Role for Nucleolin/Nsr1 in the Cellular Localization of Topoisomerase I*

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Nucleolin functions in ribosome biogenesis and contains an acidic N terminus that binds nuclear localization sequences. In previous work we showed that human nucleolin associates with the N-terminal region of human topoisomerase I (Top1). We have now mapped the topoisomerase I interaction domain of nucleolin to the N-terminal 225 amino acids. We also show that the Saccharomyces cerevisiae nucleolin ortholog, Nsr1p, physically interacts with yeast topoisomerase I, yTop1p. Studies of isogenic NSR1* and Δnsr1 strains indicate that NSR1 is important in determining the cellular localization of yTop1p. Moreover, deletion of NSR1 reduces sensitivity to camptothecin, an antineoplastic topoisomerase I inhibitor. By contrast, Δnsr1 cells are hypersensitive to the topoisomerase II-targeting drug amsacrine. These findings indicate that nucleolin/Nsr1 is involved in the cellular localization of Top1 and that this localization may be important in determining sensitivity to drugs that target topoisomerases.

Human Top1 is a nuclear protein involved in the regulation of DNA structure and is the target of an important new class of antineoplastic drugs, the camptothecins (1, 2). Studies in yeast provide convincing evidence that Top1 is the sole cellular target for camptothecins, with this knowledge facilitating attempts to understand the mechanisms by which the drug destroys cells (3–5). Top1 is a monomer protein that relaxes supercoiled DNA by creating a transient single-strand nick, with this nick involving the covalent attachment of Top1 to the DNA phosphate backbone via a phosphotyrosine bond. Recent structural studies of Top1 and the Top1-DNA covalent complex led to molecular models of the mechanism by which camptothecins inhibit the catalytic activity of Top1 (6–8). Nevertheless, formation of CPT-Top1-DNA ternary complexes is insufficient to explain the cytotoxic effects of CPT (9–11). Current models invoke interactions between ternary complexes and replication or transcriptional machinery as being important in conversion of ternary complexes to lethal forms of DNA damage (12, 13). However, it is not known whether physical interactions between Top1 and other proteins (including proteins involved in replication or transcription) are important in the cytotoxic effects of CPT. Recently, an interaction between Top1 and the SV40 T antigen helicase was shown to modulate formation of Top1-CPT-DNA ternary complexes, suggesting that similar interactions between Top1 and cellular helicases may mediate the cytotoxicity of CPT (14). In addition, Top1 rapidly redistributes from the nucleolus to the nucleus or cytoplasm and is degraded after cellular exposure to CPT (15–19). These alterations may confer transient cellular resistance to CPT and could be mediated by interactions between Top1 and other proteins.

Top1 is known to physically interact with the following proteins: HMG17 (20), casein kinase II (21), RNA polymerase I (22), nucleolin (23), SV40 T antigen (24, 25), p53 (26, 27), the TATA-binding protein (28), the splicing factors SF2/ASF (29) and PSF/p54⁹⁹ (30), and a novel RING finger protein named topos (31). Recently, two yTop1p-binding proteins were also identified (Tof1p and Tof2p) (32). Although relatively little is known regarding either protein, a Tof2p paralog, Net1p, is required for the proper nucleolar localization of Sir2 and Cdc14 (33, 34).

Since helicases may generate supercoiling problems that are resolved by topoisomerases, a need for physical interactions between these two enzyme classes is easily understood. Indeed, binding of Top1 by T antigen is one of several examples of a physical interaction between a helicase and a topoisomerase (35). The biochemical relevance of interactions between Top1 and other proteins is less clear. With regard to the nucleolin-Top1 interaction, there are three major domains in nucleolin: an acidic N terminus that contains multiple putative phosphorylation sites, a middle region containing four RNA-binding domains (RBDs), and a C-terminal glycine-arginine-rich (GAR) domain. In addition to binding RNA and DNA (36, 37), nucleolin was reported to be a helicase (38) and to function in nucleocytoplasmic transport by binding nuclear localization sequences (NLSs) (39, 40). Several lines of evidence indicate that both nucleolin and Top1 are involved in rRNA synthesis and processing (41–49). To gain insight into the cellular role of the nucleolin-Top1 interaction, we studied both a recombinant form of nucleolin and the Saccharomyces cerevisiae nucleolin ortholog NSR1. We find that the N terminus of nucleolin is necessary and sufficient for Top1 binding. Moreover, our results indicate that NSR1 is involved in the cellular localization of yTop1p and that loss of NSR1 alters cellular sensitivity to topoisomerase-targeting drugs but not to DNA damaging agents in general.
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**TABLE I**

| Strain   | Yeast strains |
|----------|---------------|
| FY251    | MATa ura3–52 leu2–Δ1 trp1–63 his3–1200 | 52 |
| TE25     | FY251 nsn1–10 :URA3 | This study |
| JW27     | FY251 Atp6-h6 and c1 | 53 |
| MRG15    | FY251 MATa ade2-100 | This study |
| TE13     | MRG15 nsn1–10 :URA3 | 3 |
| JW284    | MATa is1 ura3–52 leu2 his7 | This study |
| TE284    | JW284 nsn1–10 :URA3 | 3 |

**Strains and Plasmids—** *S. cerevisiae* strains used in this work are listed in Table I. MRG15 is a MATa derivative of MRG5 (50). TE25 was generated by transformation of FW251 with plasmid pCIS16, which is designed to replace a 0.35-kilobase region in the *SNR1* coding sequence with the *URA3* gene (51). Disruption of *NSR1* was confirmed by PCR using primers that flank the 5′ integration site (data not shown), as well as by immunoblotting using an Nsr1p antibody. An identical strategy was used to disrupt NSR1 in strains MRG13 and JW284, yielding TE13 and TE284, respectively. Immunoblotting was used to confirm loss of Nsr1p in TE13 and TE284. Yeast expression plasmids encoding nucleolin deletion fragments linked to GST were constructed using PCR (primers for this, and subsequently described PCR fragments are available upon request) with the pKG-Nuc plasmid (25) as a template. PCR products were ligated into the pKG vector (54) using the SmaI and HindIII sites in this vector. The resulting plasmids were named according to the encoded nucleolin residues and include pKG-Nuc–323, pKG-Nuc–323–707, pKG-Nuc–1–225, pKG-Nuc–224–225, pKG-Nuc–125, and pKG-Nuc–125–225. Regions of these vectors corresponding to 5′-cloning sites were sequenced to confirm that the proper recombinants had been obtained. Plasmid pKG-Nsr1 expresses Nsr1p linked to the C terminus of GST and was constructed using SmaI/HindIII restriction sites and PCR with pYCB5 (51) as a source for the *SNR1* coding sequence. Plasmid pYX-GFP expresses GFP in yeast under the control of the GAL1–10 promoter and was constructed using PCR with the pEGFP-C1 vector (CLONTECH) as a source for GFP coding sequence. The GFP PCR product was ligated into the BamHI and HindIII sites of the pYX133 vector (Ingenius Co., Oxford, United Kingdom). Similar strategies were used to generate pYX-GFP-top1 and pYX-GFP-Nop1, which express GFP-top1p and GFP-Nop1p fusion proteins, respectively. For the former vector, pYCP6StTop1 (provided by Mary-Ann Bjornsti) was used as template to obtain a PCR product containing the *S. cerevisiae* TOP1 coding sequence. The HindIII and XhoI sites in pYX-GFP were used to place TOP1 downstream from the GFP coding sequence in this vector. The same strategy and restriction sites were used to generate pYX-GFP-Nop1. The source of NOP1 coding sequence was *S. cerevisiae* genomic DNA from strain W303-1A (55). Expression of an appropriately migrating GFP fusion protein in cells transformed with the pYX-GFP-top1 or pYX-GFP-Nop1 vectors was confirmed by SDS-PAGE and immunoblotting using a GFP antibody (data not shown).

**Antibodies and Drugs—** Monoclonal antibodies were generously provided by the listed individuals for the following proteins: nucleolin, Ng-ёнг-ёng Yeh (56); Nsr1p, M. Snyder and C. Copeland2; human Top1, Y. C. Cheng (57). A polyclonal Yop1p antibody was obtained from Mary-Ann Bjornsti. Monoclonal GFP and actin antibodies were obtained from Roche Molecular Biochemicals and Amersham Pharmacia Biotech, respectively, and a polyclonal GST antibody was obtained from Amersham Pharmacia Biotech. Campthotecan lactone (CPT), methyl methane sulfonate, m-AMSA, and 1,10-phenanthroline were obtained from Sigma. Stock solutions were prepared for CPT at 5 mg/ml in Me2SO, for m-AMSA at 10 mg/ml in Me2SO, for 1,10-phenanthroline at 100 mg/ml in ethanol. Bleomycin sulfate was obtained as a 3 units/ml stock from Bristol-Myers Squibb Co.

**Gust Pull-down Assays—** Expression and purification of full-length nucleolin and nucleolin fragments using the pKG-Nuc plasmid and derivatives were performed as described previously (25). HeLa cell nuclear extracts were prepared by homogenization in 0.35% Triton X-100 followed by extraction with 0.5 M NaCl as described (58). Pull-down assays using HeLa nuclear extracts and 10 μl of glutathione beads (Amersham Pharmacia Biotech) loaded with approximately 4 μg of purified GST, GST-nucleolin, or GST-nucleolin deletion fragments were performed in 1 ml of binding buffer (phosphate-buffered saline containing 0.2% Tween 20, 1 mM EDTA, 1 mM DTT, 1 mM PMSF, 0.5 μg/ml leupeptin, and 1 μg/ml pepstatin) with approximately 20 μg of nuclear extract for 1 h. Immunoaffinity at 4°C, the beads were washed four times with binding buffer. Proteins remaining bound to the beads were examined by SDS-PAGE and immunoblotting using Top1 and GST antibodies. For pull-down assays using yeast extracts, cells were grown at 30 °C in appropriate media to an *A*<sub>600</sub> of 0.7–0.9, then pelleted and resuspended in 3 volumes of ice-cold TCA buffer (50 mM Tris-HCl, pH 7.2, 150 mM NaCl, 0.1% SDS, 1% Triton X-100, 1 mM DTT, and 1% sodium deoxycholate) with freshly added protease inhibitors (1 mM EDTA, 1 mM PMSF, 0.5 μg/ml leupeptin, and 1 μg/ml pepstatin). The suspension was vortexed with acid-washed glass beads for 10 min at 4°C and the resultant lysate cleared by centrifugation at 1,000 × *g* for 5 min. The supernatant was removed and cleared of remaining precipitate by centrifugation at 14,000 × *g* for 5 min, followed by incubation with 20 μl of glutathione beads for 2 h at 4°C. After washing the beads twice with 1 ml of RIPA buffer, bound proteins were analyzed by boiling the beads in loading dye, followed by SDS-PAGE and immunoblotting.

**Fluorescence Microscopy—** Yeast-transformed with pYX-GFP, pYX-GFP-top1, or pYX-GFP-Nop1 were first grown to an *A*<sub>600</sub> of 0.5 in minimal media lacking 2% raffinose but lacking cycloheximide. Expression of GFP fusion proteins was induced by the addition of galactose to a 2% concentration, followed by an additional 2-h incubation. 4′,6-Diamidino-2-phenylindole (DAPI) staining was performed by washing and resuspending cells in water, followed by addition of DAPI to a 0.1 μg/ml concentration. After a 10-min incubation at room temperature, the cells were washed once with 50% ethanol and once with water, then resuspended in water. Cells were co-imaged by phase contrast and fluorescent microscopy using a Zeiss Axioskop fluorescent microscope equipped with a DC-330T, high resolution CCD camera (Dage MTI, Michigan City, IN). Concurrent GFP and DAPI fluorescence imaging was accomplished using Endow GFP bandpass emission (excitation and emission wavelengths of 430–510 and 475–575 nm, respectively) and DAPI (excitation and emission wavelengths of 392–400 and 410–450 nm, respectively) filter sets (Chroma Technology, Brattleboro, VT). Images were captured digitally using Scion Image software (Scion Corp, Frederick, MD) and cropped using Adobe Photoshop.

**Preparation of Whole Cell, Nuclear, and Cytoplasmic Yeast Lysates—** Yeast whole cell lysates were prepared as described above for pull-down assays. For fractionation of nuclear and cytoplasmic proteins, yeast cell pellets were resuspended in spheroplast buffer (50 mM Tris-HCl, pH 7.5, 3 mM DTT, 10 mM MgCl<sub>2</sub>, and 1 mM sorbitol) and incubated at room temperature for 15 min. The cells were then incubated with spheroplast buffer with 2 mg/ml Zymolase 100T (Seikagaku, Tokyo) for 40 min at 37°C. The resulting spheroplasts were lysed in the following buffer: 18% Ficoll-400, 10 mM Tris-HCl, pH 7.5, 20 mM KCl, 5 mM MgCl<sub>2</sub>, 3 mM DTT, 1 mM EDTA, 1 mM PMSF, 0.5 mg/ml leupeptin, and 1 mg/ml pepstatin. The cytoplasmic lysate was filtered through 3000 molecular weight cut-off cellulose acetate filters, and the cell debris and spheroplasts. Nuclei were then pelleted by centrifugation at 20,000 × *g* for 20 min at 4°C. The supernatant, containing cytoplasmic lysate, was dialyzed against phosphate-buffered saline, then concentrated using a Centricon 30 concentrator (Amicon, Beverly, MA). Nuclei were lysed by boiling in SDS-PAGE loading dye.

**Yeast Growth and Drug Sensitivity Assays—** For growth rate assays, yeast were grown overnight in YPD medium in a shaking 30 °C water bath to stationary phase. After dilution in fresh media to an *A*<sub>600</sub> of approximately 0.03, subsequent growth at 30 °C was monitored by removal of aliquots and measurement of *A*<sub>600</sub>. If *A*<sub>600</sub> measurements were greater than 0.8, the aliquots were diluted in media 2-fold to ensure that the *A*<sub>600</sub> value remained linear with respect to cell number, with the linear range established by independent experiments. Least squares regression analysis was used to quantify the slopes of the exponential phase of growth for each strain, ascertainment by plotting log<sub>10</sub>*A*<sub>600</sub> as a function of time. Differences in slopes among the strains were examined using a two-sample t test. A p value of <0.05 was considered significant.

**Drug sensitivity assays, cells were grown overnight and adjusted to an *A*<sub>600</sub> of 2.0 using fresh YPD media. Aliquots of 4 μl obtained from serial 10-fold dilutions were spotted onto YPD plates containing various drug concentrations, with the plates then incubated at 30 °C for 2 days. Plates containing CPT were prepared with 25 μM HEPEs, pH 7.2, and 0.125% MeSO as described previously (59). In other experiments, equal numbers of cells (ranging from 200 to 400) were dispensed on YPD plates containing either no drug or various concentrations of drug.

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2 M. Snyder and C. Copeland, unpublished data.
incubation at 30 °C for 2 days, colonies greater than 1 mm in diameter were counted.

RESULTS

The N Terminus of Nucleolin Is Necessary and Sufficient for Top1 Binding—To identify regions of nucleolin involved in Top1 binding, we generated a recombinant protein consisting of nucleolin fused to the C terminus of GST. Since we were unable to express a fusion protein containing the full-length nucleolin protein in bacteria, we used a galactose-inducible yeast expression system (54). The GST-nucleolin fusion protein was purified from yeast extracts using glutathione affinity chromatography and analyzed by SDS-PAGE and immunoblotting. Purified preparations of GST-nucleolin contained a predominant Mr 126 band, which is the expected migration of a GST-nucleolin fusion protein (Fig. 1A). This Mr 126 protein was recognized by both nucleolin and GST antibodies (Fig. 1B and data not shown). To determine whether the recombinant nucleolin binds Top1, beads loaded with either purified GST-nucleolin or GST alone were incubated with HeLa cell nuclear extracts in a phosphate-buffered saline-based buffer. Beads containing GST-nucleolin, but not GST alone, bound Top1 in nuclear extracts (Fig. 1C). Taken together with our previous results using purified mammalian nucleolin (23), these data indicate that both purified and recombinant nucleolin bind Top1 in vitro.

Next, we investigated Top1 binding by a series of nucleolin deletion fragments. We expressed and purified nucleolin fragments containing the acidic N-terminal region (1–323) or portions thereof (1–125, 224–323), or the remainder of the protein, consisting of the four RBD motifs and the GAR C-terminal domain (323–707; Fig. 2A). In each case, the purified preparation contained a band of the expected mobility that was reactive with a GST antibody (Fig. 2B). In some preparations lower molecular weight bands that were reactive with the GST antibody were also detected and presumably represent degradation products (Fig. 2, A and B). In pull-down assays using HeLa nuclear extracts and GST-nucleolin fragments, an N-terminal nucleolin fragment containing residues 1–225 was identified as being necessary and sufficient for binding of Top1 (Fig. 2B). Immunoblotting with a GST antibody indicated that these results could not be explained by the presence of different quantities of GST-nucleolin fragments (Fig. 2B). Additional experiments indicated that two discrete fragments within the nucleolin N-terminal region, 1–125 and 125–225, were both capable of binding Top1, although more Top1 was bound by the 125–225 fragment than by the 1–125 fragment (Fig. 2C). Immunoblotting with a GST antibody indicated that this phenomenon was not due to the presence of a greater quantity of the 125–225 fragment relative to the 1–125 fragment (Fig. 2C).

Notably, the 125–225 nucleolin fragment has a predicted pI of 3.48, whereas the 1–125 fragment has a predicted pI of 9.45. In addition, the 224–323 fragment has a predicted pI of 3.70 and 38 sequential acidic residues (the longest uninterrupted acidic domain in the protein) and does not bind Top1. Therefore, although the acidic regions common to both the 1–125 and 125–225 nucleolin fragments may be involved in Top1-binding, global acidic charge is not sufficient to explain binding of nucleolin fragments to the relatively basic Top1 protein.

The N terminus of nucleolin is implicated in NLS binding and nucleocytoplasmic transport (39, 40). In addition, nucleolin binds an NLS-containing region in Top1 that is required for the nuclear localization of Top1 (23, 60). Taken together with our finding that the N terminus of nucleolin is sufficient for Top1 binding, these data suggest that nucleolin might function in the nucleocytoplasmic transport of Top1. While a variety of strategies might be envisioned to test this hypothesis in mammalian cells, the facility of yeast in previous studies of Top1 prompted us to investigate this question using S. cerevisiae (61).

Physical Interaction between Nsr1p and Yeast Top1p—The S. cerevisiae protein Nsr1p is similar to nucleolin in sequence and function (51, 62–64): Nsr1p has an acidic N-terminal region with several putative phosphorylation sites, a middle region with two RBDs, and a C-terminal GAR domain. To determine whether yTop1p physically interacts with Nsr1p, we initially attempted co-immunoprecipitations using yTop1p and Nsr1p antibodies, but were not able to precipitate either protein using its respective antibody. We therefore constructed a vector expressing a GST-Nsr1p fusion protein under the control of a galactose-inducible promoter. In SDS-PAGE analyses of lysates from yeast that were induced to express GST-Nsr1p, an appropriately migrating protein was recognized by an Nsr1p antibody (data not shown). Subsequently, lysates from cells expressing GST alone or the GST-Nsr1p protein were incubated with glutathione beads, with bound proteins subjected to immunoblotting using GST and yTop1p antibodies. In cells expressing GST alone, only GST was precipitated by glutathione beads (Fig. 3C). By contrast, in cells expressing GST-Nsr1p, both this protein and yTop1p were precipitated by the beads (Fig. 3C). Taken together with the prior nucleolin-Top1
data (23), these results indicate that a physical interaction between nucleolin and Top1 orthologs is detectable in both yeast and human cells.

**Altered Localization of yTop1p in a Dnsr1 Strain**—To determine whether the yTop1p-Nsr1p interaction is important in the cellular localization of yTop1p, we studied yTop1p localization in isogenic **NSR1** and **Dnsr1** strains expressing yTop1p fused to GFP. In the **NSR1** and **Dnsr1** strains, expression of the GFP protein alone was associated with diffuse cellular fluorescence (data not shown). By contrast, in the **NSR1** strain the GFP-yTop1p protein exhibited a punctate subnuclear fluorescent pattern suggestive of nucleolar localization (Fig. 4A). Similar results were obtained when GFP-yTop1p was expressed in an **NSR1** Δtop1 strain, indicating that GFP-yTop1p localization is not altered by the presence of endogenous yTop1p (data not shown). In contrast to **NSR1** cells, GFP-yTop1p fluorescence was present diffusely throughout the nucleus in Δsnr1 cells (Fig. 4A). Furthermore, in some Δsnr1 cells the GFP-yTop1p fluorescence appeared to include an area larger than the DAPI-stained nucleus, suggestive of a perinuclear distribution (Fig. 4A, arrow). These data indicate that **NSRI** is important in determining the cellular localization of a recombinant yTop1p protein.

To determine whether the alteration in GFP-yTop1p localization was related to a general disruption in nucleolar structure, we also studied the localization of a GFP-Nop1p fusion protein in the two strains (65). In contrast to the results obtained with GFP-yTop1p, GFP-Nop1p localization was similar in **NSR1** and Δsnr1 strains (Fig. 4B). As an additional control, we questioned whether the altered localization of yTop1p in

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**Fig. 2. Identification of the Top1-binding domain in nucleolin.** A, silver stain of GST-nucleolin fragments purified from yeast lysates. The nucleolin residues contained in each protein are indicated. A schematic of nucleolin is included. B, GST pull-down assays using HeLa nuclear extracts and GST or the indicated GST-nucleolin fragments were performed as described in the Fig. 1 legend. Bound proteins were analyzed by SDS-PAGE followed by sequential immunoblotting using Top1 (top panel) and GST (bottom panel) antibodies. 2 μg of HeLa nuclear lysate were loaded directly onto the gel as a control. C, results of pull-down assays using additional N-terminal fragments of nucleolin. D, schematic of the results of the binding experiments, with predicted pI values of nucleolin fragments indicated.
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yTop1p co-precipitates with Nsr1p. Lanes 1 and 2, pull-down assays were performed using 20 μl of glutathione beads and 3 mg of lysate obtained from yeast expressing either GST or GST-Nsr1p (indicated at the top of each lane). Bound proteins were analyzed by SDS-PAGE and immunoblotting using yTop1p (top panel) and GST (bottom panel) antibodies. Lanes 3 and 4, 30 μg of lysate obtained from yeast expressing either GST or GST-Nsr1p were loaded directly onto the gel. Bands representing yTop1p, GST-Nsr1p, and GST are indicated by arrows.

A. NSR1+ Δnsr1

Top1

B. NSR1+ Δnsr1

Nop1

Phase

C. NSR1+ NSR1+ Δphen

Top1

Fig. 4. Altered localization of GFP-yTop1p in Δnsr1 cells. A, S. cerevisiae strains FY251 (NSR1+) and TE25 (Δnsr1) were transformed with vectors expressing GFP-yTop1p. Two hours after induction of protein expression by addition of galactose, the cells were stained with DAPI and imaged by fluorescent and phase contrast microscopy. The arrow indicates a cell in which the GFP-yTop1p fluorescence encompasses an area larger than the DAPI-stained nucleus. B, the same strains were transformed with a vector expressing GFP-Nop1p and imaged 2 h after induction of protein expression by galactose. C, GFP-yTop1p localization in FY251 cells before and after a 25-min exposure to 100 μg/ml of the transcriptional inhibitor phenanthroline.

Δnsr1 cells was related to the transcriptional defect present in Δnsr1 cells (51). Exposure to 100 μg/ml phenanthroline (66), which is sufficient to inhibit [3H]uridine incorporation in the NSR1+ strain by approximately 50% (data not shown), did not affect the localization of GFP-yTop1p in this strain (Fig. 4C). These findings indicate that the altered distribution of GFP-
yTop1p in Δnsr1 cells is not due to gross disruption of nucleoli or transcription.

Immunoblotting experiments were performed using whole cell, nuclear, and cytoplasmic lysates to further characterize the cellular localization of GFP-yTop1p and endogenous yTop1p in NSR1+ and Δnsr1 cells. Levels of GFP-yTop1p in whole cell lysates were similar in the two strains, excluding overexpression of GFP-yTop1p as a cause for the more diffuse localization of this protein in the Δnsr1 strain (Fig. 5). Expression of endogenous yTop1p was also similar in the two strains (Fig. 5). Furthermore, GFP-yTop1p and endogenous yTop1p were detectable in nuclear but not cytoplasmic lysates in both strains (Fig. 5). GFP-yTop1p was also undetectable when the cytoplasmic lysates were immunoblotted with a GFP antibody (data not shown). Coomassie staining excluded unequal loading as a cause for the lack of detection of GFP-yTop1p in the cytoplasm of Δnsr1 cells (Fig. 5). In addition, actin was detectable in cytoplasmic but not nuclear lysates (Fig. 5). These results are consistent with those obtained using fluorescent microscopy and indicate that yTop1p remains predominantly intranuclear in Δnsr1 cells.

Loss of TOP1 Results in a Growth Defect in a NSR1+ but Not a Δnsr1 Strain—Since loss of NSR1 in yeast confers a more significant growth defect than loss of TOP1 (51, 62, 67), and our data indicate that the yTop1p and Nsr1p proteins may exist as a complex, we hypothesized that growth of a strain lacking both proteins might be similar to that of a strain lacking NSR1 alone. To test this hypothesis, strain JCW27, which lacks TOP1, and strain TE13, which lacks NSR1, were mated and tetrads from the cross were dissected. Most tetrads yielded four viable colonies, suggesting that a Δnsr1/Δtop1 strain was viable (Fig. 6A). Indeed, immunoblotting revealed that several segregants lacked both Top1p and Nsr1p expression (Fig. 6B and data not shown). The growth rates of tetratype colonies were compared at 30 °C using nonselective media. Similar to previous studies (51, 67), we found that loss of NSR1 resulted in a 1.5-fold decrease in exponential growth rate at 30 °C, whereas loss of TOP1 resulted in a detectable but lesser growth defect (Fig. 6A and data not shown). By contrast, loss of TOP1 in the Δnsr1 strain did not result in a further growth defect.
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FIG. 6. Loss of TOP1 results in a growth defect in a NSR1+ but not a ∆nsr1 strain. A, tetrads resulting from a JJC27 (Δtop1 NSR1+) × TE13 (TOP1+ ∆nsr1) cross were dissected and grown at 30 °C on nonselective media for 4 days. Growth of four tetrads is shown. B, lysates obtained from segregants D1–4 were analyzed by immunoblotting with Nsr1p (top panel) and yTop1p (bottom panel) antibodies. The inferred genotype is indicated at the top of each lane.

(Fig. 6A and data not shown). Thus, the growth rate of a strain lacking both TOP1 and NSR1 is similar to that of a strain lacking NSR1 alone.

Loss of NSR1 Results in Resistance to Camptothecin and Increased Sensitivity to m-AMSA—We questioned whether or not the alteration of yTop1p distribution in ∆nsr1 strains was associated with a change in cellular sensitivity to the topoisomerase I-targeting drug CPT. Drug sensitivity assays were performed using strains JSN284 and an isogenic ∆nsr1 derivative (TE284), which contain a drug permeability alteration that enhances cellular accumulation of CPT and other drugs (3). When serial dilutions of large numbers of cells were spotted in the absence of drug or in the presence of Me2SO alone, the growth defect conferred by loss of NSR1 was apparent (Fig. 7A). However, in the presence of 5 μg/ml CPT the growth of both strains appeared similar, with the NSR1+ strain exhibiting a 100-fold decrease in clonogenicity under these conditions and the ∆nsr1 strain exhibiting only a 10-fold decrease in clonogenicity (Fig. 7A). By contrast, the presence of 100 μg/ml of the topoisomerase II poison m-AMSA minimally affected the growth of the NSR1+ strain, whereas this drug concentration resulted in about a 10-fold decrease in clonogenicity in the ∆nsr1 strain (Fig. 7A). The strains exhibited similar decreases in clonogenicity in the presence of 0.001% of the alkylating agent methyl methane sulfonate or 0.75 milli-units/ml of the DNA-damaging agent bleomycin (Fig. 7A). To confirm that loss of NSR1 alters cellular sensitivity to CPT and m-AMSA, we also examined the survival of a few hundred isolated cells from each strain on agar plates containing different CPT and m-AMSA concentrations. Consistent with the serial dilution assays, the results of these experiments indicated that the ∆nsr1 strain was relatively resistant to CPT but hypersensitive to m-AMSA (Fig. 7B).

DISCUSSION

Based upon reports that nucleolin is a helicase, we initially hypothesized that the nucleolin-Top1 complex might be analogous to other helicase-topoisomerase interactions, such as the T antigen-Top1 and Sgs1-Top3 complexes (24, 25, 68, 69). However, our purified GST-nucleolin did not exhibit helicase activity (data not shown), and others have not been able to detect helicase activity using purified mammalian nucleolin (49). As an alternate hypothesis, we considered the possibility that nucleolin functions to recruit Top1 to sites of rDNA transcription. Consistent with this model, our data indicate that the N terminus of nucleolin is necessary and sufficient for binding to Top1. Olson and Melese originally proposed that the acidic N-terminal region of nucleolin recruits proteins important in ribosome biogenesis, while the nucleolin RBDs interact with rRNA (37, 70). Recent data confirm and extend this model of nucleolin as a scaffold protein. Nucleolin binds avidly to G quartets, which are four-stranded planar structures that form in G-rich DNA in vitro and are likely to occur in the G-rich rDNA (71). The N terminus of nucleolin is dispensable for G quartet binding (71). Similarly, an in vitro assay demonstrated that the N-terminal −300 residues of nucleolin are dispensable for rRNA binding, but required for the first step in rRNA processing, which involves cleavage of the 5′ external transcribed spacer (72). Thus, nucleolin may organize an rRNA synthesis and processing complex, with the RBDS and GAR domain involved in localizing nucleolin to rDNA transcription sites and the N terminus involved in recruitment of proteins such as Top1 (49). Interestingly, like Top1 (45, 46, 73, 74), nucleolin has been implicated in transcriptional elongation (75). Therefore, it is possible that the nucleolin-Top1 interaction is particularly important in the elongation phase of rDNA transcription.

The hypothesis that nucleolin functions to recruit Top1 to sites of rDNA transcription in the nucleolus is supported by our studies of the S. cerevisiae nucleolin ortholog Nsr1p, which indicate that Nsr1p binds yTop1p and is important in determining the subnuclear localization of yTop1p. The apparent localization of a GFP-yTop1p fusion protein to the nucleolus of wild-type yeast cells is consistent with previous studies indicating that yTop1p is important in rDNA transcription, rDNA genomic stability, and rDNA silencing (44, 47, 48, 76–78). Top1 may also be important in nucleolar RNA processing: mammalian cells treated with CPT exhibit defects in rRNA processing (41, 42) and decreased 60 S ribosomal subunits have been observed in yeast Δtop1 mutants (79). Notably, our finding that loss of TOP1 results in a growth defect in an NSR1+ but not in an isogenic ∆nsr1 strain supports a predominant role for yTop1p in the nucleolus.

Results obtained with the ∆nsr1 strain also indicate that cellular sensitivity to CPT may be modulated by proteins that bind Top1. Although previous work indicated that helicase-Top1 interactions may inhibit CPT-mediated cleavable complex formation (14), to our knowledge our studies are the first to demonstrate that loss of a Top1-binding protein may change cellular sensitivity to CPT. Since the localization of yTop1p is altered in the ∆nsr1 strain, it is tempting to speculate that this altered localization is responsible for the resistant phenotype. An intriguing possibility is that cleavable complex formation after CPT exposure is reduced in the ∆nsr1 strain as a result of mislocalized yTop1p. A plausible alternative model is that cleavable complex formation after CPT exposure is maintained in the ∆nsr1 strain, but that conversion of these complexes to
lethal DNA damage is reduced as a result of altered localization of yTop1p. Notably, a defect in topoisomerase I function in the Δnsr1 strain may also explain the hypersensitivity to m-AMSA exhibited by this strain, since increased sensitivity to topoisomerase II-targeting drugs is also found in strains in which TOP1 is disrupted, presumably resulting from a compensatory activity of Top2p (4).

While we cannot exclude the possibility that the slower growth rate of the Δnsr1 strain may also explain the hypersensitivity to m-AMSA exhibited by this strain, since increased sensitivity to topoisomerase II-targeting drugs is also found in strains in which TOPI is disrupted, presumably resulting from a compensatory activity of Top2a (4).

NSR1. Notably, drug resistance associated with altered localization of a topoisomerase has been described for several mammalian cell lines that are resistant to Top2-targeting drugs, in which the cellular localization of Top2a is cytoplasmic rather than nuclear as a result of mutations in the Top2a NLS (81–84).

Our results are also relevant to the altered localization of Top1 that occurs after cellular exposure to CPT. Recent experiments using a GFP-tagged N-terminal region of Top1 indicate that after treatment with CPT, the localization of this fusion protein changes from a nucleolar to a diffusely nuclear pattern (85). Moreover, a change in nucleolin localization from the nucleolus to the nucleus or even cytoplasm was observed after cellular exposure to mitomycin C, heat shock, or actinomycin D (86, 87). The N terminus of Top1 contains the nucleolin-binding site but is catalytically inactive and does not interact with CPT (23). Thus, it is possible that either a change in nucleolin

FIG. 7. Loss of NSR1 confers resistance to camptothecin and hypersensitivity to m-AMSA. A, isogenic NSR1+ (strain JN284) and Δnsr1 (strain TE284) colonies were grown overnight in YPD and the cultures adjusted to an A600 of 2.0. Duplicate 4-μl aliquots of serial 10-fold dilutions were then spotted onto YPD plates, which were incubated at 30 °C for 2 days. As indicated, the plates either contained no drug, 0.001% methyl methane sulfonate, 0.75 milliunit/ml bleomycin, 100 μg/ml m-AMSA, or 5 μg/ml camptothecin (in 0.125% MeSO, 25 mM HEPES, pH 7.2). As an additional control, cells were also spotted onto a YPD plate containing 0.125% MeSO and 25 mM HEPES, pH 7.2 (labeled - DMSO). B, equal numbers of either NSR1+ (open squares) or Δnsr1 (closed circles) cells were incubated for 2 days at 30 °C on YPD plates containing either no drug or various concentrations of camptothecin or m-AMSA. Results are expressed as a percentage of the number of colonies present on plates lacking drug. The mean values of duplicate independent experiments are shown, with S.E. bars included.
localization or in the interaction between nucleolin and the N terminus of Top1 is involved in the altered localization of Top1 that occurs after CPT exposure.

Acknowledgments—We thank the following individuals: Mary-Ann Bjornsti for helpful discussions as well as vectors for strain JN924, Masayuki Iioyori for pICS16 and pYCB5, John Woolford for the Nsr1p antibody, Y. C. Cheng for the human Top1 antibody, W. Steven Ward for assistance with fluorescence microscopy, Nancy C. Walworth for assistance with tetrasections, and Y. Lin and W. J. Shi for statistical analyses.

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