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The ER Repeat Protein YT521-B Localizes to a Novel Subnuclear Compartment

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Abstract. The characterization of distinct subnuclear domains suggests a dynamic nuclear framework supporting gene expression and DNA replication. Here, we show that the glutamic acid/arginine-rich domain protein YT521-B localizes to a novel subnuclear structure, the YT bodies. YT bodies are dynamic compartments, which first appear at the beginning of S-phase in the cell cycle and disperse during mitosis. Furthermore, in untreated cells of the human cell line MCF7 they were undetectable and appeared only after drug-induced differentiation. YT bodies contain transcriptionally active sites and are in close contact to other subnuclear structures such as speckles and coiled bodies. YT bodies disperse upon actinomycin D treatment, whereas other transcriptional inhibitors such as α-amanitin or DRB have little effect. On the basis of our experiments, we propose that YT521-B may participate in the assembly of genes into transcription centers, thereby allowing efficient regulation of gene expression.

Key words: subnuclear compartments • transcription • cell cycle • MCF7 differentiation • actinomycin D

Introduction

The compartmentalization of the nucleus into discrete domains contributes to the complexity of processes involved in gene expression and its regulation. Various detection methods have revealed an increasing number of distinct subnuclear structures, and the characterization of the proteins contained within these domains opens up the possibility to investigate their function. The best characterized compartment to date, the nucleolus, is the site of rRNA synthesis and pre-ribosomal assembly, whereas the functions of most other subnuclear structures are much less clear (reviewed in Matera, 1999; Spector, 1993; Nickerson et al., 1995).

Several classes of subnuclear domains have been observed. Some, such as the nucleolus and coiled bodies, fulfill particular roles in the maturation of processing RNAs, for example snRNPs or rRNA, and were therefore called nuclear factories (Matera, 1999). Other nuclear factories, such as the Oct1/PTF/transcription (OPT)[1] domain, constitute a compartment where a specific group of genes is brought together, thereby making transcriptional regulation more efficient (Pombo et al., 1998). Recently, it was shown that at least a subset of promyelocytic leukemia (PML) bodies and the perinuclear compartment (PNC) rapidly accumulate FITC-labeled nucleotides, suggesting that they may be sites of transcriptional activity (Huang et al., 1998; LaMorte et al., 1998). However, it cannot be excluded that the transcripts were initially synthesized elsewhere and then translocated into these compartments. Another domain class is formed by the human polycomb group complex (PcG), which localizes to specific heterochromatic regions, suggesting a role in the constitutive repression of transcription (Saurin et al., 1998).

Some nuclear domains are storage compartments, where certain proteins are kept in an inactive or inaccessible form. Regulatory mechanisms, such as phosphorylation, control the release of these proteins into the nucleoplasm, where they assemble into functional units. Prominent members of this class are the speckles, which are considered to

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[1]*Abbreviations used in this paper:* DRB, 5,6-dichloro-1-β-ribofuranosylbenzimidazole; EGFP, enhanced green fluorescent protein; HNTG, Hepes/NaCl/Triton X-100/Glycerol; OPT, Oct1/PTF/transcription domain; PML, promyelocytic leukemia protein; PP1, [4-amino-5-(4-methylphenyl)-7-(t-butyl) pyrazolo [3,4-d]-pyrimidine; RIPA, radioimmune precipitation assay; SAF-B, scaffold attachment factor B; SAR/MAR region, scaffold attachment/matrix attachment region; SR protein, serine/arginine-rich protein.

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be storage compartments for splicing factors (Spector, 1993; Misteli and Spector, 1998). Moreover, some transcription factors have been shown to localize in discrete dots throughout the nucleus, and it is thought that these may also represent storage compartments since they do not coincide with regions of transcriptional activity (van Steensel et al., 1995). Nuclear factories and storage compartments are dynamically linked to RNA polymerase activity. Speckles change their morphology under the influence of transcriptional inhibitors (Spector et al., 1983; Carmo-Fonseca et al., 1992; Misteli et al., 1997; Nayler et al., 1998b) and coiled bodies change their composition upon transcriptional inhibition and eventually disperse (Carmo-Fonseca et al., 1992; Matera, 1999). Recently, it was shown that transcription and replication sites, which are both active during S-phase, are found in distinct and separate subnuclear domains, and it was proposed that overlapping sites are temporally separated. This implicates that a given site is either transcriptionally active or replicates (Wei et al., 1998). Together with the characterization of novel subnuclear domains, these results provide further evidence for the existence of a dynamically regulated nuclear architecture supporting the compartmentalization of the nucleus (Nakayasu and Berezney, 1989; Jackson et al., 1993; Ma et al., 1998).

We previously identified a nuclear protein, YT521-B, as a 110-kD protein containing an amino-terminally located glutamic acid–rich domain (E-box) and a characteristic glutamic acid/arginine–rich domain (ER-domain) at the carboxy-terminal end (Hartmann et al., 1999). ER-domain proteins comprise a growing number of molecules and several family members are involved in RNA metabolism (Hartmann et al., 1999). Moreover, it was suggested that ER repeat proteins may contribute to the development of neurodegenerative diseases (Assier et al., 1999). Transiently expressed EGFP-YT521-B fusion proteins localized to the nucleus and displayed a characteristic pattern of nuclear bodies, which varied in number and size. Furthermore, transient expression of YT521-B modulated splicing of reporter minigenes in a dose dependent manner. Using immunoprecipitation and yeast two-hybrid experiments, we have shown that YT521-B interacts with scaffold attachment factor B (SAF-B) and the 68-kD Src substrate associated during mitosis (Sam68; Hartmann et al., 1999). SAF-B forms a ternary complex with RNA polymerase II and SR proteins at the so-called scaffold or matrix attachment regions (SAR/MAR; Nayler et al., 1998a). Sam68, an RNA binding protein, colocalizes with YT521-B in nuclear dots, which dispersed upon coexpression of the Src family kinase $p59^{Fyn}$ (Hartmann et al., 1999). Together, these results suggested that $p59^{Fyn}$-induced signal transduction pathways could affect RNA metabolism through SAF-B, Sam68, and YT521-B.

Here, we have investigated the localization of the endogenous YT521-B protein and show that it defines a novel subnuclear domain, termed YT bodies. YT bodies were found in a proportion of cells in a variety of cell lines, although they were undetectable in the human cell line MCF7. There, YT bodies only formed after sodium butyrate-, PMA-, or tamoxifen-induced cell differentiation. In vivo, actinomycin D, but no other transcriptional inhibitor, reversibly perturbed the localization of YT521-B into discrete domains. Our results suggest that the YT521-B protein displays novel biochemical characteristics and may participate in the organization of chromosomal domains independently of RNA polymerase activity.

Materials and Methods

Antisera

A rabbit polyclonal antiserum (PK2) was raised against a mixture of two YT521-B protein peptides (P1: RSARSVILIFSVRESGKFQCG; P2: KDGELNVLDDILTEVPEQDDECG) fused to keyhole-limpet hemocyanin. Anti-EGFP antibodies were from Boehringer. Anti–SAF-B antibodies have been described previously (Nayler et al., 1998a). Anti-SC35 antibodies were from Sigma-Aldrich. Anti-PML antibody mAb5E10 was donated by R. van Driel (Stuurman et al., 1992). The anti–p80-coilin antibody 5P10-pi (Almeida et al., 1998) was donated by Maria Carmo-Fonseca and the 66 cd4 antibody against gems (Liu et al., 1997) by U. Fischer. Secondary CY3-coupled antibodies were from Dianova.

Cell Culture

HEK293, COS7 cells, and BHK cells were maintained in DME supplemented with 10% (vol/vol) FCS (Sigma-Aldrich). MCF7 cells were maintained in RPMI 1640 supplemented with 10% (vol/vol) FCS. For immunolabeling experiments, cells were grown on glass coverslips in 6-cm cell culture dishes.

Actinomycin D (Sigma-Aldrich) was added at 0.04 μg/ml for 3 h to inhibit RNA polymerase I, or at 50 μg/ml to inhibit RNA polymerase I, II, and III (Huang et al., 1998; Perry, 1963). 5,6-dichloro-1-β-ribofuranosylbenzimidazole (DRB) was added at 75 μM for 3 h to inhibit RNA polymerase II (Sehgal et al., 1976). α-Amanitin (Sigma-Aldrich) was added at 50 μg/ml for 5 h to inhibit RNA polymerase II or at 300 μg/ml to inhibit RNA polymerase II and III (Pombo et al., 1999). Cycloheximide (Sigma-Aldrich) was added at 100 μg/ml for 5 h to inhibit protein synthesis and RNA polymerase I activity (Higashi et al., 1968). Inhibition of actinomycin D was reversed by changing the medium twice, followed by 2 h of drug-free incubation.

Cell Cycle Analysis

BHK cells were grown to 60–80% confluence and blocked at the G2/M phase with 0.5 μg/ml nocodazole for 4 h. Mitotic cells were then removed by tapping on the cell culture dish, washed, and seeded on glass coverslips (Spector et al., 1998a). The cells were incubated for 2, 4, 6, and 8 h before fixation. Staining was performed as described above, using anti–YT521-B antiserum (PK2). At each time point, 500,000 cells were trypsinized, stained with propidium iodide, and analyzed by flow cytometry as described (Spector et al., 1998a).

MCF7 Differentiation

1×10^6 MCF7 cells were seeded in 10-cm cell culture dishes containing 4 glass coverslips and grown for 24 h. Cells were then incubated with 1 μM sodium butyrate (Calbiochem-Novabiochem), 1 μg/ml Phorbol 12-myristate 13-acetate (PMA; Biomol), or 5 μM Tamoxifen (Calbiochem-Novabiochem) for a further 48 h. The glass coverslips were removed and the cells fixed in 3.7% formaldehyde and PBS for 10 min at room temperature. The cells remaining in the cell culture dish were lysed in 600 μl RIPA and benzonase as described above and 10 μl lysate supernatant was analyzed in Western blot experiments using the anti–YT521-B antiserum (PK2). Two glass coverslips were used for immunostaining with anti–YT521-B antiserum (PK2). The remaining two glass coverslips were used to determine the number of cells containing lipid droplet inclusions. They were first stained with 1% oil red O and 60% triethylphosphate (Sigma-Aldrich) for 15 min, washed in PBS, followed by 100% Haemalaun staining (Merck) for 15 min, and washed in PBS (Novikoff et al., 1980).

Immunolabeling

1×10^5 BHK cells were grown on glass coverslips in 3.5-cm culture dishes for 24 h before fixation and immunostaining. Alternatively, they were transfected 24 h after seeding as described and incubated for further 24 h. Cells were fixed in 3.7% formaldehyde and PBS for 10 min at room tem-

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perature, washed three times in PBS and 0.1% Triton X-100, and blocked in PBS, 0.1% Triton X-100, and 3% bovine serum albumine (Sigma-Aldrich) for 30–60 min at room temperature. Cells were then incubated with the appropriate antibody (diluted in PBS, 0.1% Triton X-100, and 3% bovine serum albumin) overnight at 4°C and washed three times in PBS and 0.1% Triton X-100. Cells were then incubated with CY3-coupled secondary antibody (Dianova) in PBS and 0.1% Triton X-100 for 2 h at room temperature, washed three times in PBS and 0.1% Triton X-100, and mounted on glass slides in Fluormount (Dianova). The cells were examined either by laser scanning fluorescent microscopy (Leica; Figs. 1 D, 2, 3 B, 4, and 5) or by conventional microscopy (ZEISS), followed by deconvolution using the Openlab software (Improvision; Fig. 3 A and 6).

Nucleotide Incorporation Assay

The nucleotide incorporation assay was performed using BHK cells as described (Spector et al., 1998b). Cell permeabilization was with 0.02% digitonin for 2 min, incubation time for RNA elongation was 20 min at 30°C. Br-UTP incorporation was detected with a monoclonal anti-BrdU antibody (Boehringer) and YT bodies were detected using PK2. Incubation on ice with primary and secondary antibodies was for 1 h each. Control incubations were performed in transcription cocktails containing actinomycin D (50 μg/ml). Quantitative analysis was performed using the Optimas program. The Br-UTP signal was defined as region of interest and the amount of YT521-B and Br-UTP overlap was calculated using the double standard deviation of the Br-UTP signal as threshold.

Transfections

3×10^5 HEK293 cells were seeded per 3.5-cm dish and were transiently transfected with 0.5–1 μg plasmid DNA using the calcium precipitation method as described previously (Stoss et al., 1999). In single plasmid transfections, 0.5 μg of expression plasmid was used, and in cotransfections 0.5 μg of each plasmid was used. Cells were harvested 24 h after transfection. 3×10^5 COS7 or BHK cells were seeded per 3.5-cm dish and transfected with a total of 1.5 μg plasmid DNA (0.5 μg of construct and 1 μg of empty carrier vector) and 10 μl Superfect (QIAGEN), according to the manufacturer's instructions. The expression constructs pEGFP-YT521-B (Hartmann et al., 1999) and pFlagSCA1[82] (Skinner et al., 1997) were used.

Cell Lysis and Western Blotting

6×10^5 cells were lysed for 30 min on ice in 200 μl buffer containing 50 mM Hepes, pH 7.5, 150 mM NaCl, 1% Triton X-100, 10% glycerol, 1 mM EDTA, 20 mM sodium pyrophosphate, 2 mM sodium orthovanadate, 100 mM NaF, 5 mM β-glycerophosphate, 1 mM PMSF, and 1 μg/ml aprotinin (HNTG buffer). The lysates were centrifuged at 13,000 *g* for 15 min at 4°C, 200 μl 2× SDS sample buffer was added to the supernatant, and 400 μl 2× SDS sample buffer was added to the remaining pellet. The probes were mixed, boiled for 5 min, and 15 μl of each fraction loaded onto 10% SDS–polyacrylamide gels (Nayler et al., 1997). Alternatively, cells were lysed for 30 min on ice in 200 μl RIPA buffer (0.01 M sodium phosphate, 1% NP-40, 1% sodium deoxycholate, 0.1% SDS, 0.15 M NaCl, 2 mM EDTA, 1 mM NaF, 4 mM sodium orthovanadate, 5 mM β-glycerolphosphate, 1 mM PMSF, 1 μg/ml aprotinin, and 100 U/ml benzonase; Nayler et al., 1998b). Lysates were centrifuged for 15 min at 4°C, 200 μl 2× SDS sample buffer was added to the supernatant, and 400 μl 2× SDS sample buffer was added to the pellet. 15 μl of the fractions was loaded in each lane and analyzed on 10% SDS–polyacrylamide gels. The SDS–polyacrylamide gel–separated proteins were blotted on nitrocellulose membranes (Schleicher & Schuell) using a semi-dry blotting device. The ECL system was used for immunoblot detection (Amersham Pharmacia Biotech).

Nuclear Extraction

COS7 cells were transfected with 0.5 μg pEGFP-YT521-B as described and incubated for 24 h. Nuclear extraction experiments were essentially performed as described (Patturajan et al., 1998). In brief, cells were permeabilized in TBS buffer (10 mM Tris-HCl, pH 7.4, 150 mM NaCl, and 5 mM $MgCl_2$) containing 0.5% Triton X-100 and 0.5 mM PMSF. Cells were then incubated in TBS alone, in TBS containing 20 U/ml benzonase (Sigma-Aldrich), or in TBS containing 4 U/ml DNase-free RNase (Sigma-Aldrich). Finally, cells were extracted with TBS and 0.2 M ammonium sulfate, fixed in 3.7% formaldehyde and PBS for 5 min, and incubated with 1 μg/ml Hoechst no. 33258 and PBS.

Results

The PK2 Antiserum Is Specific for YT521-B

We have previously shown that a transiently expressed EGFP fusion protein of the novel nuclear protein YT521-B concentrates in distinct nuclear foci that vary in size and number. We suggested that these dots may represent nuclear storage compartments from which the protein is released into the nucleoplasm (Hartmann et al., 1999). Similar models were recently proposed for other nuclear proteins such as SR proteins (Misteli and Spector, 1998), SAF-B (Nayler et al., 1998a), or RED (Assier et al., 1999). To address the subcellular localization of the endogenous YT521-B protein, we generated polyclonal sera raised against YT521-B peptides and tested their specificity. In Western blot experiments from HEK293 cell lysates containing transiently expressed EGFP or EGFP-YT521-B fusion protein, one of our antisera, PK2, detected a protein corresponding to the EGFP-YT521-B protein (Fig. 1, A and B). Moreover, in EGFP-YT521-B and EGFP-containing cell lysates, we detected an additional band of ~110 kD that corresponded in molecular mass to the endogenous YT521-B protein (Fig. 1 A). The signal intensities of both EGFP-YT521-B and endogenous 110-kD protein were significantly reduced when the antigenic peptide was added to the antibody solution, suggesting that the PK2 antiserum specifically recognized the YT521-B protein in Western blot experiments (Fig. 1 A). Similarly, we detected endogenous YT521-B in protein lysates of COS7 (Fig. 1 C), BHK (data not shown), and MCF7 cell lines (see below).

To investigate the subcellular localization of the protein recognized by the PK2 antiserum, we performed immunolabeling experiments in COS7 and BHK cells. In both cell lines we detected a punctuated nuclear staining in a proportion of cells (Fig. 1 D). This pattern corresponded with the previously observed localization of EGFP- and FLAG-tagged YT521-B proteins (Hartmann et al., 1999). Again, the pattern was not visible when the antigenic peptide was added to the antibody solution (data not shown). We have noticed, that the endogenous staining pattern was less pronounced than the one observed with EGFP-YT521-B fusion protein and that the observed dots displayed fuzzy boundaries. Upon higher magnification, the boundaries of overexpressed EGFP-YT521-B appeared to be quiet fuzzy, too (data not shown). Together, these experiments suggest that our PK2 antiserum specifically detects endogenous YT521-B in Western blot and immunolabeling experiments. Moreover, the subnuclear localization of the endogenous YT521-B protein in discrete subnuclear domains is similar to overexpressed fusion protein.

YT-containing Dots Are Regulated during the Cell Cycle and Appear after Tamoxifen, Sodium Butyrate, and PMA Treatment of the Human Mammary Adenocarcinoma Cell Line MCF7

We noticed a variability in the number of YT bodies among cells, suggesting that an intrinsic cellular property

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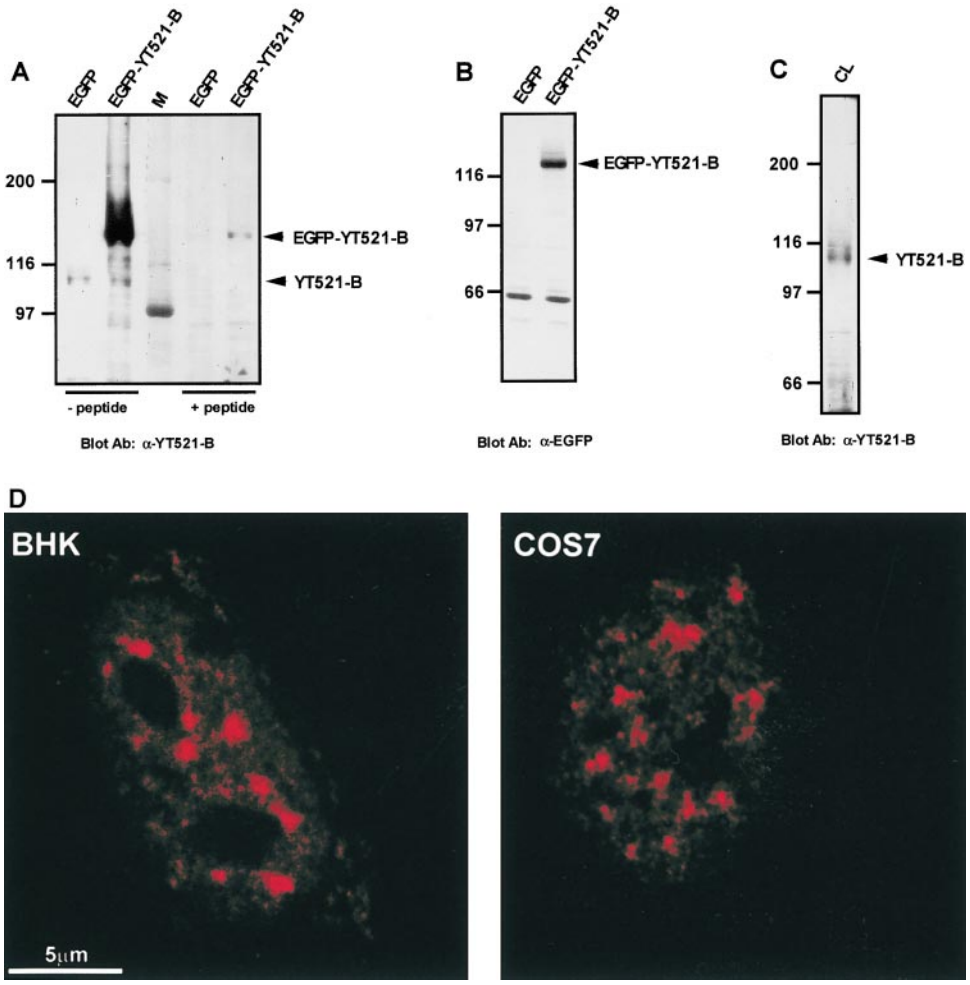

Figure 1. Specificity of the anti–YT521-B antiserum. (A) Total RIPA/benzonase cell lysates of HEK293 cells expressing EGFP or EGFP-YT521-B were loaded onto 10% SDS-PAGE gels and analyzed by Western blot using the anti–YT521-B antiserum PK2 in the absence (−peptide) or presence (+peptide) of the antigenic YT521-B peptide. (B) Reblot of (A) using an anti-EGFP antibody. (C) Total COS7 RIPA and benzonase cell lysate (CL) was analyzed by SDS-PAGE and Western blot using anti–YT521-B antiserum PK2. The YT521-B proteins detected are indicated by arrows. Molecular mass standards are given in kD. (D) BHK (left) and COS7 cells (right) were fixed in 3.7% formaldehyde and immunostained with the anti–YT521-B antiserum PK2 using a secondary CY3-coupled antibody for detection.

influences the formation of these structures. To investigate this further, we used BHK cells and endogenous YT521-B protein to test whether the formation of YT bodies is regulated during the cell cycle. The cells were blocked at the G2/M phase and then released to allow progression through G1 and S phase (Fig. 2 A). Staining at different time points illustrates that YT bodies started to form when cells entered the S-phase. In most cells, both number and distinct definition of YT bodies increased at later stages of S-phase (6 and 8 h; Fig. 2 B). These experiments demonstrate that endogenous YT bodies are regulated during the cell cycle and that their appearance correlates with the onset of transcriptional activity.

To explore this further, we employed the human cell line MCF7. This is a a mammary adenocarcinoma cell line that can be differentiated using sodium butyrate, PMA, or tamoxifen (Maas et al., 1995). After a 48-h incubation period in the presence of these drugs, MCF7 cells arrest in G1 and accumulate cytoplasmic lipid droplets, which are visualized using oil red O staining. After drug treatment, the number of MCF7 cells displaying lipid droplets clearly increased (Fig. 3 A). Less than 10% of all MCF7 cells contained lipid droplets in the absence of drugs, whereas >90% of the cells did in sodium butyrate-treated cells. 40–60% of the cells contained lipid droplets after PMA treatment and >70% of the cells after tamoxifen treatment.

In immunofluorescence studies, we detected a diffuse nuclear staining of endogenous YT521-B in untreated MCF7 cells, and no YT bodies were observed (Fig. 3 B). However, after treatment with sodium butyrate, PMA, or tamoxifen, a dramatic change in the localization of the endogenous YT521-B protein was observed. YT bodies were now very obvious in a majority of cells, suggesting that the differentiation process had also induced changes in the subnuclear structure. To rule out a change in the amount of YT521-B, we performed Western blot analysis and loaded equal amounts of RIPA lysate per lane. We detected no significant change in the amount of YT521-B protein in the presence or absence of drug treatment (Fig. 3 C).

YT521-B Localizes to a Novel Subnuclear Compartment

Endogenous YT521-B and the transiently expressed EGFP-YT521-B fusion protein are concentrated in distinct nuclear bodies. Several nuclear proteins were shown to reside in subnuclear domains such as the nucleolus, the perinuclear compartment, coiled bodies, speckles, or PML bodies (reviewed in Matera, 1999). Here, we wished to determine whether the YT521-B containing dots coincide with any of those known structures displaying a similar morphology or behavior. To address the identity of the YT521-B contain-

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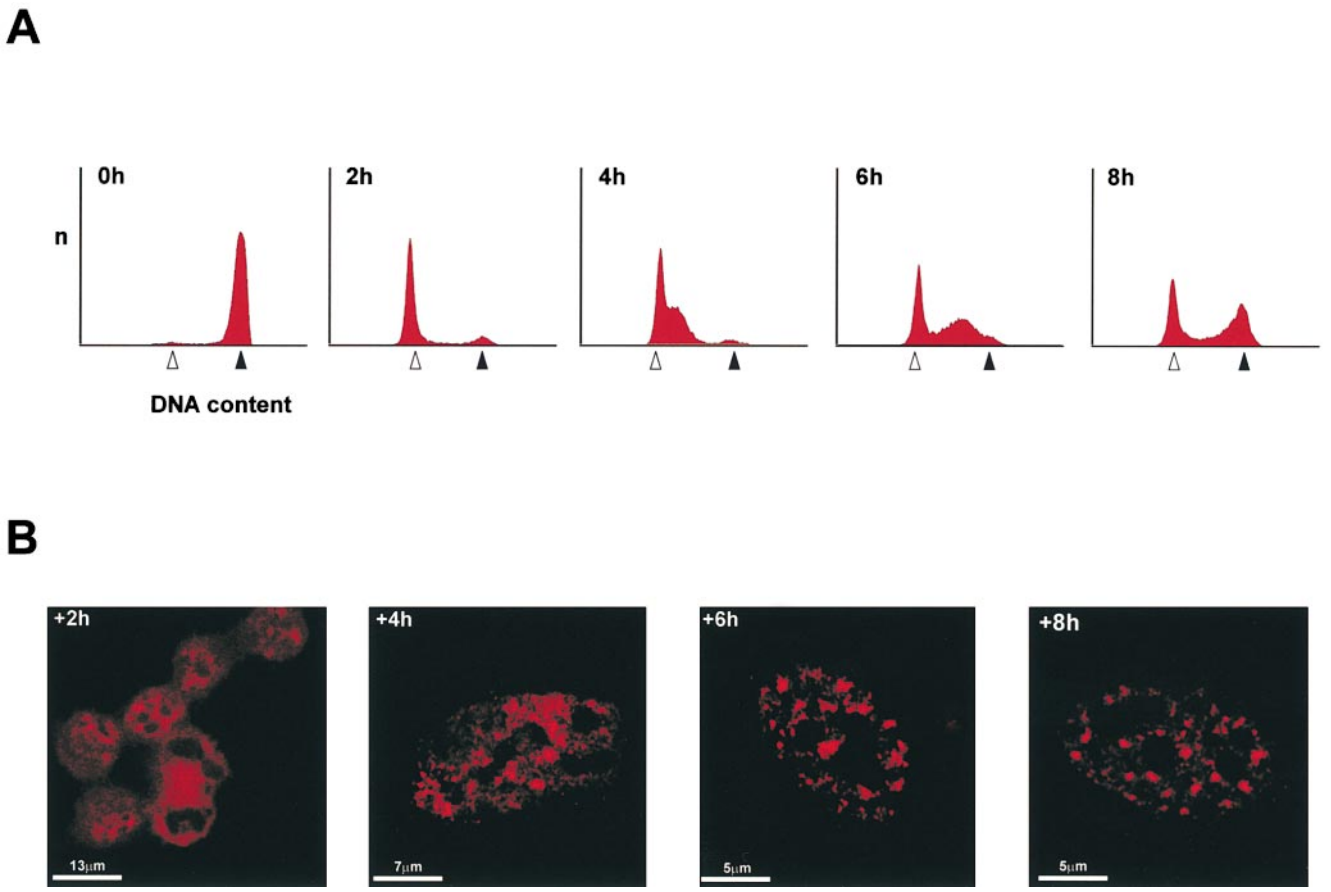

Figure 2. YT521-B structures are regulated during the cell cycle. BHK cells were arrested at the G2/M phase using nocodazole and then incubated in absence of the drug for 2, 4, 6, and 8 h (as indicated). Cells were then fixed and used for FACS analysis or stained with anti–YT521 antiserum (PK2). A. FACS analysis of the cells. An open arrow indicates the G1, a closed arrow the G2/M population of cells. (B) Images of representative cells at the various time points.

ing dots, we compared the localization of reported marker proteins with endogenous YT521-B protein in immunolabeling experiments.

Previously, we demonstrated that transiently expressed YT521-B modulates alternative splicing of reporter minigenes in a concentration-dependent manner, suggesting that it interacts with components of the splicing machinery (Hartmann et al., 1999). To this end, we first tested the splicing factor SC35 that is now widely used as a marker protein for nuclear speckles (Fu and Maniatis, 1992). Costaining of endogenous SC35 and YT521-B revealed a different staining pattern of both markers (Fig. 4 A). However, we found that SC35 and YT521-B protein localize in foci that were in close proximity (Fig. 4 A, boxed). A higher magnification (Fig. 4 B) revealed that in these areas YT521-B containing structures did not overlap, but were rather surrounding speckles, suggesting a molecular crosstalk of both compartments. Similar results have been obtained with overexpressed EGFP-YT521-B (Hartmann et al., 1999, and data not shown).

Similarly, we could not detect a colocalization of YT521-B containing dots with PML bodies (Fig. 4 C), p80-coilin-containing structures (Fig. 4 D) or nuclear gems (Fig. 4 E). However, p80-coilin–containing dots were often found in direct contact with YT521-B containing dots. Furthermore, no colocalization of either hnRNP L or RED protein with YT521-B was observed (data not shown).

The nuclear polyglutamine-containing protein SCA-1 is localized in nuclear inclusions that become much more frequent when the polyglutamine stretch is expanded (Skinner et al., 1997). Nuclear inclusions, which are formed by SCA-1 and other proteins containing expanded polyglutamine repeats, can cause neurodegenerative diseases (Hardy and Gwinn-Hardy, 1998). Strikingly, the size and number of the SCA-1(SCA-1[85]) inclusion bodies resembled the structures observed with EGFP-YT521-B fusion proteins (Hartmann et al., 1999; Skinner et al., 1997). Therefore, we investigated whether transiently expressed SCA-1[82]-Flag colocalizes with EGFP-YT521-B. As shown in Fig. 4 F, EGFP-YT521-B–containing dots did not coincide with SCA-1[82]-Flag protein structures, suggesting that, unlike other nuclear proteins, YT521-B is not drawn into aggregates formed by the SCA-1[82] protein.

In summary, we found that the YT521-B–containing dots do not coincide with any other known subnuclear structures, suggesting that the YT521-B protein forms a novel compartment, which we named YT bodies.

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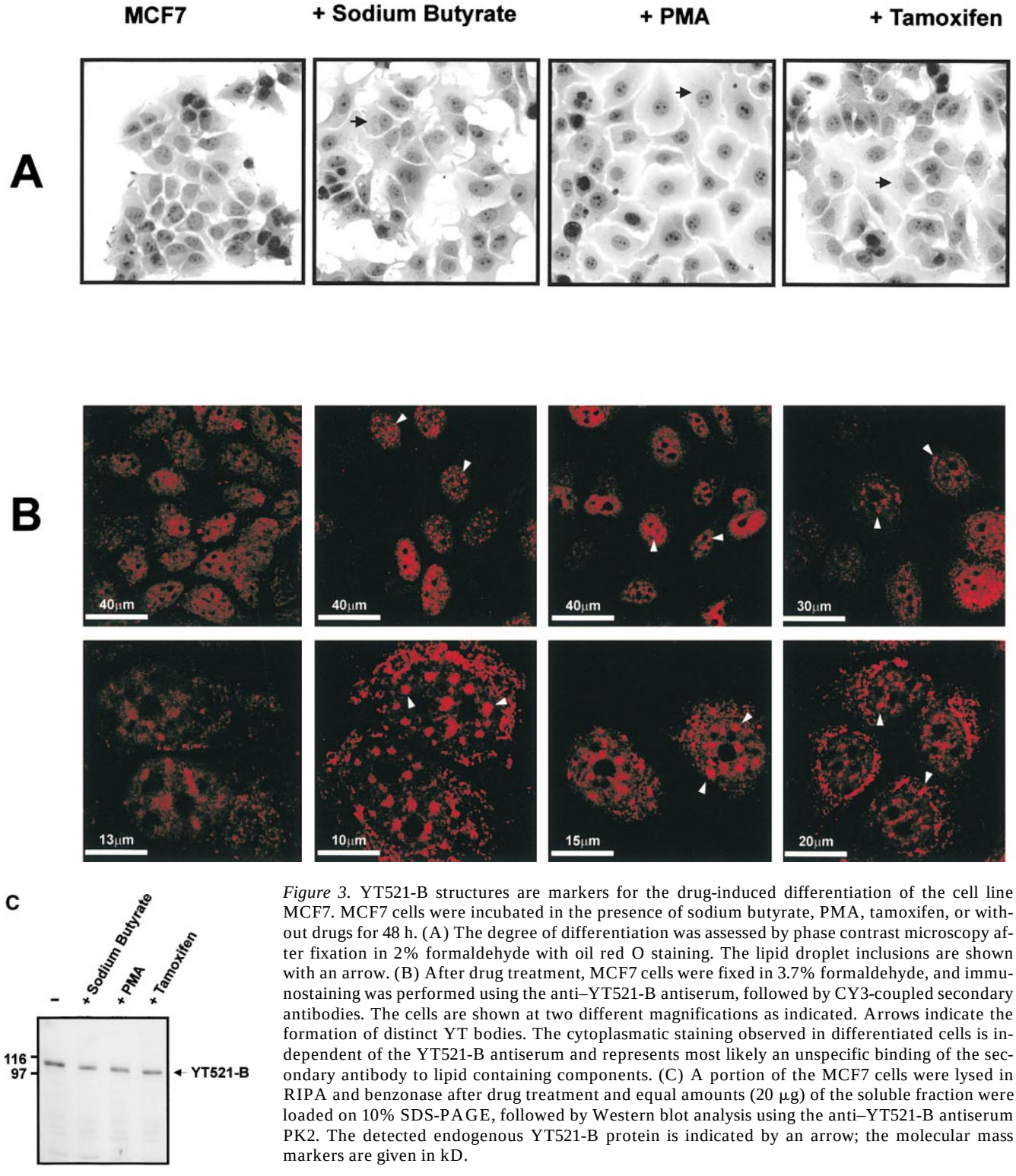

Figure 3. YT521-B structures are markers for the drug-induced differentiation of the cell line MCF7. MCF7 cells were incubated in the presence of sodium butyrate, PMA, tamoxifen, or without drugs for 48 h. (A) The degree of differentiation was assessed by phase contrast microscopy after fixation in 2% formaldehyde with oil red O staining. The lipid droplet inclusions are shown with an arrow. (B) After drug treatment, MCF7 cells were fixed in 3.7% formaldehyde, and immunostaining was performed using the anti–YT521-B antiserum, followed by CY3-coupled secondary antibodies. The cells are shown at two different magnifications as indicated. Arrows indicate the formation of distinct YT bodies. The cytoplasmatic staining observed in differentiated cells is independent of the YT521-B antiserum and represents most likely an unspecific binding of the secondary antibody to lipid containing components. (C) A portion of the MCF7 cells were lysed in RIPA and benzonase after drug treatment and equal amounts (20 μg) of the soluble fraction were loaded on 10% SDS-PAGE, followed by Western blot analysis using the anti–YT521-B antiserum PK2. The detected endogenous YT521-B protein is indicated by an arrow; the molecular mass markers are given in kD.

YT Bodies Contain Focal Sites of Transcription

Accumulating evidence supports the idea that the nucleus is organized into higher-order structures comprising segregated domains of transcriptional and replicational activity (Wei et al., 1998). However, this may not be restricted to the organization of the genome into chromosomal domains alone, but may extend to an underlying organization of the supporting nuclear architecture, which could be involved in the regulation of gene activity (Stein et al., 1998). Based on these findings, we investigated whether YT bodies colocalize with sites of RNA synthesis using in situ BrUTP incorporation (Jackson et al., 1993; Huang et al., 1998). BHK cells were permeabilized and incubated in an RNA synthesis buffer in the presence of BrUTP. After fix-

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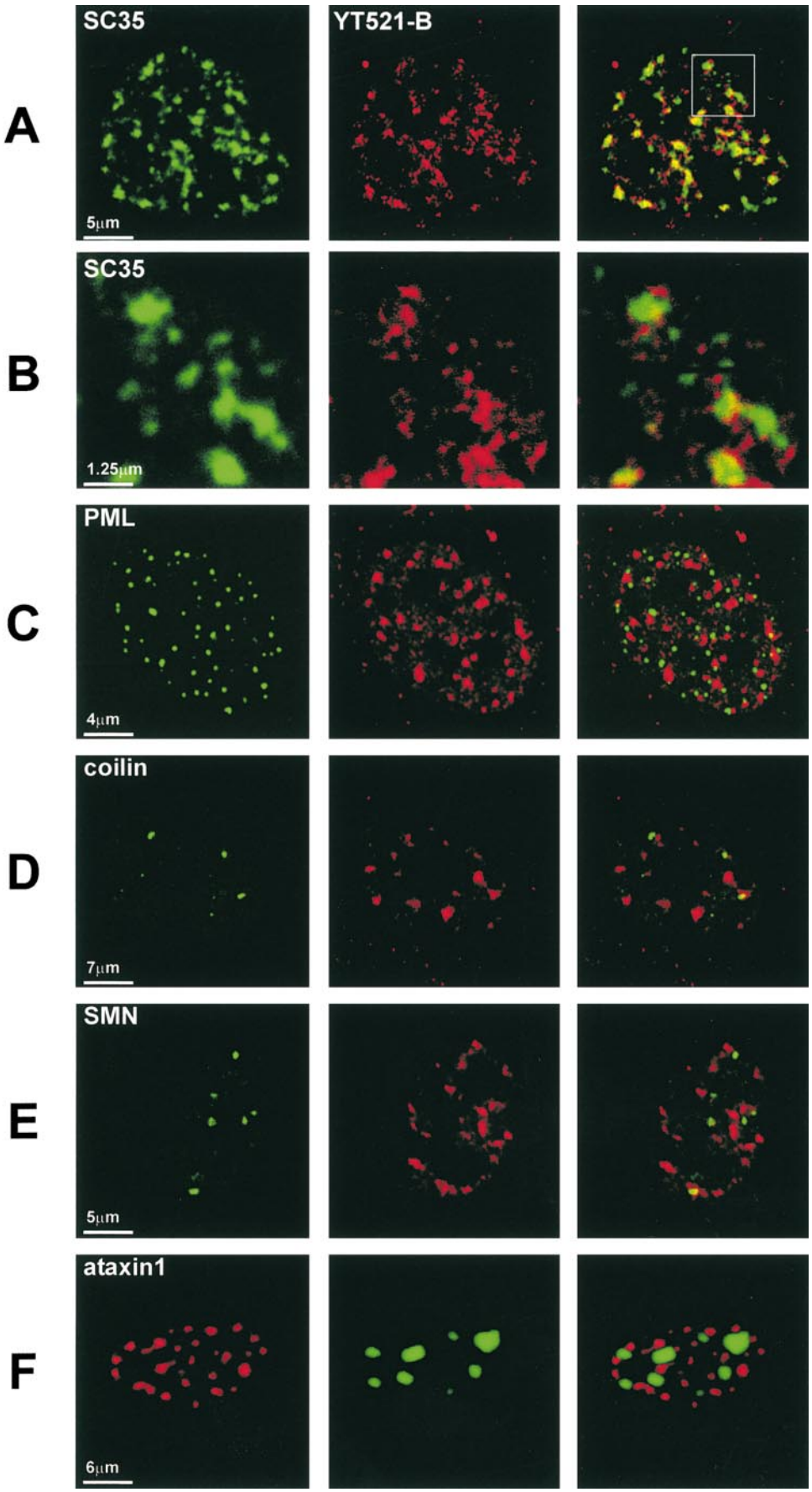

Figure 4. YT521-B protein localizes to a novel subnuclear compartment. BHK cells were fixed with 3.7% formaldehyde and immunostained with antibodies against nuclear markers (left) and YT521-B (middle). The following proteins were used: (A and B) SC35, B is an enlargement of the area indicated with a box in A; (C) PML; (D) p80-coilin; (E) SMN. In (F), cells were cotransfected with pFlagSCA1[82] (red) and pEGFP-YT521-B (green). In A–E, YT521-B was detected using a CY3-coupled secondary antibody (red) and the other nuclear component was visualized with a FITC labeled secondary antibody (green).

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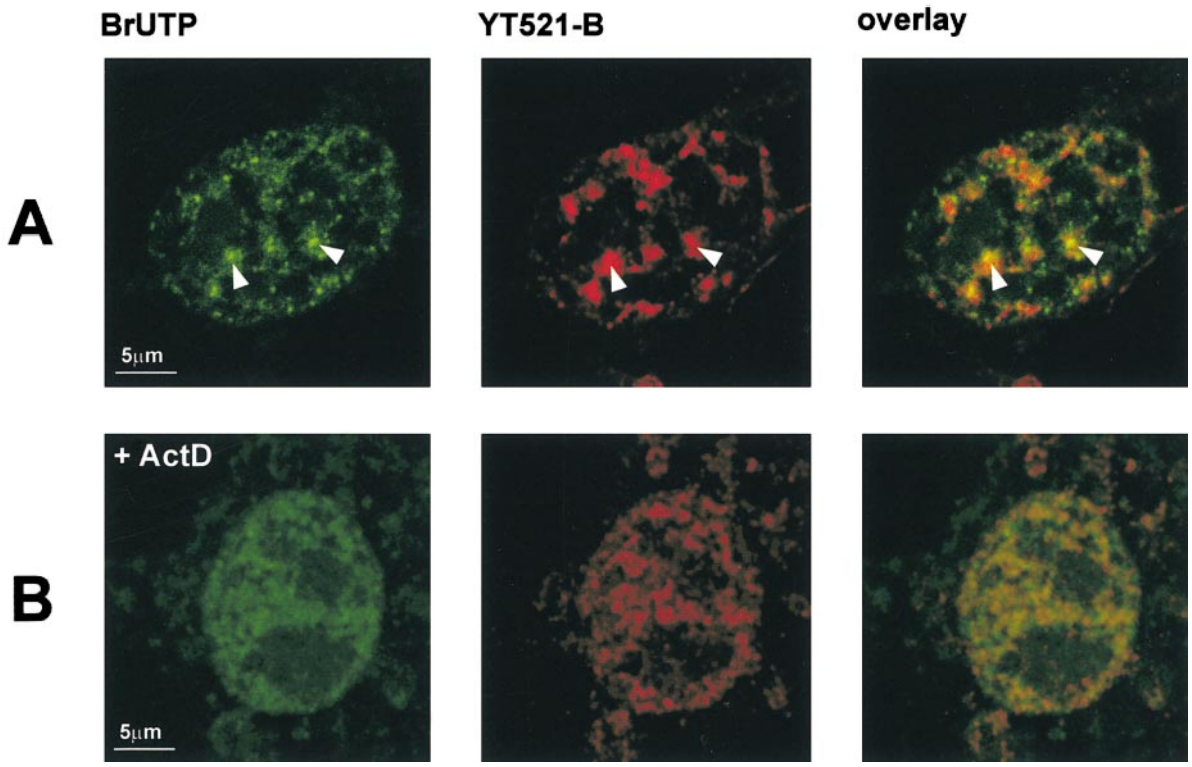

Figure 5. YT bodies contain focal sites of transcription. (A and B) BHK cells were permeabilized with digitonin. After a 20-min incubation period at 30°C in transcription buffer in the absence of transcription inhibitors (A), or 50 μg/ml actinomycin D (B), cells were fixed. BrUTP incorporation was detected using anti-BrdU antibodies and CY3-coupled secondary antibodies. YT521-B was detected with PK2 antiserum. The localization of YT521-B (red) and sites of BrUTP incorporation (green) are shown. Colocalization is visible in the overlay (orange). Some specific sites of colocalization are highlighted with an arrow. The data are the averages of three independent experiments and 150 cells were analyzed in each.

ation, the BrUTP epitope was labeled with a specific monoclonal antibody. As shown in Fig. 5 A, YT bodies contain multiple sites of BrUTP incorporation. The area containing YT bodies harbors an increased amount of BrUTP incorporation activity when compared with the surrounding nucleoplasma. A statistical analysis shows that 37.5 (±3.5%) of BrUTP containing dots are present in YT bodies. To investigate the specificity of the labeling we performed BrUTP incorporation experiments in presence of the transcriptional inhibitor actinomycin D and detected no BrUTP incorporation (Fig. 5 B). Furthermore, actinomycin D treatment also dissolved YT bodies. These experiments show that YT bodies coincide with focal sites of transcription and suggest that the formation of YT bodies may depend on the activity of RNA polymerases.

YT Bodies Are Sensitive to Actinomycin D Treatment

YT bodies contain focal sites of transcription and are formed during S-phase of the cell cycle. This suggests that YT bodies are dynamic structures that could be involved in gene expression. Therefore, we studied the dynamic behavior of YT bodies under the influence of known transcriptional or translational inhibitors more closely. We used COS7 cells transiently expressing EGFP-YT521-B and, after drug treatment, cells were analyzed and quantified by fluorescence microscopy.

Initially, cells were treated for 5 h with 50 μg/ml α-amanitin, which blocks transcription through its direct inhibition of RNA polymerase II (Weinman et al., 1974). As seen in Fig. 6, α-amanitin had no visible effect on the subnuclear domains defined by EGFP-YT521-B, and caused only a modest reduction in the percentage of cells displaying YT bodies (41% in untreated and 31% in α-amanitin–treated cells). Similarly, DRB, which blocks transcription through inhibition of an upstream kinase of RNA polymerase II (Sehgal et al., 1976; Zandomeni and Weinmann, 1984), had little effect on the quality or quantity of the YT bodies (32% in DRB-treated cells). A very different picture was observed after a 3-h treatment with actinomycin D at a concentration of 50 μg/ml (high ActD). Actinomycin D binds to GC-rich double stranded DNA, permitting RNA initiation, but blocking RNA elongation (Perry, 1963). At a concentration of 50 μg/ml, actinomycin D inhibits all three RNA polymerases, and we observed a complete loss of YT bodies. However, the dispersed dots reappeared after a 2-h incubation period after removing the inhibitor (high ActD + resc.), suggesting that the effect is fully reversible. The same effect of actinomycin D was observed when endogenous YT521-B was analyzed (data not shown). At a lower actinomycin D (low ActD) concentration (0.04 μg/ml actinomycin D) or after cycloheximide (100 μg/ml) treatment (Cyclo), only RNA polymerase I is blocked (Perry, 1963; Higashi et al., 1968) and no changes

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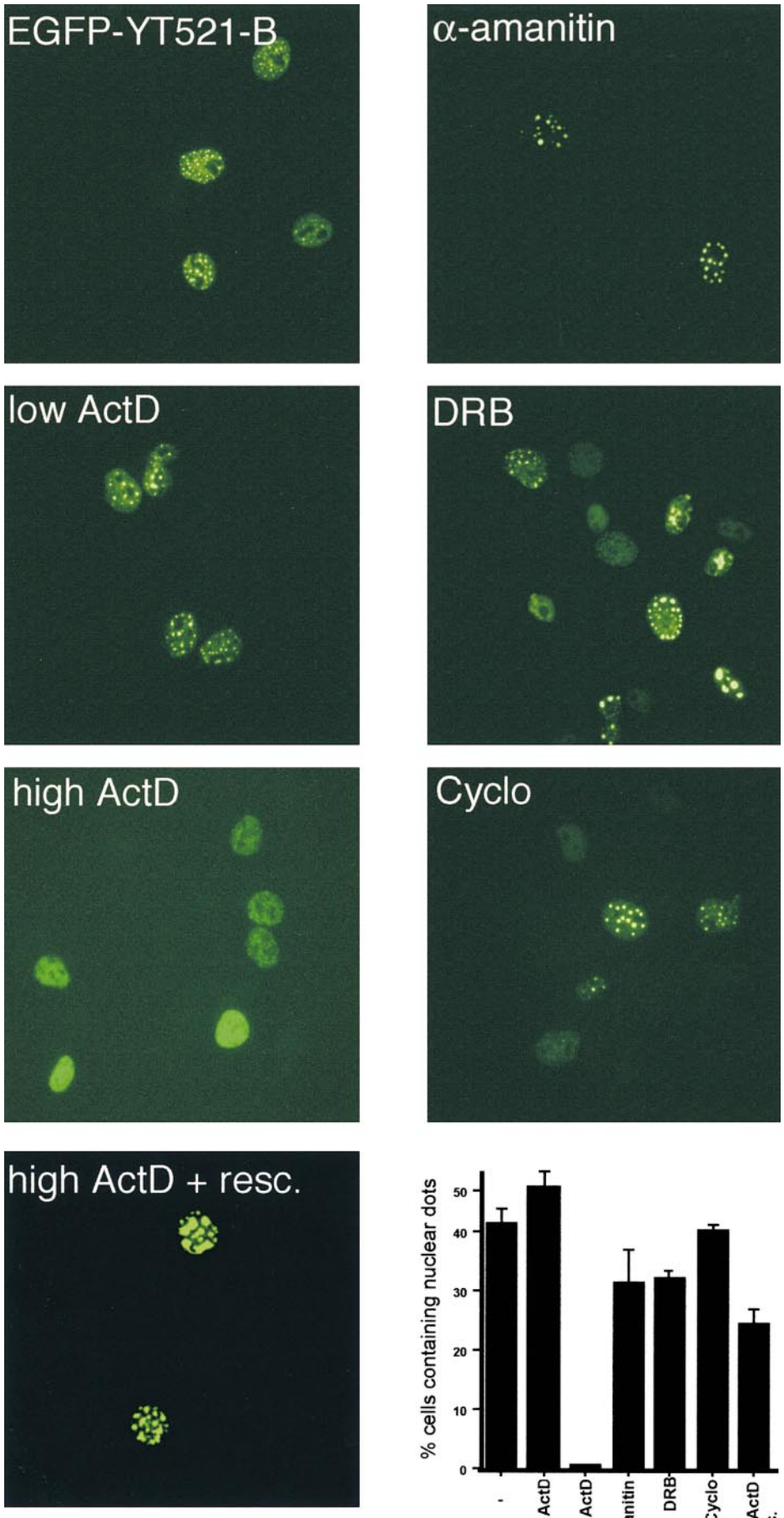

Figure 6. Actinomycin D reversibly dissolves YT bodies. COS7 cells were transiently transfected with pEGFP-YT521-B and treated with 50 μg/ml α-amanitin for 5 h (α-amanitin), 0.04 μg/ml actinomycin D for 3 h (low ActD), 75 μM DRB for 3 h (DRB), 50 μg/ml actinomycin D for 3 h (high ActD), 100 μg/ml cycloheximide for 5 h (Cyclo), or they were treated with 50 μg/ml actinomycin D, followed by a 2-h incubation period in the absence of the drug (high ActD + resc.). The percentage of cells containing YT521-B domains was determined by counting individual cells in three independent experiments. The data are the averages of three independent experiments (±SD) and 150 cells were counted in each.

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in the number of cells containing YT bodies were observed (45% in low actinomycin D–treated cells and 40% in cycloheximide-treated cells). Additionally, to eliminate the possibility that the observed effect was due to RNA polymerase III inhibition, we incubated the cells with 300 μg/ml α-amanitin (Weinman et al., 1974) and detected no significant effects (data not shown).

YT Bodies Are Dynamic, Triton X-100–soluble Structures

Several recently described subnuclear domains are associated with an insoluble, proteinaceous scaffold, the nuclear matrix. It was proposed that some of those structures may constitute storage compartments from which proteins are released into the nucleoplasma, whereas others represent functional units for gene expression (reviewed in Lamond and Earnshaw, 1998; Spector, 1993; Nickerson et al., 1995; Huang, 2000). It was also shown that the phosphorylation of proteins regulates their release or uptake into nuclear compartments and that transcriptional activity can influence the balance of compartmentalized versus noncompartmentalized proteins (Colwill et al., 1996; Lyon et al., 1997; Huang et al., 1998; Misteli and Spector, 1998; Nayler et al., 1998b; Hartmann et al., 1999). YT bodies dispersed after actinomycin D treatment, but there was little effect after incubation with other transcriptional inhibitors (see above). On the basis of our previously established protocol to separate nuclear proteins into a soluble nucleoplasmic and insoluble fraction (Nayler et al., 1998b), we investigated the solubility of transiently expressed EGFP-YT521-B protein in the presence or absence of actinomycin D, and compared it with the solubility of a previously characterized nuclear matrix associated protein SAF-B (Renz and Fackelmayer, 1996; Oesterreich et al., 1997; Nayler et al., 1998a; Weighardt et al., 1999). The EGFP-YT521-B protein was detected in the pellet (P) fraction and in the soluble (S) fraction of a 1% Triton X-100–based cell lysis buffer (HNTG) in absence of actinomycin D (Fig. 7 A). However, upon actinomycin D treatment, the amount of HNTG soluble protein was significantly reduced and the amount of protein in the corresponding pellet fraction increased (Fig. 7 A). In contrast, when we used a RIPA/benzonase lysis buffer, which releases most nuclear proteins, we detected almost all endogenous YT521-B protein in the soluble fraction and there was no change observed after actinomycin D treatment. The HNTG solubility of the endogenous nuclear matrix-associated protein SAF-B was not affected by actinomycin D (Fig. 7 B), un-

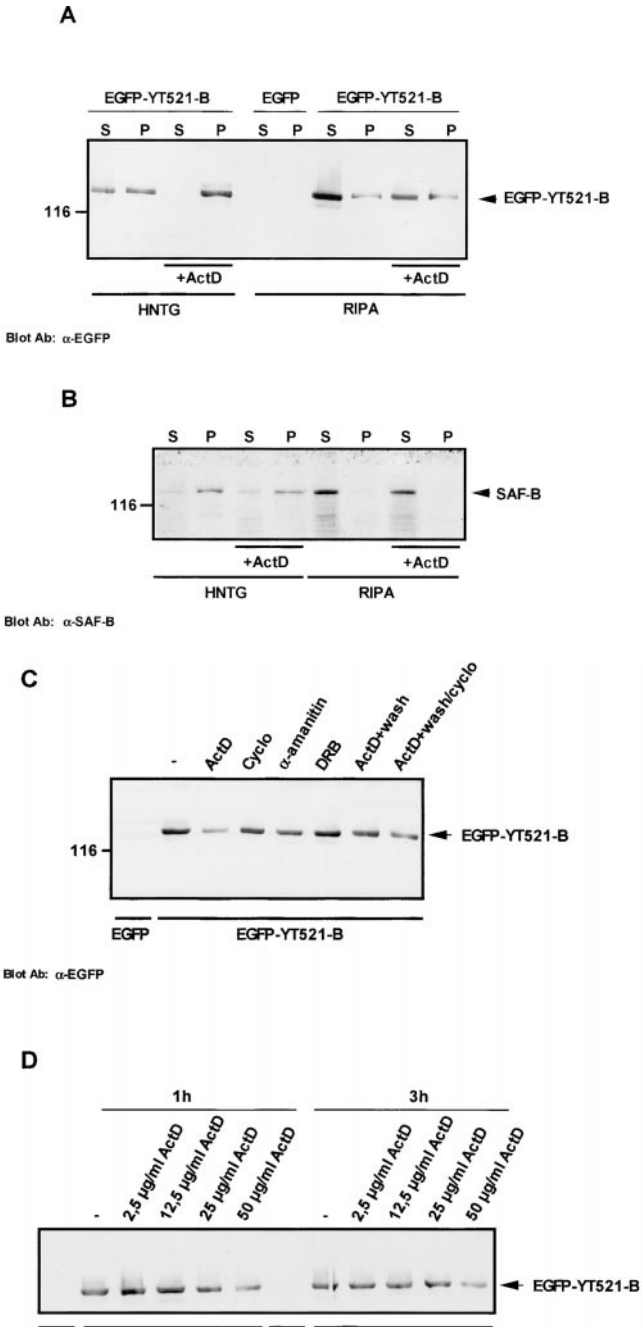

Figure 7. YT bodies are dynamic, Triton X-100–soluble compartments. (A) HEK293 cells were transiently transfected with pEGFP-YT521-B, incubated with 50 μg/ml actinomycin D for 3 h, and lysed in RIPA and benzonase or HNTG. Equal amounts of the soluble fraction (S) or redissolved pellet fraction (P) were loaded onto 10% SDS-PAGE gels. Proteins were analyzed by Western blot and ECL using the anti-EGFP antibody. (B) HEK 293 cells incubated with 50 μg/ml actinomycin D for 3 h and lysed in RIPA and benzonase or HNTG. Equal amounts of the soluble fraction (S) or redissolved pellet fraction (P) were loaded onto 10% SDS-PAGE gels. Proteins were analyzed by Western blot and ECL using the anti–SAF-B antibody. (C) HEK293 cells were transiently transfected with EGFP (control) or pEGFP-YT521-B. The pEGFP-YT521-B transfected cells were then incubated with 50 μg/ml actinomycin D for 3 h (ActD), 100 μg/ml cycloheximide for 5 h (Cyclo), 50 μg/ml α-amanitin for 5 h (α-amanitin), 75 μM DRB for 3 h, or they were incubated with 50 μg/ml actinomycin D for 3 h, followed by a 2-h actinomycin D–free incubation in the presence (ActD+wash/cyclo) or the absence (actD+wash) of 100 μg/ml cycloheximide. Equal amounts of the soluble fractions of HNTG cell lysates were analyzed on 10% SDS-PAGE and Western blotting using the anti-EGFP antibody. (D) HEK293 cells were transiently transfected with EGF (control) or pEGFP-YT521-B. The pEGFP-YT521-B–transfected cells were then incubated with increasing amounts of actinomycin D for 1 or 3 h. Equal amounts of the soluble fractions of HNTG cell lysates were analyzed on 10% SDS-PAGE and Western blot using the anti-EGFP antibody. The detected proteins are indicated by arrows; the molecular mass is indicated in kD.

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derlining the specificity of the actinomycin D effect for YT521-B, as most of the SAF-B protein was only soluble in RIPA/benzonase as previously described (Nayler et al., 1998a). To investigate the solubility of the YT521-B protein further we incubated EGFP-YT521-B expressing cells with actinomycin D, α-amanitin, DRB, or cycloheximide and lysed them in HNTG (Fig. 7 C). Again, we see a reversible reduction of soluble YT521-B protein upon actinomycin D treatment (ActD and ActD+wash), whereas no effects were detected using the other transcription inhibitors α-amanitin and DRB, or the translation inhibitor cycloheximide. Moreover, the reappearance of YT521-B protein in the soluble fraction after removal of actinomycin D was not dependent on de novo protein synthesis, since cycloheximide did not suppress this effect during the recovery phase (ActD+wash/cyclo). The removal of YT521-B protein from the soluble fraction after actinomycin D treatment was dose dependent and detectable at concentrations of 50 μg/ml actinomycin D after only 1 h of incubation. Lower concentrations of actinomycin D had no visible effect in Western blot experiments, even after 3 h of treatment (Fig. 7 D).

Together, our biochemical and immunolocalization experiments showed that the YT521-B protein resides in two separable compartments, an HNTG soluble and an HNTG insoluble nuclear fraction. In addition, these experiments also suggested that there is a dynamic exchange between these compartments and, most interestingly, that YT bodies comprise the soluble fraction.

Discussion

Recently, we analyzed the novel nuclear YT521-B protein (Hartmann et al., 1999) and observed the formation of nuclear bodies in a proportion of cells expressing EGFP-YT521-B or Flag-tagged YT521-B protein. However, these structures may reflect unphysiological protein deposits caused by overexpressed and misfolded proteins or, alternatively, they may enlarge pre-existing compartments of the endogenous protein. To clarify this situation, we investigated the localization and biochemical characteristics of endogenous YT521-B protein using specific antisera raised against YT521-B peptides. Immunofluorescence experiments, described in this study, revealed that the distribution of transiently expressed EGFP-YT521-B or Flag-tagged YT521-B protein agrees with the endogenous protein, which is concentrated in discrete compartments or dots in a proportion of cells in different cell lines. However, it appeared that the endogenous structures are usually smaller in size and not as clearly defined as the structures seen with EGFP-YT521-B. Endogenous YT521-B bodies more closely resemble the pattern observed with overexpressed Flag-tagged YT521-B constructs (Hartmann et al., 1999).

We observed a variability of YT bodies in unsynchronized cells and could demonstrate that the formation of YT bodies is regulated during the cell cycle. They appeared when cells entered the S phase and persist throughout it. This feature is shared by several dynamic subnuclear structures (Ferreira et al., 1994; Matera, 1998), although, in contrast, heterochromatin-associated PcG domains persist during mitosis (Saurin et al., 1998). These findings support our results that YT bodies may be functionally significant during the G1/S-phase, when transcription is active (Wei et al., 1998). We have investigated this further and examined transformed cells which often display changes in their nuclear architecture, reflecting their capacity for unlimited growth and metastatic potential (Nickerson, 1998). Remarkably, we found that the appearance of endogenous YT bodies was a reliable indicator of sodium butyrate, PMA, or tamoxifen-induced differentiation of the human cell line MCF7. MCF7 cells are a mammary adenocarcinoma cell line and can be differentiated in vitro using tamoxifen, sodium butyrate, or PMA. All three drugs induce MCF7 differentiation, visualized by the appearance of cytoplasmatic lipid droplets and arrest the cells in G1 (Guilbaud et al., 1990; Maas et al., 1995; Alblas et al., 1998). Considering the cell cycle–regulated formation of YT bodies in normal cell lines (see above), this could explain the pronounced morphology of the YT bodies in G1 arrested MCF7 cells. Moreover, since sodium butyrate is known to induce changes in the methylation pattern of CpG islands (Cosgrove and Cox, 1990), which is often altered in breast cancer cells (Huang et al., 1999; Pilat et al., 1998), it is interesting to note that YT bodies are most pronounced in sodium butyrate–treated cells. Methylation of CpG dinucleotides is implicated in transcriptional control and serves as a recognition site for structural proteins that assemble chromatin (Ashraf and Ip, 1998). However, whether YT521-B is involved in these processes or plays a role in the G1- to S-phase transition remains to be investigated.

An increasing number of reports describe proteins that localize to distinct subnuclear domains, and current views suggest that the nucleus is divided into functional units and storage compartments for nuclear proteins (summarized in Lamond and Eanshaw, 1998; Spector, 1993; Strouboulis and Wolffe, 1996; Matera, 1999). When we compared the localization of YT521-B protein with several other well-characterized structures, we found that YT521-B defines a novel subnuclear compartment and that these compartments contain focal sites of transcription. We have termed these novel structures YT bodies. Previously, it was shown that transcriptional activity occurred in the vicinity of speckles, allowing splice factors to be recruited into the transcriptosomal machinery (Misteli et al., 1997). Our results demonstrate that speckles and coiled bodies are often found in close proximity to YT bodies, suggesting that these structures maybe functionally linked and possibly exchange factors between them. Furthermore, we suggest that YT bodies are sites of transcriptional activity.

The morphology of various subnuclear structures is influenced by transcriptional activity and inhibition of transcription by α-amanitin, DRB, or actinomycin D cause an enlargement of structures, or may cause them to disperse (Spector, 1993; Haaf and Ward, 1996; Schul et al., 1996; Misteli et al., 1997; Huang et al., 1998; Nayler et al., 1998b; Pombo et al., 1998). For example, the PNC changed its structure upon DRB, α-amanitin, or actinomycin D treatment (Huang et al., 1998) and the number of coiled bodies decreased (Schul et al., 1996). Moreover, the OPT domain is not affected by short exposures to α-amanitin, but DRB and actinomycin D significantly reduced the number of cells containing OPT structures (Pombo et al., 1998). Fi-

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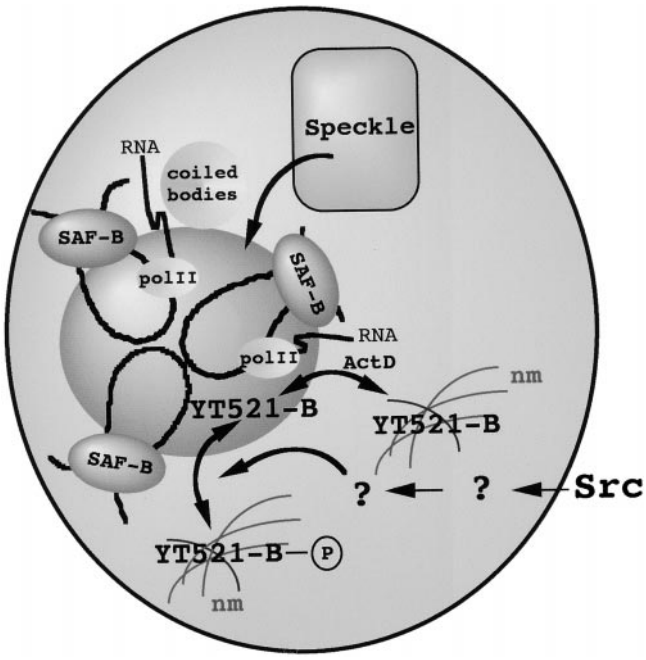

Figure 8. YT bodies define focal sites of transcription in the absence of RNA polymerase activity: a model. YT bodies (indicated in dark gray) combine different chromosomal loops into focal transcription sites in the absence of RNA polymerase activity, possibly through interaction with scaffold attachment factor-B (SAF-B) and DNA. Coiled bodies or speckles (which are often found in close contact with YT bodies) serve as accessory domains supplying the transcriptionally active site with necessary helper factors. The YT-body is dynamically regulated by signal transduction pathways emanating from members of the Src kinase family. nm, Nuclear matrix.

nally, speckles lose connections between them, round up and grow after inhibition of transcription with actinomycin D, α-amanitin, or DRB (Spector et al., 1983; Misteli et al., 1997; Nayler et al., 1998b). It is thus conceivable that storage compartments generally accumulate proteins in the absence of transcription, whereas functional units disperse. We have investigated the localization of YT521-B in the presence or absence of different transcriptional inhibitors and have found a selective effect of actinomycin D on the morphology of YT bodies. At the same time, we found that YT521-B changes its solubility upon actinomycin D treatment, suggesting that the YT521-B protein shuttles between a soluble compartment, the YT bodies, and an insoluble compartment, where the YT521-B protein is diffusely distributed. To our knowledge, these characteristics are novel and distinguish YT bodies from previously reported nuclear structures. Furthermore, the experiments using actinomycin D, α-amanitin, or DRB suggest that the integrity of the YT bodies does not depend directly on the activity of RNA polymerases. Instead, our results indicate that YT bodies form independently of transcriptional activity, possibly through a direct or indirect DNA–protein interaction. Indeed, in vitro nucleotide binding of YT-521-B to polyG-agarose was observed in preliminary experiments (Hartmann, A., unpublished results).

In summary, we describe a novel dynamic subnuclear domain, the YT bodies, which contain focal sites of transcription. Furthermore, our results suggest that the formation of these structures is independent of RNA polymerase activity and may instead depend on the integrity of a transcriptional complex at the sites of RNA initiation. To our knowledge, there is no other subnuclear structure displaying similar properties, and, in analogy to models proposed for the OPT domain (Pombo et al., 1998) and the nucleolus, we suggest that YT521-B and its molecular binding partners may group genes into higher-order structures. The proximity to speckles and coiled bodies would thereby lead to the formation of efficient gene expression centers (Fig. 8). In addition, signal transduction pathways emanating from the Src protein kinase family might regulate the formation of this subnuclear domain (Hartmann et al., 1999). Finally, future studies will clarify whether the formation of YT bodies seen upon MCF7 cell differentiation can provide a novel cancer diagnostic tool.

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