The EMBO Journal vol.4 no.5 pp.1237-1243, 1985

Eukaryotic type I topoisomerase is enriched in the nucleolus and catalytically active on ribosomal DNA

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Communicated by N.M.Wilkie

The distribution of eukaryotic DNA topoisomerase I in the cell has been analyzed at four levels: (i) at the level of the nuclear matrix; (ii) at the cytological level by immunofluorescence of whole cells; (iii) at the electron microscopic level using the protein A/colloidal gold technique; and (iv) at the level of DNA to identify *in situ* the sequence upon which topoisomerase I is catalytically active. Although topoisomerase I is clearly distributed non-randomly in the nucleus, the unique distribution of the enzyme is not related to the nuclear matrix. The data support the conclusion that topoisomerase I is heavily concentrated in the nucleolus of the cell; furthermore, particular regions within the nucleolus are depleted of topoisomerase. A technique has been developed which allows isolation and analysis of the cellular DNA sequences covalently attached to topoisomerase. Ribosomal DNA sequences are at least 20-fold enriched in topoisomerase/DNA complexes isolated directly from a chromosomal setting, relative to total DNA. This is the first direct evidence that topoisomerase I is catalytically active on ribosomal DNA *in vivo*.

Key words: DNA type I topoisomerase/nucleolus/ribosomal DNA

Introduction

DNA topoisomerases are enzymes that transiently break the phosphodiester backbone of DNA and then reseal the free DNA ends (for reviews, see Champoux, 1978; Cozzarelli, 1980; Wang, 1981; Gellert, 1981; Liu, 1983). The cycle of breakage and rejoining of DNA allows for strand passage and alterations in DNA topology. These enzymes have been implicated in a variety of events such as transcription (Akrigg and Cook, 1980; Sternglanz *et al.*, 1981; Weisbrod, 1982a, 1982b; Gocke *et al.*, 1983), recombination (Kikuchi and Nash, 1979), transposition (Sternglanz *et al.*, 1981), DNA replication (Liu *et al.*, 1980) and segregation of daughter DNA molecules after S phase (DiNardo *et al.*, 1984).

The interphase nucleus of the eukaryotic cell is highly organized with respect to structure and function. An association of topoisomerases with a particular nuclear structure might lead to a better understanding of the primary function of topoisomerase I (topo I) in chromatin. For example, identifiable organelles such as the nucleolus produce rRNA which is assembled and transported to the cytosol (Busch and Smetana, 1970). In addition a protein framework is thought to be responsible for organizing the nucleus into domains (loops) defined by the attachment sites of DNA to the nuclear matrix (Hancock, 1982; Berezney, 1984).

We have examined the distribution of topo I in the nucleus. We show here that this enzyme is heavily concentrated in the nucleolus, suggesting a primary role in transcription of ribosomal genes. Furthermore, we have isolated covalent topo/DNA complexes directly from nuclei and have shown an association with rDNA sequences. These data constitute rigorous proof that topo I is active at these sites in chromatin.

Results

Characterization of antibody to topoisomerase I

In our analysis of the distribution of topo I in the nucleus, we used anti-topo I antibody. A polyclonal antibody was prepared and tested for its ability to neutralize enzymatic activity. Since the binding of topo I to DNA is non-specifically inhibited by serum proteins (unpublished observation), we purified the IgG from rabbit serum. Pre-incubating purified topo I (Trask and Muller, 1983) with the IgG effectively blocks the relaxation of supercoiled DNA whereas pre-immune IgG does not (Figure 1). When analyzed on immunoblots, the IgG reacts with a 105-kd polypeptide (Figure 2A) in addition to trace amounts of lower mol. wt. forms of topo I. The appearance of these forms can be minimized by including a broad spectrum of proteinase inhibitors. Additional characterizations of the IgG (not shown) were carried out as follows. Pre-incubation of the enzyme with the antibody prevented the covalent binding of topoisomerase to DNA. When covalent topo/DNA complexes are formed using labeled DNA (Trask *et al.*, 1984) those DNA molecules bound to topo (but not free DNA) are immunoprecipitated using the IgG and protein A-Sepharose (see also Figure 5 below). The 105-kd peptide is recovered in the radioimmunoprecipitate of [^{35}S]methionine-labeled MSB-1 nuclear proteins using the IgG. The IgG did not react with avian erythrocyte histones or with

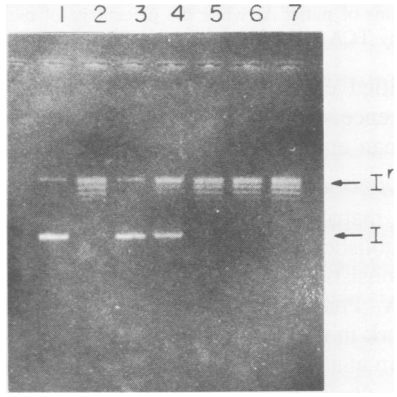

Fig. 1. Characterization of antibody to topoisomerase I. A fixed amount of topoisomerase I (10 units) was pre-incubated for 60 min on ice with various dilutions of anti-topoisomerase I IgG, prior to addition of 0.1 μg pBR322 DNA substrate. The ability of the enzyme to relax supercoiled pBR322 DNA was then measured by a 30 min incubation at 30°C followed by gel electrophoresis as described previously (Trask *et al.*, 1984). **Lane 1**: supercoiled pBR32 DNA marker; **lane 2**: relaxation of DNA with topoisomerase I; **lanes 3–6**: as in **lane 2** but topoisomerase was pre-incubated with 24, 12, 6 and 3 ng, respectively, of anti-topoisomerase I IgG; **lane 7**: as in **lane 2** but the enzyme was pre-incubated with 24 ng pre-immune IgG. Supercoiled (I) and relaxed (I^r) DNA are marked.

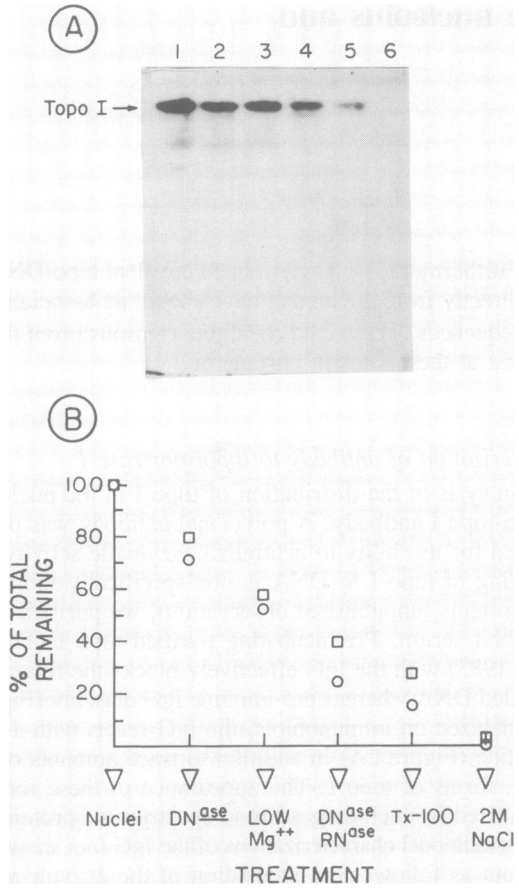

Fig. 2. Extraction of topoisomerase I during preparation of the nuclear matrix. Nuclear matrices were prepared from [^{3}H]thymidine-labeled MSB-1 cells as described in Materials and methods. In **panel A**, samples of nuclei (**lane 1**) and the other residual structures obtained after the sequential treatments using DNase I (**lane 2**), low magnesium buffer (**lane 3**), DNase I/RNase A (**lane 4**), Triton X-100 (**lane 5**), and 2 M NaCl (**lane 6**) were assayed for topoisomerase I using immunoblots. The position of the 105-kd form of topo I is indicated. **Panel B** shows the relationship between the extraction of topo I and the extraction of DNA. The percentage of the total topo I remaining in the residual structures was determined using densitometric scans of **panel A**, while the percentage of the total DNA remaining was by TCA precipitation.

partially purified chicken topoisomerase II. Based upon immunofluorescence, the avian antibody reacted weakly with monkey, human and mouse cell nuclei.

Analysis of topoisomerase I and the nuclear matrix

The nuclear matrix was prepared using a modification of previously published procedures (Berezney, 1979). The final preparation consists of 2% of the total nuclear proteins and 1% of total DNA. Phase contrast microscopy revealed a typical fibrous network in the residual structures devoid of the nuclear membrane. An analysis of proteins by SDS-PAGE showed that the structures contained a primary collection of peptides of 45–75 kd with a few minor peptides of 20–30 kd.

Conditions used to isolate the nuclear matrix resulted in the extraction of topo I. The 105-kd form of the enzyme was monitored by immunoblots of residual structures obtained after treatments described in Figure 2. Virtually all of the topoisomerase was extracted with 2 M NaCl (Figure 2A, lane 6). This observation is consistent with the fact that topo I is efficiently extracted from nuclei by treatment with considerably lower levels of salt (Trask and Muller, 1983). Prior to the 2 M extraction step, successive treatments with DNase, $MgCl_2$ and detergent removed 30%, 50% and >80%, respectively, of the enzyme (Figure 2B). The extraction of topo I, therefore, closely parallels the removal of total DNA from nuclei (Figure 2B).

Enrichment of topoisomerase I in the nucleolus

Incubation of anti-topo IgG with cells resulted in the fluorescent labeling of the nucleolar region (Figure 3). The pattern of fluorescence is very intense (panels B, D) and is not observed with control IgG (panel F). In addition, when anti-topo IgG was pre-incubated with purified topoisomerase I (but not topoisomerase II or histones), fluorescent labeling was abolished. In the process of preparing cells for fluorescent staining, topo I was not extracted (as assayed by immunoblots); thus, the results are not biased by selective extraction of the antigen. Efficient extraction of topo I requires much higher salt (Trask and Muller, 1983).

In interphase MSB-1 cells one usually observes one or two phase-dense nucleolar regions (Figure 3, panel A). Under all conditions tested, we observed intense staining of the nucleolar regions and only background levels of cytoplasmic staining. In nucleoplasmic regions a small amount of staining was generally observed. This staining was very distinctive in being somewhat punctiform; as shown in Figure 3B, pinpoint fluorescence can be seen in the nucleoplasm of the cell in the upper right corner of the photograph (marked with a small arrowhead). Visualization of nucleoplasmic fluorescence on all cells in a given field was difficult since focusing was critical. In a small fraction of nuclei on a given slide, phase dense material was not observed presumably because these cells were in early prophase (Ochs *et al.*, 1983). In these nuclei, neither nucleolar nor pinpoint fluorescence could be detected; however, more generalized and very diffuse staining patterns were observed. Similarly, metaphase cells displayed a generalized fluorescence (data not shown).

Immunoelectron microscopic localization of topoisomerase I

Electron microscopy was used to locate more precisely the enzyme in the nucleolus. Thin sections of MSB-1 cells were incubated with anti-topo IgG and immune complexes visualized with protein A/colloidal gold (Bendayan, 1984). Consistent with the immunofluorescent data, the nucleolus is enriched in gold particles (Figure 4A). Controls using pre-immune serum did not show the same nucleolar clustering of particles (Figure 4B).

The absence of gold particles in areas of low electron density within the nucleolus is striking (Figure 4A). These regions correspond to fibrillar centers (FC, Figure 4A) (Jordan, 1984) although fibrilles are not particularly well elaborated in these cells. The fibrillar shell surrounding the FC and the perinucleolar chromatin (PC) are indicated; however, a localization of particles in these areas was not statistically significant. A statistical analysis was carried out on the relative frequency of gold particles in three regions of the cell: (i) the cytoplasm; (2) the nucleoplasm and (iii) the nucleolus. Randomly selected areas (in different thin sections) revealed significant distribution differences between the three regions. Numbers of gold particles per unit area were greater in the nucleolus relative to nucleoplasm ($p<0.001$, by t test) or cytoplasm ($p<0.001$). Within the nucleolus, a deficiency of particles inside the FC was also statistically significant ($p<0.001$). In addition, numbers of particles in the nucleoplasm were significantly greater than in the cytoplasm ($p<0.005$). These observations only apply to sections treated with anti-topo IgG and not pre-immune IgG.

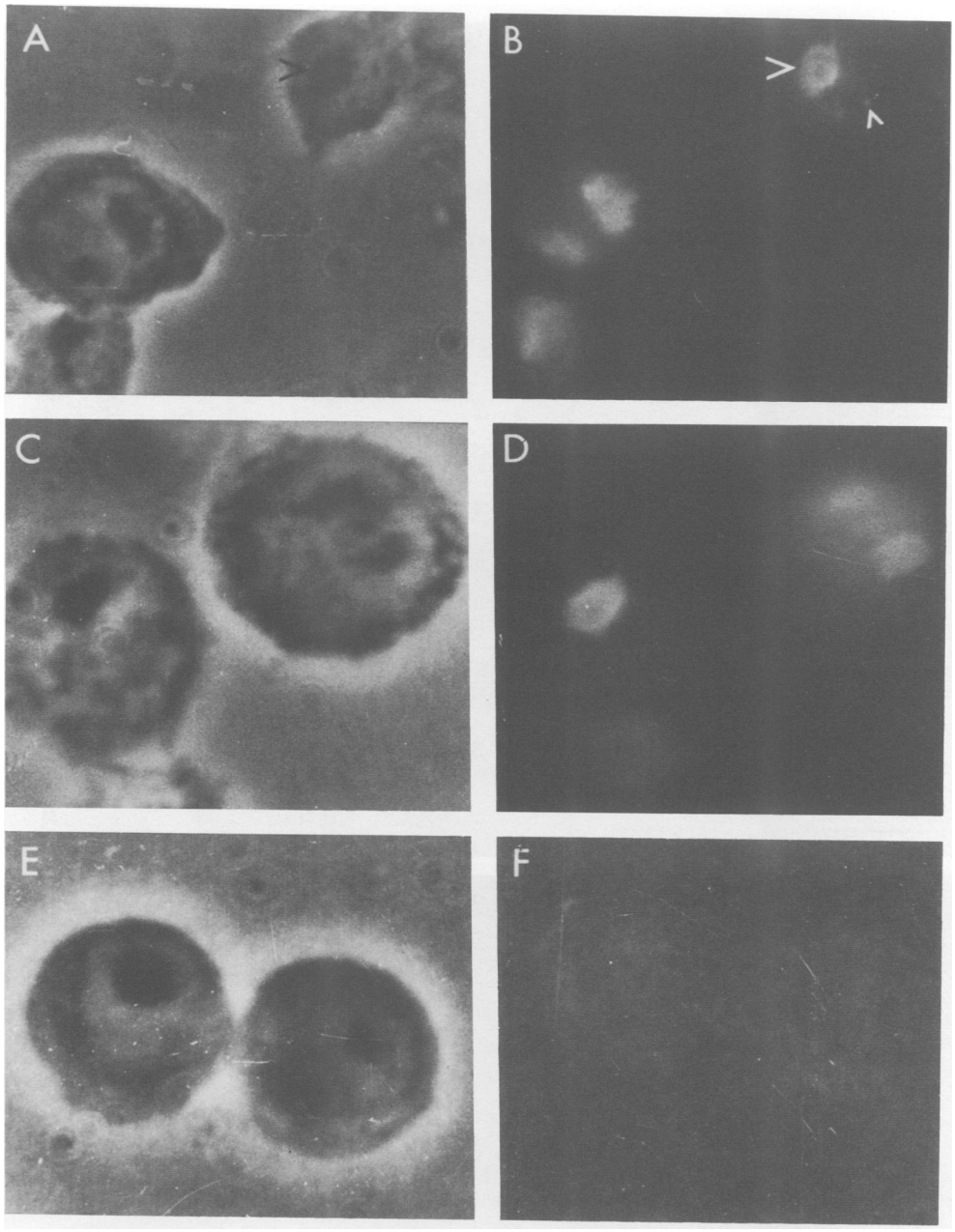

Fig. 3. Localization of topoisomerase I using indirect immunofluorescence. Phase contrast images (**panels A, C** and **E**) and the corresponding immunofluorescent images (**panels B, D** and **F**) of interphase MSB-1 cells are shown. Cells were stained using anti-topoisomerase I IgG (**panels B** and **D**) and pre-immune IgG (**panel F**). Large arrow heads (**panels A, B**) indicate the location of the nucleolus, and the small arrow head (**panel B**) indicates pinpoint fluorescence within the nucleoplasm. Magnification is ~3800 x.

Association of topoisomerase I with rDNA

Based upon a number of experiments employing purified enzyme and DNA, we previously reported that topo I becomes trapped in a covalent complex with DNA when the catalytic reaction sequence is interrupted with SDS (Trask *et al.*, 1984). The observations raise the possibility that topo/DNA cross-linking might be useful as a means of identifying the DNA site(s) upon which the endogenous enzyme is catalytically active. To test this, MSB-1 nuclei (labeled with [^{3}H]TdR) were incubated under conditions previously shown to be ideal for detection of covalent complexes (Trask *et al.*, 1984) and lysed with detergent to arrest the topo/DNA covalent intermediate. The lysate was layered onto a step CsCl gradient (Shaw *et al.*, 1975) to separate DNA from protein. Immunoblotting was then performed across the gradient to locate topoisomerase I. As shown in Figure 5, a fraction of the topoisomerase remains bound to DNA under these rather harsh conditions (detergent, high salt) indicating that the enzyme is covalently coupled to the helix. Digestion of the lysed nuclei with proteinase K prior to loading the gradient abolished the signal on the immunoblot showing that a tightly bound protein and not radiolabeled DNA is responsible for the immunoblot signal. These data show that it is possible to obtain topoisomerase/DNA complexes mixed with total DNA directly from nuclei. When free topoisomerase is denatured and added to labeled DNA and the mixture analyzed on CsCl, topoisomerase is only detectable at the top of the gradient with free protein (data not shown);

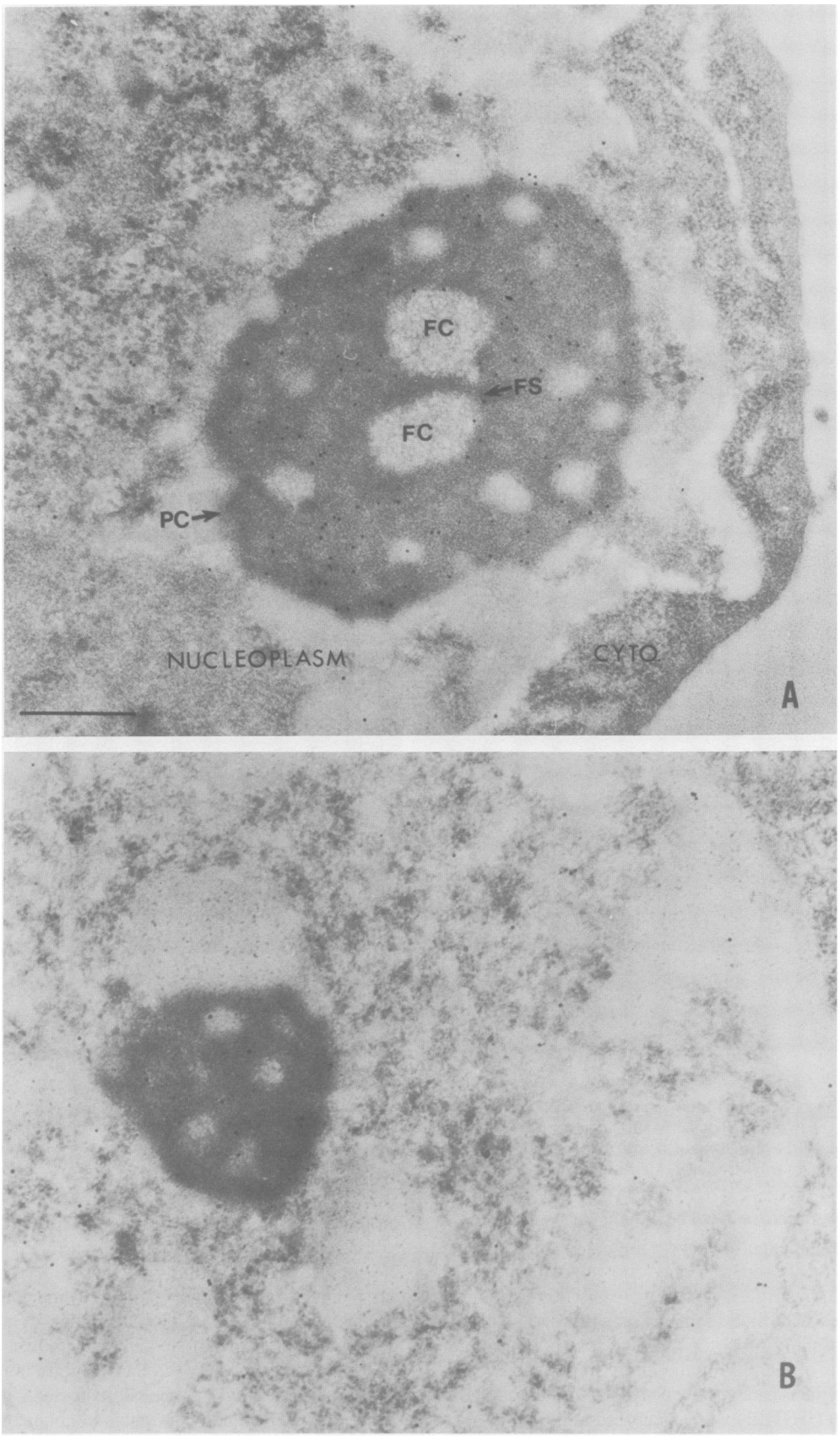

Fig. 4 Electron microscopic localization of topoisiomerase I within the nucleolus. Thin sections through MSB-1 nucleoli were treated with either anti-topoisomerase I IgG (**panel A**) or pre-immune IgG (**panel B**) followed by labeling with protein A/colloidal gold as described. Fibrillar centers (FC) are indicated. The assignment of the fibrillar shell (FS) is based solely upon its location relative to the FC (see Spector *et al.*, 1984); however, the FS is not particularly well elaborated in these cells. Perinucleolar chromatin (PC) surrounding the nucleolus is marked. The bar represents 500 nm.

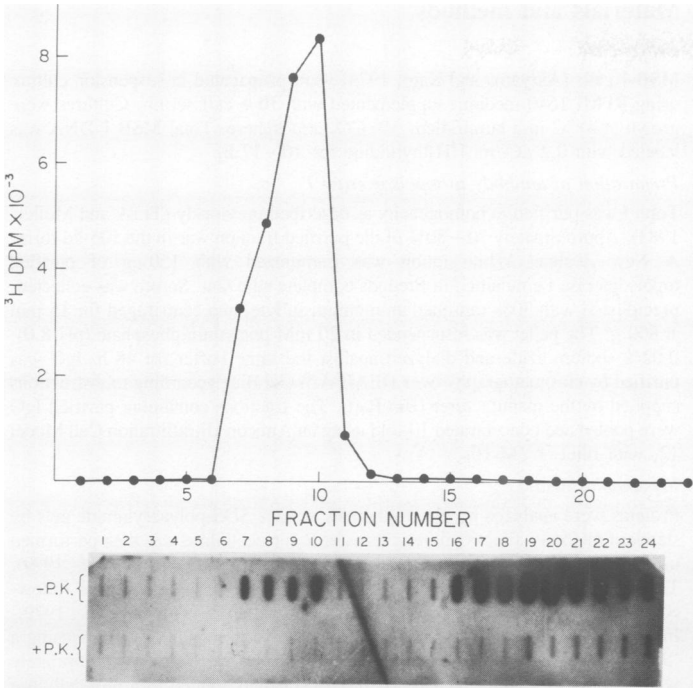

Fig. 5. Isolation of topo/DNA complexes on CsCl gradients. Nuclei were prepared from [^{3}H]thymidine-labeled MSB-1 cells, lysed and 5 x 10^8 nuclei layered on a step CsCl gradient which was centrifuged as described in Materials and methods. The position of labeled DNA was determined by TCA precipitation and is shown in the upper graph. Aliquots (20 μl) of each fraction across the gradient were analyzed for topo content by immunoblotting (lower autoradiograph). For negative controls, the nuclear lysate was digested with 100 μg proteinase K/ml for 5 h at 37°C and phenol extracted prior to layering on the gradient.

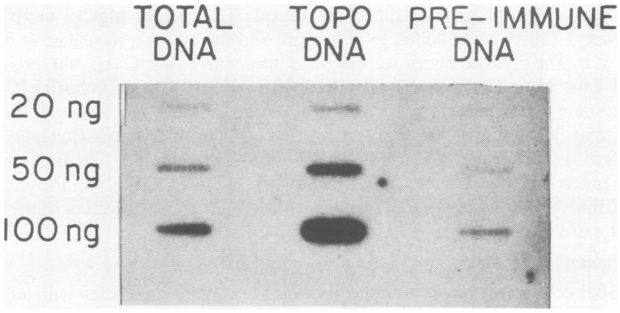

Fig. 6. Enrichment of rDNA sequences associated with topo. DNA fragments stably coupled to topo were immunologically selected from the total DNA peak of the CsCl gradient as described in Figure 5 and in Materials and methods. Various amount of DNA (20, 50 or 100 ng) were hybridized to pX1r 101A. The samples on the blot are: **left column**, total DNA; **middle column**, DNA associated with topo; **right column**, DNA recovered after reaction with the same amount of pre-immune IgG.

therefore, the method allows separation of free and DNA-bound topoisomerase. We estimate that ~10% of the endogenous topo is coupled to DNA under these conditions. All attempts to increase the efficiency of cross-linking, for example by altering conditions of temperature, pH, ionic strength, etc., were negative. If the half life of the *in vivo* topo/DNA intermediate is of short duration, one might expect that <100% of the enzyme would be trapped in the covalent complex. Furthermore, it appears that most endogenous enzyme is intimately associated with DNA in nuclei, since significantly more topo is coupled by u.v.-induced cross-linking (data not shown). This rather indirect evidence suggests that the efficiency of cross-linking is ~10% as opposed to the possibility that only 1 in 10 enzyme molecules is catalytically active *in vivo*.

To investigate the association of rDNA sequences with topoisomerase I, we used isopycnic separation (described above) in conjunction with an immunoaffinity fractionation using anti-topo IgG and protein A-Sepharose (Gilmour and Lis, 1984). Briefly, total DNA plus DNA/topo complexes were isolated from the gradient, dialyzed and digested with *Eco*RI. The reactions were then incubated with antibody (either anti-topo IgG or pre-immune IgG) and immune complexes recovered using protein A-Sepharose. DNA was recovered from the complexes retained on the affinity column and hybridized to the ribosomal DNA repeat of *Xenopus laevis* (kindly provided by R.Reeder). As shown in Figure 6, such an analysis reveals a striking enrichment of rDNA sequences associated wtih topoisomerase I compared with total DNA. We estimate a 20-fold enrichment of ribosomal sequences in the fraction reacting with the antibody to topoisomerase I. As expected, a control hybridization of DNA selected with pre-immune IgG revealed that rDNA sequences were not enriched. The amount of DNA recovered using pre-immune IgG was substantially less than with anti-topo IgG making accurate estimates of DNA concentration problematic. As a result, our estimate of total DNA concentration in this sample was off by about a factor of two. Although the pre-immune DNA appears slightly depleted in rDNA sequences compared with total DNA, there was in fact no significant difference.

Discussion

In this work, we show that the majority of eukaryotic topo I is intimately associated with the nucleolus. The following observations are mutually consistent with this conclusion. First, the distribution of topo I in the cell is clearly non-random: intense fluorescent staining of the nucleolar region is observed with anti-topo I IgG. Second, during the normal cell cycle, when identifiable nucleoli disperse (prophase, metaphase cells for example), so does the intense fluorescence. Third, we have relied on electron microscopy to demonstrate that the distribution within the nucleolus is not uniform. Finally, we have demonstrated that topo I is associated directly with rDNA in a way to suggest that the enzyme is catalytically active at these sites.

Topo I is not stably bound to the nuclear matrix; thus, conditions used to isolate the matrix result in extraction of the enzyme. For instance, the final step used to isolate the matrix (2 M NaCl) causes dissociation of native DNA/topo complexes in chromatin (Trask and Muller, 1983). Prior to the final step, however, the release of topo I closely parallels release of DNA from the nucleus. Active ribosomal genes lack a typical nucleosomal structure and are highly sensitive to cleavage by nucleases (Davis *et al.*, 1983; Sogo *et al.*, 1984). Thus, a parallel release of nucleolar DNA and topo might be anticipated based upon a heavy concentration of the endogenous enzyme in the nucleolus. The immunoelectron micrographs did not show a particular enrichment of topo I in the nuclear membrane or periphery of the nucleus. Proteins of the nuclear periphery such as lamins appear to interact with DNA (Gerace and Blobel, 1981; Lebkowski and Laemmli, 1982; Hancock, 1982) and the topological/spatial organization of the matrix has been proposed to be peripheral (Hancock, 1982; Berezney, 1984). While we cannot exclude an association of topo I with the matrix, the collective data show that the enzyme is concentrated in other regions of the nucleus.

Within the nucleolus, a striking absence of topo I was noted in the fibrillar center (nomenclature of Jordan, 1984) which has been reported to contain transcriptionally quiescient rDNA (Mirre

and Stahl, 1978a, 1978b). Although high transcriptional activity has been proposed to occur at the periphery of the fibrillar center in a dense fibrillar component (Mirre and Stahl, 1978a; Goessens and Lepoint, 1979; Hernandez-Verdun and Bouteille, 1979), Scheer and Rose (1984) showed that RNA polymerase I is located within fibrillar centers and proposed that active rRNA genes are located within these regions or perhaps more toward the edge of the fibrillar center. In any case, it is difficult to reconcile the results from different laboratories with ours. At present we can only draw significance to the observation that the distribution of topo I within the nucleolus is not uniform.

The heavy concentration of topo I in the nucleolus raises several questions along with some speculation. Notably, why is topo enriched on ribosomal genes? One possibility that stands out is that topo I is enriched at all active genes, although single copy genes transcribed by RNA polymerase II would be more difficult to detect by cytological assays employed here. However, the pinpoint nucleoplasmic fluorescence observed in Figure 3 probably represents sites of nucleoplasmic transcription. Detection of high levels of topo I in the nucleolus is most likely due to a need for the enzyme in transcription (Higashinakagawa *et al.*, 1977; Gocke *et al.*, 1983; Flesihmann *et al.*, 1984; Muller and Trask, unpublished observations) and the clustering of a large number of highly active genes in one region. Why does topo I associate with active genes? We envisage that the enzyme is involved in some aspect of transcription as opposed to a passive role in nucleolar chromatin organization. It was recently reported that intercalating agents can influence the RNA polymerase I promoter selection (Pruitt and Reeder, 1985). A logical extension of this observation is the possibility that topo I, through its effect on DNA supercoiling, is involved in discriminating one promoter from the next. This possibility is analogous to the proposed role of *Escherichia coli* topo I in gene regulation (Menzel and Gellert, 1983; Gellert *et al.*, 1983).

Topo I was previously identified in nucleolar chromatin (Higashinakagawa *et al.*, 1977). The present data show that the association is related to the interaction between topo and specific DNA sequences as opposed to an association with proteins of the transcriptional apparatus. For example, an association between HMG proteins and topo I (Weisbrod, 1982a, 1982b; Javaherian and Liu, 1983) has been reported. Recently, single strand nicks reminiscent of topo-induced cleavages were identified in rDNA of *Tetrahymena* (Gocke *et al.*, 1983). We have immunologically selected covalent DNA/topo complexes, isolated directly from nuclei, to demonstrate that topo I is stably (or covalently) bound to rDNA sequences. From these observations we conclude that topo at these sites must have been catalytically active on rDNA. This conclusion follows from previous work showing that SDS treatment traps a functional topo/DNA intermediate (Trask *et al.*, 1984).

It is likely that topo I is catalytically active on transcriptionally active genes outside the nucleolus (Weisbrod, 1982a, 1982b; Fleishmann *et al.*, 1984). The punctiform immunofluorescence observed in the nucleoplasm (Figure 3) is consistent with this possibility. Moreover, when topo/DNA complexes were isolated from erythrocytes and the DNA hybridized to transcriptionally active (globin) *versus* inactive (ovalbumin) gene probes, the active gene was significantly enriched (D.Trask and M.Muller, in preparation). These experiments indicate a role for topo I in RNA polymerase I and II transcriptional events.

Materials and methods

Cell culture

MSB-1 cells (Akiyama and Kato, 1974) were propagated in suspension culture using RPMI 1640 medium supplemented with 10% calf serum. Cultures were grown at 41°C in a humifidied, 5% CO_2 atmosphere. Total MSB-1 DNA was labeled with 0.2 μCi/ml [^{3}H]thymidine for 10 – 12 h.

Preparation of antibody to topoisomerase I

Topo I was purified to homogeneity as described previously (Trask and Muller, 1983). Approximately 70 – 80% of the purified fraction was in the 105-kd form. A New Zealand White rabbit was immunized with 150 μg of purified topoisomerase I emulsified in Freunds complete adjuvant. Serum was collected, precipitated with 30% saturated ammonium sulfate, and centrifuged for 15 min at 800 *g*. The pellet was resuspended in 20 mM potassium phosphate (pH 8.0), 0.02% sodium azide and dialyzed against the same buffer for 48 h. IgG was purified by chromatography over DEAE-Affi Gel Blue according to instructions supplied by the manufacturer (Bio Rad). The fractions containing purified IgG were pooled and concentrated 10-fold using an Amicon Ultrafiltration Cell Model 12, with filter # YM-10.

Immunoblot assays

Proteins were analyzed by electrophoresis in 12% SDS-polyacrylamide gels by standard methods. Electrophoretic transfer to nitrocellulose was then performed using a Trans-Blot Transfer Cell (Bio Rad) at 0.8 A for 3 h (Towbin *et al.*, 1979). The nitrocellulose filter was treated with a 1:600 dilution of purified IgG followed by washing and labeling with 2.5 μCi [^{125}I]protein A (Towbin *et al.*, 1979). In some cases, the antigen was applied directly to nitrocellulose filters using a 'Slot Blot' device (Schleicher and Schuell). Samples were diluted appropriately with 10 mM Tris-HCl (pH 7.6), 1 mM EDTA and applied to a nitrocellulose filter which had been pre-soaked in 160 mM NaCl, 15 mM sodium citrate (pH 7.0). Each sample well was then washed with 400 μl 25 mM sodium phosphate (pH 6.5) before the nitrocellulose was dried and processed as described above.

Nuclear matrix isolation

One liter of exponentially growing MSB-1 cells were harvested by centrifugation and resuspended to 1 x 10^8/ml in buffer A [250 mM sucrose, 50 mM Tris-HCl (pH 7.4), 5 mM $MgCl_2$]. The cells were then centrifuged, resuspended in buffer A and incubated at 4°C for 10 min. Nuclei were released with 8 – 10 strokes of a Dounce homogenizer using a tight fitting pestle, centrifuged (1500 *g*, 10 min) and washed once in buffer A before being resuspended in buffer B [146 mM sucrose, 100 mM KCl, 10 mM Tris-HC (pH 7.3), 5 mM $MgCl_2$] to 10^8/ml. DNase I (Sigma) was added to 500 μg/ml and the reaction incubated at 37°C for 2 h. The digested nuclei were washed once with buffer C (10 mM Tris-HCl pH 7.6), 250 mM sucrose, 0.2 mM $MgCl_2$) and resuspended in buffer B. The digestion was repeated for 1 h with 500 μg/ml DNase I and 100 μg RNase A (Sigma). The resulting nuclei were then extracted with 10 mM Tris-HCl (pH 7.6), 250 mM sucrose, 0.2 mM $MgCl_2$, 1.0% (v/v) Triton X-100. The final step of the procedure was extraction with 10 mM Tris-HCl (pH 7.6), 250 mM sucrose, 0.2 mM $MgCl_2$, 2 M NaCl. The pellet obtained after centrifugation of the high salt extraction is designated the nuclear matrix.

Immunofluorescence

MSB-1 cells were allowed to settle onto glass coverslips pre-coated with poly-L-lysine (Sigma). The attached cells were then processed as described by Ochs *et al.* (1983) using anti-topoisomerase IgG or pre-immune IgG as the primary antibody. Fluorescein-conjugated goat anti-rabbit IgG (Cappel) was used as second antibody. Fluorescence was visualized using a Zeiss microscope equipped with epifluorescence illumination.

Immunoelectron microscopy

MSB-1 cells were fixed with 1.5% glutaraldehyde at 0°C for 90 min and dehydrated according to the following schedule: 30% ethanol for 30 min at 0°C; 50% ethanol, 60 min at −20°C; 70% ethanol, 60 min at −20°C; two changes of 90% ethanol, each for 2 h at −20°C. Infiltration was performed at −20°C using 90% ethanol/Lowicryl K4M (1:1) for 60 min, followed by 90% ethanol/Lowicryl K4M (1:2) for 15 h and Lowicryl K4M (undiluted) for 24 h. Samples were then placed in BEEM capsules, polymerized and immunolabeled according to a previously published procedure (Bendayan, 1984).

Isolation of topo/DNA covalent complexes

Nuclei were prepared from MSB-1 cells as described above except that 0.5% Nonidet P-40 (NP-40) was included in buffer A. After washing with the same buffer, the nuclei were suspended to 5 x 10^8/ml in buffer A plus 0.5% NP-40. Following incubation at 37°C for 30 min, sarkosyl was added to 2% and rapidly

mixed. The lysate was layered onto a CsCl step gradient (Shaw *et al.*, 1975) and centrifuged in an SW41 rotor (33 000 r.p.m., 18 h). The DNA peak fractions were recovered, dialyzed against 10 mM Tris-HCl (pH 7.9), 1 mM EDTA and digested with *Eco*RI. DNA/topo complexes were immunologically selected using a modification of the procedure reported by Gilmour and Lis (1984). IgG (0.1 volume) was added to the *Eco*RI-digested DNA and incubated on ice for 3 h. A slurry of protein A-Sepharose [50% w/v in 50 mM Tris-HCl (pH 8), 150 mM NaCl] was then added (0.2 volumes) and the mixture incubated on a rotary shaker for 2 h at 4°C. The protein A-Sepharose was pelleted in a microfuge for 30 s and washed 10 times with 1 ml of 0.1 M Tris-HCl (pH 9), 1% NP-40, 1% deoxycholate and 0.5 M LiCl. The immune complexes were eluted with four washes (0.2 ml each) of 50 mM $NaHCO_3$, 1% SDS at room temperature. The eluate was adjusted to pH 8.0 with M Tris-HCl (pH 7.0) and digested with proteinase K (0.1 mg/ml) 12 h at 37°C. DNA was purified by phenol extractions and ethanol precipitation.

DNA blotting and hybridization

The DNA was denatured and applied to pre-wetted nitrocellulose on a Slot Blot device as recommended by the manufacturer (Schleicher and Schuell). The ribosomal repeat from *X. laevis*, pX1r 101A (Bakken *et al.*, 1982) was used to probe the blot. The probe was labeled as described (James and Leffak, 1984) and hybridization conditions were performed according to standard procedures (Maniatis *et al.*, 1982) except that *E. coli* DNA was used as heterologous DNA.

Acknowledgements

We thank Dr. Viviane Hardy for her kind assistance with the protein A/gold technique and Dr. Robert Pfister for additional assistance with the electron microscopy. The rDNA plasmid was provided by Dr. Ronald Reeder and is gratefully acknowledged. We acknowledge helpful discussions with Dr. Robert Ochs. This work was supported in part by grant number R01 GM31640-02 from the National Institute of Health and by a Basil O'Connor Starter Research Grant No. 5-427 to MTM from the March of Dimes Birth Defects Foundation.

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Received on 22 January 1985; revised on 15 March 1985