″) and stimulated (**b–b″, c–c″**) peripheral blood lymphocytes prepared by Method 1. Cells were labelled with anti-fibrillarin (a–c) and Aff 29 (a′–c′), and were counterstained with DAPI (a″–c″). ×1,050.

specific antibodies used in this study. The question remains as to what accounts for the generalized nucleolar labelling observed with 8F8. One possibility, as suggested for Aff 29, is that 8F8 detects a population of Topo IIβ that is redistributed into the nucleolus during sample preparation. We consider this unlikely because, unlike Aff 29, 8F8 labelling did not vary with fixation protocol, detecting nucleoli in every cell of every sample examined. A second possibility is that 8F8 is detecting a subset of Topo IIβ that is concentrated in, and distributed throughout, the nucleolus. Although we cannot rule out this possibility, we consider it also to be unlikely since none of the other Topo IIβ-specific antibodies showed this pattern of nucleolar staining. Furthermore, a uniform nucleolar distribution of Topo IIβ, as indicated with 8F8, is difficult to reconcile with the known localization of nucleolar DNA solely in the fibrillar subcompartment [Scheer and Weisenberger, 1994]. A third possibility that should be considered is that, in addition to nucleoplasmic Topo IIβ, 8F8 is detecting a non-Topo II nucleolar antigen in microscopic preparations which is not recognized by 8F8 on immunoblots.

3. *Serum 20, Serum 21, and Monoclonal 5A7.* The labelling pattern produced by these Topo IIβ-specific antibodies was consistent, regardless of cell type and sample preparation, and was also consistent with the known function of topoisomerases in altering chromatin topology. We consider, therefore, that the distribution of Topo IIβ indicated by these antibodies is most likely to be representative of the organization of this isozyme in vivo.

On the basis of this study, therefore, we must conclude that, like Topo IIα, Topo IIβ is also primarily a nucleoplasmic protein, but that unlike Topo IIα, small amounts are also associated with intranucleolar chromatin. Whether this interpretation of Topo IIβ localization is indeed correct will first require the definition of its precise function in mammalian nuclei.

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