Requirement of the carboxy-terminal domain of RNA polymerase II for the transcriptional activation of chromosomal *c-fos* and *hsp*70A genes

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Abstract The carboxy-terminal domain of the large subunit of mouse and human RNA polymerase II contains 52 repeats of a heptapeptide which are the targets for a variety of kinases. We have used an α-amanitin resistant form of the large subunit of pol II to study the role of the carboxy-terminal domain in the expression of chromosomal genes. The large subunit of RNA polymerase II and deletion mutants thereof, which contain only 31 (LSΔ31) and 5 (LSΔ5) repeats, were expressed in 293 cells. Subsequently, the endogenous large subunit of RNA polymerase II was inhibited by α-amanitin and the induction of chromosomal c-*fos* and *hsp*70A genes was determined. Cells expressing the large subunit of RNA polymerase II and LSΔ31 were able to transcribe the c-*fos* and *hsp*70A genes after treatment with the phorbolester TPA and after heat-shock, respectively. In contrast, cells expressing LSΔ5 failed to induce expression of both genes.

Key words: RNA polymerase II; Carboxy-terminal domain; α-Amanitin; Transcription

1. Introduction

Eukaryotic RNA polymerase I, II and III are multi subunit enzymes consisting of more than 10 subunits. The large subunit is highly conserved between the three polymerases and shows also striking homology to the large subunit of *Escherichia coli* RNA polymerase [1]. The large subunit of RNA polymearase II (pol II) has a particularly structured carboxy-terminal domain (CTD) that is not present in pol I and pol III. This CTD comprises multiple copies of a heptapeptide repeat with the consensus repeat Ser-Pro-Thr-Ser-Pro-Ser-Tyr. The number of repeats varies from 26 in yeast to 52 in mouse and human cells [2–4]. It has been suggested that the CTD fulfils multiple functions during the transcription process and that these functions are regulated by its phosphorylation. In cells, two forms of pol II are detectable containing a hypophosphorylated CTD (pol IIa) and hyperphosphorylated CTD (pol IIo).

Phosphorylation of CTD in mammalian cells is catalyzed by a variety of kinases including the cyclin dependent kinases cdk7, cdk8, cdk9, casein kinase II, cdc2, map kinases and others. These kinases are either stably associated with the pol II holoenzyme as subunits of the basal transcription factor TFII-H and the SRB/mediator complex or associate to and phosphorylate CTD, e.g. upon appropriate stimuli [5–7]. Even though CTD phosphorylation is far from being understood, studies with different promoters suggested that the hypophosphorylated form of pol II, pol IIa, is involved in establishing the initiation complex. Hyperphosphorylation of CTD occurs at later time points, e.g. when pol II undergoes the transition into a processive transcription mode [5,7–11]. However, recent work in yeast indicated that phosphorylation of CTD by SRB10/11 (the homologues of cdk8 and cyclin C) has an inhibitory effect on transcription of genes involved in cell type specificity, meiosis and sugar utilization [12]. Thus, CTD phosphorylation may affect the gene activity in a positive and negative way.

Deletion mutants have revealed insight into possible functions of CTD. Deletion of CTD did not affect the basal transcription by pol II in in vitro assays [13]. In transient transfection experiments, transcription from promoters driven by SP1, a factor that typically activates housekeeping genes from positions proximal to the initiation sites, was also not affected [14]. However, deletion of CTD abolished the transcriptional activation by several enhancers [14]. Interestingly, the CTD appears also to be critical for the efficient processing of the primary transcript. In transient transfection assays, processes such as RNA 5′ end capping, splicing as well as 3′ end formation and termination of transcription were all inhibited when the CTD was deleted. This is explained by the observation that enzymes responsible for 5′ capping (RNA guanylyltransferase and RNA guanine-7-methyltransferase), splicing factors and cleavage-polyadenylation factors (CPSF and CstF) are all found to be associated with the hyperphosphorylated, but not hypophosphorylated, form of CTD [15–17]. Thus, a large complex may exist which mediates transcription, splicing and cleavage-polyadenylation of mRNA precursors [5].

In this study we investigated, whether pol II with a truncated CTD is able to express chromosomal genes.

2. Materials and methods

2.1. DNA constructs

Plasmids expressing the α-amanitin resistant large subunit of murine pol II (LSwt), a CTD deletion mutant with 31 repeats (LSΔ31) and with 5 repeats (LSΔ5) were kindly provided by W. Schaffner [14]. All recombinant proteins were haemagglutinin-(HA)-tagged at the amino-terminus.

2.2. Cell lines and transfection

Human kidney 293 cells were maintained before and after transfection in RPMI 1640 medium supplemented with 10% fetal bovine serum, 100 U/ml penicillin, 100 mg/ml streptomycin and 2 mM L-glutamine (Gibco BRL). For the expression of the various recombinant large subunits, 40% confluent 293 cells in 145 cm^2 tissue culture dishes (Nunc) were transfected in Optimem I medium (Gibco BRL) with 10 μg of plasmid DNA by the Lipofectamine method according to the manufacturer (Life Technologies). α-amanitin (1 mg/ml in H_2O, 2 μg/ml final concentration (Boehringer Mannheim) was added to the medium to inhibit the endogenous pol II. The *c-fos* gene was activated for 1 h by 12-*o*-tetra-decanoylphorbol-13-acetate (TPA) (200 μg/ml in

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DMSO, 100 ng/ml final concentration: Sigma). The *hsp*70A gene was activated by incubating the 293 cells in the tissue culture dishes covered with 10 ml medium and no lid at 43°C for 2 h.

2.3. Western blots

Lysates containing total cellular protein were analyzed by Western blotting after SDS-PAGE (6%) [18]. To verify that equal amounts of protein were transferred, the membrane (Immobilon-P, Millipore) was stained with Ponceau S. The endogenous large subunit of pol II was detected by an anti-CTD specific monoclonal antibody (8WG16), LSwt and deletion mutants thereof by an anti-HA specific monoclonal antibody (12CA5). Immunocomplexes were visualized by enhanced chemiluminescence (Amersham) using goat anti-mouse IgG horse radish peroxidase conjugate (Promega) as a secondary antibody.

2.4. Northern blots

Total RNA was isolated using the RNeasy Midi Kit (Qiagen). Northern blot analysis was performed as described elsewhere [18]. 10 μg of total RNA was loaded per lane. Probes were generated as follows: mouse *c-fos*, a 506 bp PCR product from exon 4 using 5′-TGCTTTGCAGACCGAGATTGC-3′ and 5′-GGTAGGTGAAGACAAAGGAAGACG-3′ as primers; human *hsp*70A, a 2.4 kb *Eco*RI fragment from pUCHsp70A (StressGene).

3. Results and discussion

LSwt, LSΔ31 and LSΔ5 (Fig. 1A) were transiently transfected into 293 cells to study the effect of CTD deletions on the inducibility of the chromosomal c-*fos* and *hsp*70A genes. The same vectors have been used previously to study the effect of CTD deletions on the expression of transiently transfected reporter gene constructs [14–16]. After transfection, cells were cultivated for 10 h to allow the expression of each subunit. Subsequently, α-amanitin was added and expression of each subunit was analyzed. Expression of LSwt, LSΔ31, and LSΔ5 was clearly detectable in cellular extracts of 293 cells 14 h and

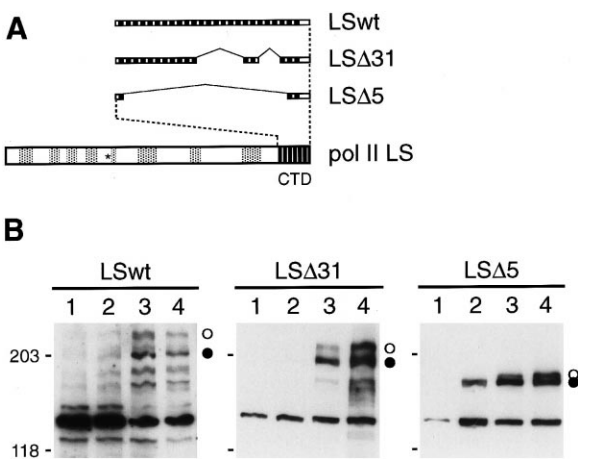

Fig. 1. Expression of the recombinant large subunit of pol II in transiently transfected 293 cells. A: The large subunit of pol II (polIILS) with its CTD is shown. Shadowed regions are evolutionary highly conserved. The point mutation conferring α-amanitin resistance is marked by an asteriks. The full size CTD (LSwt) as well as LSΔ31 and LSΔ5 are shown. B: Cells were transfected with the expression vectors for LSwt, LSΔ31 and LSΔ5. Protein lysates were prepared from untransfected cells (1) and from cells transfected for 10 h (2), 24 h (3) and 48 h (4). α-Amanitin was added after 10 h. Equal amounts of proteins were separated by SDS-PAGE, blotted onto nylon membranes and probed with a HA-specific antibody. The hypophosphorylated form of the large subunit is marked by ●, the hyperphosphorylated form by ○. The exposure time for the X-ray film for LSwt was 5 min, for LSΔ31 and LSΔ5 15 s.

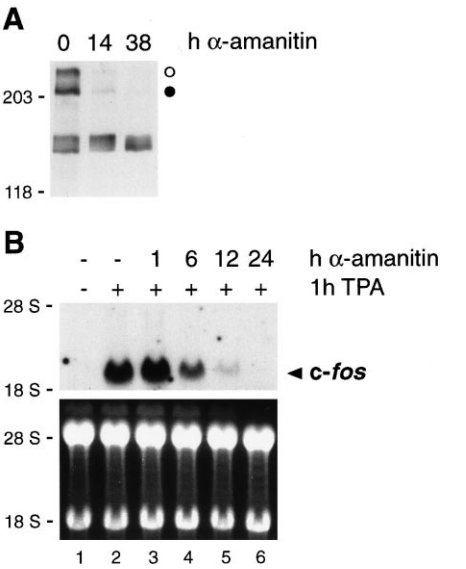

Fig. 2. Effect of α-amanitin on steady state levels of the large subunit of pol II and on c-*fos* gene induction by TPA. A: 293 cells were treated for 0, 14 and 38 h with α-amanitin. Cellular extracts were prepared and analyzed with a CTD-specific antibody by Western analysis. ● and ○ designate the hypo and hyperphosphorylated forms of the large subunit of pol II. B: 293 cells were treated with α-amanitin (lanes 3–6) and after the indicated times TPA was added for 1 h. Total RNA was purifed and c-*fos* expression was studied by Northern analysis (upper pannel). The lower pannel shows the ethidium bromide stained RNA before blotting.

38 h after addition of α-amanitin (Fig. 1B, lanes 3 and 4). Two bands were visible corresponding to the hypo and hyperphosphorylated forms of the CTD. As expected, the bands for LSΔ31 and LSΔ5 migrated with reduced molecular sizes.

We next studied, whether α-amanitin affected the expression of the endogenous large subunit of pol II (eLS) in 293 cells. The levels of eLS were strongly reduced 14 h after the addition of α-amanitin and no longer detectable after 38 h (Fig. 2A). This observation is in agreement with previous reports that α-amanitin does not only inhibit the activity of pol II but also leads to a rapid degradation of the large subunit of pol II [19]. We also examined, whether traces of eLS, which could still be present in 293 cells 24 h after treatment with α-amanitin, are capable to confer any transcriptional activity. Cells were treated with α-amanitin for the indicated times and subsequently activated for 1 h with the phorbolester TPA. TPA rapidly induces several immediate early genes including the proto-oncogene c-*fos*. TPA strongly induced the c-*fos* gene in 293 cells and in cells treated for 1 h with α-amanitin. In cells treated for 12 h with α-amanitin, c-*fos* induction was significantly reduced and in cells treated for 24 h, no longer detectable (Fig. 2B). Therefore, gene induction experiments with the LSwt and CTD deletions thereof were all carried out at least 24 h after addition of α-amanitin. At this time, eLS was almost entirely replaced by a recombinant large subunit.

We now examined, whether LSwt was capable of transcribing the chromosomal c-*fos* gene in 293 cells upon TPA stimulation. The c-*fos* gene carries a promoter proximal stalled pol II with a pause site approximately 20 bases downstream of the RNA cap site [20,21]. As shown in Fig. 3A, lane 2, treatment of 293 cells with α-amanitin for 48 h blocked the TPA-induced c-*fos* expression entirely. Transfection of the expression

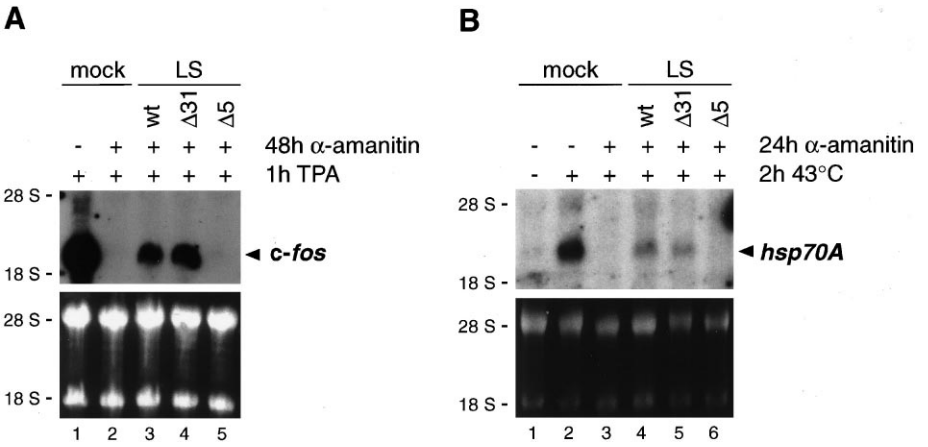

Fig. 3. Induction of the c-*fos* and *hsp*70A genes by pol II with a recombinant large subunit. A: 293 cells were transfected with the expression vectors for LSwt, LSΔ31 and LSΔ5. After 16 h, cells were grown with α-amanitin for an additional 48 h. Cells were then induced with TPA for 1 h. B: 293 cells were transfected with LSwt, LSΔ31 and LSΔ5. After 20 h, cells were grown with α-amanitin for an additional 24 h. Cells were then heat-shocked at 43°C for 2 h. Subsequently, total RNA was isolated and c-*fos* and *hsp*70A gene expression was analyzed as described in Fig. 2.

vector for LSwt rescued c-*fos* induction in 293 cells (lane 3). The intensity of the signal produced by LSwt was approximately 5–10% of the signal obtained with the eLS in 293 cells (lane 1). This was consistent with the transfection rate for the LSwt DNA construct, which was also in the range of 5–10% (controlled by co-transfection of a green fluorescence protein expression vector). Thus, expression of LSwt could restore the activity of the eLS. Transfection experiments with LSΔ31 revealed results very similar to those obtained with LSwt (lane 4) suggesting that the deletion of 21 repeats of the CTD did not significantly affect the inducibility of c-*fos* gene. However, as shown above, Western analysis consistently showed a less abundant expression of LSwt compared to LSΔ31 and LSΔ5 (Fig. 1B). Therefore, we cannot rule out a partial deficiency of LSΔ31 in c-*fos* induction. Even though LSΔ5 was well expressed (Fig. 1B), it could not restore the induction of the c-*fos* gene in 293 cells (Fig. 3A, lane 5). Long exposures of the autoradiogram gave no indication for c-*fos* mRNA after treatment of 293 cells with TPA (data not shown).

As a second gene, we studied the heat-shock inducible *hsp*70A gene in 293 cells. Similar to c-*fos*, transcription of the *hsp*70A gene is regulated by a promoter proximal pausing of pol II [22,23]. *Hsp*70A shows a low level of basal expression in 293 cells (Fig. 3B, lane 1) which disappeared 24 h after treatment of cells with α-amanitin (lane 3). In transfection experiments, expression of LSwt and of the mutants Δ31 and Δ5 gave in principal the same results as obtained for the c-*fos* gene. LSwt and LSΔ31 transcribed the *hsp*70A gene upon heat-shock (lane 3 and 4, respectively), whereas LSΔ5 could not (lane 6).

We have shown here that the recombinant LSwt and LSΔ31 can substitute the endogenous large subunit of pol II for induction of chromosomal c-*fos* and *hsp*70A genes in 293 cells. Both genes are regulated by a promoter proximal pausing of pol II. In isolated nuclei, pol II is tightly associated with its pause site and does not dissociate in the presence of 0.5% sarcosyl [22]. The turnover of pol II at its pause site in the c-*fos* gene in vivo is unknown. Our data indicate that the endogenous paused pol II has to be replaced by a pol II with a recombinant large subunit. This replacement may occur either by destabilization of the transcription complex at the pause site or after premature termination of transcription downstream of the pause site. The latter assumption suggests that pausing of pol II occurs only transiently. In fact, transient pausing of pol II and premature termination of transcription have recently been described for a c-*fos* gene on stably transfected episomes [21]. The stability of the paused pol II complex at the *hsp*70A promoter has not yet been studied. Our data support the assumption that promoter proximal paused pol II complexes have a turnover, even if a gene is non-transcribed.

The CTD deletion mutants of the large subunit of pol II behaved differently in the induction experiments. Transfection experiments with the LSΔ31 mutant gave similar results as obtained with a LSwt. This may suggest that a CTD with 31 repeats can establish the platform for all the factors required for activation of the transcriptional machinery and the subsequent modifications and processing of the primary transcript. In contrast, using a large subunit with only 5 repeats of the CTD failed to induce c-*fos* and *hsp*70A gene transcription. The molecular level for this failure is not yet clear. The LSΔ5 mutant used in this study has previously been reported to have defects in expression of transiently transfected genes. This mutant failed to activate promoters in an enhancer-dependent manner [14] and to process the primary transcript [16,17]. Therefore, we cannot rule out that LSΔ5 is able to transcribe the c-*fos* and *hsp*70A genes in 293 cells, but the primary transcripts are not properly processed and fail to be transported into the cytoplasm. This question can only be answered with the help of nuclear run on experiments. Unfortunately, these experiments are difficult to perform in transiently transfected cells. However, in the process of studying the function of CTD in the transcription of chromosomal genes, we have established two cell lines expressing conditionally LSwt and LSΔ5. In these cell lines, LSwt but not LSΔ5 produced c-*fos* gene specific nuclear run on transcripts after induction with TPA (data not shown). Thus, different to transiently transfected reporter constructs [15], LSΔ5 appears to have a defect at the level of initiation and/or RNA elongation on chromosomal c-*fos* templates.

In yeast, the major CTD kinase is a subunit of the general transcription factor TFIIH and is encoded by an essential

gene, *kin*28. The general importance of CTD phosphorylation by Kin28 for transcription in yeast has been suggested because for all genes tested, transcription is inhibited at the non-permissive temperature in temperature-sensitive *kin*28 mutants [24]. However, recent experiments showed that the CUP1 gene is still highly inducible at the non-permissive temperature [23]. This is in line with the observation that the CUP1 gene is still transcribed by a CTD-less pol II (D.L. Bentley, personal communication). Thus, pol II possibly does not require the CTD for transcription of all genes. The transient transfection assay described here will be quite useful to study this questions for individual chromosomal genes.

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