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Transition from initiation to promoter proximal pausing requires the CTD of RNA polymerase II

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ABSTRACT

The C-terminal domain (CTD) of mammalian RNA polymerase II consists of 52 repeats of the consensus hepta-peptide YSPTSPS, and links transcription to the processing of pre-mRNA. Although Pol II with a CTD shortened to five repeats (Pol II Δ5) is transcriptionally inactive on chromatin templates, it is not clear whether CTD is required for promoter recognition *in vivo*. Here, we demonstrate that in the context of chromatin, Pol II Δ5 can bind to the c-*myc* promoter with the same efficiency as wild type Pol II. However, Pol II Δ5 does not form a stable initiation complex, and does not transcribe promoter proximal sequences. Fluorescence recovery after photobleaching (FRAP) experiments with cells expressing enhanced green fluorescent protein (EGFP)-tagged Δ5 or wildtype Pol II revealed a single, highly mobile Pol II Δ5 fraction whereas wildtype Pol II yielded less mobile fractions. These data suggest that CTD is not required for promoter recognition, but rather for subsequent formation of a stable initiation complex and isomerization to an elongation competent complex.

INTRODUCTION

The large subunit of eukaryotic RNA polymerase II (Pol II LS) harbours a unique C-terminal domain (CTD) consisting of repeats of the consensus hepta-peptide sequence YSPTSPS (1). The consensus sequence is highly conserved across organisms, but the number of repeats appears to have increased through evolution (2).

The phosphorylation status of the CTD is essential for the regulation of transcription [reviewed in (3,4)]. *In vivo*, only the non-phosphorylated (IIA) form of Pol II can participate in the formation of a pre-initiation complex (PIC), while CTD phosphorylation is essential for transcriptional elongation (the IIO form) (3,4). The effectors of this regulation include several cyclin-dependent-, and stress-activated kinases, whose activities during certain stages of the transcription cycle may serve to regulate initiation, elongation and the binding of pre-mRNA processing factors to Pol II [for reviews see (5–8)]. The phosphorylation of non-engaged Pol II by kinases, such as ERK or CDK8/cyclin C may function to down regulate the transcription by preventing the formation of new PICs (9–11), suggesting that the CTD could be involved in controlling early steps of initiation. Phosphorylation of Pol II during the transition from initiation to elongation by CDK7/cyclin H of the general transcription factor TFIIH, and CDK9/cyclins T and K of the elongation factor P-TEFb, may relieve the inhibitory effects of the DSIF, NELF and Mediator complexes. Hence, Pol II with phosphorylated CTD (Pol IIO) has a specific defect in the initiation on chromatin templates, while a polymerase with an unphosphorylated CTD can properly initiate. This leaves open the question whether an unphosphorylated CTD is required for promoter recognition, or if a CTD-deleted Pol II can recognize a chromatin-packaged promoter as well.

Here, we show that the recognition of the c-*myc* promoter by Pol II with a CTD truncated to five repeats (Pol II Δ5) is not affected in the context of chromatin, while subsequent steps like stable PIC formation and isomerization to an elongation competent complex are severely impaired. This is in agreement with our further observation that Pol II Δ5 is highly mobile in fluorescence recovery after photobleaching (FRAP) experiments and not stably associated with nuclear structures.

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The authors wish it to be known that, in their opinion, the first two authors should be regarded as joint First Authors

MATERIALS AND METHODS

Cell culture and cell lines

Cell lines were obtained by stable transfection of the Burkitt's lymphoma cell line Raji with DNA encoding the 8.1 kb HindIII–EcoRI c-*myc* gene locus on the episomal, self-replicating Epstein–Barr virus (EBV)-derived vector KH375, and selected with hygromycin. Subsequently, cells were transfected with LSmock, Pol II wt or Pol II Δ5 carrying a tet-off regulatable promoter (12), and selected with neomycin. HeLa cells were transfected with the plasmid pSV40-H2B-mRFP1 and stable single cell clones expressing H2B-MRFP were isolated. Positive cell clones were subsequently transfected with plasmids expressing α-amanitin resistant fusions of the large subunit of Pol II wt or Pol II Δ5 and enhanced green fluorescent protein (EGFP) (13) and selected with neomycin. If indicated, cells were treated with final concentrations of 2 μg/ml α-amanitin or 3 mM/ml sodium butyrate (SoB).

Nuclear run-on assay, S1 analysis

Isolation of nuclei, purification and hybridization of labelled RNA to membrane-bound oligonucleotides, the washing procedure of membranes including the digestion of single-stranded RNA with RNAse A, oligonucleotides complementary to the human antisense c-*myc* strand, *in vitro* transcribed T7 control RNA, as well as S1 analysis have been described in detail elsewhere (14).

Chromatin immunoprecipitation (ChIP) analysis

Cells were formaldehyde cross-linked and immunoprecipitated as described previously (14). Antibodies against HA-tag (3F10, Roche) and isotype control (Santa Cruz) were applied. Antibody/protein/DNA complexes were isolated by immunoprecipitation with blocked protein A positive Staph A cells. Following extensive washing, bound DNA fragments were eluted and analysed by subsequent PCR. Each reaction contained 3 μl of immunoprecipitated DNA, 1× Taq reaction buffer (Promega), 1.5 mM $MgCl_2$, 50 ng of each primer, 1.7 U *Taq* polymerase (Promega) and 200 μM each dNTP (Boehringer Mannheim) (including 1 μCi [^{32}P]dCTP) in a final reaction volume of 20 μl. PCRs were amplified for 1 cycle at 95°C for 5 min, annealing temperature of the primers for 5 min, 72°C for 3 min and 27 cycles at 95°C for 1 min, annealing temperature of the primers for 2 min, 72°C for 1.5 min. PCR products were separated by electrophoresis through a 6% polyacrylamide gel, and visualized by autoradiography. The following primers were used for the c-*myc* promoter region and insulin gene:

Myc-1: 5′-GGTCTGGACGGCTGAGGACCCCCG-3′;
Myc-2: 5′-CTCTCGCTGGAATTACTACAGCG3′;
Insulin 1: 5′-GCTGGTAGAGGGAGCAGATG-3′;
Insulin 2: 5′-CCCTGACTGTGTCCTCCTGT-3′.

FRAP analysis

FRAP analysis was performed on HeLa cells expressing H2B-MRFP, and Pol II wt-EGFP or Pol II Δ5-EGFP. Cells were seeded on round coverslips fitting in the temperature controlled (37°C) POC-Chamber (LaCon, Germany). Live cell microscopy was performed with a Zeiss LSM510 Meta confocal microscope equipped with 63×/1.4 NA Plan apochromat oil objective. An Ar laser (488 nm, 35 mW) and a HeNe laser (543 nm, 1 mW) were used to excite the fluorescent proteins. Image acquisition before and after bleaching was performed at low laser power (5%). For FRAP analysis a region of interest was selected and photobleached by an intense 488 nm Ar laser beam (set to 100%) for 5 s, after which confocal image series were recorded at 1 s time intervals for 1 min and thereafter at 5 s time intervals for ∼4 min. Mean fluorescence intensities of the bleached region were corrected for background and for total nuclear loss of fluorescence over the time course. FRAP data of at least eight nuclei were averaged and the mean curve as well as the standard deviations were plotted. As a control fixed specimen (4% paraformaldehyde, 10 min) of the same cell lines mounted in Vectashield (Vector, USA) were subjected to FRAP analysis and plotted as well.

RESULTS AND DISCUSSION

CTD is not required for recognition and binding to the c-*myc* promoter *in vivo*

A requirement for the CTD in the control of promoter escape and maturation of mRNA *in vivo* has been documented in detail before. Its role in promoter recognition, formation of a stable initiation complex and isomerization to an elongation competent complex, however, is still elusive. The c-*myc* promoter is an ideal tool to study this question, since Pol II is stalled immediately after the isomerization step proximal to the promoter. At this position Pol II is easily detectable in run-on transcription assays.

We have previously reconstituted transcriptional regulation of the c-*myc* gene on stably transfected episomal vectors in the B cell line, Raji. The episomal c-*myc* establishes a chromatin structure undistinguishable from the structure of an endogenous c-*myc* to the nucleosomes positioned upstream and downstream of the c-*myc* promoter. Importantly, episomes carry a stalled Pol II immediately downstream of the major c-*myc* P2 promoter as it is observed for the endogenous c-*myc* (14–18). We used Raji cells with a reconstituted c-*myc* chromatin on episome KH375 (Figure 1A), and introduced an additional episome carrying a tetracycline-regulatable, α-amanitin resistant large subunit of Pol II into these cells (Figure 1B). Cell lines were established carrying only the vector (mock), Pol II with the complete CTD (Pol II wt), or with internal deletions of 21 (Pol II Δ31; deletion of 23–36 + 39–47) and 47 repeats (Pol II Δ5; deletion of 4–50) (12,19). The copy number of episomes as determined by Southern analysis turned out to be similar for the c-*myc* and Pol II constructs in all cell lines (Figure 1C). Similar levels of the large subunit of Pol II were induced in all cell lines after removal of tetracycline (Figure 1D). Pol II wt and Pol II Δ31 displayed both the hyperphosphorylated (Pol II0) and hypophosphorylated (Pol IIa) forms while Pol II Δ5 migrated as a single small band in gel electrophoresis. Pol II Δ31 behaved like Pol II wt and is not further shown in the following experiments.

ChIP experiments were performed to measure the binding of the Pol II wt and Pol II Δ5 to the c-*myc* promoter. Both RNA polymerases cross-linked to the c-*myc* promoter with the same high efficiency (Figure 1E, lanes 3 and 11). No cross-linking to the c-*myc* promoter could be observed in the mock cell line (lane 7), or to the promoter of the insulin gene, which is not

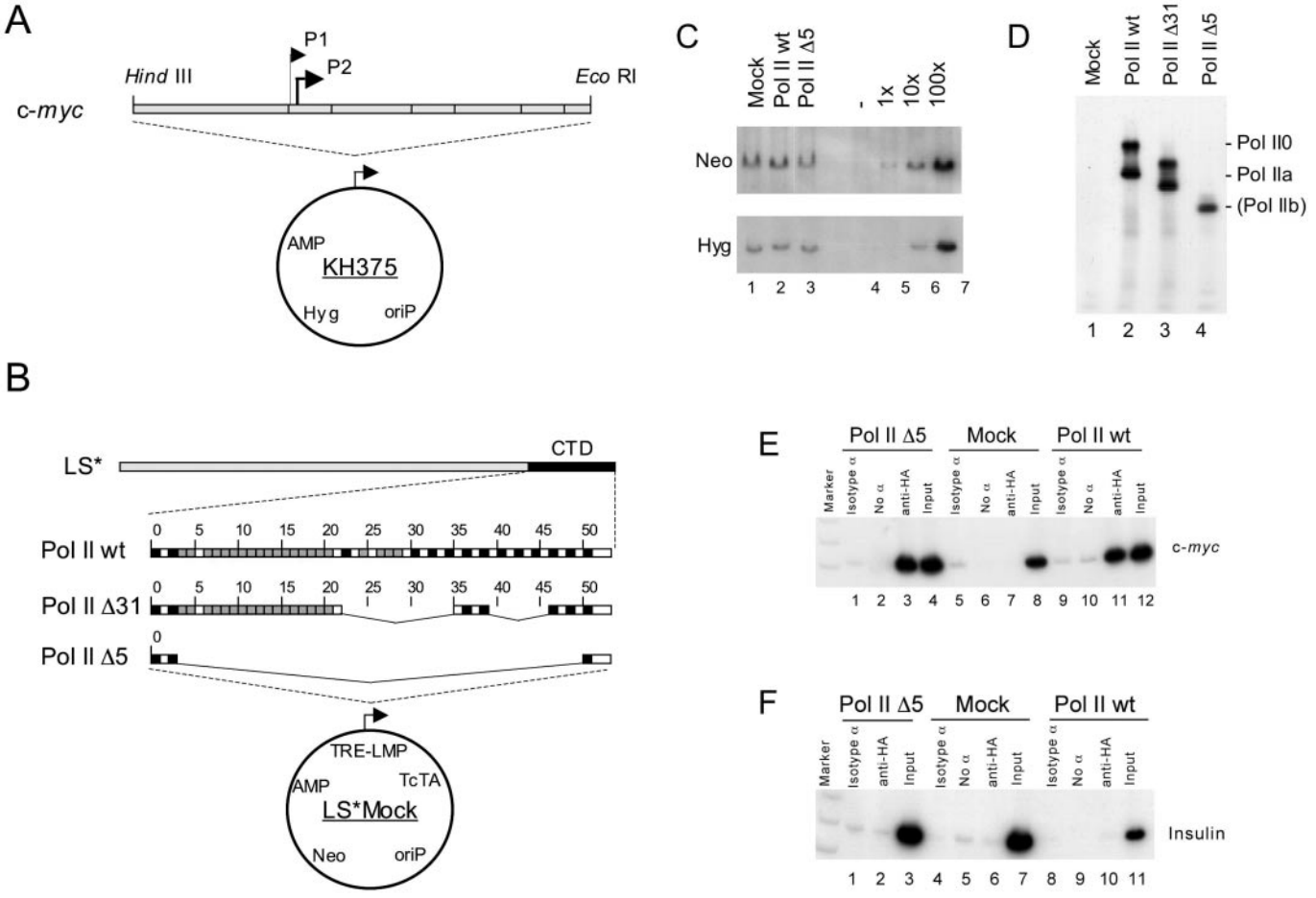

Figure 1. Efficient binding of Pol II Δ5 to the c-*myc* promoter. (**A**) Raji cell lines carrying the episomal c-*myc* gene locus, and in addition (**B**) either LS*Mock (vector control), Pol II wt, or Pol II Δ5 were established. (**C**) The copy number of constructs was determined by Southern analysis with specific probes for the neomycin and hygromycin resistance genes. 1×, 10× and 100× correspond to 1, 10 and 100 copies/genome, respectively. (**D**) Proper expression of Pol II wt and Pol II Δ5 after removal of tetracycline was controlled by western blot analysis using anti-HA antibodies. Pol II Δ31 served as size control. (**E**) ChIP analysis revealed similar binding of Pol II Δ5 and Pol II wt to the c-*myc* promoter (lanes 3 and 11, respectively), while binding was not detectable in the Mock cell line (lane 7), or (**F**) to a non-transcribed region in the insulin gene.

transcribed in B cells (Figure 1F, lanes 2 and 10). Cross-linking of E2F to the c-*myc* promoter (14) was seen in all cell lines and is therefore not affected by the expression of Pol II wt, and Pol II Δ5 (data not shown). We conclude that Pol II Δ5 binds and cross-links to the c-*myc* promoter with the same efficiency as Pol II wt, and that the CTD has neither a positive or negative effect on c-*myc* promoter recognition. Whether the hypo- and hyperphosphorylated Pol IIo can recognize the c-*myc* promoter with different efficiencies, as reported previously for the adenovirus major late promoter (20), cannot be analysed in this assay, and remains unclear.

CTD is required for stable PIC formation and/or isomerization of Pol II at the c-*myc* promoter

The episomal c-*myc* is induced after inhibition of histone deacetylase (HDAC) activity (14). We next tested whether Pol II wt and Pol II Δ5 can transcribe the c-*myc* gene after inhibition of HDAC activity by SoB. Cells were induced by removal of tetracycline, and treated with α-amanitin and SoB as indicated in Figure 2A. Expression of the episomal c-*myc* by SoB is induced in Pol II wt cells (Figure 2B, lane 9) but not in Mock and Pol II Δ5 cells (lanes 1 and 5). Note that transcription of the translocated, Ig-enhancer driven c-*myc* gene is repressed by SoB in Raji cells, leaving the signal for c-*myc* mRNA in lane 9 unchanged (14). However, induction of the episomal and repression of the translocated c-*myc* is distinguishable by S1 analysis. P1t and P2t mRNAs are derived from the translocated c-*myc* (lane 14) and P2 mRNA is derived from the episomal c-*myc* (lane 13).

Importantly, the failure of Pol II Δ5 to induce c-*myc* expression is not solely caused by its inability to support maturation of c-*myc* mRNA. Nuclear run-on experiments showed that Pol II Δ5 is defective in the initiation of transcription at the c-*myc* promoter and cannot transcribe promoter proximal sequences (Figure 2C, lane 2). In contrast, transcription by Pol II wt produced strong transcription signals on oligonucleotides E, F and G downstream of the c-*myc* P2 promoter (Figure 2C, lane 4). These signals were not detectable for Pol II Δ5 and mock cells (lanes 2 and 3). Since pausing of Pol II at the translocated c-*myc* promoter is abolished in Burkitt's lymphoma cells (21), including Raji cells (22), the detected run-on signals are almost derived exclusively from episomal c-*myc* alleles. From these data we conclude that Pol II Δ5 is defective

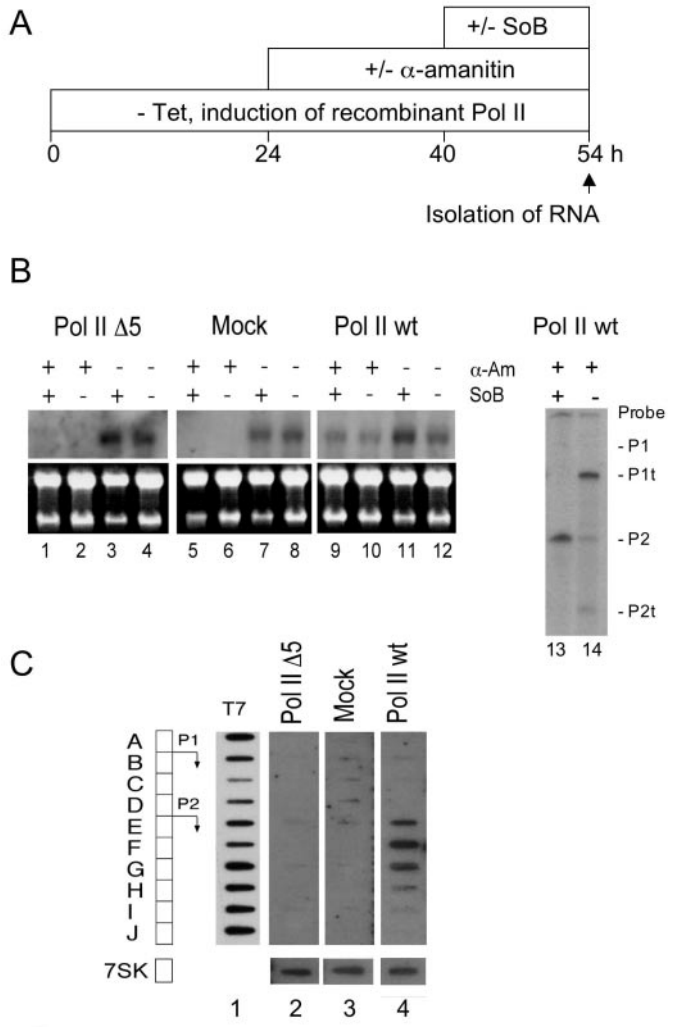

Figure 2. Transcription of the c-*myc* promoter by recombinant Pol II. (**A**) Tetracycline was washed out from cell lines at time point 0 h to allow expression of Pol II wt and Pol II Δ5. α-amanitin was added 24 h later to achieve quantitative inhibition of the endogenous Pol II. After 16 h, transcription of the episomal c-*myc* was induced by treatment of cells with SoB for 14 h. (**B**) northern analysis of c-*myc* RNA (left hand site), discrimination of endogenous and episomal c-*myc* RNA by S1 analysis (right hand site). P1t/P2t mark RNA from the endogenous, translocated c-*myc* gene (lane 14), P1/P2 RNA from the episomal c-*myc* (lane 13). (**C**) Nuclear run-on activity of Pol II Δ5 and Pol II wt at the episomal c-*myc* promoter. Cells were treated as described in (A), but nuclei were isolated 24 h after addition of α-amanitin, cells were not induced with SoB. (A–J) Long antisense oligonucleotides (50 nt) covering the c-*myc* promoter region; 7SK: antisense oligonucleotide for Pol III transcribed 7SK gene; T7: T7 RNA polymerase transcribed, uniformely labelled RNA of the c-*myc* promoter region.

either in the formation of a stable PIC, or in the execution of the subsequent isomerization step to an elongation competent complex. The strong cross-linking of Pol II Δ5 to the c-*myc* promoter argues that Pol II Δ5 is present at the c-*myc* promoter at least as frequently as Pol II wt, but is defective in initiating transcription.

Nuclear mobility of EGFP-tagged Pol II wt versus -Pol II Δ5

To study the nuclear mobility of Pol II wt and Pol II Δ5 in living cells, we fused EGFP to the carboxy-terminus of both molecules (Figure 3A), and expressed them under the control of the thymidine-kinase promoter in HeLa cells stably expressing a histone 2B-MRFP. HeLa cells expressing Pol II wt-EGFP turned out to be viable and could be cultured and expanded for months in the presence of α-amanitin. In contrast, Pol II Δ5-EGFP expressing cells could be cultured in the presence of α-amanitin for only three days before significant cell death was observed. Therefore, cells were selected with neomycin for 10 days before α-amanitin was added and FRAP experiments were performed. Cloning of EGFP to the carboxy-terminus of the large subunit of Pol II wt and Pol II Δ5 resulted in stable fusion proteins. Importantly, the stability of CTD is controlled by repeats 1–3 and repeat 52 (last repeat) *in vivo* (13,23). These repeats are present in Pol II Δ5. Moreover, we could recently show that fusion of EGFP to the C-terminus of Pol II mutants lacking repeats 1–3 and repeat 52 fully rescued the stability of CTD deletion mutants (23).

Both Pol II wt-EGFP and Pol II Δ5-EGFP displayed nuclear staining with the exclusion of the nucleoli (Figure 3B, panel 2 and 3). In Figure 3B panel 1 is an example of the EGFP signal of a fixed HeLa H2B-mRFP nucleus, stably transfected with Pol II wt-EGFP. Bleaching a region of interest results in a loss of EGFP fluorescence and an imprinted hole, observable over the whole time course and serving as a positive control for an effective bleaching event. Panel 4 in Figure 3B is an example of an monomeric red fluorescent protein (MRFP) signal of unfixed HeLa H2B-MRFP, Pol II Δ5-EGFP cells. Similar to the fixed cells, the MRFP signal shows only little recovery during the 350 s observation time. The mean intensities of such a bleached region calculated over the whole observation are plotted in Figure 3C. In case of the fixed control a loss of fluorescence to ~30% is observed (Figure 3C, blue curve). The same result was obtained when performing FRAP analysis on fixed cells stably transfected withPol II Δ5-EGFP (data not shown).

FRAP analysis performed on living HeLa cells expressing Pol II wt-EGFP (exemplary nucleus shown in Figure 3B, panel 2) reveals a recovery curve reaching a plateau after ~200 s (Figure 3C, green curve). The recovery behaviour of Pol II wt-EGFP is in accordance with data reporting several populations of Pol II with differing kinetics (24,25). At least three populations of Pol II exist, based on their differing kinetics. The largest fraction ought to be a free diffusing pool enabling the rapid recovery observable over the first 50 s. The other two fractions ought to be either bound but inactive, or bound and elongating polymerase-II-GFP molecules, which recover much slower and lead to the slightly accelerated recovery in the subsequent observation.

In contrast, performing FRAP analysis on HeLa cells stably expressing Pol II Δ5-EGFP (exemplary nucleus shown in Figure 3B, panel 3) reveals a much faster recovery (Figure 3C, red curve). This argues for a high take off rate, and an increase of the free diffusing population of Pol II Δ5 correlating with the loss of 47/52 repeats of the CTD. We note that the bulk dynamics observed for the different polymerases may not reflect the situation at particular loci.

CONCLUSIONS

The finding that CTD is dispensable for the recognition of the c-*myc* promoter in the context of chromatin, but required for

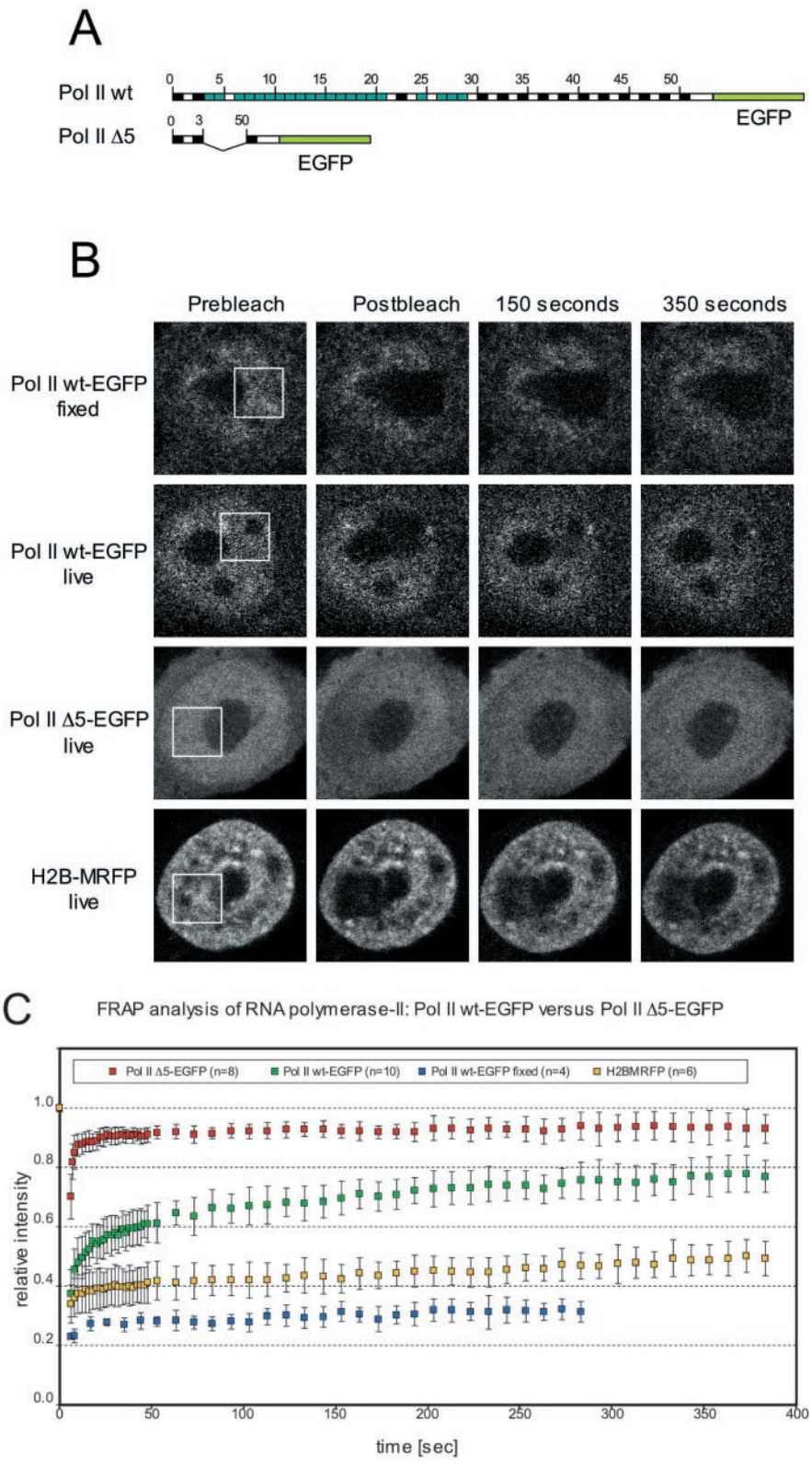

Figure 3. FRAP analysis of EGFP-tagged Pol II. (**A**) Pol II wt and Pol II Δ5 were tagged with EGFP at the carboxy-terminus. (**B**) Pol II-EGFP and H2B-MRFP signals of exemplary nuclei subjected to FRAP analysis were shown: fixed Pol II wt-EGFP (panel 1); live Pol II wt-EGFP (panel 2), live Pol II Δ5-EGFP (panel 3) and live H2B-MRFP (panel 4). The images show the signal distribution before ('Prebleach'), right after the bleaching ('Postbleach') and after a recovery period of 150 and 350 s. The squares in the Prebleach images indicate the bleached region of interest. (**C**) The plotted curves represent the mean relative intensities measured in the bleached regions over time. The averaged values of 4, 10, 8 and 6 nuclei (fixed Pol II wt-EGFP, live Pol II wt-EGFP, live Pol II Δ5-EGFP and live H2B-MRFP, respectively) and accordingly the standard deviations were plotted for each time point. Fixed samples served as positive controls and confirmed the effectiveness of the bleaching indicated by a drastic drop of the curve to ∼30% and a subsequent stay at this level (blue curve).

the isomerization of Pol II into an elongating enzyme underscores our notion that CTD is not only important for the control of RNA elongation, but also for the process of initiation. For this step, the CTD of Pol II probably exist must in its hypophosphorylated form. The inability of a CTD-deleted polymerase to initiate and to transcribe to promoter proximal pause sites may also be a safety mechanism. We could recently show that the integrity of Pol II *in vivo* can be controlled by a protease that cleaves the CTD from the large subunit (13,23). Removal of the CTD would not only disconnect such polymerases from the CTD-associated RNA-processing machinery, but also would prevent further rounds of transcription by such polymerases.

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