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The cdk7-Cyclin H-MAT1 Complex Associated with TFIIH Is Localized in Coiled Bodies

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TFIIH is a general transcription factor for RNA polymerase II that in addition is involved in DNA excision repair. TFIIH is composed of eight or nine subunits and we show that at least four of them, namely, cdk7, cyclin H, MAT1, and p62 are localized in the coiled body, a distinct subnuclear structure that is transcription dependent and highly enriched in small nuclear ribonucleoproteins. Although coiled bodies do not correspond to sites of transcription, in vivo incorporation of bromo-UTP shows that they are surrounded by transcription foci. Immunofluorescence analysis using antibodies directed against the essential repair factors proliferating cell nuclear antigen and XPG did not reveal labeling of the coiled body in either untreated cells or cells irradiated with UV light, arguing that coiled bodies are probably not involved in DNA repair mechanisms. The localization of cyclin H in the coiled body was predominantly detected during the G_1 and S-phases of the cell cycle, whereas in G_2 coiled bodies were very small or not detected. Finally, both cyclin H and cdk7 did not colocalize with p80 coilin after disruption of the coiled body, indicating that these proteins are specifically targeted to the small nuclear ribonucleoprotein-containing domain.

INTRODUCTION

Initiation of transcription by RNA polymerase II is a complex process that requires a set of concerted interactions between a family of protein factors collectively known as general transcriptions factors. These include TFIID, TFIIA, TFIIB, TFIIF, TFIIE, and TFIIH. TFIIH in particular has been the subject of intense research after the discovery that it also plays a role in nucleotide excision repair (for recent reviews, see Zawel and Reinberg, 1995; Friedberg, 1996; Hoeijmakers *et al.*, 1996). TFIIH is a multisubunit complex composed of eight or nine polypeptides. The human TFIIH cloned genes include ERCC3 (XPB), ERCC2 (XPD), p62, p52, p44, p34, cdk7, cyclin H, and MAT1, and the corresponding yeast homologues are named SSL2, RAD3, TFB1, TFB2, SSL1, TFB4, KIN28, CCL1, and TFB3. The function of TFIIH in excision repair involves ATPase and helicase activities associated with the XPD and XPB subunits (see Friedberg, 1996; Sancar, 1996), whereas cdk7-cyclin H-MAT1 form a subcomplex with kinase activity (reviewed by Nigg, 1996). Cdk7 has been recently shown to phosphorylate the carboxyl-terminal domain (CTD) of the large subunit of polymerase II (Roy *et al.*, 1994) and this is thought to trigger the release of the polymerase from interactions with promoter-bound factors such as the TATA-binding protein (see Maldonado and Reinberg, 1995). Thus, the kinase activity of TFIIH appears to have a role in promoter clearance and/or elongation of polymerase II transcription (reviewed by Friedberg, 1996).

In the present work, we asked whether the dual transcription and repair functions of TFIIH are associated with a particular pattern of subnuclear localization in mammalian cells. Surprisingly, the results indicate that at least four subunits of TFIIH, namely, cdk7, cyclin H, MAT1, and p62, are present in coiled bodies, a distinct subnuclear organelle enriched in splicing small nuclear ribonucleoproteins (snRNPs; for reviews, see Lamond and Carmo-Fonseca, 1993; Bohmann *et al.*, 1995b).

Coiled bodies have been observed in almost all cell types examined, ranging from mammals to plants. A specific component of the human coiled body is a

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protein with a molecular weight (M_r) of approximately 80×10^3 called p80 coilin (Andrade *et al.*, 1991), and more recently a homologue protein named SPH-1 was identified in *Xenopus laevis* (Tuma *et al.*, 1993). Coiled bodies are intimately associated with the nucleolus, and in fact they were first described as "nucleolar accessory bodies" by Ramón y Cajal in 1903. Although coiled bodies are apparently deprived of rRNA, they contain some nucleolar proteins such as fibrillarin (Raska *et al.*, 1990, 1991), Nopp140, NAP57 (Meier and Blobel, 1994), and ribosomal protein S6 (Jiménez-Garcia *et al.*, 1994). Furthermore, it has been recently shown that overexpression of mutated forms of the human p80 coilin gene induces a disorganization of both the coiled body and the nucleolus, demonstrating that the two structures share either a common structural framework or a common assembly pathway (Bohmann *et al.*, 1995a). In addition to nucleolar proteins, coiled bodies contain the U1, U2, U4, U5, and U6 spliceosomal snRNPs (reviewed in Lamond and Carmo-Fonseca, 1993), the U7 snRNP involved in histone pre-mRNA 3'-end processing, and the U3 and U8 small nucleolar RNPs involved in rRNA maturation (Wu *et al.*, 1993; Bauer *et al.*, 1994; Jiménez-Garcia *et al.*, 1994; Frey and Matera, 1995). Thus, it is thought that coiled bodies may play a role in the maturation, transport, or recycling of snRNPs in the nucleus, and the finding that cdk7, cyclin H, MAT1, and p62 are targeted to this subnuclear compartment raises the view that TFIIH may have an unexpected involvement in snRNP metabolism.

MATERIALS AND METHODS

Cells

HeLa cells were grown as monolayers in Eagle's minimum essential medium supplemented with 10% fetal calf serum (Life Technologies, Gaithersburg, MD), glutamine, and nonessential amino acids and maintained mycoplasm free. Human lymphocytes isolated from peripheral blood of a healthy volunteer donor were incubated for 48 h in RPMI medium containing 10% fetal calf serum and 5 μg/ml phytohemagglutinin. A sample of normal human liver was obtained from a biopsy after informed consent. For inhibition of transcription, dichlororibofuranosylbenzimidazole (DRB; Sigma, St. Louis, MO) was added to the culture medium of HeLa cells to a final concentration of 75 μM and incubated for 1 to 2 h. Alternatively, cells were incubated with actinomycin D (Sigma) at a final concentration of 5 μg/ml for 1 to 2 h. Infection of HeLa cells with adenovirus 2 was done as described (Rebelo *et al.* 1996).

Immunofluorescence

For indirect immunofluorescence HeLa cells were grown on glass coverslips (10 × 10 mm) and harvested at 60–80% confluency. Coverslips with attached cells were washed twice in phosphate-buffered saline (PBS) and treated according to one of the following alternative protocols: 1) immediate fixation with 3.7% formaldehyde in PBS for 10 min and subsequent permeabilization with 0.5% Triton X-100 in PBS for 15 min; 2) simultaneous fixation and permeabilization with 3.7% formaldehyde and 0.5% Triton X-100 in HPEM buffer [30 mM HEPES, 65 mM piperazine-*N*,*N*'-bis(2-ethanesulfonic acid), 2 mM $MgCl_2$, 10 mM EGTA acid, pH 6.9] for 15 min; 3) permeabilization with 0.5% Triton X-100 in CSK buffer (Fey *et al.*, 1986) containing 0.1 mM phenylmethylsulfonyl fluoride for 1 min on ice and subsequent fixation with 3.7% formaldehyde in CSK for 10 min; 4) fixation with 3.7% formaldehyde in HPEM buffer for 10 min and subsequent permeabilization with 0.05% SDS in HPEM for 10 min; 5) fixation in methanol for 10 min at −20°C. All formaldehyde solutions were freshly prepared from paraformaldehyde.

Primary lymphocytes were allowed to adhere to poly(L-lysine)-coated coverslips and treated with 3.7% formaldehyde and 0.5% Triton X-100 in HPEM buffer for 15 min. Human liver samples were fixed by immersion in 2% formaldehyde in 0.2 M HEPES at pH 7.4 and trimmed into blocks of ~1 mm × 1 mm. The blocks were infused with 2.3 M sucrose in PBS (three 15-min incubations), mounted on copper stubs, and plunged into liquid nitrogen, as described by Griffiths *et al.* (1993). Semithin 1-μm-thick sections were obtained with a cryo-ultramicrotome (MT7/CR21, RMC, Tuscon, AZ), using glass knives. The sections were collected in 2.3 M sucrose with a wire loop and placed on microscope slides coated with 0.01% Alcian blue.

For immunofluorescence, the cells were rinsed in PBS containing 0.05% Tween 20 (PBS-T), incubated for 30 min with primary antibodies diluted in PBS-T, washed, and incubated for 30 min with the appropriate secondary antibodies conjugated to either fluorescein isothiocyanate (FITC) or Texas Red (Vector Laboratories, Peterborough, United Kingdom). Finally, the coverslips were mounted in VectaShield (Vector) and sealed with nail polish. Cryosections were rinsed once in PBS, incubated for 1 h in PBS/0.1% glycine, washed for three 15-min periods in PBS/0.1% Tween 20 (PBS/Tween), and blocked for 15 min with 2% gelatin in PBS/Tween. Incubation with both primary and secondary antibodies was for 1 h.

The following antibodies were used: affinity-purified rabbit antibodies against cyclin H (Shiekhattar *et al.* 1995), mouse monoclonal MO-1.1 against cdk7 (Tassan *et al.* 1994), mouse monoclonal 3C9 against p62 (Fischer *et al.* 1992), rabbit antiserum against MAT1 (Tassan *et al.*, 1995), rabbit antiserum 204.3 against human p80-coilin (Bohmann *et al.* 1995a), mouse monoclonal 1D4-δ against human p80-coilin (Rebelo *et al.* 1996), monoclonal antibody 8H7 against the human XPG endonuclease (O'Donovan *et al.* 1994), monoclonal antibody PC10 against proliferating cell nuclear antigen (PCNA; Dako, Dakopatts, Glostrup, Denmark), and rabbit serum directed against the adenoviral protein DNA-binding protein (DBP; Linné *et al.*, 1977).

Cell Cycle Analysis

To identify each stage of the cell cycle, unsynchronized HeLa cells were pulse labeled with 10 μM bromodeoxyuridine (BrdUrd) for 10 to 15 min. After the pulse, the cells were rinsed several times with fresh medium and further incubated for up to 13 h. To detect the incorporated BrdUrd, the cells were extracted with Triton X-100 and fixed with formaldehyde as described above. The DNA was then mildly denatured by incubation in 0.1 N HCl/150 mM NaCl for 15 min at room temperature. After extensive washing in PBS, the cellular DNA was partially digested for 30 min at 37°C with 100 U/ml *Eco*RI and 300 U/ml exonuclease III in digestion buffer [66 mM Tris-HCl, pH 7.5, 6.6 mM $MgCl_2$, 1 mM dithiothreitol]. The cells were then washed for three 5-min periods in PBS containing 5 mM EDTA and double labeled with a monoclonal anti-BrdUrd antibody (Boehringer Mannheim, Mannheim, Germany) and either anti-p80-coilin or anti-cyclin H rabbit serum.

Visualization of Transcription Sites

For the detection of nucleoplasmic transcription 5-bromo-2'-uridine 5'-triphosphate (BrdUTP, Sigma; 5 mM) was microinjected into the cytoplasm of HeLa cells. The medium was replaced and cells were further incubated for 5 to 10 min at 37°C. Cells were then rinsed in PBS and simultaneously fixed and extracted in 3.7% formaldehyde

and 0.5% Triton X-100 in HPEM buffer (see above). The incorporated bromouridine was detected with a monoclonal antibody directed against bromodeoxyuridine (Boehringer Mannheim) and a secondary antibody labeled with fluorescein. As a control, α-amanitin (0.5 μg/μl) was coinjected with BrdUTP (see Carmo-Fonseca *et al.*, 1996). Cells were microinjected by using the Eppendorf microinjector 5242. Micropipettes obtained from Clark Electromedical Instruments (Pangbourne, United Kingdom) were freshly prepared on a P-87 puller (Sutter Instruments, Novato, CA).

Microscopy

All samples were analyzed using the laser scanning microscope Zeiss LSM410. For double-labeling experiments, the specimens were sequentially scanned with an argon ion laser (488 nm) to excite FITC fluorescence and a helium-neon laser (543 nm) to excite Texas Red fluorescence. Pseudocolored images of both signals were generated and superimposed. The data files were directly printed on a Kodak XLS 8300 digital printer.

Immunoblotting

A crude nuclear pellet was isolated from HeLa cells and mixed with SDS sample buffer. Alternatively, total cellular proteins from uninfected HeLa cells or cells infected with adenovirus 2 for 24 h were analyzed. The proteins were separated in 8% polyacrylamide minigels (Bio-Rad Laboratories, Richmond, CA), electroblotted to a nitrocellulose membrane, and probed with antibodies, as previously described (Jordan *et al.*, 1996)

RESULTS

The TFIIH Subunits cdk7, Cyclin H, and p62 Are Localized in Coiled Bodies

Indirect immunofluorescence experiments were performed with antibodies directed against the TFIIH subunits cdk7, cyclin H, and p62 (Fischer *et al.* 1992; Tassan *et al.* 1994; Shiekhattar *et al.* 1995). The results show that each of these proteins is diffusely distributed in the nucleoplasm and is excluded from nucleoli (Figure 1). However, within the nucleoplasm the labeling is highly concentrated in one to four foci (Figure 1, A–C) and double-labeling experiments using either anti-cdk7 and anti-cyclin H antibodies (Figure 2A) or anti-cyclin H and anti-p62 antibodies (our unpublished results) demonstrate that the three proteins colocalize in the same foci. Similar results were observed in cells fixed and extracted by distinct protocols as described in MATERIALS AND METHODS, although the staining of foci was most prominent in cells exposed to detergents either before or simultaneously with fixation.

Since the nucleoplasmic foci enriched in TFIIH subunits were reminiscent of coiled bodies, double-labeling experiments were performed with antibodies to the coiled body protein p80 coilin (Andrade *et al.*, 1991). As depicted in Figure 2B, the foci labeled by anti-cdk7 monoclonal antibody colocalize with the foci labeled by anti-coilin antibodies, and similar results were obtained with anti-cyclin H and anti-p62 antibodies. Furthermore, similar results were observed in human primary lymphocytes from peripheral blood and hepatocytes from a liver biopsy (Figure 2, C–E). Finally, an immunoblot analysis of total nuclear proteins from HeLa cells confirmed the specificity of each antibody used (Figure 3). On the basis of these data, we conclude that the TFIIH subunits cdk7, cyclin H, and p62 are localized in coiled bodies.

MAT1 Is Also Present in the Coiled Body

MAT1 is a 36-kDa ring-finger protein that has been recently identified as a component of TFIIH (Tassan *et al.*, 1995). As MAT1 is strongly associated with the cdk7-cyclin H complex and stimulates its kinase activ-

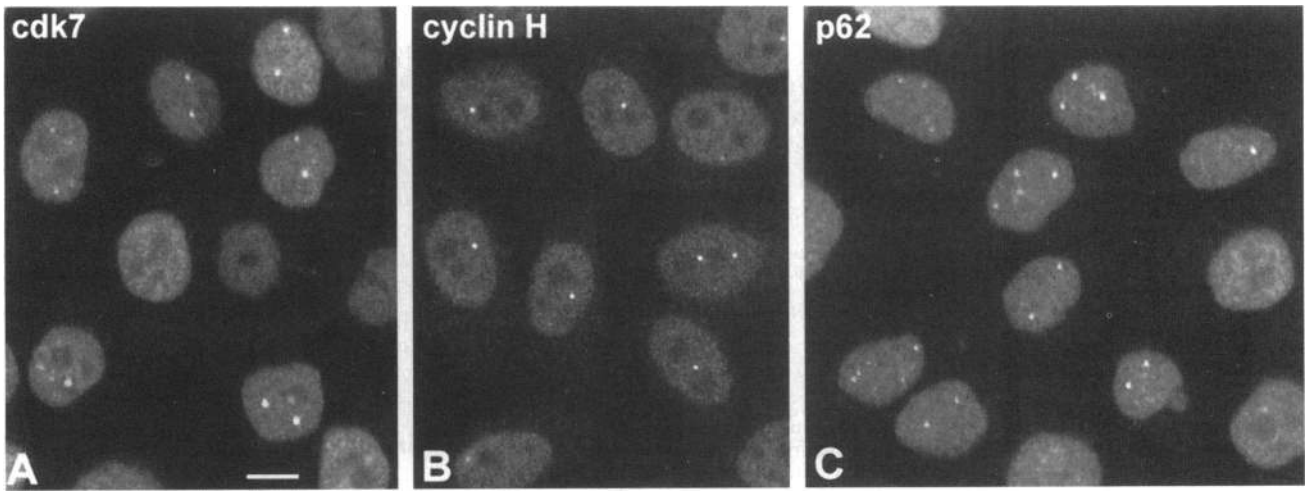

Figure 1. TFIIH subunits cdk7, cyclin H, and p62 are localized in nuclear foci. HeLa cells were extracted with Triton X-100, fixed in formaldehyde, and immunostained with antibodies directed against cdk7 (A), cyclin H (B), or p62 (C). Note that under these fixation/permeabilization conditions, labeling by anti-p62 antibody does not seem to be completely excluded from the nucleoli. Bar, 10 μm.

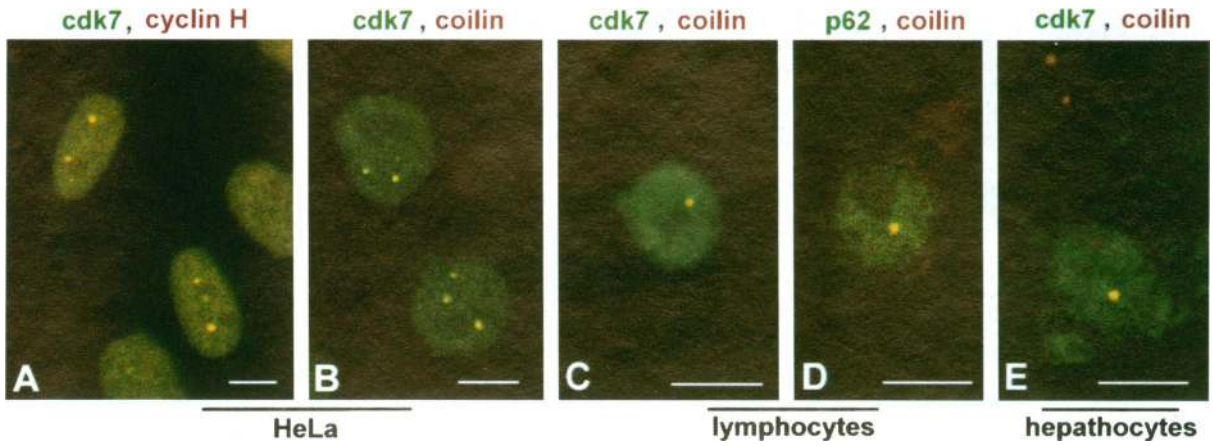

Figure 2. Cdk7, cyclin H, and p62 colocalize in coiled bodies. Double-labeling experiments were performed with anti-cdk7 and anti-cyclin H antibodies (A), anti-cdk7 and anti-coilin antibodies (B, C, and E), or anti-p62 and anti-coilin antibodies (D). cdk7 and p62 were detected with anti-mouse IgG coupled to FITC (green staining), whereas cyclin H and coilin were visualized with anti-rabbit secondary antibodies coupled to Texas Red (red staining). The panel depicts pseudocolor overlays of the corresponding green and red images. HeLa cells were permeabilized with Triton X-100 before fixation in formaldehyde (A and B). Human lymphocytes (C and D) and liver tissue (E) were prepared as described in MATERIALS AND METHODS. Bar, 10 μm.

ity (Adamczewski *et al.*, 1996), we were interested in determining whether it also localizes in coiled bodies. Thus, indirect immunofluorescence was performed with rabbit antibodies raised against a glutathionine *S*-transferase-p36 fusion protein expressed in *Escherichia coli* (Tassan *et al.*, 1995). As previously described, MAT1 is predominantly localized in the nucleus (Tassan *et al.*, 1995; Figure 4). However, in addition to the diffuse nucleoplasmic staining, we observed concentration of labeling in a few foci that correspond to coiled bodies stained by anti-coilin or anti-cdk7 antibodies (Figure 4, B and C). If we take into account that MAT1, cyclin H, and cdk7 fractionate as an active kinase complex (Tassan *et al.*, 1994; Adamczewski *et al.*, 1996), our results suggest that coiled bodies contain TFIIH kinase activity.

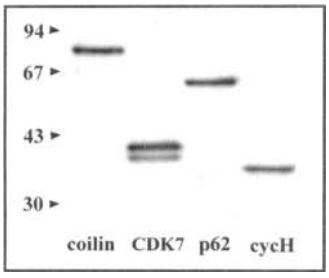

Figure 3. Proteins from HeLa nuclei were separated in a 8% polyacrylamide gel, electroblotted to a nitrocellulose membrane, and probed with anti-coilin (mAb 1D4-δ), anti-cdk7 (mAb MO-1.1), anti-p62 (mAb 3C9), and anti-cyclin H antibodies. Molecular mass markers (kDa) are indicated on the left. Each antibody reacts specifically with polypeptides of approximately 80, 40, 62, and 32 kDa, respectively.

Coiled Bodies Are Surrounded by Sites of Transcription

Since TFIIH is an essential component of the RNA polymerase II initiation complex, a simple explanation for the observed concentration of TFIIH in coiled bodies would be that these subnuclear domains represent specialized transcription sites. Although early electron microscopic studies failed to detect newly synthesized RNA in coiled bodies (see Fakan, 1994), we decided to investigate the spatial distribution of coiled bodies with respect to transcription sites as visualized by laser scanning confocal microscopy. Living HeLa cells were microinjected with BrdUTP and the incorporated nucleotides were detected with fluorochrome-conjugated antibodies. Newly synthesized RNA was visualized as a multitude of microfoci scattered throughout the nucleoplasm, as previously observed in permeabilized cells (Jackson *et al.*, 1993). However, some transcription foci surround coiled bodies (Figure 5, A–C, arrows). As genes tend to occupy defined positions in the nucleus (Carmo-Fonseca *et al.*, 1996), this could indicate that a specific subset of loci are transcribed in the vicinity of coiled bodies. Accordingly, coiled bodies are often associated with the U1, U2, and histone gene loci (Frey and Matera, 1995), but clearly further studies are needed to address this point.

DNA Repair Factors Are Not Detected in the Coiled Body

To study whether the presence of TFIIH in coiled bodies correlates with DNA repair activity, we analyzed the subnuclear localization of two other factors

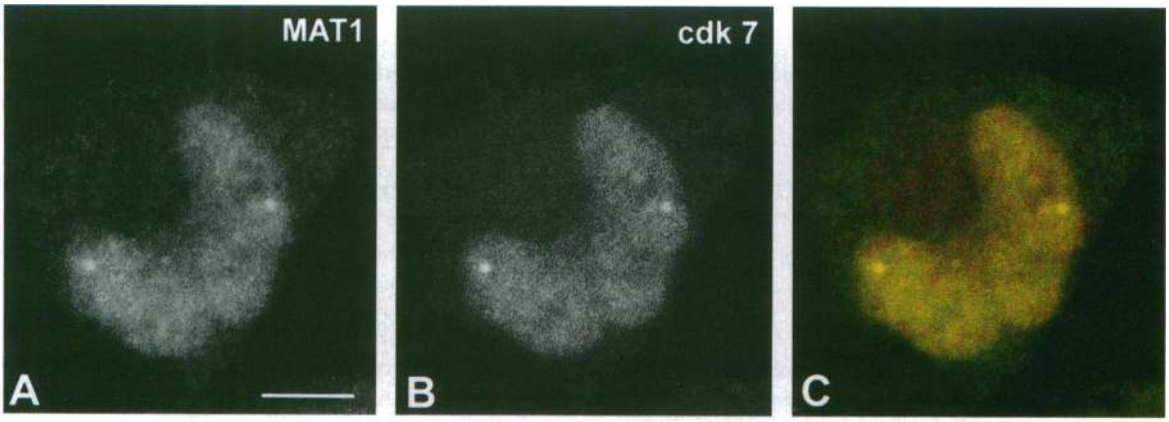

Figure 4. MAT1 colocalizes with cdk7 in coiled bodies. HeLa cells were fixed with formaldehyde, permeabilized with 0.05% SDS, and double labeled with mouse monoclonal MO-1.1 against cdk7 and rabbit polyclonal antibodies against MAT1. MAT1 was visualized with a secondary antibody coupled to FITC (A), and cdk7 was detected using anti-mouse IgG coupled to Texas Red (B). (C) Pseudocolor overlay of the two images. Bar, 10 μm.

involved in the nucleotide excision repair pathway, XPG and PCNA. XPG is an endonuclease that is responsible for the 3′ incision of the damaged site, and the PCNA is an auxillary factor for DNA polymerase ϵ or δ involved in replacing the damaged DNA (reviewed by Sancar, 1996). By indirect immunofluorescence, anti-PCNA monoclonal antibody predominantly labels sites of DNA replication and, therefore, stains only cells in S phase (Bravo and Macdonald-Bravo, 1987; Figure 6A), whereas XPG appears diffusely distributed in the nucleoplasm of all cells (Figure 6C). Double-labeling experiments using anti-cyclin H antibodies show that neither XPG nor PCNA are detected in coiled bodies, regardless of the method used for fixation and permeabilization of cells (Figure 6, A–D). In addition, antibodies directed against the

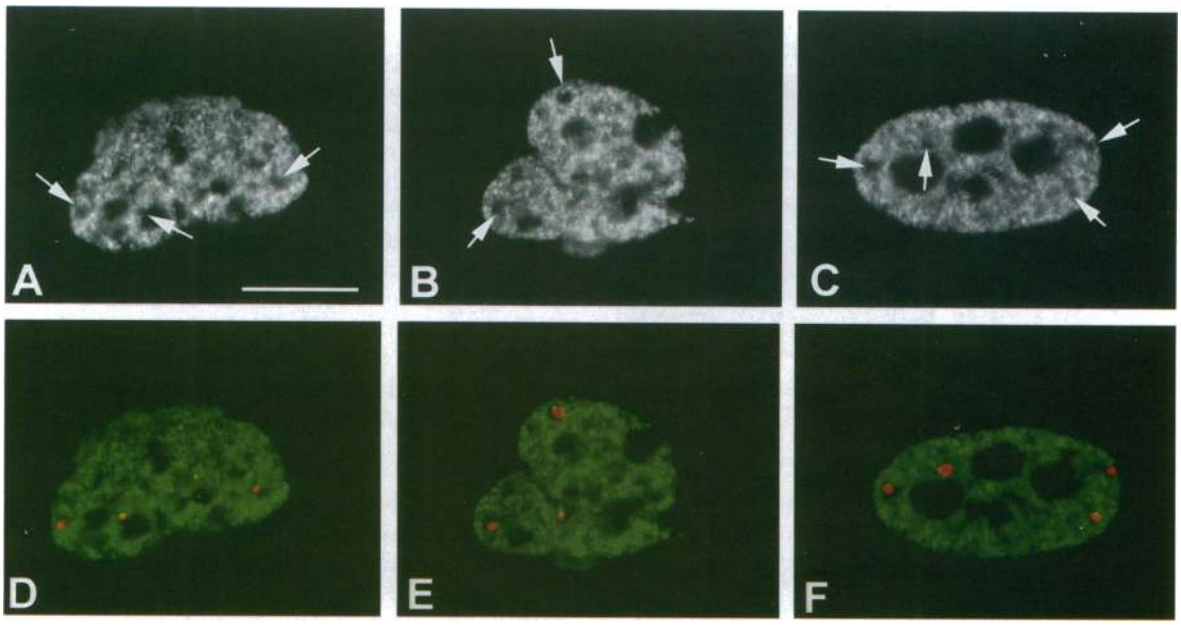

Figure 5. Coiled bodies are surrounded by transcription sites. BrdUTP was microinjected into the cytoplasm of living HeLa cells. Approximately 10 min after injection, the cells were fixed in 3.7% formaldehyde containing 0.5% Triton X-100. The incorporated bromouridine was detected with a monoclonal antibody directed against BrdUrd and a secondary antibody coupled to FITC (A–C; D–F, green staining); coiled bodies were identified with rabbit anti-coilin serum and a secondary antibody coupled to Texas Red (D–F, red staining). (D–F) Pseudocolor overlays of the corresponding green and red images. Note that coiled bodies do not incorporate BrdUrd; however, each coiled body is surrounded by transcription foci (A–C, arrows). Bar, 10 μm.

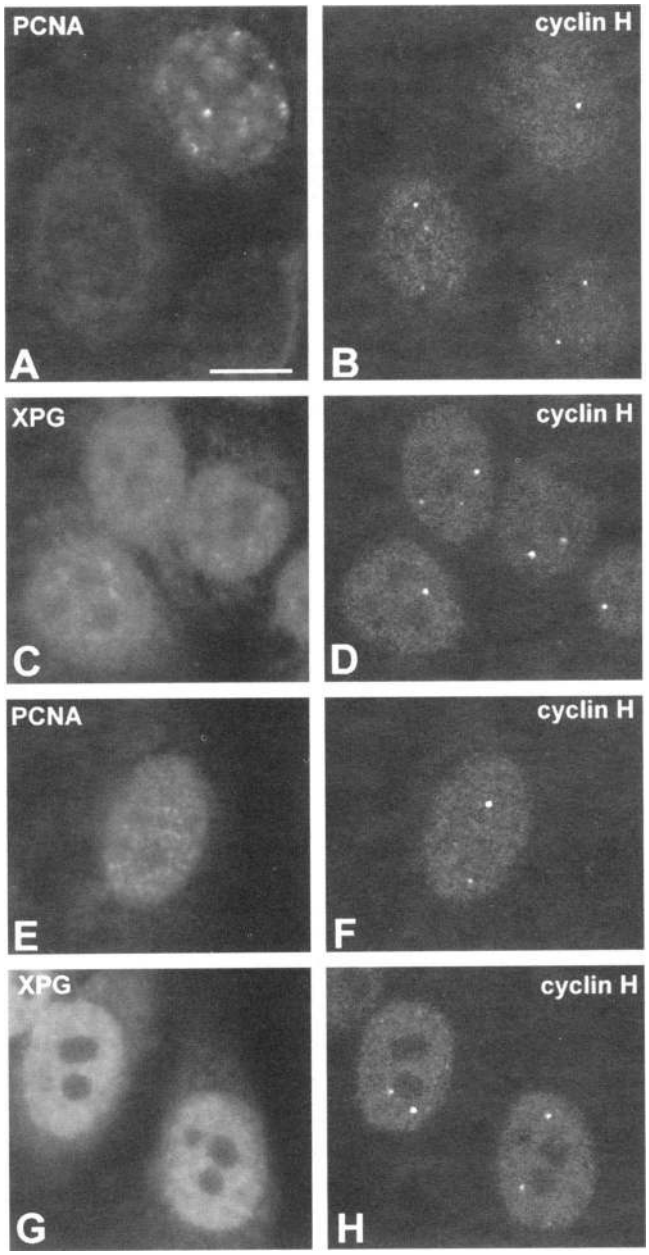

Figure 6. DNA repair factors are not detected in coiled bodies. HeLa cells were double labeled with anti-PCNA and anti-cyclin H antibodies (A, B, E, and F) or anti-XPG and anti-cyclin H antibodies (C, D, G, and H). For labeling with anti-PCNA monoclonal antibody PC10, cells were permeabilized with 0.5% Triton X-100 in CSK buffer for 1 min on ice and subsequently fixed in methanol for 10 min at −20°C (see Landberg and Roos, 1991). For labeling with anti-XPG monoclonal antibody 8H7, cells were permeabilized with Triton X-100 and then fixed with formaldehyde. Cells depicted in E–H were irradiated with UV light for 5 s from a 12-W UV lamp at a distance of 50 cm and then incubated in conditioned medium for 5 h at 37°C. In nonirradiated cells, PCNA is only detected in S-phase cells, whereas after irradiation most of the cells are stained. Neither PCNA nor XPG are detected in coiled bodies either before or after irradiation. Note that UV irradiation does not affect the localization of cyclin H in coiled bodies. Bar, 10 μm.

TFIIH repair subunits ERCC2 and ERCC3 produced a diffuse staining of the nucleoplasm, with no concentration in coiled bodies (our unpublished results).

To further investigate a possible involvement of coiled bodies in DNA repair, HeLa cells were irradiated with UV light for 5 s and double labeled with anti-cyclin H and either anti-PCNA or anti-XPG antibodies (Figure 6, E–H). In contrast to nonirradiated cells where the anti-PCNA antibody reacted only with S-phase nuclei, after UV treatment the antibody labeled a multitude of microfoci in the nucleus of most cells (Figure 6E), which have been shown to correspond to sites of DNA repair (Jackson *et al.*, 1994). As depicted in Figure 6, no major changes were detected in the distribution of either XPG or cyclin H after UV irradiation. Furthermore, immunolocalization of coilin showed no apparent changes in coiled bodies induced by UV irradiation. Thus, it is unlikely that coiled bodies participate in DNA repair.

Cyclin H Localizes in the Coiled Body Predominantly during G_1 and S-Phases

The cdk7-containing complex activates cyclin-dependent kinases and was, therefore, thought to be implicated in cell cycle control. Because coiled bodies are very dynamic structures that vary in size and number throughout the cell cycle (Andrade *et al.*, 1993; Ferreira *et al.*, 1994), we were interested in determining whether the localization of cdk7 and cyclin H in coiled bodies correlates with cell cycle progression. Because HeLa cells proved difficult to synchronize, the identification of specific stages of the cell division cycle was established by pulse-labeling asynchronous cultures in vivo with 10 μM BrdUrd for 10 to 15 min. The cells were then allowed to grow in fresh BrdUrd-free medium and analyzed at hourly intervals. On the basis of the percentage of labeled metaphases at each time point, we estimated that the duration of S-phase in these cells is approximately 8 h (Figure 7). Because DNA replicates asynchronously during the S-phase, different patterns of BrdUrd staining can be observed depending on the region of chromatin that is replicating in each cell during the pulse with BrdUrd. Because specific chromatin regions replicate at defined times, it is possible to identify cells in early, middle, and late S-phase based on the pattern of BrdUrd staining (reviewed by Spector, 1993). Figure 8A depicts a typical late S-phase-labeling pattern; i.e., the BrdUrd staining is concentrated in large clumps dispersed in the nucleoplasm (O'Keefe *et al.*, 1992). As this staining pattern is observed in 9.4% of all pulse-labeled cells, we estimate that this stage of S-phase lasts approximately 45 min. Once replicated, each chromatin domain retains its specific spatial position in the nucleus, and therefore, the cells that have been labeled in late S-phase during the pulse can be easily identified (Fer-

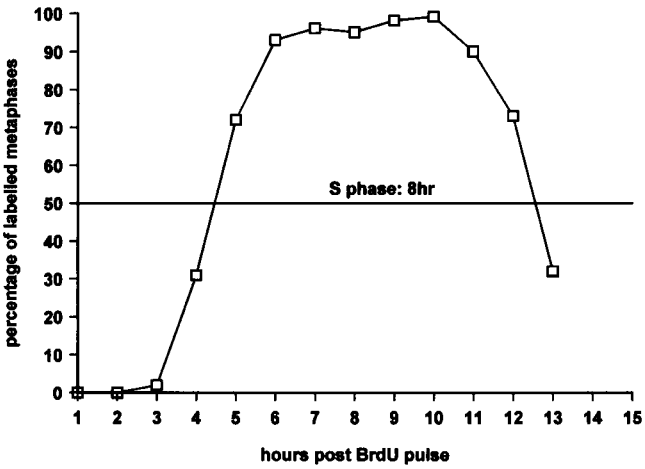

Figure 7. To determine the average length of S-phase (S period) of the HeLa cells used in this study, the cells were given a pulse of BrdUrd (BrdU) in vivo and sampled at hourly intervals, and then the proportion of cells labeled mitosis was counted. The average S period is taken as the time between the two points where 50% of the mitosis are labeled (see Mitchison, 1971).

reira, personal communication). Thus, these data allow us to conclude that at 1 h after the pulse, the cells containing nucleoplasmic clumps of BrdUrd are in transition between S and G_2 phase, whereas at 2 h all cells containing nucleoplasmic clumps of BrdUrd are in G_2. At 3 h after the pulse, some labeled cells were detected in metaphase, and at 7 h after the pulse, the BrdUrd clumps were detected in early G_1 cells, indicating that the incorporation of BrdUrd did not affect progression through the cell division cycle (Figure 8, G–I).

Coiled bodies in late S-phase are clearly stained by anti-cyclin H antibodies (Figure 8, A–D), whereas during G_2 staining of coiled bodies by anti-cyclin H antibodies is rarely observed. To clarify whether coiled bodies are still present in G_2 cells, parallel experiments were performed with anti-coilin antibodies. The results show that in G_2 cells coiled bodies are either not detected or significantly smaller than those observed in G_1-S cells (Figure 8, E and F). This contrasts with the observation that coiled bodies are largest in size in the S and G_2 phases of HeLa cells synchronized by double thymidine block and nocodazole arrest (Andrade *et al.*, 1993) and the reason for this discrepancy is unclear, although it may reflect physiological differences between distinct HeLa cell populations. Coiled bodies stained by anti-cyclin H antibodies are also observed shortly after mitosis (Figure 8, G–I), in G_1 phase, and in all stages of S-phase. On the basis of the relative intensity of the immunofluorescent signals, the results indicate that the amount of cyclin H present in coiled bodies is scant shortly after mitosis, peaks during G_1 and S-phases and decreases drastically in G_2 phase, in parallel with a sharp reduction in the coiled body size.

cdk7 and Cyclin H Do Not Colocalize with p80-Coilin after Disruption of Coiled Bodies

Because the kinase activity of TFIIH is responsible for the phosphorylation of the CTD of polymerase II and the transcription inhibitor DRB inhibits CTD phosphorylation in vivo and TFIIH kinase in vitro (see Bentley, 1995), we asked whether DRB affects the localization of cdk7 and cyclin H in vivo. Cells were treated with 75 μM DRB for 1 to 2 h and then double labeled with anti-cyclin H and anti-coilin antibodies (Figure 9, A and B) or anti-cdk7 and anti-coilin antibodies (Figure 9, C and D). The results show that DRB induces a disruption of coiled bodies with an accumulation of p80 coilin around the nucleoli, as previously observed in cells treated with other transcription inhibitors such as actinomycin D and α-amanitin (see Lamond and Carmo-Fonseca, 1993). This effect is visible in some cells after 1 h of treatment and is observed in the vast majority of cells after 2 h of treatment. Because the perinucleolar structures stained by anti-coilin antibodies are thought to represent precursor forms of the coiled body (Ferreira and Carmo-Fonseca, 1995) and neither cyclin H nor cdk7 are seen to colocalize with coilin at the periphery of the nucleolus (Figure 9, A–D), these results suggest that the cyclin H–cdk7 complex associates specifically with mature coiled bodies. To further address this question, we have analyzed the distribution of cyclin H and cdk7 in cells infected with adenovirus, which is known to cause a disruption of coiled bodies (Rebelo *et al.*, 1996). At 18 h after viral infection, coilin is detected in numerous microfoci (Figure 9F) that do not have the typical coiled body morphology when viewed with the electron microscope and that do not contain snRNPs (Rebelo *et al.*, 1996). Double-labeling experiments using anti-coilin and anti-cdk7 (Figure 9, E and F) or anti-coilin and anti-cyclin H antibodies show that the cyclin H–cdk7 complex is not present in the coilin microfoci. Interestingly, cdk7 is redistributed in the nucleus of infected cells where it colocalizes with the sites labeled with an antibody directed against the adenovirus DBP (Figure 9, G and H). Furthermore, double labeling of infected cells using anti-cdk7 and anti-cyclin H antibodies shows that both cdk7 and cyclin H are recruited to the sites of viral DNA accumulation. Finally, the specificity of the anti-cdk7 antibody was confirmed by immunoblot analysis of total proteins from infected HeLa cells (our unpublished results).

Thus the data indicate that both cyclin H and cdk7 associate specifically with snRNP-containing coiled bodies and not with precursor coilin structures.

DISCUSSION

In this work we show that antibodies specific for cdk7, cyclin H, MAT1, and p62 label the coiled body in the

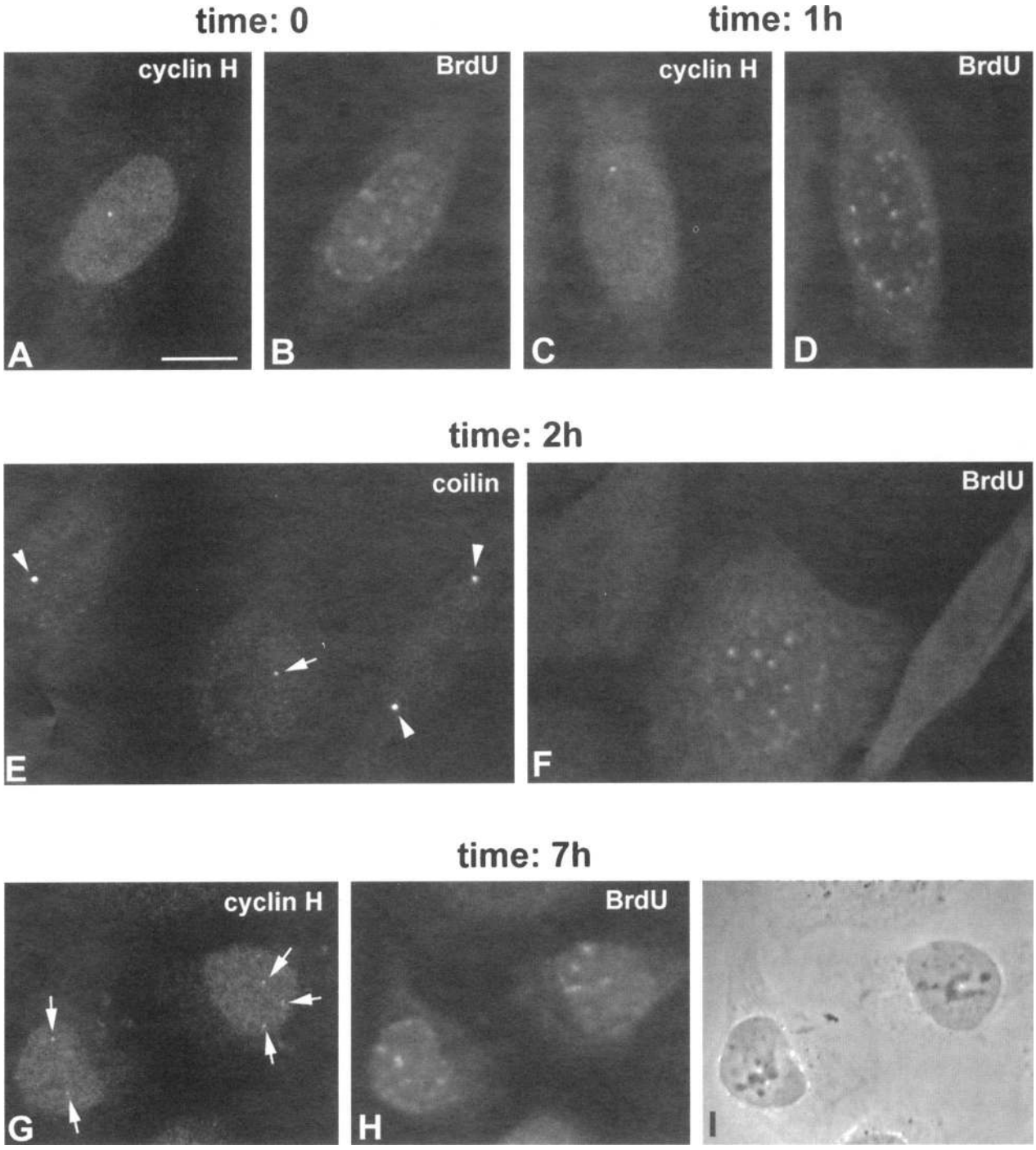

Figure 8. Localization of cyclin H in coiled bodies during the cell cycle. Asynchronous cultures were pulse labeled in vivo with 10 μM BrdUrd for 10 to 15 min, and the cells were then allowed to grow in fresh BrdUrd-free medium. Pulse-labeled cells have distinct patterns of BrdUrd staining depending on the region of chromatin that is replicating. Because nucleoplasmic regions of heterochromatin are the last to replicate, cells in late S-phase are identified by BrdUrd staining of large clumps dispersed in the nucleoplasm (A; see O'Keefe *et al.*, 1992). In these cells cyclin H is detected in coiled bodies (B). At 1 h after the pulse, the cells containing nucleoplasmic clumps of BrdUrd (which are in transition between S and G_2 phases) still contain cyclin H associated with coiled bodies (C and D). At 2 h (E and F), when all cells containing nucleoplasmic clumps of BrdUrd are in G_2, coiled bodies are significantly smaller (arrow in F, compare with coiled bodies indicated by arrowheads in cells unlabeled by BrdUrd, which therefore, are in G_1 or early S-phase). At 7 h after the pulse, the BrdUrd clumps are detected in cells that have just completed mitosis (H and I; note that the two daughter cells are still connected by the midbody); in these nuclei the anti-cyclin H antibodies produce a faint staining associated with small coiled bodies (G, arrows). Note that under the fixation/permeabili-

nucleus of both HeLa and primary human cells. cdk7, a member of the cdc2 superfamily of protein kinases, is responsible for the TFIIH-associated kinase activity implicated in CTD phosphorylation (see Nigg, 1996) and was originally identified as the catalytic subunit of the cdk-activating kinase (CAK) required for the phosphorylation of key cell cycle regulatory kinases (reviewed by Morgan, 1995; Nigg, 1995). Cdk 7 interacts tightly with cyclin H and MAT1 to form a complex that fractionates as an active kinase complex (Tassan *et al.*, 1994; Adamczewski *et al.*, 1996; Drapkin *et al.*, 1996; Reardon *et al.*, 1996). Thus, the finding that cdk7, cyclin H, and MAT1 colocalize in the coiled body suggests that CAK is active in this subnuclear organelle. Interestingly, other kinases have been previously localized in the coiled body, namely the cAMP-dependent protein kinase, PKA (Trinczek *et al.* 1993) and the double-stranded RNA-activated protein kinase, DAI (Jiménez-Garcia *et al.*, 1993).

Cdk 7, cyclin H and MAT1 form a ternary complex which can be separated from TFIIH as free CAK (Tassan *et al.*, 1995; Adamczewski *et al.*, 1996; Drapkin *et al.*, 1996; Reardon *et al.*, 1996), and it remains an open issue whether or not TFIIH-associated and free CAKs are functionally equivalent. Because p62 is also localized in the coiled body, it is more likely that the cdk 7, cyclin H, and MAT1 detected in this structure represent TFIIH-associated rather then free CAK. Nevertheless, antibodies raised against the TFIIH subunits ERCC2, ERCC3, and p44 produced a diffuse staining of the nucleoplasm with no apparent concentration in coiled bodies, and similar results were obtained with antibodies against two essential repair factors, PCNA and XPG. Because ERCC2 and ERCC3 are essential for nucleotide excision repair, these data indicate that the CAK and DNA repair components of TFIIH are differentially compartmentalized in the nucleus and suggest that the presence of CAK in the coiled body is unrelated to DNA excision repair. Consistent with the view that the kinase and repair activities of TFIIH may be functionally separated, there is recent genetic evidence suggesting that the cdk7 yeast homologue KIN28 gene is not essential for nucleotide excision repair (see Friedberg, 1996). A puzzling result, however, is the finding that the yeast homologues of p62 and p44, TFB1 and SSL1, are involved in DNA repair (Wang *et al.*, 1995), though their precise roles in mammalian cells remain to be elucidated.

Although CAK is responsible for TFIIH-associated phosphorylation of the CTD of RNA polymerase II, there is no evidence that polymerase II is localized in the

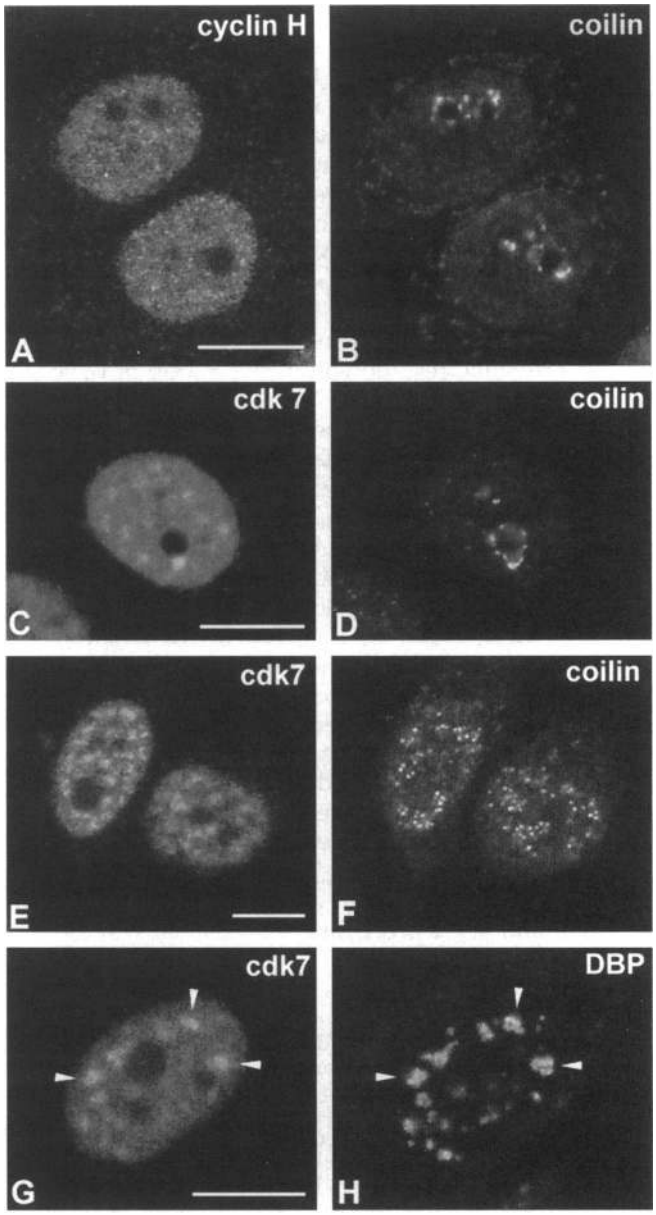

Figure 9. cdk7 and cyclin H do not colocalize with coilin after disruption of the coiled body. (A–D) HeLa cells were treated with 75 μM DRB for 2 h and double labeled with anti-cyclin H and anti-coilin (monoclonal antibody 1D4-δ) antibodies (A and B) or anti-cdk7 (monoclonal antibody MO-1.1) and anti-coilin (rabbit antibody 204.3) antibodies (C and D). Coiled bodies are not visible in these cells; instead, coilin is detected in small patches around the periphery of the nucleolus that are not labeled by either anti-cyclin H or anti-cdk7 antibodies. (G–H) HeLa cells were infected with adenovirus 2 for 17 h and double labeled with anti-cdk7 (monoclonal antibody MO-1.1) and anti-coilin (rabbit antibody 204.3) antibodies (E and F) or anti-cdk7 (monoclonal antibody MO-1.1) and anti-DBP antibodies (G and H). Coilin is detected in numerous microfoci that do not correspond to coiled bodies (see Rebelo *et al.*, 1996) and that are not labeled by anti-cdk7 antibodies (cf. E and F). In these cells cdk7 redistributes and colocalizes with the sites of adenoviral DNA accumulation labeled by anti-DBP antibodies (cf. G and H, arrowheads). Bar, 10 μm.

Figure 8 (cont). zation conditions required for BrdUrd detection, the labeling of coiled bodies by anti-cyclin H antibody is less efficient than labeling by anti-coilin antibody. Therefore, the relative size of coiled bodies can only be analyzed within each experimental sample. Bar, 10 μm.

coiled body (Du and Warren, 1997), and therefore, it is unlikely that phosphorylation of the CTD occurs in this structure. However, coiled bodies are surrounded by transcription foci and there is evidence suggesting that coiled bodies may preferentially associate with a subset of gene loci (Frey and Matera, 1995). Furthermore, data from in vitro systems suggest that not all genes require TFIIH (see Sancar, 1996). Thus, the compartmentalization of CAK in the coiled body could facilitate access of TFIIH to the promoters of specific genes localized in the vicinity of this structure.

In addition to the CTD, cdk7 phosphorylates a conserved threonine residue within the T loop of the cdc2, cdk2, and cdk4 kinases (see Nigg, 1995), and possibly it may also phosphorylate other, as yet unidentified, substrates that are present in the coiled body. One obvious candidate is p80-coilin, a protein that is differentially phosphorylated in interphase and mitosis (Carmo-Fonseca *et al.*, 1993). Because CAK associates with snRNP-containing coiled bodies but not with p80-coilin aggregates induced by transcription inhibitors or adenovirus infection, CAK could be involved in the assembly or maturation of the coiled body. Alternatively (or additionally), given that coiled bodies are highly enriched in snRNPs, CAK could play a role in the phosphorylation of snRNP proteins. In the light of recent data demonstrating that the C-terminal domain of RNA polymerase II is required for efficient pre-mRNA splicing and polyadenylation (Du and Warren, 1997; McCracken *et al.*, 1997), the colocalization of TFIIH and snRNPs in the coiled body may represent another link between transcription and RNA processing.

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